

DETECTION OF ENTEROTOXIGENIC *Escherichia coli* BY  
COLONY DNA HYBRIDIZATION IN THAILAND

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**OBJECTIVES :** To determine the applicability and limitations of examining specimens with a DNA hybridization technique in which genes encoding for enterotoxins are detected.

**BACKGROUND :** Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of diarrheal disease throughout the world (1,12,14). ETEC contain plasmids, transferable extrachromosomal segments of DNA, which carry the genetic information for the production of heat-labile (LT) and heat-stable (ST) enterotoxins (15). Gyles et al (7) found plasmids associated with LT and ST synthesis were reasonably homogeneous whether they were isolated from livestock or man. Plasmids of porcine origin which encoded for only ST were more heterogeneous both in size and guanine and cytosine content. So et al (16) subsequently demonstrated that the genes coding for ST production were located on a transposon on plasmid CLS-2 which suggested genes involved in ST synthesis might be found on a variety of plasmids.

The structural genes for both LT and ST have been isolated and characterized (3,17) and used as probes to identify and compare their homology with clinical isolates of ETEC (11,17). To determine the applicability and limitations of the DNA hybridization technique of identifying ETEC, we established and applied this technique in Bangkok. LT+ST+, LT+ST-, and LT-ST+ *E. coli* isolated from patients with diarrhea at different locations in Thailand were examined with the probes for LT and ST previously used in Dacca (11). A second probe for ST, recently provided by Nigel Harford (8), (Smith-Kline-Rit S.A., Rixensart, Belgium) was also employed. Stools from patients with diarrhea were examined simultaneously with both the DNA hybridization technique and by testing *E. coli* isolated from each stool with the Y-1 adrenal (13) and suckling mouse (4) assays. Lastly the sensitivity of the colony hybridization assay was determined by mixing LT+ST+ *E. coli* with non-enterotoxigenic bacteria or contaminated water.

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## METHODS :

Bacterial strains : ETEC had been previously isolated from children and adults with diarrhea at Children's Hospital, and Bamrasnaradura Infectious Disease Hospital, Bangkok; Soongnern Hospital, Soongnern; and Peace Corps volunteers at four different locations in rural Thailand. Strains from animals were isolated in a rural village in Soongnern district. Ten lactose positive colonies, selected from a MacConkey agar culture of stool, had been stored on nutrient agar slants and tested for LT and ST in the Y-1 adrenal cell (13), and suckling mouse assays (4).

*E. coli* K12 C600 (EWD 299) containing a multicopy recombinant plasmid encoding for LT (3), *E. coli* K12 C600 (CLS-2) containing a multicopy recombinant plasmid encoding for ST originally isolated from an ETEC of porcine origin (referred to as ST<sub>P</sub>) (9), and *E. coli* C600 (004) containing a recombinant plasmid encoding for ST originally isolated from an ETEC of human origin (referred to as ST<sub>H</sub>) (8) have previously been described and were provided by S. Falkow and N. Harford.

Preparation of P<sup>32</sup> labelled probes for LT, ST<sub>P</sub>, and ST<sub>H</sub> : Plasmid DNA was isolated as described by So et al (19). Enzymatic cleavage of plasmid DNA was carried out under conditions specified by the supplier (New England Biolabs Inc., Beverly, MA, P-L Biochemicals Inc., Milwaukee, WI). LT probe DNA was prepared from EWD 299 and consisted of a 0.5 megadalton *Hind* III generated fragment encoding predominantly for the A fragment of the LT molecule. ST<sub>P</sub> probe DNA was a 157 base pair *Hinf* I fragment of CLS-2. DNA fragments from each digest were isolated, cut out of the gel, and the DNA removed by electroelution. The ST<sub>H</sub> probe DNA was prepared from 004 by first digestion with *EcoR* I and *Hind* III, separating these fragments as described above, and then digesting the appropriate fragment with *Hpa* II. The ST<sub>H</sub> probe consisted of a 2000 base pair fragment. The isolated DNA fragments which were used as probes were phenol extracted, ethanol precipitated, and labelled *in vitro* with  $\alpha$ -P<sup>32</sup> deoxynucleotide triphosphate by nick translation (17) New England Nuclear, Boston, MA).

Hybridization of specimens on filters : Hybridization of filters was performed as described by Mosely et al (11) with modifications. Sterile nitrocellulose discs (90 mm, BA-85, Schleicher and Schuell, Keene, NH) were placed on MacConkey agar and inoculated with ETEC, *E. coli* K12 (negative control), or spotted with diarrheal stools. After overnight incubation at 37°C the filters were removed from the MacConkey agar and placed on a double layer of Whatman No. 3 paper saturated with 0.5 M NaOH for ten minutes. The filters were then transferred to Whatman paper saturated with 1.0 M ammonium acetate, 0.02 M NaOH for one minute. This process was repeated four more times. After the last transfer the filter was kept on the saturated Whatman paper for 10 minutes. The filter was then removed, dried, and baked overnight at 65°C.

