

IDENTIFICATION OF ENTEROTOXIGENIC *Escherichia coli* IN HOMES
OF CHILDREN WITH DIARRHEA BY DNA HYBRIDIZATION WITH
THREE ENTEROTOXIN GENE PROBES

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OBJECTIVE : To use the DNA hybridization technique to detect genes encoding for *Escherichia coli* enterotoxin to identify enterotoxigenic *Escherichia coli* (ETEC) in household contacts, food, and water in homes of children with diarrhea in Thailand.

BACKGROUND : Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea worldwide (1-3). In developing countries ETEC is one of the most common enteric pathogens in young children (4,5). However because standard assays used to identify ETEC are cumbersome and time consuming, it has been difficult to identify sources of this enteric pathogen. To identify ETEC in a large number of specimens, we applied molecular genetic and deoxyribonucleic acid technology to study the epidemiology of this infectious disease. Specimens collected from food, and water in homes of children with diarrhea and from their household and neighborhood contacts were examined for DNA homologous with genes coding for *E. coli* enterotoxin.

METHODS :

Patient selection : From February 15 to July 31, 1982, the first two children under five years of age with diarrhea of less than 48 hours duration who were seen at an outpatient clinic of children's hospital in Bangkok, Thailand were enrolled in the study. Diarrhea was defined as the passage of three or more loose stools in less than 24 hours. On the day of the clinic visit two nurses visited the child's home and the home of a family with children under five years of age living nearby. Rectal swabs were collected from all family members and neighbors. Individuals were asked if they or their children had developed diarrhea in the preceding week. Specimens of water were collected in sterile open mouthed glass bottles from clay jars used to store drinking and bathing water, and from the source of the drinking water in both homes. Two specimens of food which had been recently eaten by the child with diarrhea and two similar foods from neighbor's home were also collected. Lastly hand imprints were collected on MacConkey agar from the mothers in the diarrhea and neighboring houses.

Processing of specimens : Two rectal swabs were obtained from each person, inoculated into Gary-Blair media, and processed within four hours of collection. One swab was cultured on MacConkey media and the resultant growth inoculated into six different pieces of nitrocellulose paper, (Schleicher & Schuell,

Keene, NH) layered on MacConkey agar. After incubation at 37°C overnight, the DNA of the resultant bacterial growth was fixed and examined in the DNA hybridization assay (6). Ten *E. coli* were also selected from the initial MacConkey media and stored on nutrient agar slants. Colonies from specimens which hybridized with one or more of the three DNA probes for genes coding for enterotoxins {LT (7), ST-H (8), and ST-P (9)} were tested for heat-labile (LT) and heat-stable (ST) enterotoxin production in the Y-1 adrenal (10) and suckling mouse assays (11), (referred to as the standard assays). All toxigenic isolates were serotyped (12), and assayed for colonization antigens by mannose-resistant hemagglutination of human and bovine erythrocytes and agglutination by antisera to CFA I, CFA II and *E. coli* E8775 (13-15). The second swab was cultured on thiosulfate citrate bile salts (TCBS) agar before and after enrichment in alkaline peptone water pH = 8.0. Food samples were homogenized in sterile glass pestles with two ml of brain heart infusion broth and processed in the same manner as the rectal swabs. Six 100 ml aliquots of each water specimen were passed through 0.45 µ nitrocellulose filters and processed in the DNA hybridization assay (6). Two additional 100 ml aliquots of water were passed through 0.45 µ millipore filters (Millipore Corp., Bedford, MA) one of which was layered on MacConkey and the other on TCBS agar. After incubation overnight at 37°C, ten *E. coli* selected from the MacConkey agar were saved on nutrient agar slants for testing in the standard assays. Vibrios were identified from filters layered on TCBS media (16). Bacteria isolated on MacConkey agar from imprints of the mothers' hands were examined with the DNA hybridization assay. Ten *E. coli*, if present, were picked from food, water, and hand cultures to be stored on nutrient agar slants. Colonies from samples which hybridized with the enterotoxin gene probes were tested for ETEC in the standard assays.

Bacterial growth from all specimens was fixed on nitrocellulose paper and examined with two different preparations of each of the three α-³²P labelled enterotoxin gene probes (6). *E. coli* K12 C600 (pRIT 10036) and *E. coli* K12 C600 (pSLM 004) containing multicopy recombinant plasmids from which the ST-P and ST-H probes were derived, LT and ST producing ETEC B2C which hybridized with the LT and ST-H probes, and non-enterotoxigenic *E. coli* K12 Xac were included as probe controls on each sheet of nitrocellulose paper.

Comparison of the DNA hybridization technique with the standard assays : Specimens collected from 80 children with diarrhea, 240 family contacts, and 256 neighbors were examined by both the DNA hybridization and the standard assays. A total of 320 food and 443 water specimens collected from case and adjacent homes were also analyzed in this manner. ETEC was detected in the same 72 samples by both methods. The LT gene probe detected five additional specimens which were not detected by testing *E. coli* from specimens in the Y-1 adrenal cell assay (10), while the ST-H probe detected an additional ETEC infection which was not detected by the standard suckling mouse assay (11). In 1480 specimens tested with both assays, 78 specimens were positive using the DNA hybridization assay. ETEC as identified by the standard methods was isolated from 92 percent (72/78) of specimens which were positive in the DNA hybridization assay.

