

DETECTION OF ENTEROTOXIGENIC *Escherichia coli* IN WATER  
BY FILTER HYBRIDIZATION WITH THREE  
ENTEROTOXIN GENE PROBES

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**OBJECTIVE :** To use the DNA hybridization assay for genes encoding for *Escherichia coli* enterotoxins to examine water specimens in Thailand.

**BACKGROUND :** Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of diarrheal disease throughout the world (2,8,15). Heat-labile toxin-producing *E. coli* (LT *E. coli*) are usually identified by testing culture supernatants of single isolates in one of several tissue culture assays (7,14), whereas heat-stable toxin-producing *E. coli* (ST *E. coli*) are identified by injecting culture supernatants intragastrically in suckling mice (4). Since these techniques to identify LT and ST *E. coli* are time consuming and require a large number of suckling mice, it is difficult to screen large numbers of isolates.

We have recently employed recombinant DNA technology to overcome this problem. ETEC contain extra chromosomal segments of DNA which carry the genes coding for the production of LT and ST *E. coli* (16). The structural genes for these enterotoxins have been cloned (3,10,17) and used as probes to identify ETEC in diarrheal stools spotted on nitrocellulose paper (11,13). Once established, this technique provides a method to screen large numbers of environmental samples to identify ETEC or other bacteria which contain similar genes for enterotoxins. In this report, the sensitivity of the DNA hybridization assay in detecting ETEC in water was compared with testing *E. coli* isolates in the Y-1 adrenal (14) and suckling mouse (4) assays. This novel approach was used to detect organisms which carried the structural genes for enterotoxin production in water collected from homes in rural Thailand.

**METHODS :**

Filter hybridization assay : Water to be examined for ETEC or other organism which carry the genes for enterotoxin production were passed through a pre-boiled 0.45  $\mu$ m nitrocellulose filter (Schleicher & Schuell Co., Keene, N.H.) and placed on MacConkey medium. After overnight incubation at 37°C, the filters were removed from the agar and placed on a double thickness of Whatman no.3 paper saturated with 0.5 N NaOH for 10 min. The filters were then transferred to Whatman paper saturated with 1.0 M ammonium acetate 0.02 N NaOH for 1 min. This process was repeated four more times. After the last transfer, the filter was kept on the saturated Whatman paper for 10 min. The filter was then removed, air dried, and baked overnight at 65°C. Filters were stored in metal boxes until examined with the  $\alpha$ -<sup>32</sup>P labelled DNA probes (13). Three filters were processed for each specimen.

The derivations of the LT, ST-H, and ST-P probe DNAs have previously been described (11,13). The isolated DNA fragments which were used as probes were phenol extracted, ethanol precipitated, and labeled *in vitro* with  $\alpha$ -<sup>32</sup>P deoxynucleotide triphosphates by nick translation. After 75 to 100 nitrocellulose filters had been prepared, they were immersed in enough hybridization solution (13) to wet each filter, wrapped in plastic, and incubated at 37°C for 3 h. The filters were then transferred to fresh hybridization solution containing approximately 10 cpm of one of the three heat-denatured DNA probes per ml and 75 µg of sheared, heat-denatured, calf thymus DNA per ml and incubated at 37°C overnight. The filters were then washed in 5 x SSC (1 x SSC = 0.15 M sodium chloride plus 0.015 M sodium citrate)- 0.1% sodium dodecyl sulfate for 45 min. at 65°C, rinsed in 2 x SSC at room temperature, and air dried. The filters were exposed to Kodak X-Omat-R X-ray film (Eastman Kodak, Rochester, N.Y.) with a single Cronex Lightening-Plus intensification screen (E.I. DuPont de Nemours & Co., Inc., Wilmington, Del.) for 24 h. at -70°C. The films were developed according to the manufacturer's instructions.