After five to ten filters had been prepared they were immersed in enough hybridization solution (50% formamide; 5X SSC, (1X SSC 0.15 M NaCl and 0.015 M Na citrate); 0.1% sodium dodecyl sulfate; 1mM EDTA and Denhardt's solution (5)), to wet each filter, wrapped in plastic (Saran wrap), and incubated at

37°C for three hours. The filters were then transferred to fresh hybridization solution containing approximately  $10^5$  cpm/ml probe DNA and 75 µg/ml of sheared calf thymus DNA and incubated at 37°C overnight. The filters were then washed in 0.7 M NaCl, 0.075 M sodium acetate, 0.1% SDS for 45 minutes at 65°C, rinsed in 2X SSC at room temperature, and dried. The filters were exposed to Kodak X-omat-R X-ray film (Eastman Kodak, Rochester, NY) with a single Cronex lightening-Plus intensification screen (DuPont De Nemours, Wilmington, Del.) for 24 hours at -70°C. The film was developed according to the manufacturer's instructions.

Comparison of standard assays with spotted stools : Stool or rectal swabs were collected from children less than ten years of age with watery diarrhea of less than 72 hours duration at Children's Hospital, Bangkok during one week in April 1981. Specimens were cultured simultaneously on MacConkey agar and spotted on nitrocellulose filters. After overnight incubation at 37°C ten lactose positive colonies were selected from the MacConkey plate and tested within two weeks of isolation for LT with the Y-1 adrenal cell (13) and ST with the suckling mouse assays (4). Nitrocellulose filters were hybridized with the LT, ST<sub>p</sub> and ST<sub>H</sub> probes as previously described.

Effect of other bacteria on detecting ETEC by DNA hybridization : LT+ST+ *E. coli* B2C, non-enterotoxigenic *E. coli*, or *Aeromonas hydrophila*, the most common organism found in drinking and bathing water in Thailand (unpublished observation), were spotted in different proportions on nitrocellulose filters, placed on MacConkey agar, and incubated at 37°C overnight. Filters were processed and examined with the DNA hybridization assay.

Detection of ETEC in klong water : Klong (canal) water was serially diluted in sterile PBS and cultured on MacConkey, Heckteon, and blood agar (DIFCO, Detroit, MI) at 37°C for 24 hours. All bacterial species were identified and quantitated.  $10^9$  LT+ST+ *E. coli* B2C was serially diluted in either klong water or PBS. Ten mls of each dilution was passed through a preboiled 0.45 µ millipore filter (Millipore Corp, Bedford, MA). The filters were then placed on MacConkey agar, incubated at 37°C overnight, and examined with the DNA hybridization assay employing all three probes.

## RESULTS :

Homology of Thai ETEC with DNA probes : Twenty-four LT+ST+, 17 LT+ST- and 22 LT-ST+ *E. coli* were examined for homology with the LT, ST<sub>p</sub>, and ST<sub>H</sub> DNA probes. The sources of these isolates are given in Table 1. All 41 LT+ST+ and LT+ST- *E. coli* were homologous with the LT probe. Five *E. coli*, not included in the analysis, were initially considered to be positive in the Y-1 adrenal cell assay, but were not identified with the LT probe. Sterile culture filtrates of these five *E. coli* isolates caused rounding of Y-1 adrenal tissue cultures which was not heat-labile or inhibited by *V. cholerae* human convalescent serum (NIH Research Reagent Cat. No. G005-501-572). These isolates presumably produced either a cytotoxin, or a toxin which did not crossreact with *V. cholerae* enterotoxin.

All of the LT+ST+ and LT-ST+ *E. coli* were detected with either the ST<sub>P</sub> and/or the ST<sub>H</sub> probes. Among LT+ST+ isolates eleven were homologous with ST<sub>P</sub>, eleven with ST<sub>H</sub> and two with both. Of 22 LT-ST+ *E. coli* examined 13 were detected with the ST<sub>H</sub>, six with the ST<sub>P</sub> and three with both probes.

Genes homologous with either the ST<sub>P</sub> or ST<sub>H</sub> probes were distributed among isolates of LT+ST+ *E. coli* from patients from different sources. LT-ST+ *E. coli*, homologous with ST<sub>H</sub> but not ST<sub>P</sub>, were only found in individuals with diarrhea in rural Thailand. The difference between the proportion of LT-ST+ *E. coli* homologous with the ST<sub>H</sub> probe from rural vs urban Thailand. (9/9 vs 4/12) was significant ( $p=0.03^H$  (Fisher's exact test)), Table 2. All seven of the LT-ST+ strains isolated from children with diarrhea in urban or rural Thailand were homologous with only the ST<sub>H</sub> probe.

