JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1985, p. 576–581
0095-1137/85/100576-06$02.00/0

Vol. 22, No. 4

Enterotoxigenic *Escherichia coli* in a Population of Infants with Diarrhea in Chile

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Received 22 March 1985/Accepted 9 July 1985

The incidence of enterotoxigenic *Escherichia coli* (ETEC) was investigated in 95 *E. coli* strains isolated from 48 infants with diarrhea in Santiago, Chile. By using standard biological assays and DNA-DNA hybridization procedures, ETEC was found in 31.2% of the cases: 14 strains produced heat-stable enterotoxin (ST) only, three strains produced heat-labile enterotoxin (LT) and ST, and two strains produced LT only. DNA probes detected all enterotoxin producers except one ST-producing strain. The ST strains hybridized with one or both of the human ST probes (ST Ib and ST A2). Two of the LT-ST strains hybridized with the ST Ia and ST Ib probes, and the third strain did not hybridize with any of the ST probes. Only the ST group expressed multiple resistance (85.7%) and colonization factor antigen I (CFA I) (92.8%); CFA II was found in two of three LT-ST strains. The O153:H45 serotype was found in 10 of 14 ST strains, and O6:K15:H16 was found in one LT strain and in two LT-ST strains. These findings suggest that ETEC, especially strains that produce ST, may be an important cause of diarrhea among Chilean infants.

Several studies undertaken in different developing countries have determined that enterotoxigenic *Escherichia coli* (ETEC) plays an important role in the etiology of diarrhea in young children and travellers to these regions (3, 5, 13, 19, 25–28, 31, 46, 54). Work performed mainly on Asian and African strains has provided insight into the epidemiology and ecology of these strains and has also demonstrated interesting aspects of the genetic heterogeneity of the heat-stable enterotoxin (ST) genes (12–15, 36–38, 49). Investigations into the incidence of ETEC in South America are limited, probably because of the complexity of the biological assays required to detect enterotoxins, which make them impractical for use in clinical laboratories and field studies. Investigations in Brazil have reported an increased frequency of ETEC in children with diarrhea (26, 27), but they have also demonstrated heat-labile enterotoxin-producing (LT) strains in a population of healthy children (44). Acute diarrhea is an important health problem among Chilean infants and is the main cause of hospitalization in this age group during the warm season. A recent investigation in Chile revealed that LT and LT-ST strains might be important etiologic agents in children with diarrhea, but only LT strains were tested for the production of ST (43). We believe that a complete knowledge of the frequency of the different causes of diarrhea and their mode of transmission in different countries is needed before immunoprophylactic measures can be implemented at the local level. In the present study, we characterized a population of ETEC isolated from infants with diarrhea in Chile as a preliminary step in determining the enteropathogenic role of ETEC in Chile. This characterization was achieved by standard biological assays and by DNA-DNA hybridization analysis with one LT and three ST DNA probes. The presence of colonization factor antigens (CFA), antibiotic resistance, and the bioserotype of these strains was also determined.

MATERIALS AND METHODS

Strains. This study included 95 *E. coli* strains isolated from 48 infants with diarrhea in Santiago, Chile, during the warm season, between November 1982 and March 1983. The infants ranged in age from 15 days to 22 months and were either attended to in the outpatient clinics of the L. C. Mackenna Hospital or were hospitalized at that institution. Stool samples were obtained at the time of consultation or within 24 h of admission by using a sterile cotton swab and were transported in Cary Blair transport medium. Specimens were cultured in MacConkey agar, salmonella-shigella agar, and Selenite-F Enrichment broth and were analyzed for the presence of bacterial enteropathogens, including "classic" enteropathogenic *E. coli* serogroups, enteroinvasive *E. coli* serogroups, and *Salmonella*, *Shigella*, and *Campylobacter* species (data not shown). Two lactose-positive colonies, identified as *E. coli* by standard biochemical tests, were selected at random from each infant in whom no other known bacterial or parasitic enteropathogens were isolated and were shipped for study (16). *E. coli* C600(pEWD299) containing the cloned LT gene and *E. coli* HB101(pSLM004) containing a human ST gene were obtained from S. Moseley, Stanford University, Stanford, Calif. *E. coli* C600(pRIT10036) containing a cloned porcine ST gene was kindly supplied by N. Harford, Smith, Kline-RIT, S. A., Rixensart, Belgium, and *E. coli* MM294(pNG10) containing a cloned human ST gene was provided by D. Taylor, Smith, Kline & French Laboratories, Philadelphia, Pa. Positive ETEC strains, used as controls, were kindly provided by W. Maas, New York University Medical Center, New York, N.Y.

