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Alkaline Phosphatase-Conjugated Oligonucleotide Probes for Enterotoxigenic *Escherichia coli* in Travelers to South America and West Africa

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Several studies have demonstrated the usefulness of ^{32}P-labeled recombinant DNA probes for identifying enterotoxigenic *Escherichia coli* (ETEC). The use of radioisotopes and X-ray development, however, severely handicaps the utility of DNA probes in most clinical laboratories. In this study, enzyme-labeled oligonucleotide probes for ETEC LT (heat-labile toxin) and ST (heat-stable toxin) genes were compared with the standard Y1 adrenal cell and suckling mouse assays for their ability to identify ETEC in a population of American adults experiencing acute episodes of diarrhea in South America and West Africa. The LT probe hybridized with 12% (64 of 529) of the *E. coli* colonies tested, whereas 11% (57 of 529) were positive by Y1 adrenal cell assay. DNA from 9% (47 of 529) of the *E. coli* colonies tested hybridized with the ST probe, whereas only 5% (28 of 529) produced ST as measured by the suckling mouse bioassay. For the patient samples tested, correlation between probe and bioassay for LT was 97%, or three discrepancies in 111 patients tested. Overall concordance of the ST probe and bioassay was 95%, or five discrepancies in 111 patients. Enzyme-labeled oligonucleotide probes represent a major advance in the diagnosis of ETEC-associated diarrheal disease and may be used in laboratories with minimal equipment.

Escherichia coli produces toxins which cause diarrhea in humans. Enterotoxigenic *E. coli* (ETEC) is among the most frequent enteric pathogens associated with diarrheal disease episodes in developing countries. Studies of travelers to less developed areas have shown that an average of 36% of diarrhea cases are associated with ETEC (2). ETEC infections also occur in a high proportion of children in developing countries (10, 11, 21). Methods for detection of ETEC include the suckling mouse assay for heat-stable toxin (ST) and the Y1 adrenal cell assay for heat-labile toxin (LT). Both bioassays can be costly, time consuming, and difficult to run in a hospital or clinical laboratory.

Since 1982, the usefulness of recombinant DNA probes for identifying ETEC has been demonstrated. These probes are strands of DNA which carry unique segments that code for ST and LT and can hybridize with complementary strands from ETEC. Several studies have shown that cloned polynucleotide DNA probes are quite sensitive and specific for both ETEC toxin genes and compare well with other methods (5, 6, 13, 16, 19). These initial probes were labeled with ^{32}P and detected by autoradiography; the need for unstable radioisotopes and X-ray development, however, severely handicaps their use in clinical laboratories. Radioactive labels have been replaced with biotin-avidin systems in which alkaline phosphatase is used to produce a colorimetric signal (1, 18).

The development of synthetic oligonucleotides of only 20 to 30 bases has the potential to simplify and speed up probe detection assays. Comparisons of ^{32}P-labeled oligonucleotide probes and cloned polynucleotide probes with standard assays for ETEC have shown promise (7, 8, 15). Recently, oligonucleotide probes specific for ST and LT have been conjugated directly with alkaline phosphatase, thus making a "cold" probe which does not require special equipment and can be visually interpreted (17).

In this study, two enzyme-labeled oligonucleotide probes were compared with the standard suckling mouse and Y1 assays for their ability to identify ETEC in a population of North American adult males experiencing acute episodes of diarrhea in South America and West Africa. This group also provided an opportunity to evaluate these probes by using a collection of *E. coli* isolates from several diverse geographic regions.

MATERIALS AND METHODS

***E. coli* isolates.** *E. coli* strains were isolated from U.S. Navy and Marine Corps personnel participating in a clinical drug treatment trial for traveler's diarrhea. This trial was performed during three exercises taking place in one site in Ecuador and major port cities in South America and West Africa. Stools from 111 acute diarrhea cases were streaked onto MacConkey agar plates. Five *E. coli* colonies per patient were saved in vials containing Trypticase soy agar with a mineral oil overlay. A sample was considered positive if at least one colony out of five tested was ETEC positive.

Preparation of cell-free supernatants. *E. coli* strains were grown overnight in brucella broth in flasks at 37°C in a shaking incubator. Polymyxin B was then added at a final concentration of 0.1 mg/ml, and the flasks were incubated for an additional 10 min. This material was then centrifuged at 25,000 × *g* for 20 min, and the supernatant was removed for testing. Portions of the supernatant from each patient were pooled for initial testing.

Y1 adrenal cell assay for LT. The test for LT followed the method of Donta et al. (4).

Suckling mouse assay for ST. Samples were tested for the production of ST by the method of Dean et al., as described elsewhere (3).