Detection of ETEC infections in patients with diarrhea : Stools collected from 110 children at Children's Hospital were examined simultaneously with the DNA hybridization assay and by testing ten lactose positive colonies for LT and ST. The same eight children were found to be infected with ETEC by both methods. Two children were infected with LT+ST+, five with LT+ST-, and one with LT-ST+ *E. coli*. The number of ETEC detected by the Y-1 adrenal (13) and suckling mouse (4) assays varied from one to ten. In three stools the LT probe detected DNA coding for LT when only one of ten isolates tested from the stool culture was positive in the Y-1 adrenal cell assay. The ST<sub>H</sub> probe which detected all of the stools containing ST producing colonies was also able to detect a stool in which only one of ten colonies tested produced ST. None of the stools were positive with the ST<sub>P</sub> probe.

Effect of other bacteria on detecting ETEC : Different proportions of tox- *E. coli* or *A. hydrophila* were mixed with LT+ST+ *E. coli* B2C (LT and ST<sub>H</sub> probe positive) to determine if the DNA from the other bacteria could interfere in detecting genes coding for LT and ST. The DNA hybridization assay was positive when  $10^9$  *A. hydrophila* or tox- *E. coli* were mixed with  $10^5$  LT+ST+ *E. coli* B2C or a clinical isolate LT+ST+ *E. coli* 12C-2 (LT, ST<sub>H</sub>, and ST<sub>P</sub> probe positive), were spotted on a nitrocellulose filters which were placed on MacConkey agar, and incubated at 37°C for 24 hours.

Detection of ETEC in contaminated water : To determine the sensitivity of the DNA probe assay in detecting ETEC in water containing other species of bacteria tenfold dilutions of LT+ST+ *E. coli* B2C were made in klong water and sterile PBS. One ml of klong water contained  $2.1 \times 10^5$  *Proteus*,  $1.5 \times 10^5$  *Enterobacter*,  $1.3 \times 10^5$  *Aeromonas*,  $6.0 \times 10^4$  *E. coli*,  $6.0 \times 10^4$  *Klebsiella*, and  $3.2 \times 10^4$  *Pseudomonas*. The DNA probe assay was positive with both the LT and ST<sub>H</sub> probes when ETEC were diluted in both PBS and klong water to contain ten ETEC/ml.

This study is complete.

Table 1. Source of enterotoxigenic *Escherichia coli* isolated from individuals with diarrhea

<u>No. of ETEC</u>	<u>Source</u>
24 LT+ST+	10 <sup>+</sup> Children, Children's Hospital, Bangkok
	6 Children, Soongnern Hospital, Soongnern
	4 Adults, Bamrasnaradura Hospital, Bangkok
	3 Peace Corps volunteers, rural Thailand
	1 Adult, Soongnern Hospital, Soongnern
17 LT+ST-	7 Children, Children's Hospital, Bangkok
	3 Children, Soongnern Hospital, Soongnern
	3 Adults, Soongnern Hospital, Soongnern
	2 Peace Corps volunteers, rural Thailand
	1 Buffalo, Soongnern
	1 Pig, Soongnern
22 LT-ST+	10 Adults, Bamrasnaradura Hospital, Bangkok
	5 Children, Soongnern Hospital, Soongnern
	3 Adults, Soongnern Hospital, Soongnern
	2 Children, Children's Hospital, Bangkok
	1 Peace Corps volunteers, rural Thailand
	1 Pig, Soongnern

<sup>+</sup> Number of individuals from whom ETEC were isolated (one ETEC/individual)

Table 2. Homology of ST<sub>P</sub> and ST<sub>H</sub> with LT+ST+ and LT-ST+ *E. coli* isolated from patients with diarrhea in urban and rural Thailand

<u>Source</u>	<u>No. of ETEC isolated</u>	<u>No. of colonies homologous with</u>		
		<u>ST<sub>P</sub></u>	<u>ST<sub>H</sub></u>	<u>ST<sub>P</sub> + ST<sub>H</sub></u>
Urban †	14 LT+ST+	5	8	1
	12 LT-ST+	5	4	3
Rural Δ	10 LT+ST+	6	3	1
	9 LT-ST+	0	9	0
TOTAL	24 LT+ST+	11	11	2
	21 LT-ST+	5	13	3

LT+ = produce heat-labile toxin

ST+ = produce heat-stable toxin

† Urban refers to metropolitan Bangkok

Δ Rural refers to specimens collected from Thais in Soongnern and American Peace Corps volunteers at U'thong, Pranburi, Inburi, and Sri Prachan

One LT-ST+ *E. coli* isolated from a pig in rural Thailand was only homologous with ST<sub>P</sub>.

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