Enterotoxin assays. All *E. coli* strains were assayed for the production of LT and ST. LT production was determined by the Y1 adrenal tumor cell tissue culture technique (10, 11,

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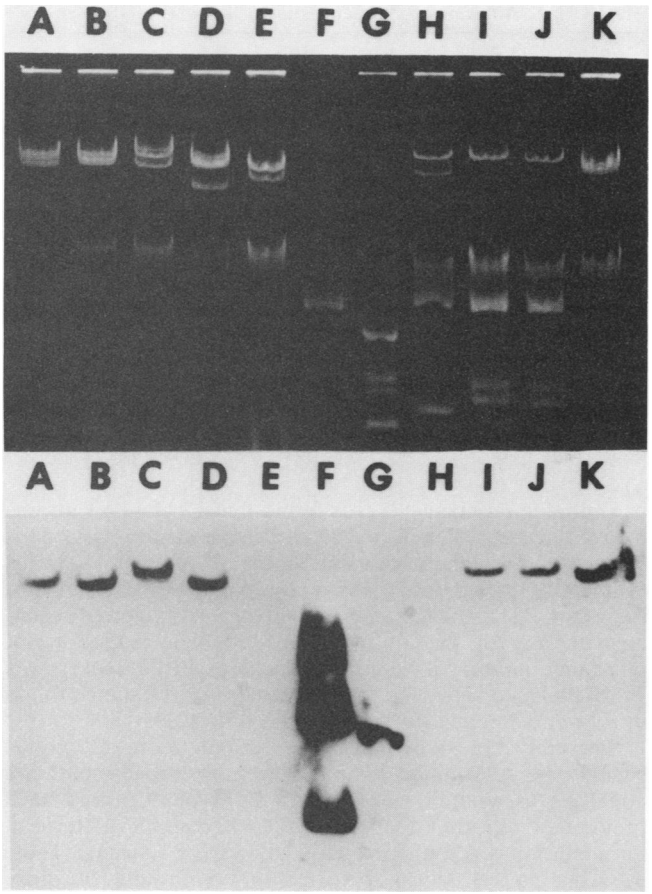

FIG. 1. Agarose gel electrophoresis of plasmid DNA from some ST strains (top) and the corresponding autoradiograph that shows plasmids containing DNA sequences which hybridize with the ST A2 probe (bottom). Lanes: A, *E. coli* 11; B, *E. coli* 42; C, *E. coli* 52; D, *E. coli* 53; E, known control that does not produce ST; F, *E. coli* MM294(pNG10) recombinant plasmid containing the ST A2 probe (three molecular species of this hybrid plasmid can be observed in the agarose gel and in the autoradiograph); G, *E. coli* 56; H, *E. coli* 69 (both non-ETEC); I, *E. coli* 98; J. *E. coli* 97 (identical to *E. coli* 98); K, *E. coli* 100.

47). ST production was determined by the infant mouse model described by Dean et al. (8), as modified by Giannella (24). Ratios of gut weight to remaining carcass weight that were greater than 0.083 were considered positive (24). Positive and negative control bacteria were always included in LT and ST assays.

