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Comparative Study of Synthetic Oligonucleotide and Cloned Polynucleotide Enterotoxin Gene Probes to Identify Enterotoxigenic *Escherichia coli*

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***Escherichia coli* isolated from 2,126 children in Thailand and the Philippines was examined for enterotoxin production and for DNA hybridization with synthetic oligonucleotide and cloned polynucleotide enterotoxin gene probes. A total of 233 infections with *E. coli* that were detected by one or more of these assays were identified. Of the infections, 75% (164/233) were identified by all three methods. An additional 18% (43/233) were identified by two of three methods. Isolates from 10% (19/183) of infections with *E. coli* that hybridized with both the oligonucleotide and cloned enterotoxin gene probes were nontoxigenic, as determined by the Y1 adrenal cell and suckling mouse assays. Although synthetic oligonucleotide probes to detect enterotoxigenic *E. coli* are more uniform and easier to use than cloned enterotoxin gene probes, the heat-labile toxin oligo probe used in this study did not identify 13% (11/87) of infections with *E. coli* that produced heat-labile toxin, as identified with the Y1 adrenal cell assay and the cloned enterotoxin gene probe. Synthetic oligonucleotide probes enable laboratories with only minimal equipment to use DNA hybridization assays to identify enterotoxigenic *E. coli*.**

Enterotoxigenic *Escherichia coli* (ETEC) is a frequent cause of diarrhea in children and nonimmune adults in tropical developing countries (1, 2, 13, 14). ETEC is usually identified by testing isolates for enterotoxin production in bioassays or by serological tests that detect phenotypic expression of the genes coding for heat-labile enterotoxins (LT) and heat-stable enterotoxins (ST) (5, 9, 17, 20, 23). Alternatively, ETEC can be identified by detecting genes coding for these enterotoxins by DNA hybridization (6, 7, 14, 18). This method has been particularly useful in detecting ETEC in large numbers of specimens.

The genes coding for LT and two genetically distinct forms of ST-A (ST-A1 [ST-P, porcine] and ST-A2 [ST-H, human]) have been cloned and sequenced (4, 11, 15, 22). Specific DNA fragments of plasmids containing the cloned enterotoxin genes have been used as probes to identify ETEC (14, 16, 18). These endonuclease restriction fragment probes are isolated electrophoretically and separated from the cloning vector to obtain a specific probe. This process is time consuming and technically difficult. Furthermore, these probes may contain cloning vector DNA and be nonspecific (7).

Two synthetic oligonucleotide probes to detect genes coding for LT and ST-A have been constructed (R. D. Smith, C. M. Trepod, and E. Tu, Fed. Proc. **44**:527, 1985). The sequences chosen for these synthetic oligonucleotides were taken from the DNA sequences of the previously determined sequences of the LT and ST-A1 genes (4, 11, 15, 22). The DNA sequence of the LT oligo probe spans the A1 and A2 regions of the *toxA* gene, is homologous to the LT-H (human) gene, and differs from the LT-P (porcine) gene by a single base change. The ST oligo probe is homologous to the ST-A2 DNA sequence and differs from the ST-A1 sequence by two base changes. Both oligo probes are 26 bases long and are synthesized by using phosphoramidite chemistry on a DNA synthesizer (3).

E. coli isolated from children in Thailand and the Philippines was examined to compare the identification of ETEC by testing isolates for enterotoxin production with hybridization with cloned enterotoxin gene and synthetic oligonucleotide probes.

MATERIALS AND METHODS

Fecal specimens were collected from children during studies of diarrheal disease in Thailand and the Philippines in 1985. Five to ten *E. coli* colonies were selected from specimens cultured on MacConkey agar and saved on nutrient agar slants. Isolates were tested for LT and ST-A production in the Y1 adrenal cell and suckling mouse assays (standard assays) within 3 months of isolation and simultaneously spotted on Whatman 541 papers (Whatman, Inc.) (5, 12, 17). These filters were examined for hybridization with the radiolabeled LT and ST oligonucleotide probes (oligo probes) and the LT, ST-A1, and ST-A2 cloned enterotoxin gene probes (cloned probes) as previously described (6, 7, 18). *E. coli* that produced enterotoxins (ETEC) or contained genes coding for enterotoxins is referred to as enterotoxin gene-containing *E. coli* (Ent+ *E. coli*).

