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Simultaneous Detection of *Escherichia coli* Heat-Stable and Heat-Labile Enterotoxin Genes with a Single RNA Probe

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A single RNA probe was synthesized and used to detect simultaneously the methanol-soluble heat-stable enterotoxin and heat-labile enterotoxin genes in *Escherichia coli* strains. The results with the biotinylated or radioactive probe correlated 100% with the biological assay results for both toxins. The RNA probe detected the three known heat-stable enterotoxin A alleles.

Enterotoxigenic *Escherichia coli* (ETEC) strains are a major cause of childhood diarrheal disease in developing countries and a frequent cause of enteric illness among travelers to those countries (2, 12, 24). ETEC is identified by detecting serologically or by a bioassay the heat-stable enterotoxins (ST) and/or the heat-labile enterotoxins (LT) in *E. coli* cultures. The methanol-soluble ST (ST_A) is assayed by fluid accumulation in ligated rabbit (9) or pig (27) intestinal loops, by the suckling mouse model (7), or by an enzyme-linked immunosorbent assay (31); LT is detected in adrenal (23) or Chinese hamster ovary (CHO) (11) cell cultures, by immunological assays (33), or by the rabbit ligated ileal loop model. The cost and inconvenience of the animal models limit their use to a few research laboratories.

Recently, alternative approaches have been developed for detection of the three ST_A alleles (*estA1*, *estA2*, and *estA3*; 14) or the LT genes (*eltA* and *eltB*; 6) with either RNA (4, 5) or DNA (1, 8, 18, 21, 26, 28) gene probes. The use of a single probe that could simultaneously detect genes of both ST and LT as an initial screening for ETEC strains could facilitate large epidemiological studies. Small laboratories could identify ETEC strains by using a single nonradioactive test. We discuss here the use of an *estA-eltB* gene fusion set under the SP6 RNA polymerase-driven system to generate a single hybrid RNA probe and the use of this probe to detect ETEC strains. The fused gene probe identified all the colonies that were detected by the individual *estA* or *eltB* probes. The results are discussed in relation to the use of the hybrid *estA-eltB* RNA probe in the detection of ETEC.

E. coli HB101 (13) harboring different plasmids was the laboratory strain used. Plasmids pSP64 and pSP65 (Promega Biotec, Madison, Wis.) are identical ColE1 derivatives that contain in one or the opposite orientation a polylinker region in proximity to the SP6 promoter (20). pYK158 and pYK159 were constructed by cloning into pSP64 and pSP65, respectively, 911 base pairs (bp) (Fig. 1) of DNA where the regulatory elements and the signal peptide of *eltB* are replaced by *estA2* DNA, devoid of transcription and translation stop signals (13); pYK164 was generated by cloning into pSP65 a 530-bp *eltB* fragment derived from pEWD299 (6). The multicopy plasmids pGK22 and pGK40 are *estA2* and *eltB* pUC8 derivatives, respectively (13); pYK007 is an *estA3*, ampicillin resistance, low-copy-number plasmid isolated from an ST-producing clinical ETEC strain (30). Also used were HB101(pBR322) (3), HB101(pUC8) (32), HB101 (pSP64), and HB101(pSP65); strain SA53(pCP3727), provided by Randall Holmes (Uniformed Services University of the Health Sciences, Bethesda, Md.), is an LT II_a producer that does not cross-react with an LT B-I probe (22). A total of 30 ETEC strains (16 ST, 10 LT, and 4 ST and LT [ST/LT] producers) and 8 non-ETEC strains isolated from children with acute diarrhea were provided by Jorge Olarte (Hospital Infantil de Mexico, Mexico D.F., Mexico). Seven ETEC strains (two ST, two LT, and three ST/LT producers) were provided by Bradley Sack (Johns Hopkins University School of Medicine, Baltimore, Md.). The *estA1* strains were provided by Donald Robertson (University of Kansas, Lawrence), and the *estA2* strain was provided by Nigel Harford (Smith Kline MIT, Rixensart, Belgium). A total of 250 *E. coli* colonies (5 colonies per patient) isolated from 50 children with acute diarrhea at Children's Medical Center, Dallas, Tex., in 1987 and 1988 were also tested. Bacterial strains were grown in L broth and on L agar (13) supplemented when necessary with ampicillin (100 μg/ml).