Sensitivity of the DNA hybridization assay : Canal water was serially diluted in sterile phosphate-buffered saline and cultured on MacConkey, Hektoen, and blood agar (BBL Microbiology Systems, Cockeysville, MD) at 37°C for 24 h. All bacterial species were identified and quantitated and water was shown not to contain ETEC by testing 10 *E. coli* colonies in the Y-1 adrenal (14) and suckling mouse (4) assays (referred to as standard assays) and testing filtrates of 100 ml of the canal water in the DNA hybridization assay. ETEC B2C (homologous with the LT and ST-H probes) (10<sup>9</sup>/ml) was serially diluted in 100 ml of canal water in duplicate. One set of dilutions was passed through 0.45 µm filters (Millipore Corp. Bedford, Mass.), placed on mFc medium (1), and incubated anaerobically at 37°C for 24 h. The method of incubating mFc medium at 44°C to select for coliform bacteria (1) was not used because of the concern that plasmids coding for enterotoxin might be lost at this temperature. Because 37°C was used, plates were incubated under anaerobic conditions to inhibit non-coliform bacterial growth. Although anaerobiosis did not inhibit all such bacterial growth, it did suppress the majority of non-enterobacteria present in the water. Ten lactose positive colonies with the typical appearance of *E. coli* were selected from a culture of each dilution on mFc agar and tested for LT and ST *E. coli* in the standard assays. A similar set of dilutions of ETEC B2C in canal water was passed through 0.45 µm nitrocellulose filters and examined with the DNA probes homologous with genes coding for enterotoxin production.

Identification of ETEC in environmental samples : Between 1 July and 31 December 1981, water specimens were collected from the homes of 88 patients with diarrhea treated at the hospital in Soongnern, Nakornrajsima, in north-eastern Thailand. The patients all had diarrhea for less than 48 h., and the water was collected from the patients' homes within six h. of the time the patient arrived at the hospital. One liter of water was collected in open-mouth sterile glass bottles from the large earthen jars used in Thailand to store drinking and bathing water. Rural Thais use a small can of bathing water to wash the perineum after defecation. Water specimens were also collected from the home immediately adjacent to the home of the patient with diarrhea. All specimens were processed within 2 h. of collection. Samples of water 100 ml each) were passed through two separate 0.45 µm filters and three

separate preboiled 0.45  $\mu$ m nitrocellulose filters. One filter was placed on mFc medium and incubated anaerobically at 37°C for 24 h., and the other was placed on thiosulfate-citrate-bile-salts sucrose (TCBS) medium (Difco Laboratories, Detroit, Mich.) and incubated aerobically at 37°C for 24 h. The nitrocellulose filters were examined with the DNA probes as previously described. Nitrocellulose filters with ETEC homologous with the three enterotoxin gene probes and non-ETEC were included with each DNA hybridization assay. Ten lactose-positive colonies with the typical appearance of *E. coli* were selected from the mFc medium and stored on nutrient agar slants. *E. coli* isolated from samples found to contain bacteria which were homologous with the DNA probes for genes coding for enterotoxin production (BHEGP) were tested for LT and ST in the standard assays within two months of isolation. TCSG cultures were examined for serotype O1 and non-O1 *Vibrio cholerae*.

Rectal swabs collected from patients with diarrhea were cultured on MacConkey medium, which was incubated at 37°C for 24 h. Ten lactose-positive colonies with the typical appearance of *E. coli* were selected from the MacConkey agar and tested for ETEC in the standard assays.

Sensitivity of the DNA hybridization assay versus the standard assays in the detection of ETEC in water : ETEC B2C was serially diluted in canal water which contained  $5.4 \times 10^7$  *Aeromonas hydrophila*,  $5 \times 10^4$  *E. coli*,  $2 \times 10^4$  *Enterobacter agglomerans*, and  $1 \times 10^4$  *Klebsiella pneumoniae* per ml. As shown in Table 1, ETEC was detected in 100 ml of canal water in which ETEC B2C had been diluted to contain  $10^5$ /ml by picking 10 lactose-positive colonies and testing them in the standard assays. Fewer ETEC in canal water were not detected with the standard assays. In contrast, colonies which hybridized with the LT or ST-H probes were detected in canal water which contained only 1 ETEC per ml. Since 100 ml of water had been passed through a filter, there were 100 ETEC on each nitrocellulose filter. The results of the DNA hybridization assay are shown in Fig. 1.

