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Identification of Enterotoxigenic *Escherichia coli* in Patients with Diarrhea in Asia with Three Enterotoxin Gene Probes

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A total of 984 enterotoxigenic *Escherichia coli* (ETEC) and 733 non-ETEC isolated from patients with diarrhea in Asia (one isolate per patient) were examined for homology with radiolabeled fragments of DNA encoding heat-labile toxin (LT) or heat-stable toxin of porcine (ST-P) or human (ST-H) origin. A total of 246 ETEC that produced LT and ST as determined by the Y-1 adrenal and suckling mouse assays were homologous with the LT probe. Of these 246 LTST ETEC, 156 (63%) were homologous with the ST-H, 46 (19%) were homologous with the ST-P, and 44 (18%) were homologous with both probes. A total of 401 LT ETEC were homologous with the LT probe. Of 337 ST ETEC identified by the suckling mouse assay, 244 (72%) were homologous with the ST-H, 84 (25%) were homologous with the ST-P, and 9 (3%) were homologous with both probes. None of the 733 isolates that were non-enterotoxigenic as determined by the Y-1 adrenal and suckling mouse assays was homologous with genes coding for enterotoxin. Four isolates (not included among the 984 ETEC examined) that were initially considered to produce LT because sterile culture supernatants produced rounding of Y-1 adrenal cells were not homologous with the LT probe. The sterile culture supernatants of these four isolates caused rounding after 8 h and subsequent destruction after 24 h of Y-1 adrenal tissue cultures. This effect was not inhibited by convalescent human cholera antiserum, Swiss Serum Institute cholera antitoxin, or antiserum to purified LT. These isolates were also negative in the Biken test previously used to identify LT-producing *E. coli*. The DNA hybridization technique with three enterotoxin gene probes was developed as a specific technique to identify ETEC in large numbers of specimens in Asia.

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea in Asia (1, 4, 9, 12). ETEC is usually identified by testing culture supernatants of *E. coli* in one of several tissue culture assays for heat-labile enterotoxin (LT) (6, 13) and in the suckling mouse assay (3) for heat-stable toxin (ST). Neither of these standard assays is convenient to use in tropical, developing countries. Both the Y-1 adrenal and CHO cell assay require special media and CO_2 atmospheres, as well as sterility, which is sometimes difficult to maintain in tropical laboratories. Suckling mice are difficult to obtain in tropical countries for a variety of reasons. Hot climates decrease both breeding efficiency and the size of litters, and breeding is difficult to avoid without ready access to new breeders.

The ability of ETEC to produce LT, ST, or both is plasmid mediated (15). The genes for these enterotoxins have been cloned (2, 8, 11, 16) and used to identify ETEC directly in bacterial growth from stool (11) or water (5). Once established, the DNA hybridization assay (11) provides a means of examining a large number of colonies for the ability to produce enterotoxins. ETEC and non-ETEC from Asia which had previously been identified by either the Y-1 adrenal (13), CHO cell (6), or suckling mouse (3) assays were examined for hybridization with the LT, ST-H (from an ETEC of human origin), and ST-P (from an ETEC of porcine origin) enterotoxin gene probes (11). This study was performed to determine whether ETEC identified by the standard assays were identifiable with this newly developed technique and whether *E. coli* which was considered to be non-enterotoxigenic in the standard assays contained genes coding for enterotoxin.

MATERIALS AND METHODS

Source of specimens. *E. coli* from patients with diarrhea in Bangladesh, Indonesia, the Philippines,

TABLE 1. Numbers of ETEC isolated in Asia hybridizing with the LT, ST-H, and ST-P gene probes

Country of origin (total)	No. of ETEC hybridizing with DNA probe coding for genes coding for:						
	LT ST-H ST-P	LT ST-H	LT ST-P	LT	ST-H	ST-P	ST-H ST-P
Bangladesh (233)	24	59	26	28	86	10	0
Indonesia (5)	1	0	0	0	4	0	0
Philippines (38)	1	17	0	13	6	1	0
Thailand (708)	18	80	20	360	148	73	9
Total (984)	44	156	46	401	244	84	9

and Thailand were isolated from stools or rectal swabs cultured on MacConkey media. *E. coli* selected from these cultures were stored on nutrient agar slants. A total of 235 isolates from Dhaka, Bangladesh had previously been identified as ETEC by the CHO cell (6) or suckling mouse (3) assays or both at the International Diarrheal Disease Research Center in Bangladesh in 1981. *E. coli* isolated from patients with diarrhea by Naval Medical Research Unit No. 2 laboratories in Manila, the Philippines, and Jakarta, Indonesia, in 1980 to 1983 were sent to the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand, and tested for enterotoxin production with the Y-1 adrenal (13) and suckling mouse (3) assays. ETEC, identified with the same assays (3, 13) in Thailand, were collected from patients with diarrhea in Bangkok in 1979 to 1982. A total of 370 *E. coli* from Thailand and 363 from the Philippines (total, 733) that had been isolated from patients with diarrhea (one per patient) and were negative in the Y-1 adrenal (13) and suckling mouse (3) assays were also examined in the DNA hybridization assay (11).

