

IDENTIFICATION OF ENTEROTOXIGENIC *Escherichia coli* IN  
PATIENTS WITH DIARRHEA IN ASIA WITH THREE  
ENTEROTOXIN GENE PROBES

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OBJECTIVE : To apply the DNA hybridization assay in identifying ETEC in Asia.

BACKGROUND : Nine hundred and eighty-four enterotoxigenic *Escherichia coli* (ETEC) and 733 non-ETEC isolated from patients with diarrhea in Asia (one isolate/patient) were examined for homology with radiolabelled fragments of DNA encoding heat-labile toxin (LT), or heat-stable toxin of porcine origin (ST-P) or of human origin (ST-H). Two hundred and forty-six 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%) with the ST-P, and 44 (18%) with both probes. Four hundred and one 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%) with the ST-P, and nine (3%) with both probes. None of the 733 isolates that were non-enterotoxigenic as determined by the Y-1 adrenal and suckling mouse assays were 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. Sterile culture supernatants of these four isolates caused rounding after eight hours and subsequent destruction after 24 hours 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 is a specific technique to identify ETEC in a large number of specimens in Asia.

METHODS :

Source of specimens : *E. coli* isolated from patients with diarrhea in Bangladesh, Indonesia, the Philippines, 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. Two hundred and thirty-five isolated from Dhaka, Bangladesh had previously been identified as ETEC by the CHO cell (6) and/or suckling mouse (3) assays 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 1980-1983 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 1979-1982. Three hundred and seventy *E. coli* from Thailand and 363 from the Philippines (total 733) that had been isolated from patients with diarrhea (one/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* K12 (C600) containing multicopy recombinant plasmids encoding for enterotoxin; these included EWD 299 coding for LT (2), pRIT 10036 (previously designated CLS-2) coding for ST originally isolated from an ETEC of porcine origin (ST-P) (8), and pSLM 004 coding for ST isolated from an ETEC of human origin (ST-H) (10).

ETEC 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 two ml of trypticase soy broth (BBL, Cockeysville, MD) with 0.6 percent yeast extract (Difco, Detroit, MI) in 13 mm x 100 mm tubes and incubated stationary at 37°C for 48 hours. Fifty µl of human cholera convalescent serum (NIH Reference Reagent G005-501-572), Swiss Serum Institute cholera antitoxin, or anti-LT (7) for 30 minutes 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 minutes. Y-1 adrenal cells were examined after eight and 24 hours.

Hybridization with the enterotoxin gene probes : ETEC to be examined for their homology with the enterotoxin gene probes were inoculated on three separate 6 x 7 cm pieces of nitrocellulose paper (BA-85, Schleicher and Schuell Co., Keene, NH) layered on MacConkey agar, and incubated at 37°C overnight. Twenty ETEC were inoculated on each nitrocellulose paper as well as *E. coli* K12 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 <sup>32</sup>P labelled DNA fragments encoding for either LT or ST and exposed to X-Omat-R X-ray film (Eastman Kodak, Rochester, NY) with a single Cronex Lightening-Plus intensification screen (EI DuPont de Nemours and Co., Inc., Wilmington, Del.) for 48 hours at -70°C. Films were developed according to the manufacturer's instruction. Replicates of each filter were examined with each of the three radiolabelled 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 (ie. 600 colonies) in each hybridization reaction).

Hybridization of ETEC with three enterotoxin gene probes : The number of ETEC isolated from four countries in Asia that hybridized with the LT, ST-H, and ST-P enterotoxin gene probes are shown in Table 1. An example of these results is shown in Figure 1. All 246 LTST ETEC hybridized with the LT probe. Sixty-three percent (156/246) hybridized with the ST-H, 19 percent (46/246) with the ST-P, and 18 percent (44/246) with both probes. Of 401 LT ETEC isolated from patients with diarrhea in Bangladesh, the Philippines, and Thailand all hybridized with the LT probe. Seventy-two percent (244/337) of ST ETEC hybridized with ST-H probe, 25 percent (84/337) with the ST-P probes, and 3 percent (9/335) with both. ST ETEC that hybridized with the ST-P, but not the ST-H probe was more prevalent in Thailand (73/221) than Bangladesh (10/96) ( $p < 0.001$ ), 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* which cause rounding of Y-1 adrenal cells : Sterile culture supernatants of four *E. coli* isolated 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 eight hours and destruction of these cells after 24 hours. This effect was destroyed by heating of 100°C for 30 minutes, but was not inhibited by human cholera convalescent serum, cholera antitoxin, or anti-LT (7). These four organisms were negative in the Biken test (7).

Nine hundred and eighty four ETEC isolated from patients with diarrhea in Asia hybridized with one or more of the three enterotoxin gene probes. Eighteen percent of LTST *E. coli* and three percent of ST *E. coli* hybridized with both the ST-H and ST-P probes indicating ST producing ETEC may carry more than one nucleotide sequence coding for ST on different plasmids in LTST *E. coli* H 10407.

The difference in the proportion of ST ETEC which hybridized with either the ST-H or ST-P probes between Bangladesh and Thailand indicate that differences in nucleotide sequences of genes coding for ST may serve as an additional marker in addition to 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 strains isolated in Bangkok hybridized with either the ST-H or ST-P probe or both (11).