A total of 4191 specimens from people, food, and water were collected in and around homes of 221 children with diarrhea. When examined by the gene

probe assay, 89 were found to contain DNA which hybridized with one or more of the three probes coding for *E. coli* enterotoxin (Table 1). ETEC as identified by standard assays was isolated from 69 of these 89 specimens. Isolates from four specimens died after storage on nutrient agar slants and no *E. coli* was isolated from two hand cultures because of the overgrowth of other species of bacteria. ETEC was found in 83 percent (69/83) of specimens which hybridized with the enterotoxin gene probes, and from which *E. coli* was available for testing in the standard assay.

ETEC was recovered from 28 of 30 (93%) children with diarrhea, 19 of 22 (86%) family members, 13 of 22 (59%) neighbors, seven of seven waters, one of one hand culture, and one of one food specimen which hybridized with the enterotoxin gene probes and from which *E. coli* was available for testing in the standard assays. With the 69 specimens in which the results of the standard assays agreed with those of the DNA hybridization assay. *E. coli* was tested in the standard assays sooner after isolation than with the 14 specimens in which only the DNA hybridization assay detected evidence of ETEC (mean 103, \pm 64 days vs mean 137, \pm 82 days) { $p = <0.05$ (one tailed t-test)}.

Detection of enterotoxigenic *Escherichia coli* in household contacts : The DNA hybridization assay identified ETEC in eight (9%) of 88 household contacts in 30 homes of children with ETEC associated diarrhea, five (5%) of 101 of their neighbors, and 32 (2%) of 1379 individuals from 382 homes not associated with children with ETEC associated diarrhea. Forty-two percent (5/12) of children under five years of age living in homes of children with ETEC associated diarrhea and five percent (2/42) of neighborhood children of the same age were infected with ETEC ($p < 0.001$). Two of these seven infected children had diarrhea. None of the six older household contacts with ETEC infections had diarrhea.

In one home both parents and a child were infected with the same LT ETEC (07:H-). The other six family contacts of children with ETEC associated diarrhea were infected with ETEC of different serotypes as the child with diarrhea. LTST ETEC 078:H12 was isolated from a child with diarrhea and a 19 year old girl in an adjacent home who frequently cared for this child.

Source of enterotoxigenic *Escherichia coli* : In six (20%) of 30 homes of children with ETEC associated diarrhea evidence of this enteric pathogen was found either in drinking water, bathing water, food, or on mothers' hands (Table 3). Evidence of ETEC was more frequently found in homes of children with ETEC infections than in homes of children without infections ($p < 0.001$). The bathing waters in homes adjacent to two homes of children with diarrhea contained similar ETEC as was found in the children with diarrhea (LTST ETEC 0128:H12 and LT ETEC 07:H-).

In this study the DNA hybridization assay (6) was shown to be a sensitive method of detecting bacteria containing genes coding for *E. coli* enterotoxin. All specimens that contained ETEC as determined by the standard methods (10,11) hybridized with the enterotoxin gene probes. ETEC was not detected in 20 specimens which hybridized with the LT (16), ST-H (3), or LT and ST-H (1) probes by the more time consuming task of selecting, storing, and subsequently testing individual colonies in the standard assays.

There may be several reasons why the DNA hybridization technique detected more ETEC than the standard assays. First, since enterotoxin production is plasmid mediated (17), ETEC may have lost plasmids coding for enterotoxin (ent) during storage. Closer agreement between the results of the two techniques was noted when standard assays were performed early on suggesting that loss of ent over time was a factor. In the enterotoxin gene probe assay bacterial DNA is fixed onto nitrocellulose within 48 hours. Once fixed it has been found to be extremely stable. When nitrocellulose papers containing ETEC colonies from an earlier study (6) were re-examined, enterotoxin genes were still detectable after one year. Second, other bacteria may interfere with the isolation of *E. coli* especially in asymptomatic carriers and on hand imprints. ETEC may be few in comparison to other bacteria, such that ETEC are obscured and not selected for testing in the standard assays. In contrast DNA from non-enterotoxigenic bacteria do not interfere with the DNA hybridization assay (18). Third, this newer method is at least 10,000 times more sensitive in detecting ETEC in water (18) and 1,000 times more sensitive in stool (6) which are fixed directly on nitrocellulose paper than testing ten individual from the same specimen in the standard assays (10,11). Finally although bacteria other than *E. coli* have been reported to produce enterotoxin, other species of bacteria were not tested in the standard assays (19,20). It is possible that other genera of bacteria carried genes coding for *E. coli* enterotoxin. Vibrios can share some degree of homology with the LT probe (21); however, it is unlikely that similar, but not identical DNA sequences were detected by the enterotoxin gene probes under the DNA hybridization conditions used in this study. Furthermore no vibrios were found in the 17 specimens which were positive with the LT probe and from which no LT producing ETEC was isolated.