Enterotoxin gene probes were prepared from *E. coli* K-12 (C600) containing multicopy recombinant plasmids encoding for enterotoxin; these included EWD299 coding for LT (2), pRIT10036 (previously designated CLS-2) coding for ST-P (8), and pSLM004 coding for ST-H (11).

ETEC specimens were retested in the Y-1 adrenal cell (13) and suckling mouse assays (3) immediately before being examined for their homology with the three enterotoxin gene probes (LT, ST-H, and ST-P). Organisms which had become non-enterotoxigenic were not examined in this study.

Four *E. coli* which caused rounding of Y-1 adrenal cells, but which did not hybridize with the LT probe, were tested in the Biken test (7). These organisms were inoculated into 2 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 0.6% yeast extract (Difco Laboratories, Detroit, Mich.) in tubes (13 by 100 mm) and incubated without shaking at 37°C for 48 h. A 50-μl amount of sterile culture supernatants was incubated with 50 μl of either convalescent human cholera serum (NIH Reference Reagent G005-501-572), Swiss Serum Institute cholera antitoxin, or anti-LT (7) for 30 min at 37°C and then tested in the Y-1 adrenal cell assay (13). Supernatants were also tested before and after heating at 100°C for 30 min. Y-1 adrenal cells were examined after 8 and 24 h.

Hybridization with enterotoxin gene probes. *E. coli* specimens to be examined for their homology with the enterotoxin gene probes were inoculated on three separate pieces of nitrocellulose paper (BA-85, 6 by 7 cm; Schleicher & Schuell Co., Keene, N.H.), layered on MacConkey agar, and incubated at 37°C overnight. Twenty *E. coli* specimens were inoculated on each nitrocellulose paper, as was *E. coli* K-12 C600 containing the multicopy plasmids from which the enterotoxin gene probes were derived. DNA of the resultant bacterial growth was fixed on the nitrocellulose paper with 0.5 N NaOH and 1.0 M ammonium acetate–0.02 N NaOH as previously described (11). The filters were then air dried and baked overnight at 65°C. DNA fixed on the nitrocellulose paper was then hybridized with [α-^{32}P]DNA fragments encoding for either LT or ST and exposed to X-Omat-R X-ray film (Eastman Kodak, Rochester, N.Y.) with a single Cronex Lightning-Plus intensification screen (Du Pont Co., Wilmington, Del.) for 48 h at −70°C. The films were developed according to the manufacturer's instruction. Replicates of each filter were examined with each of the three radiolabeled DNA probes coding for enterotoxins (LT, ST-H, and ST-P). (It was possible to test as many as 25 papers containing 24 colonies each, i.e., 600 colonies, in each hybridization reaction.)

RESULTS

Hybridization of ETEC with three enterotoxin gene probes. The numbers of ETEC isolated from four countries in Asia that hybridized with the LT, ST-H, and ST-P gene probes are shown in Table 1. An example of these results is shown in Fig. 1. All 246 LTST ETEC hybridized with the LT probe. A total of 63% (156 of 246) hybridized with the ST-H, 19% (46 of 246) hybridized with the ST-P, and 18% (44 of 246) hybridized with both probes. Of 401 LT ETEC isolated from patients with diarrhea in Bangladesh, the Philippines, and Thailand, all hybridized with the LT probe. A total of 72% (244 of 337) of ST ETEC hybridized with ST-H probe, 25% (84 of 337) hybridized with the ST-P probe, and 3% (9 of 335) hybridized with both. ST ETEC that hybridized with the ST-P, but not the ST-H probe was more prevalent in Thailand (73 of 221) than in Bangladesh (10 of 95) ($P < 0.001$), a Muslim country in which swine are relatively rare. None of the 733 non-ETEC, as identified by the Y-1 adrenal (13) and suckling mouse (3) assays, hybridized with the LT, ST-H, or ST-P enterotoxin gene probes.