The significance of the four *E. coli* whose culture supernatants produced rounding of Y-1 adrenal cells which has 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 hours. It is possible that these organisms also produced a toxin which was antigenetically and genetically distinct from LT. Similar *E. coli* have previously been reported from Thailand (11) and attempts to further characterize these isolates are being pursued.

Table 1. Number of enterotoxigenic *Escherichia coli* isolated in Asia which hybridized with the LT, ST-H, and ST-P enterotoxin gene probes.

<u>Enterotoxin</u> <u>gene probes</u>	<u>Number of ETEC which hybridized with</u> <u>enterotoxin gene probes</u>				Total
	Bangladesh <sup>+</sup>	Indonesia	Philippines	Thailand	
<u>LT-ST ETEC</u>					
<u>LT ST-H ST-P</u>	24	1	1	18	44
<u>LT ST-H</u>	59	0	17	80	156
<u>LT ST-P</u>	26	0	0	20	46
<u>LT ETEC</u>					
<u>LT</u>	28	0	13	360	401
<u>ST ETEC</u>					
<u>ST-H</u>	86	4	6	148	244
<u>ST-P</u>	10	0	1	73	84
<u>ST-H ST-P</u>	0	0	0	9	9
Total	233	5	38	708	984

LT-ST ETEC = Heat-labile and heat-stable producing enterotoxigenic *Escherichia coli* ; LT ETEC = Heat-labile producing enterotoxigenic *Escherichia coli* ; ST ETEC = Heat-stable producing enterotoxigenic *Escherichia coli* ; + = Origin of isolates; LT = DNA probe coding for genes coding for heat-labile toxin; ST-H = DNA probe coding for genes coding for heat-stable toxin, probe originates from heat-stable toxin producing enterotoxigenic *E. coli* of human origin; ST-P = DNA probe coding for genes coding for ST toxin, probe originates from heat-stable producing enterotoxigenic *E. coli* porcine origin.

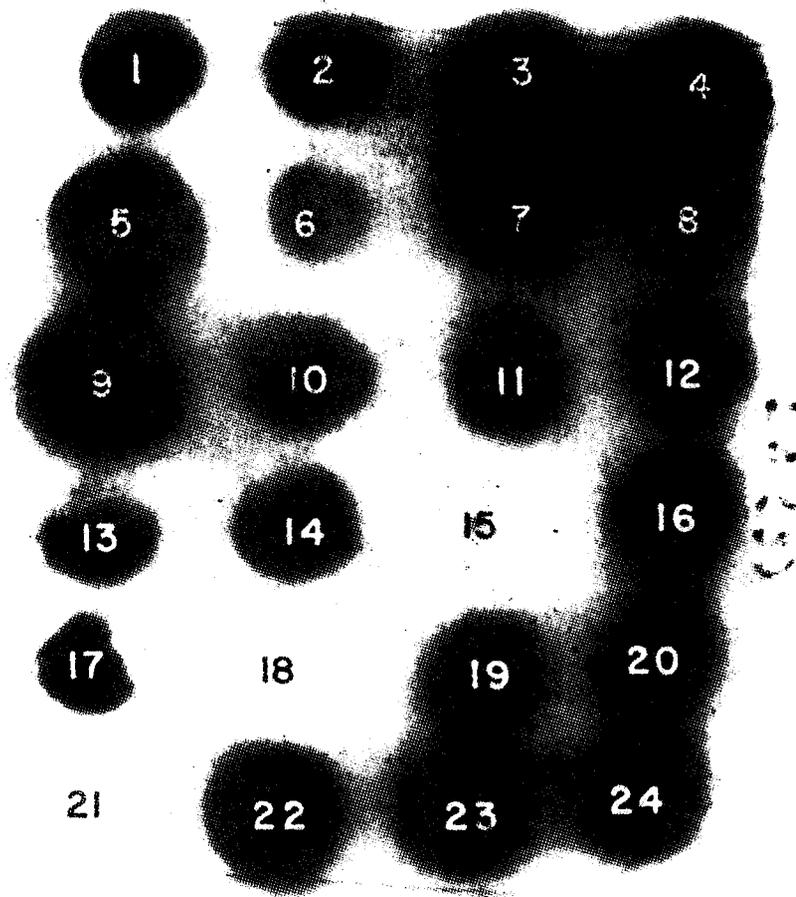


Fig. 1. Autoradiogram demonstrating DNA hybridization with ETEC that were grown on nitrocellulose paper. The paper was overlaid with autoradiographic film after hybridization with radio-labeled, LT enterotoxin gene-specific DNA. This autoradiogram is of 22 ETEC; ETEC B2C, isolate 17, which produces LT 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).

Maintaining tissue culture and animal facilities to perform either the Y-1 adrenal (13) or CHO cell (6), and the suckling mouse (3) assays to identify ETEC are difficult in tropical countries. While the DNA hybridization assay does require 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, specimens can be fixed directly on nitrocellulose paper and mailed.

A double blind prospective study is currently being performed between AFRIMS and another laboratory in Asia to determine if examining DNA of bacterial growth of stool or *E. coli* fixed directly on nitrocellulose paper with the hybridization assay in one laboratory agrees 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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