The methods for plasmid isolation, restriction enzyme analysis of DNA fragments, and agarose gel electrophoresis were described previously (13, 19). To protect RNA from RNases, we baked all the glassware at 250°C for 6 h and made all the solutions with distilled water treated for 12 h with 0.1% diethylpyrocarbonate and then autoclaved.

Plasmid pYK159 (*estA-eltB*) (Fig. 1) was first linearized with *Hin*dIII and then transcribed with SP6 RNA polymerase in accordance with manufacturer recommendations (Promega Biotec; 25). RNA transcripts were stored in ethanol and pelleted by centrifugation immediately before use. Biotinylated RNA probes were generated by in vitro transcription with synthetic UTP containing biotin attached at position 5 of the pyrimidine base by an 11-atom linker (biotin-11-UTP; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The reaction conditions used were those suggested by the manufacturer.

The *eltB* RNA probe was generated with plasmid pYK164 (13) previously linearized with *Hin*dIII and transcribed with SP6 RNA polymerase. To detect ST-producing strains, we radiolabeled the 20-oligomer TTACAACACAATTCACA GCA complementary to positions 323 to 342 from the *Eco*RI site of the wild-type *estA* gene (14) by incubation with polynucleotide kinase and [γ-^{32}P]ATP as described previously (13). This DNA region is identical in *estA2* and *estA3*,

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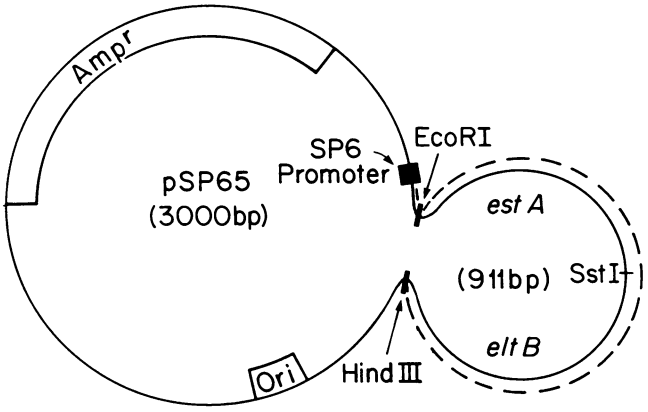

FIG. 1. Plasmid pYK159, source of the fused *estA-eltB* probe, was obtained by cloning a 911-bp *Eco*I-*Hin*dIII DNA fragment containing the fused genes into plasmid pSP65 treated with the same enzymes. The plasmid was linearized with *Hin*dIII prior to incubation with SP6 polymerase and radioactive or biotinylated ribonucleotides. Amp^r, Ampicillin resistance; Ori, origin of replication. The dotted line indicates the RNA transcript used as a probe for the detection of both toxin genes.

and two nucleotides differ in *estA1* (positions 679 and 681; 29).

Plasmid DNAs were prepared for slot blots and applied through a 24-well filtration manifold (Hybri-slot; Bethesda Research Laboratories) to a 0.45-μm-pore nitrocellulose membrane (BA85; Schleicher & Schuell, Inc., Keene, N.H.). The membrane was baked for 2 h at 80°C in a vacuum oven and then hybridized with the radioactive and/or biotinylated probes as described previously (19).

Colony hybridization and radioactive detection were performed as described by Maniatis et al. (19). Detection of the genes in colonies with the biotinylated probe included, when indicated, incubation of the filters on Whatman papers saturated with 10% sodium dodecyl sulfate (SDS) for 3 min. This procedure preceded the colony lysis method (15), which included proteinase K (200 ng/ml) treatment and ethanol and chloroform washes of the filters (10).

The hybridization solutions described by Maniatis et al. (19) were modified by excluding formamide. Either 20 ng of radiolabeled RNA probe per ml (2×10^6 cpm/ml) or 200 ng of biotinylated RNA probe per ml was added, and the mixture was incubated overnight at 37°C. The filters were washed three times (20 min each time) at room temperature with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% SDS and then three times (20 min each time) at 56°C with 0.1× SSC–0.5% SDS. The radiolabeled filters were exposed to films and developed (13).