Characterization of LT probe-negative *E. coli* causing rounding of Y-1 adrenal cells. Sterile culture supernatants of four *E. coli* isolates from two patients with diarrhea in the Philippines and

two patients with diarrhea in Bangladesh were not homologous with the LT probe. Sterile culture supernatants of these isolates caused rounding of Y-1 adrenal cells after 8 h and destruction of these cells after 24 h. This effect was destroyed by heating at 100°C for 30 min, but was not inhibited by convalescent human cholera serum, cholera antitoxin, or anti-LT (7). These four organisms were negative in the Biken test (7).

DISCUSSION

A total of 984 ETEC isolated from patients with diarrhea in Asia hybridized with one or more of the three enterotoxin gene probes. A total of 18% of LTST *E. coli* and 3% of ST *E. coli* hybridized with both the ST-H and ST-P probes, indicating that ST-producing ETEC may carry more than one nucleotide sequence coding for ST. This observation agrees with the work of Yamamoto and Yokota (17), who identified two different nucelotide sequences coding for ST on different plasmids in LTST *E. coli* H10407.

The difference in the proportion of ST ETEC which hybridized with either the ST-H or ST-P probes between Bangladesh and Thailand indicates that differences in nucleotide sequences of genes coding for ST may serve as an additional marker (besides serotyping) with which to characterize ETEC. In an earlier study, strains of ST ETEC isolated in rural Thailand were homologous with the ST-H probe only, whereas those isolated in Bangkok hybridized with either the ST-H or the ST-P probe or both (11).

The significance of the four *E. coli* specimens whose culture supernatants produced rounding of Y-1 adrenal cells which was not inhibited by convalescent human cholera antiserum, Swiss Serum Institute cholera antitoxin, or anti-LT and that were not homologous with the LT probe under the conditions used in this study is not clear. These isolates were shown to produce a cytotoxin since Y-1 adrenal monolayers were destroyed after 24 h. It is possible that these organisms also produced a toxin which was antigenically and genetically distinct from LT. Similar *E. coli* specimens have previously been reported in Thailand (11), and attempts to further characterize these isolates are being made.

Maintaining tissue culture and animal facilities to perform either the Y-1 adrenal (13) or CHO cell (6) assay and the suckling mouse (3) assay to identify ETEC is difficult in tropical countries. Although the DNA hybridization assay requires expensive equipment and reagents (ultracentrifuge, electrophoresis equipment, enzymes, chemicals, etc.), once established, it enables a central laboratory to identify ETEC in a large collection of *E. coli*. Rather than send *E. coli* isolates on nutrient agar slants to a central laboratory, workers can mail specimens fixed directly on nitrocellulose paper.

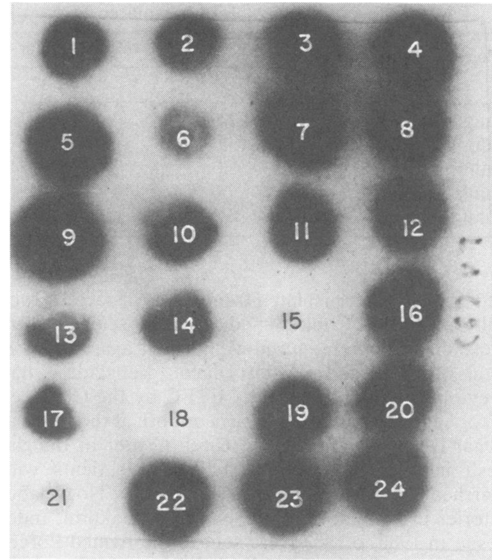

FIG. 1. Autoradiogram demonstrating DNA hybridization with ETEC that were grown on nitrocellulose paper. The paper was overlaid with autoradiographic film after hybridization with radiolabeled, LT enterotoxin gene-specific DNA. This autoradiogram is of 22 ETEC; ETEC B2C, isolate 17, which produces LT and ST enterotoxin; and non-ETEC K-12 Xac, isolate 21. Isolates 15 and 18 produced ST only (3), whereas isolates 1 to 14, 16 and 17, 19 and 20, and 22 to 24 produced LT toxin as determined by the Y-1 adrenal cell assay (13).

A double-blind prospective study is currently being performed between the Armed Forces Research Institute of Medical Sciences and another laboratory in Asia to determine whether the results of examining DNA from bacterial growth of stool or *E. coli* fixed directly on nitrocellulose paper by the hybridization assay in one laboratory agree with the standard methods of identifying ETEC as performed in another laboratory. This study suggests that the vast majority of ETEC, as identified by the standard assays, are homologous and thus identifiable with the LT, ST-H, and ST-P enterotoxin gene probes.

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