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Biochemical identification of strains. All isolates were confirmed as *E. coli* by using standard bacteriological methods (9) and the API 20E strip (Analytab Products, Plainview, N.Y.).

Bacterial DNA extraction. Replicate *E. coli* colonies (five per patient) were spotted onto Zeta-Probe membranes (Bio-Rad Laboratories, Richmond, Calif.). DNA was extracted on these membranes by using 1 mg of proteinase K per ml–0.1% sodium dodecyl sulfate in 1× standard saline citrate (SSC) and 0.5 M NaOH–1.5 M NaCl. Membranes were then neutralized with 2 M ammonium acetate and rinsed with 6× SSC, and the DNA was fixed onto the membranes by baking at 80°C.

Hybridization. Hybridization of alkaline phosphatase-conjugated oligonucleotide probes (Molecular Biosystems, Inc., San Diego, Calif.) was carried out at 45°C for 15 min for LT and 60°C for 15 min for ST (ST-h) probes; the hybridization buffer contained: 5× SSC, 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone, and 1% sodium dodecyl sulfate. Membranes were then washed in 1× SSC–1% sodium dodecyl sulfate. A second wash in 1× SSC–1% Triton X-100 was carried out for 5 min at hybridization temperature. A final wash in 1× SSC was done, and membranes were placed in substrate (Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl phosphate). Positive hybridization yielded a dark precipitate.

RESULTS

Comparison of colonies tested in the oligonucleotide probe and bioassays. A total of 529 *E. coli* colonies from 111 patients in three regions were tested for LT and ST by bioassay and oligonucleotide probe. A total of 47 (9%) colonies were positive for LT by Y1 adrenal cell assay, and 63 (12%) were positive by LT oligonucleotide probe. When assayed with the ST oligonucleotide probe, 46 (9%) of the colonies hybridized, whereas only 26 (5%) were positive for ST as measured by the suckling mouse bioassay. The LT oligonucleotide probe detected significantly (McNemar test) more positive colonies than the Y1 adrenal cell assay (63 of 72 versus 47 of 72; $P < 0.05$). Significantly more ST-positive colonies were detected by oligonucleotide probe than by mouse bioassay (46 of 46 versus 26 of 46; $P < 0.001$).

Probe and bioassay test results are compared in Table 1. The LT oligonucleotide probe hybridized with 83% (40 of 48) of LT-only colonies, whereas the ST probe hybridized with 100% (23 of 23) of ST-only colonies. There was no significant difference in identification of LT-only colonies by the oligonucleotide probe (40 of 48) and by bioassay (35 of 48) ($P > 0.05$). However, significantly more ST-only colonies hybridized with the probe than were identified by bioassay (23 of 23 versus 14 of 23; $P < 0.001$). Overall, oligonucleotide probes hybridized with 91% (86 of 95) of LT or ST ETEC as identified by bioassay.

Identification of ETEC infections in patients by testing isolates in oligonucleotide probe and bioassays. Probe and bioassay results were more comparable when correlated with patient diagnoses. For LT, a discrepancy of 3% (3 of 111) in patient diagnoses was found. All 3 discrepancies in LT diagnoses were in patient samples from West Africa. Of 15 patients diagnosed as ST positive by oligonucleotide probe, 10 (66%) were also diagnosed as ST positive by suckling mouse bioassay. All bioassay-diagnosed patients were also positive by probe. The ST probe diagnosed significantly more positive patients than the bioassay (15 of 15 versus 10 of 15; $P < 0.01$). This difference was consistent for samples from all three regions. All bioassay-negative, probe-positive cases remained negative when tested individually by suckling mouse assay.

TABLE 1. Comparison of results for detection of ETEC in 529 colonies tested by oligonucleotide probe and bioassay

Test results (probe/Y1-SMA[a])	No. of colonies detected (%) for ETEC type:			
	LT	ST	LT-ST	Total
+/+	27 (56)	14 (61)	11 (46)	52 (55)
+/−	13 (27)	9 (39)	12 (50)	34 (36)
−/+	8 (17)	0 (0)	1 (4)	9 (9)

[a] SMA, Suckling mouse assay.

A total of 27 cases of ETEC were identified from 111 patients (24%). Of these, 12 (44%) were positive for LT only, 5 (19%) were positive for ST only, and 10 (37%) had combined LT and ST infections (Table 2). Of 27 ETEC-positive patients, 20 (74%) were identified by both oligonucleotide probe and bioassay. Patient diagnoses by LT oligonucleotide probe and Y1 adrenal cell bioassay did not differ significantly (11 of 12 versus 11 of 12; $P > 0.1$) for ETEC LT-only cases. Three ST-only cases were positive by bioassay and oligonucleotide probe; an additional two cases were diagnosed by ST oligonucleotide probe hybridization only. All LT-ST infections were identified by oligonucleotide probes; however, 3 of 10 (30%) did not produce both LT and ST in respective bioassays.