Detection of the biotinylated probe-target hybrid (16, 17) was carried out by incubating the filters with streptavidin coupled to alkaline phosphatase and developing them with 5-bromo-4-chloro-3-indolyl phosphate as the substrate and Nitro Blue Tetrazolium as the chromogen (ENZO Biochem, Inc., New York, N.Y.) in accordance with the recommendations of the manufacturer.

The hybridization conditions used for the LT RNA probe were identical to those used for the hybrid probe except that the temperature was decreased to 50°C in the last three washes. For hybridization with the ST DNA oligoprobe we used 6× SSC–5× Denhardt solution–0.5% SDS–10 mM EDTA–heat-denatured salmon testis DNA (100 μg/ml)–radiolabeled ST DNA oligoprobe (20 ng/ml) (13); the washing conditions were the same as those described for the LT RNA probe.

TABLE 1. Detection of ETEC by colony hybridization

Enterotoxin	No. of isolates tested	No. of isolates identified as positive by colony hybridization with:			
		Radioactive probes			Biotinylated probes (ST/LT)
		ST	LT	ST/LT	
ST	20	18	0	18	18
LT	13	0	13	13	13
ST/LT	9	9	9	9	9
None	13	0	0	0	0

Transcription by SP6 RNA polymerase of the plasmids that harbor the SP6 promoter preceding the cloned enterotoxin genes yielded radioactive RNA probes with specific activities of 3×10^8 to 8×10^8 cpm/μg; typically, more than 80% of the radioisotope was incorporated into the RNA. In the case of biotin-11-UTP, the reaction conditions yielded 1.5 μg of RNA per μg of DNA template. In either case, enough radioactive or biotinylated probe was obtained to perform at least eight hybridization reactions.

Various quantities of pGK22 (*estA2*), pGK40 (*eltB*), pUC8, or pSP64 plasmid DNA were fixed to nitrocellulose membranes and hybridized to either the radioactive or the biotinylated hybrid RNA probe. When 100 ng of pUC8 or pSP65 DNA was applied to the membrane as a negative control, no hybridization was detected with either probe. The radioactive probe detected 25 pg of the target DNA (either pGK22 or pGK40); it was 10 times more sensitive than the biotinylated probe, which detected 250 pg of either plasmid (data not shown).

A total of 55 *E. coli* strains (10 laboratory strains and 45 clinical isolates) previously characterized as producing ST, LT, or ST/LT or as being non-ETEC by bioassays were analyzed by the colony hybridization method with either the radioactive or the biotinylated hybrid RNA probe. The radioactive probe identified 9 ST/LT-producing strains, 13 LT-producing strains, and 18 of 20 ST-producing strains; none of the 13 non-ETEC strains gave a positive signal (Table 1). Comparative detection of *estA* and *elt* strains with the fused *estA-eltB* RNA probe and with individual probes directed against the two genes is shown in Fig. 2. All strains identified by the hybrid probe as *estA*$^+$ or *eltB*$^+$ were also identified by the two separate gene probes. The two ST-producing strains that did not give a positive signal with the hybrid RNA probe were also negative with the *estA* oligoprobe (Fig. 2A and C, positions 22 and 23). When these two strains were tested in the suckling mouse model, they were no longer positive. These results are summarized in Table 1. The three alleles *estA1*, *estA2*, and *estA3* (Fig. 2C, D, and E, positions 47, 43, and 46) were recognized by the *estA-eltB* RNA probe and by the 20-mer *estA* oligoprobe (Fig. 2A); strain SA53(pCP3727) harbors an LT allele (LT II_a) not recognized by the *eltB* probes used in this study (Fig. 2B, position 40).

The specificity of the biotinylated probe was enhanced by treatment of the nitrocellulose membrane with SDS before implementation of the colony hybridization procedure. Nonspecific binding (Fig. 2D) was greatly diminished when the SDS step was included (Fig. 2E). With this modification, all the negative strains gave a ringlike pattern (clear center and enhancement of the edges) (Fig. 2E). The positive control strains and several clinical strains gave a stronger blue-

