Identification of Enterotoxigenic Escherichia coli by Colony Hybridization with Nonradioactive Digoxigenin-Labeled DNA Probes

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Enterotoxigenic Escherichia coli (ETEC) strains were readily identified in pure and mixed cultures with nonradioactive, digoxigenin-labeled DNA probes coding for heat-labile (LT) and heat-stable (STaI, STaII, and STb) enterotoxins. Digoxigenin-labeled ETEC fragments were more sensitive than and exhibited less nonspecific background contamination than biotinylated ETEC probes.

The role of enterotoxigenic Escherichia coli (ETEC) strains as etiologic agents of diarrheal disease in neonatal animals and humans is well recognized. Four types of E. coli enterotoxins have been extensively studied: a methanol-soluble, heat-stable toxin isolated from animals and humans (STaI); a methanol-soluble, heat-stable toxin isolated only from humans (STaII); a heat-labile toxin (LT); and a methanol-insoluble, heat-stable toxin (STb). Identification of ETEC has traditionally relied on conventional bacteriologic culture followed by bioassays or serologic tests for the presence of toxin antigens (3, 11, 13, 16, 17). More recently, hybridization assays using 32P-radiolabeled cloned toxin sequences have been used to distinguish toxigenic from nonnontoxigenic E. coli (8, 10). These assays have the advantage of being very specific and sensitive, and they detect bacterial genotypes without relying on toxin expression. The disadvantages of these isotopic assays for routine diagnosis in clinical laboratories have been the expense, the short half-life, and the biohazards involved in handling radioactive DNA.

To circumvent these difficulties, alkaline phosphatase-conjugated synthetic oligonucleotides (12) and biotinylated cloned DNA fragments (1, 4) coding for ETEC sequences have been used as gene probes. Seriwatana et al. (12) demonstrated that single 26-base-pair ETEC oligonucleotide probes, labeled by attaching alkaline phosphatase directly to thymidine bases, were less sensitive than radiolabeled counterparts, thus limiting their apparent utility. Cloned DNA fragments used for detection of ETEC sequences are significantly larger than oligonucleotide probes, ranging in size from 157 to 850 base pairs. The increased size of the probes allows use of random primed or nick translation labeling techniques, which cannot be successfully used with oligonucleotides. Probes labeled by these methods contain more labeled bases per molecule and, theoretically, exhibit increased sensitivity over synthetic oligonucleotide probes. Attempts to utilize this strategy for nonradioactive detection of ETEC with biotin-labeled cloned ETEC DNA fragments yielded low signal-to-noise ratios. High background levels are apparently due to nonspecific binding of the streptavidin to membrane supports, a finding which has been reported by other investigators who used biotinylated DNA probes (15, 18). Recently, digoxigenin-labeled dUTP has become commercially available (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and has been reported to have minimal nonspecific binding properties. In this study, we examined the use of digoxigenin-labeled cloned DNA fragments for identification of ETEC in colony hybridizations and compared these results with results obtained with biotinylated probes to determine whether digoxigenin-labeled probes could be utilized to overcome the limitations of biotinylated probes while taking advantage of the increased sensitivity of large cloned DNA fragments as probes. Our results indicated that digoxigenin-labeled probes showed greater signal intensity and much less background noise than biotinylated probes did, effectively improving assay sensitivity.

Plasmids and strains used in this study are listed in Table 1. Recombinant plasmids harboring sequences for ETEC toxins, provided by S. Moseley (University of Washington, Seattle), were as follows: pDAS100, encoding a 215-base-pair fragment of STaII (9); pDAS101, encoding a 157-base-pair fragment of STaI from a porcine isolate (5, 9); pDAS102, encoding an 850-base-pair HindIII fragment of LT (2); and pDAS103, encoding a 460-base-pair HindIII fragment of STb, inserted at the Smal site (6). Toxin-specific sequences were isolated by cleavage of recombinant plasmids with appropriate restriction enzymes (14) and elution from agarose gels (7). Purified DNA fragments were labeled by random primed incorporation of digoxigenin-labeled dUTP, as recommended by the kit manufacturer (DNA labeling and detection kit [Genius Kit]; Boehringer Mannheim Biochemicals). Unincorporated label was removed by ethanol precipitation.

Standard human and porcine ETEC strains of known phenotype were provided by D. Robertson (University of Kansas, Lawrence). Clinical isolates from animals with gastroenteritis were obtained through the University of Missouri Veterinary School. All isolates were previously tested for the presence of enterotoxins by standard bioassays or hybridization assays with radioactive probes. Bacterial strains to be tested with nonradioactive probes were inoculated onto nitrocellulose (Millipore Corp., Bedford, Mass.) or nylon (Nytran; Schleicher & Schuell, Inc., Keene, N.H.) membranes, placed on Luria agar plates, and incubated overnight at 37°C. Nitrocellulose membranes were processed as described by Maniatis et al. (7). Nytran membranes were prepared identically except that some membranes were steamed during the alkali denaturation step to determine whether steaming enhanced the signal-to-noise...
ratio. Because nitrocellulose membranes decompose at elevated temperatures under alkali conditions, nitrocellulose filters were not steamed. After processing, membranes were baked for 20 min at 80°C, washed for 45 min at 37°C in three changes of prewashed solution (1 M NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 50 mM Tris hydrochloride [pH 8.0]), and deproteinized at 37°C for 1 h in a solution of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), 0.1% sodium dodecyl sulfate, and 50 μg of proteinase K per ml. Membranes were prehybridized, hybridized, and visualized as described by the manufacturer. Bound probes were detected by sequential incubations with antidigoxigenin antibody conjugated to alkaline phosphatase and developed in a solution of Nitro Blue Tetracium and 5-bromo-4-chloro-3-indolylphosphate toluidinium salt. Blots were allowed to develop for 15 to 30 min.