In addition, bacterial growth on MacConkey agar from fecal specimens collected from children with diarrhea in Thailand was spotted on five different Whatman 541 filters and examined with the two oligo and the three cloned probes. Bacterial growth from fecal specimens from children in the Philippines was spotted on Whatman 541 filters and examined with the LT and ST oligo probes. The bacterial growth from stools spotted on Whatman 541 filters was referred to as "stool blots."

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TABLE 1. Ent+ *E. coli* infections indentified by the standard bioassay and the cloned and oligo probes

Ent+ *E. coli*[a]	No. of infections identified by:		
	Bio[a]	Cloned[c]	Oligo[d]
LT/ST	40	41	38
LT	89	97	86
ST	65	77	71

[a] LT/ST Ent+ *E. coli*, *E. coli* that produced LT and ST or contained genes coding for LT and ST. LT, *E. coli* that produced LT or contained genes coding for LT. ST, *E. coli* that produced ST or contained genes coding for ST.
[b] Bio, Bioassay; Y1 adrenal cell assay to detect *E. coli* LT and the suckling mouse assay to detect ST (5, 17).
[c] Cloned, Hybridization with cloned probes (15).
[d] Oligo, Hybridization with oligo probes (7).

RESULTS

A total of 233 Ent+ *E. coli* infections were identified in 225 (11%) of 2,126 children by one or more of the three assays. A total of 10% (77/734) of children with diarrhea and 4% (17/410) of children without diarrhea in the preceding 2 weeks in Thailand and 18% (81/458) of children with diarrhea and 9% (50/524) of controls without diarrhea within 3 days of their examination in the Philippines were infected with Ent+ *E. coli*. Seven children had two and one child had three different Ent+ infections. A total of 194 Ent+ infections were identified by the standard enterotoxin assays, 215 were identified by the cloned probes, and 195 were identified by the oligo probes (Table 1). The three methods are compared in Table 2. A total of 75% (164/233) of Ent+ infections were identified by all three methods. An additional 18% (43/233) were identified by two of three methods. Isolates from 10% (19/183) of infections with *E. coli* that hybridized with both the oligo and cloned probes were nontoxigenic, as determined by the standard assays. A total of 26 infections were identified by only one method: 65% (17/26) by the cloned probes, 23% (6/26) by the standard assays, and 11% (3/26) by the oligo probes.

Among 194 children from whom ETEC *E. coli* was identified by testing isolates for enterotoxin production in the Y1 adrenal cell and suckling mouse assays, the oligo probes identified a similar number of LT/ST and ST infections as the cloned probes (Table 3). Significantly more LT-only ETEC infections were identified with the LT cloned probe than with the LT oligo probe (87/89 versus 76/89; $P < 0.005$ [McNemar test]). The ST oligo probe hybridized with 261 (98%) of 265 ST-A2 and 22 (92%) of 24 ST-A1 *E. coli* that produced ST, as measured in the suckling mouse assay. The difference between the ability of the ST oligo probe to hybridize with ST-A1 and ST-A2 enterotoxin-producing *E. coli* was not significant ($P < 0.3$).

Stool blots collected from 324 children in Thailand were examined for hybridization with the oligo and cloned probes. Blots collected from 77% (24/32) of children from whom ETEC was isolated and from none of 293 children from whom ETEC was not isolated hybridized with both the oligo and cloned probes. Stool blots prepared in the Philippines were examined from 825 children. Blots from 77% (75/98) of children infected with ETEC and from 2% (17/727) of children from whom ETEC was not isolated hybridized with the oligo probes. With specimens from both Thailand and the Philippines, 83% (20/24) of children infected with LT/ST ETEC, 75% (42/56) of children infected with LT-only ETEC, and 76% (39/51) of children infected with ST-only ETEC were identified by examination of stool blots with oligo probes.

DISCUSSION

DNA probes for the detection of ETEC must be able to hybridize with DNA sequences coding for enterotoxins in target bacteria and not hybridize with other DNAs. Hybridization occurs when there is sufficient homology between single strands of probe DNA and single strands of target DNA to permit stable hydrogen bonding between nucleotide bases and the formation of a double-stranded complex. The radiolabeled cloned enterotoxin gene probes are 154 (ST-A2), 215 (ST-A1), and 1,300 (LT) bases long and hybridize under stringent conditions with target DNA that is 80% homologous with these probes (14). The synthetic oligonucleotide probes are 27 bases long and may not hybridize to target sequences when only one nucleotide is mismatched within the sequence (8). Oligonucleotide probes are, therefore, very specific in detecting selected DNA sequences but may not detect single-base mutations in target cell DNA, a change that might still preserve toxin production.