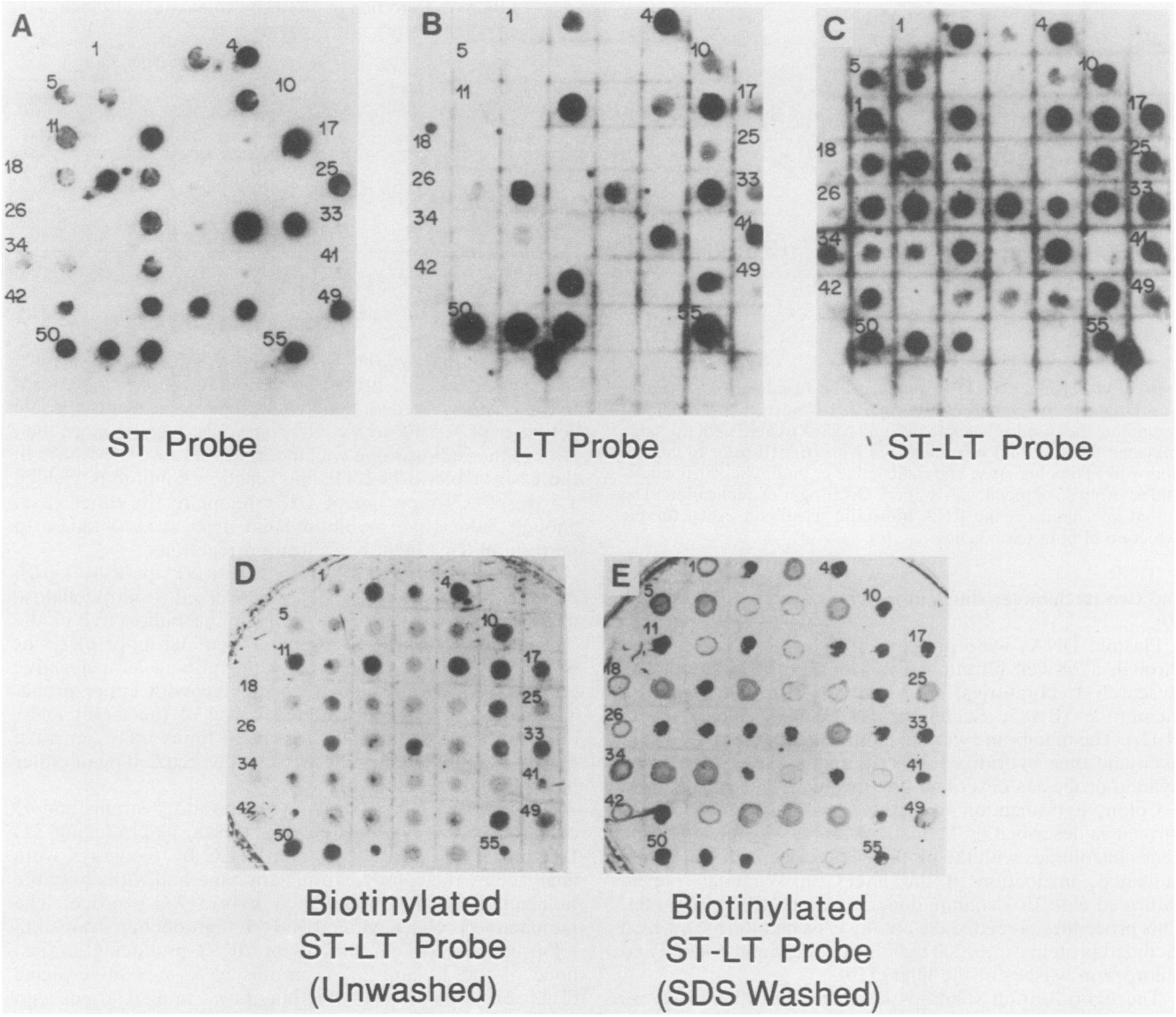

FIG. 2. Detection of ETEC strains by RNA colony hybridization with the radioactive 20-mer *estA* oligoprobe (A), the radioactive 580-bp *eltB* RNA probe (B), the radiolabeled hybrid RNA probe (C), the biotinylated hybrid RNA probe (D), and the biotinylated hybrid RNA probe with SDS treatment of the filter (E). Positions 3, 5, 6, 9, 11, 19, 20, 21, 25, 29, 31, 34, 35, 37, and 49 are ST-producing clinical strains; position 43 is *E. coli* HB101(pGK22), a multicopy ST_{A2}-producing strain; position 46 is CA8000(pYK007), a low-copy ST_{A3}-producing strain; and position 47 is an ST_{A1}-producing clinical strain. Positions 2, 10, 15, 17, 24, 27, 28, 30, 33, 36, 39, and 48 are LT-producing clinical strains; position 41 is *E. coli* HB101(pGK40), a multicopy LT_B-producing strain. Positions 4, 13, 16, 32, 45, 50, and 51 are ST/LT-producing clinical strains; positions 52 and 55 are *E. coli* HB101(pYK158) and HB101(pYK159), respectively, both multicopy ST/LT-producing strains. Positions 1, 7, 8, 12, 14, 18, 22, 23, 26, and 38 are non-ETEC clinical strains; position 40 is *E. coli* SA53(pCP3727), an LT II_a-producing strain; and positions 42, 44, 53, and 54 are *E. coli* HB101 harboring plasmids pBR322, pUC8, pSP64, and pSP65, respectively, all non-ETEC control strains.