In preliminary experiments, bacterial strains inoculated on nylon membranes gave much more intense signals than did identical samples applied to nitrocellulose (data not shown). Signal intensity on nylon membranes was also markedly improved by steaming during alkaline denaturation (data not shown). On the basis of these findings, all subsequent experiments were performed with nylon membranes which were processed by steam treatment during alkaline denaturation.

Digoxigenin-labeled ETEC probes specifically identified strains known to harbor genes coding for heat-stable enterotoxins and LTI toxins (Fig. 1). Probes differentiated porcine STaI, human STaII, LTI, and STb isolates. Very little background noise was discernible on the membrane or associated with toxin-negative strains. Digoxigenin-labeled probes failed to identify only one ETEC isolate, a clinical isolate from a canine which was positive for STaI by radiolabeled probe assay (Fig. 1, sample 4B). It is unclear why the digoxigenin-labeled probes failed to detect the enterotoxin sequences in this isolate.

To ascertain whether digoxigenin-labeled probes would allow identification of ETEC in mixed cultures containing common intestinal flora, broth cultures of *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and toxigenic *E. coli* 263 were saturated over night and combined, and the mixture was inoculated onto MacConkey agar plates. Plates were incubated overnight at 37°C and photographed to delineate locations of isolated colonies (Fig. 2A). Replicas of bacterial streak plates were transferred onto nylon supports by placing membranes directly onto the surfaces of bacterial colonies, as reported elsewhere (7). Membranes were processed, and hybridizations were carried out as described above. Streak plates showed three readily distinguishable phenotypes: light pink, very mucoid colonies, indicative of lactose-fermenting *K. pneumoniae*; dark pink, less mucoid colonies, indicative of lactose-positive *E. coli*; and clear, lactose-negative colonies indicative of either *P. mirabilis* or *E. cloacae*. Biochemical tests on representative colonies confirmed bacterial identification. Blots probed with digoxigenin-labeled LTI sequences yielded positive signals which corresponded to the location of *E. coli* 263 colonies (Fig. 2B).
FIG. 3. Detection of ETEC colonies in stool blots. Duplicate blots of ETEC mixed with normal fecal flora (row 1) and of normal fecal flora alone (row 2) were prepared on nylon membranes, and ETEC colonies were detected by using digoxigenin-labeled probes.

2B). No signals were visible for isolated colonies of any other phenotype. These data indicate that digoxigenin-labeled hybridization assays can be used to detect ETEC in mixed cultures grown directly from intestinal or rectal swabs.

To ascertain whether digoxigenin-labeled probes can also be utilized for detection of ETEC in macrocolonies containing total bacterial growth from stools (stool blots), rat fecal material containing 2.8 x 10⁵ normal flora bacteria was spiked with various amounts of *E. coli* 263, botted onto nylon membranes, and incubated overnight on MacConkey plates to allow bacterial proliferation. Controls were processed identically, except that no ETEC was added to normal flora inoculum. Membranes were processed, hybridized with digoxigenin-labeled STb⁺ probe, and visualized as described above. Stool blots detected ETEC when an inoculum of as few as 10⁴ ETEC was used (Fig. 3). Signals were never observed in controls inoculated with normal flora only, indicating that nonspecific background problems with digoxigenin-labeled probes are minimal. The ability to detect ETEC in mixed-colony and stool blots with digoxigenin-labeled probes eliminates the preparation of pure cultures, a step required for serological and biological testing for ETEC.

To compare biotin- and digoxigenin-labeled ETEC DNA probes, purified LTI gene sequences were labeled as described above, except that for biotinylated probes, digoxigenin was replaced by biotin-11-dUTP at concentrations recommended by the kit manufacturer (Blugene nonradioactive nucleic acid detection system; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Procedures were as described above, with the exception that formamide concentrations were decreased from 50 to 45% in hybridization reactions containing biotinylated probes, according to protocols of the manufacturer. Biotinylated probes were visualized by incubation with streptavidin-biotin conjugate and developed in a solution of Nitro Blue Tetrizolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium salt. Digoxigenin-labeled probes readily detected the control strain, pDAS102, from which probe sequences were isolated, as well as clinical ETEC strains of known phenotypes (Fig. 4A). Identical blots with biotinylated probes showed weaker signal intensity and increased background contamination (Fig. 4B) when either nitrocellulose or nylon supports were used.

Probes labeled with digoxigenin and stored at -20°C appeared stable for longer than 6 months. While the kit costs are significantly higher for digoxigenin-labeled probes than for biotin-labeled probes, experiments in our laboratory showed that hybridization solutions containing digoxigenin-labeled probes could be stored at -20°C after use and reutilized up to four times without significant decrease in sensitivity. The ability to reuse hybridization solutions multiple times significantly decreased the expense of digoxigenin use.

In summary, we have shown that colony hybridization using digoxigenin-labeled probes is a sensitive, specific procedure for detection of ETEC in either pure or mixed cultures. These probes eliminate the disadvantages of radioactive probes, and signal-to-noise ratios appear superior to those obtained with biotinylated probes.

FIG. 4. Comparison of digoxigenin-labeled (A) and biotin-labeled (B) DNA probes for detection of ETEC. Identical blots were prepared on nylon membranes and hybridized with labeled ETEC DNA probes. Row 1, pDAS102; row 2, 263; row 3, 1362; row 4, 334.

LITERATURE CITED