Although oligo probes detected a similar number of LT/ST and ST-only ETEC infections as the cloned probes, the LT oligo probe did not detect 11 LT-only ETEC infections that were detected with the LT cloned probe and the standard assays. This suggested that the 37 LT-only strains in these 11 infections contained DNA sequences that were not homolo-

TABLE 2. Identification of *E. coli* infections by testing colonies for enterotoxin production by hybridization with oligo and cloned probes

Test results			No. of Ent+ *E. coli* infections		
Bio[a]	Cloned probe[b]	Oligo probe[c]	LT/ST	LT	ST
+	+	+	31	76	57
+	+	−	3	11	1
+	−	+	3	0	6
−	+	+	4	9	6
+	−	−	3	2	1
−	+	−	3	1	13
−	−	+	0	1	2

[a] Bio, Bioassay, testing *E. coli* for enterotoxin production (5, 17).
[b] Cloned probe, Hybridization with cloned probe (15).
[c] Oligo probe, Hybridization with oligo probe (17).

TABLE 3. Identification of 194 ETEC infections by testing isolates for hybridization with the cloned and oligo probes

ETEC type[a]	No. of children infected[b]	No. of children with *E. coli* with genes coding for enterotoxins as determined by hybridization with:	
		Cloned probes (%)	Oligo probes (%)
LT/ST	40	34 (85)	34 (85)
LT	89	87[c] (98)	76[c] (85)
ST	65	58[d] (89)	63[d] (97)
Total		179 (92)	173 (89)

[a] LT/ST ETEC, *E. coli* that produced LT and ST; LT ETEC, *E. coli* that produced LT only; ST ETEC, *E. coli* that produced ST only.
[b] As determined by testing isolates for enterotoxin production in the Y1 adrenal cell and suckling mouse assays (5, 17).
[c] $P < 0.001$ (McNemar test).
[d] $P > 0.05$ (McNemar test).

gous with the DNA sequences chosen as the LT oligo probe. Differences have been found between the genes coding for LT in isolates from human and animal sources (21). The difficulty in detecting these heterogenous LT genes might be overcome by using another sequence for the LT oligo probe or using two oligo probes with sequences chosen from nonoverlapping regions of the LT gene. The ST oligo probe used in this study was homologous to DNA sequences coding for ST-A2 but differed from the ST-A1 sequence by two base changes. This oligo probe hybridized with 98% of ST-A2 and 92% of ST-A1 ETEC examined.

Because the greatest use of DNA probes for detection of ETEC has been in epidemiologic studies, the use of oligo probes for different sequences chosen from sequenced genes coding for LT, ST-A1, and ST-A2 would be useful (5). This would enable investigators to use oligo probes to detect different DNA sequences coding for similar enterotoxins. This information as well as serotyping of strains could be used as epidemiologic markers.

E. coli from 10% of infections with strains that hybridized with both the oligo and cloned probes was nontoxigenic. These strains either contained genes coding for enterotoxins that were not expressed, probably caused by changes in regulatory genes, or produced small amounts of toxins that were not detected. In earlier studies, infections with *E. coli* that contained genes coding for enterotoxins that were not expressed were epidemiologically related (6).

Nonradioactive methods of labeling DNA probes must be developed before DNA hybridization techniques can be used by clinical laboratories or by research laboratories in developing countries. It is possible to substitute biotinylated nucleotides for radiolabeled nucleotides in nick translating a large cloned gene probe. A biotinylated 17-kilobase DNA probe has been used in the identification of enteroinvasive *E. coli* and *Shigella* sp. by colony and specimen hybridization (19). Biotinylated oligonucleotide probes for ETEC have also been developed and appear as sensitive and specific as radiolabeled probes in detecting enterotoxin genes in cell lysates but are not sensitive enough to be used to detect genes coding for enterotoxins in colonies or stools fixed on nitrocellulose or Whatman 541 filters (7). This is a major disadvantage in examining large numbers of colonies.

Since most laboratories do not have the equipment or the expertise to construct specific enterotoxin gene probes, oligonucleotide probes that can be commercially produced and sent lyophilized by mail appear more practical. This would enable laboratories with only minimal equipment to use DNA hybridization assays to identify ETEC. Once nonradioactive markers for oligonucleotide probes are developed, the use of DNA hybridization assays to identify ETEC would be possible in laboratories without reliance on frequent shipments of radioactively labeled nucleotides.

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