Search for ETEC in water in homes in rural Thailand : Of 88 patients with diarrhea whose homes were studied, 10 (11%) were infected with ETEC as determined by testing 10 individual colonies from each patient in the standard assays (4LTST *E. coli*, 4LT *E. coli*, and 2ST *E. coli*). No patients were infected with serotype O1 or non-O1 *V. cholerae*. In 2 of 10 homes of patients infected with ETEC, BHEGP was found in both the drinking and bathing water. These bacteria were not found in water collected from control homes adjacent to those of patients with ETEC infections.

Water specimens collected from 78 homes of patients with diarrhea without ETEC infections as well as water from 77 control homes were examined in the DNA hybridization assay. BHEGP was identified in 6% (5 of 78) of drinking water specimens collected from homes of patients with diarrhea without identifiable ETEC infections and a similar proportion (5 of 77) of drinking water samples collected from control homes. Fourteen percent (11 of 78) of bathing water specimens from homes of patients without ETEC associated diarrhea and 8% (6 of 77) of bathing water collected from control homes contained BHEGP. BHEGP was found in both drinking and bathing water samples collected from 4 homes of 78 patients not associated with ETEC infections and in 1 of 77 control homes.

Table 1. Comparison of detection of ETEC in the standard assays versus the DNA hybridization assay

No. of ETEC B2C per ml of canal water*	Standard assays (no. positive/ no. tested)		DNA hybridization assay	
	Y-1 adrenal assay	Suckling mouse assay	LT probe	ST-H probe
10	10/10	10/10	+	+
10	10/10	10/10	+	+
10	10/10	10/10	+	+
10	2/10	2/10	+	+
10	0/10	0/10	+	+
10	0/10	0/10	+	+
10	0/10	0/10	+	+
10	0/10	0/10	+	+
1	0/10	0/10	+	+
0	0/10	0/10	-	-

\* Canal water contained  $5.4 \times 10^4$  *A. hydrophila*,  $5 \times 10^4$  non-enterotoxi-  
genic *E. coli*,  $2 \times 10^4$  *E. agglomerans*, and  $1 \times 10^4$  *K. pneumoniae*  
per ml.

Ten *E. coli* from each of 31 water specimens which contained BHEGP were tested in the Y-1 adrenal and suckling mouse assays (4,14). In two homes of patients infected with LTST *E. coli*, this enteric pathogen was identified by both the standard and the DNA hybridization assays in drinking and bathing water samples. The other 27 water specimens would not have been found to contain bacteria which contained genes coding for enterotoxin if only the standard assays had been used. None of the water containing BHEGP contained O1 or non-O1 *V. cholerae*.

In a reconstruction experiment, the DNA filter hybridization assay was at least  $10^4$  times more sensitive than testing 10 individual *E. coli* in the standard assays in identifying water containing ETEC. In two previous studies (11,13) the DNA hybridization assay had been shown to be a sensitive and specific tool in identifying ETEC in diarrheal stools. This study suggests that the DNA hybridization assay is a considerably more sensitive method of detecting ETEC in water than the standard assays. Presumably, this observation also applies to other environmental specimens. There was variation in the number of ETEC identified with the DNA hybridization assay. For example, in Fig. 1, row A, filter 8, on which were presumably 100 ETEC, more colonies hybridized with the LT probe than on a filter with the same number of colonies diluted in the same canal water (Fig. 1, row B, filter 8). This variation was presumably due to interference with either the growth of *E. coli* or attachment of the DNA by differing amounts of soil or other debris in the water.

Bacteria other than ETEC have been reported to produce heat-labile enterotoxins which are similar to LT *E. coli* (8,9,12,18). It is possible that a number of species of bacteria might possess DNA homologous with the DNA probes for genes coding for enterotoxin or that some organisms contain the structural genes for enterotoxin which are not expressed and thus not identified in the standard assays. To date, however, the only bacterial species which have been reported to contain DNA homologous with the enterotoxin gene probes for LT or ST under the high stringency conditions used in this study have been ETEC, although partial homology has been demonstrated between the LT probe and vibrios under conditions of lesser degrees of stringency (9,12).