In homes of children with ETEC associated diarrhea in Thailand the DNA hybridization technique identified ETEC in nine percent of family contacts and the rate of infection was greatest in siblings under five years of age. These results are similar to a study in Bangladesh in which 11 percent of household contacts were infected and both the rate of infection and proportion of infected persons with diarrhea decreased with increasing age (22).

Food and water have previously been identified as sources of ETEC in epidemics of diarrhea in the United States and Japan (23-27). In two homes drinking water and powdered milk were identified as likely sources of infections. In the other 28 homes studied these sources were not implicated either because the responsible food or water was no longer available for testing, or the infection was acquired elsewhere.

There are two difficulties in defining the epidemiology of ETEC, one is separating *E. coli* from the biomass present in most environmental samples and the other is testing large numbers of these colonies for enterotoxin production once these *E. coli* have been isolated. The DNA hybridization assay employing cloned genes for LT and ST is an effective tool to identify ETEC among a large number of specimens, and in conjunction with serotyping of isolates, will be useful in further defining the epidemiology of this enteric pathogen.

Table 1. Number of specimens which hybridized with the enterotoxin gene probes and from which ETEC were identified by the standard assays

	DNA probe assay*		Standard assay ⁺	
Children with diarrhea (221)**	<u>LTST-H</u>	4++	LTST	4/4
	<u>LT</u>	17	LT	15/17
	<u>ST-H</u>	8	ST	8/8
	<u>ST-P</u>	1	ST	1/1
	Total	30		28/30
Family members (681)	<u>LTST-H</u>	1	LTST	1/1
	<u>LTST-P</u>	3	LTST	3/3
	<u>LT</u>	16	LT	13/16
	<u>ST-H</u>	3	ST	2/2
	Total	23		19/22
Neighbors (735)	<u>LTST-H</u>	3	LTST	2/3
	<u>LTST-P</u>	1	LTST	1/1
	<u>LT</u>	18	LT	9/15
	<u>ST-H</u>	2	None	0/2
	<u>ST-P</u>	1	ST	1/1
	Total	25		13/22
Drinking water (790)	<u>LT</u>	1	LT	1/1
	<u>ST-H</u>	1	ST	1/1
	Total	2		2/2
Bathing water (438)	<u>LTST-H</u>	1	LTST	1/1
	<u>LT</u>	2	LT	2/2
	<u>ST-H</u>	2	ST	2/2
	Total	5		5/5
Hand cultures (442)	<u>LT</u>	3	LT	1/1
	Total	3		1/1
Food (884)	<u>ST-H</u>	1	ST	1/1
	Total	1		1/1

Table 1 (cont'd)

All specimens (4191)	<u>LTST-H</u>	9	LTST	8/9
	<u>LTST-P</u>	4	LTST	4/4
	<u>LT</u>	57	LT	41/52
	<u>ST-H</u>	17	ST	14/16
	<u>ST-P</u>	2	ST	2/2
Total		89		69/83

* DNA probe assay refers to the technique in which the DNA of the specimen is fixed on nitrocellulose paper and tested for homology with the three enterotoxin gene probes LT, ST-H and ST-P; + = Standard assay refers to the results of testing *E. coli* for heat-labile toxin (LT) in the Y-1 adrenal (7) and for heat-stable toxin (ST) in suckling mouse assays (8); ** Number of specimens examined; ++ Number of specimens with ETEC/number of specimens from which *E. coli* were available for testing in the standard assays.

Table 2. Evidence of ETEC infections among inhabitants in homes of children with diarrhea with ETEC infections, in homes of their neighbors', and in homes not associated with ETEC infections

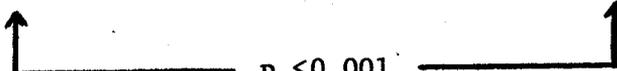
Age of contacts (years)	Inhabitants in 30 homes of children with ETEC infections		Neighbors in 30 homes adjacent to homes with ETEC infections		Inhabitants in 382 homes not associated with ETEC infections	
	No. contacts	No. infected*	No. contacts	No. infected	No. contacts	No. infected
0-1	2	1(50)**	29	0	314	8(3)
2-4	10	4(40)	14	2(14)	197	11(6)
5-19	17	1(6)	15	2(13)	263	6(2)
20-29	27	2(7)	25	1(4)	338	5(1)
30+	32	0	18	0	267	2(1)
Total	88	8(9)	101	5(5)	1,379	32(2)

* As determined by the DNA hybridization assay (6)

** Percent

Table 3. Evidence of ETEC in drinking water and bathing water, food, and on mother's hand in homes of children with ETEC associated diarrhea, in homes of their neighbors and in homes not associated with ETEC infections

Specimens	30 homes of children with ETEC infections	30 homes of matched neighboring homes	382 homes not associated with ETEC infections
Source of drinking water	0/30	0/30	0/382
Drinking water	1/30	0/30	1/382
Bathing water	1/30	2+/30	2/382
Food	1/60	0/60	0/762
Mother's hand	3/30	0/30	0/382
Total	6/180	2/180	3/2,290



 $p < 0.001$

+ Both neighboring homes' bathing water contained ETEC of the same serotype found in the child with diarrhea.

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