purple signal than did other positive clinical strains. When the filters were SDS treated, the biotinylated probe identified as positive the same colonies as did the radioactive probe.

Of the 250 *E. coli* isolates from 50 patients with acute diarrhea at Children's Medical Center, 18 (7.2%) positive colonies were identified. The positive isolates were recovered from five cases [two patients each with all five isolates positive, one with four, one with three, and one with only one isolate(s) positive]. These strains were confirmed to be ST producers by specific *estA2* and *eltB* probes and by the suckling mouse assay.

The biotinylated hybrid RNA probe has been stable for at least 10 months; longer-term stability is being investigated.

The use of single-stranded RNA probes offers an easy and effective approach for detecting desired DNA sequences. RNA probes are easy to prepare and increase the sensitivity of and reduce the background in the detection of complementary genes because the stability of the DNA-RNA duplexes formed allows more stringency in the experimental hybridization conditions (5). These characteristics of RNA probes must be contrasted with the additional precautions needed to avoid RNase activity in the solutions. The SP6

RNA polymerase system yielded enough specific RNA probes to perform at least eight hybridization assays per microgram of template DNA. Similar results have been obtained by other workers (20). This system has been used to detect ST-producing ETEC strains (4). In this study, we successfully synthesized and used an RNA hybrid probe to detect *E. coli* strains that have the genetic potential for producing ST, LT, or both.

The 100% specificity (13 of 13 negative strains) obtained as compared with those of standard assays makes this approach a suitable alternative and a rapid and reliable diagnostic test. The apparent 95% sensitivity (40 of 42 positive strains) occurred because the two ST-producing clinical strains that gave apparently false-negative results when retested had lost the ability to produce ST; these phenotypic changes were probably due to the loss of the *estA* plasmids. Thus, the sensitivity (40 of 40 positive strains) and specificity (15 of 15 negative strains) were actually each 100%.

The three known *estA* alleles (14, 29) were identified as strong positive signals by the 20-mer ST DNA oligoprobe (Fig. 2A, positions 43, 46, and 47); similarly, the fused *estA-eltB* RNA probe identified these colonies as ST producers, but *estA1* and *estA3* yielded weaker signals (Fig. 2E, positions 46 and 47). Nevertheless, these signals were clearly distinguishable from those of the negative controls (Fig. 2E, positions 42, 44, 53, and 54).

The availability of nonradioactive labeling methods obviates the problems related to radioactive probes. The method described by Haas and Fleming (15) offers a suitable colony lysis technique to decrease the nonspecific binding of biotinylated probes to nitrocellulose filters. However, Huovinen et al. (16) obtained 21% false-positive and 27% false-negative reactions with that method. Incorporation of an initial SDS step improves the results considerably, since it makes easy the removal of cellular debris, proteins, and interfering substances once the filter is exposed to protease treatment, and in our experiments it made the assay 100% reliable.

As far as we know this is the first study in which a single probe has been used to detect simultaneously two genes encoding different toxins. In epidemiological studies in which it is convenient to analyze large numbers of isolates simultaneously the hybridization technique has obvious advantages: each nitrocellulose membrane is capable of harboring DNA representing up to hundreds of samples and the membranes can be stored for several months at room temperature and sent by mail to the relevant laboratory for analysis (28). Combining these advantages with the availability of this stable, nonradioactively labeled hybrid RNA probe and the possibility of detecting both *E. coli* enterotoxin genes simultaneously with a single probe should provide a simple, inexpensive, and accurate tool for large-scale epidemiological studies.

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