BHEGP was found in 9% (31 of 350) of water samples examined with the DNA hybridization assay. Although BHEGP was more frequently found in water in homes of patients with identified ETEC infections than in water collected from their neighbors' homes, these potential enteric pathogens were widespread in water storage jars in rural Thailand. Since 10 ETEC are required to cause diarrhea, at least in healthy volunteers (5), and colonization factors are presumably also required to elicit clinical illness (6), the widespread distribution of low concentrations of ETEC in water in rural Thailand may not actually pose a serious health hazard. Prospective studies are in progress to identify BHEGP in water, food, people with and without diarrhea, and animals to define the epidemiology of these organisms.

When known quantities of ETEC were diluted with bacteriologically defined canal water, the DNA hybridization assay was positive with water containing ETEC in a ratio of 1 ETEC per 10,000 non-enterotoxigenic coliforms. In this preliminary study in rural Thailand, BHEGP was found in 20% of water samples collected from homes of patients with ETEC associated diarrhea. BHEGP was

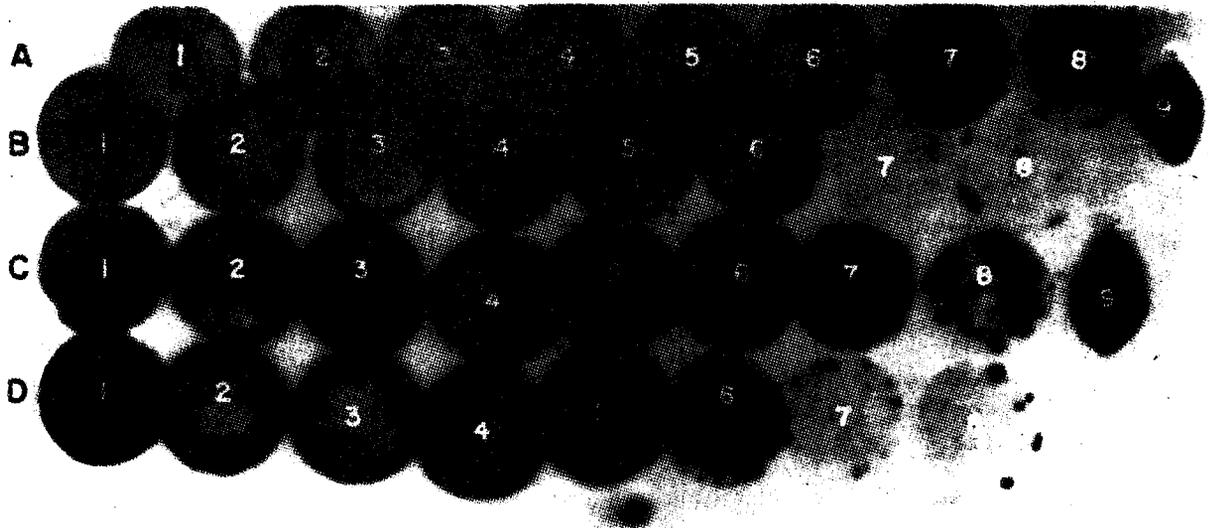


Fig. 1. Autoradiographs demonstrating radiolabeled gene-specific DNA hybridization with bacteria recovered from 100 ml of canal water containing different concentrations of ETEC B2C per milliliter. Rows A and B are filtrates of water containing  $1, 10^7$ ;  $1, 10^6$ ;  $1, 10^5$ ;  $1, 10^4$ ;  $1, 10^3$ ;  $1, 10^2$ ;  $1, 10^1$ ; and 8, 1 ETEC B2C/ml; row B, no. 9, is the canal water to which ETEC was not added. Rows C and D are filtrates of the same waters containing similar concentrations of ETEC B2C examined with the ST-H probe. Row D, no. 9, is a filtrate of canal water to which ETEC was not added; the filter was examined with the ST-H probe.

also found in a smaller proportion of water samples collected in homes of patients with non-ETEC associated diarrhea and samples collected from control homes. Further studies employing the sensitive DNA hybridization assay to identify ETEC or BHEGP may redefine the epidemiology of this important enteric pathogen. It is intriguing to speculate how an understanding of the epidemiology of ETEC might further increase if specific DNA probes for colonization factors could be constructed by using current recombinant DNA technology.

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