Serial dilutions of bacteria were then spotted onto Zeta-Probe to determine the limit of detection for the LT and ST oligonucleotide probes. The limit of detection for the LT oligonucleotide probe was 4.58×10^7 cells per ml and 10^7 copies per ml of purified plasmid DNA. The ST oligonucleotide probe was able to detect 10^7 copies per ml of plasmid DNA and 4.84×10^7 cells per ml of ETEC.

DISCUSSION

Alkaline phosphatase-labeled, synthetic oligonucleotide probes were able to specifically identify target bacteria in this study. The LT probe used was complementary to conserved sequences within the ETEC LT gene (20). The ST probe was homologous to DNA sequences within the ST-A2 (STh) region which differs from ST-A1 (STp) sequences by two bases (8, 14). This oligonucleotide has been reported to hybridize with 98% of ST-A2 and 92% of ST-A1 ETEC tested (8).

Our data indicated that there were no significant differences in patient diagnoses by LT probe, whereas the ST probe identified significantly more patients. LT correlation by colony and patient was 100% for samples from South America, including Ecuador, but was not 100% for samples from West Africa. Consistent variation in ST test results

TABLE 2. Comparison of test results for the detection of ETEC in 111 patients tested

Test results (probe/Y1-SMA[a])	No. of positive patient samples (%) for ETEC type:			
	LT	ST	LT-ST	Total
+/+	10 (84)	3 (60)	7 (70)	20 (74)
+/−	1 (8)	2 (40)	3 (30)	6 (22)
−/+	1 (8)	0 (0)	0 (0)	1 (4)

[a] SMA, Suckling mouse assay.

occurred in samples from all three regions. This suggests that regional differences in ETEC may occur, resulting in differing abilities to diagnose patients by each method. In every case, however, an equal or greater number of patients were diagnosed by oligonucleotide probe.

One concern in using oligonucleotide probes as diagnostic tools is their increased specificity. These probes may not hybridize to target sequences mismatched by more than one base (15). This would result in a number of false-negatives, since it is possible for toxin genes to vary by more than one base and still produce faithful polypeptides. A second concern is that oligonucleotide probes may be too sensitive, hybridizing to genes which contain partial deletions and therefore are unable to produce functional toxins. This event would result in false-positive identifications.

The greater number of positives diagnosed by oligonucleotide probe versus bioassay may be because of either differential expression of the bacterial toxin or failure of either test system, resulting in bioassay false-negatives or probe false-positives (7, 19). In these studies, no other etiologic agent was isolated from three of the five ST-probe-positive, bioassay-negative patients. Preliminary data has shown that these three samples were positive for ST by enzyme immunoassay (12), and the remaining two were negative. This indicates that the increased number of ST cases identified by oligonucleotide probe were not false-positives but reflected the greater sensitivity and accuracy of oligonucleotide probes.

The mechanism by which ETEC was probe positive and bioassay negative was not determined. It is possible that probe-positive, bioassay-negative ETEC has intact toxin genes but does not produce toxin through the fault or dislocation of the gene promoter. Toxin production might be lower than the limit of detection in a bioassay, the sensitivity of which may vary. These bacteria may contain toxin genes rendered defective by a deletion mutation. Toxin genes containing a deletion mutation could still be hybridizable with a short oligonucleotide probe complementary to sequences in a region upstream or downstream of the deletion. Bacteria with deletion mutations could also produce a partially intact protein, portions of which might be reactive in an enzyme-linked immunosorbent assay. This question will be resolved by DNA sequence analysis.

Non-radioactively labeled oligonucleotide probes represent a major advance in the diagnosis of ETEC-associated diarrheal disease. More specimens may be accurately diagnosed more quickly and cheaply. Although detection of unexpressed genes might result in a low percentage of false-positives, this may be important information to epidemiologists. Alkaline phosphatase-conjugated oligonucleotide probes may be used in laboratories with minimal equipment. Signal development requires only the addition of enzyme substrate. In using these probes, bacterial DNA was extracted directly on nylon 66 membranes with proteinase K-sodium dodecyl sulfate and NaOH-NaCl. This protocol proved to be an efficient method for processing large numbers of samples and produced an unequivocal signal. Oligonucleotide probes are an accurate and consistent method of identifying ETEC in lieu of bioassays.

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