Colony and Southern hybridization assays. Simultaneously with the investigation of the production of enterotoxins by biological assays, *E. coli* strains were screened for the presence of genes encoding for enterotoxins by using the colony hybridization technique (38). Bacteria were grown overnight on 9-cm-diameter nitrocellulose filters (no. BA-85; Schleicher & Schuell, Inc., Keene, N.H.) placed onto MacConkey agar plates (Difco Laboratories, Detroit, Mich); the filters were then treated as described by Moseley et al. (36, 38). The LT probe consisted of a *Hin*dIII fragment from plasmid pEWD299 encoding primarily for the B subunit of the LT molecule (7). The ST probes used consisted of one probe obtained from a porcine strain and two probes of human origin. The ST Ia probe, obtained from a porcine strain, consisted of a 157-base-pair *Hin*fI fragment from pRIT10036 (30, 36); the ST Ib probe of human origin consisted of a 215-base-pair *Hpa*II fragment from pSLM004 (37); and the ST A2 probe, obtained from a human ST strain (characterized at Smith, Kline-RIT, S.A., Rixensart, Belgium), consisted of a 510-base-pair *Eco*RI fragment from pNG10 (10, 29). DNA probes were labeled with α-^{32}P-labeled deoxynucleotides (Amersham Corp. Arlington Heights, Ill.) by nick translation (34). Plasmid DNA of all the strains which gave positive results in the colony hybridization assay were extracted by the method of Birnboim and Doly (2), electrophoresed in slab agarose gels, transferred to nitrocellulose filters (50), and hybridized with ^{32}P-labeded DNA probes. Filters were then exposed to X-Omat X-ray film (Eastman Kodak Co., Rochester, N.Y.) for 24 h at −70°C as described previously (33).

Plasmid molecular weight. Plasmid molecular weights were determined by comparison with known molecular weight plasmids, by the DNA extraction method of Birnboim and Doly (2), and by electrophoresis in parallel with plasmid DNA from the strains being studied. The controls were *E. coli* strains harboring the following plasmids: ColV FC201 [80 megadaltons (MDa)], R6-5 (66.6 MDa), *E. coli* TR438 containing a 64-MDa and a 42.6-MDa plasmid, and *E. coli* V517 harboring eight plasmid species that ranged in size from 1.36 to 35.8 MDa (32).

Determination of CFA. Bacteria grown on Casamino Acids (Difco Laboratories, Detroit, Mich.)-yeast extract-salts agar plates (18) were investigated for hemagglutination of human group A and bovine erythrocytes suspended in phosphate-buffered saline containing 1% mannose to determine CFA I and II, respectively (17, 20).

Detection of antibiotic resistance. The *E. coli* strains were studied for antibiotic resistance by using the Bauer et al. assay (1). The antibiotics tested were chloramphenicol, ampicillin, carbenicillin, gentamicin, streptomycin, kanamycin, cephalothin, and tetracycline.

Biotyping ETEC. *E. coli* strains which were positive for the production of enterotoxins were subjected to various fermentation assays, as described previously (35, 40, 45).

Serotyping. All ETEC strains were serotyped at the International Center of Escherichia and Klebsiella, Statens Seruminstitut, Denmark (39).

RESULTS

Frequency of ETEC. ETEC strains were isolated from 15 of 48 infants (31.2%). Of the 95 *E. coli* strains studied (an average of two strains per patient), 19 different ETEC strains were isolated, as determined by biological assays, hybridization procedures, and the pattern of plasmid DNA content. These were 14 ST, three LT-ST, and two LT strains. In eight patients, one of the two strains isolated was ETEC; in three patients, both strains isolated were the same ETEC strain; and in four patients, each individual isolate corresponded to two different ETEC strains. No differences in age or severity of diarrhea were found between infants whose stools were positive for ETEC and those in whom ETEC strains were not found. ETEC strains were isolated in 9 of 28 infants who needed to be hospitalized and in 6 of 20 infants treated on an outpatient basis.

Hybridization with ^{32}P-labeled DNA sequences encoding for enterotoxins. We found that all but one ETEC strain hybridized with one or more of the DNA probes used. Of the 14 strains determined to be ST producers by the infant mouse model, 10 hybridized with both human ST probes (ST Ib and ST A2), three hybridized only with the ST A2 probe, and one

hybridized with the ST Ib probe (Fig. 1 and Table 1). Work done by others has shown that these two human ST probes cross-hybridize under stringent conditions in the colony assay but that certain ST strains give stronger reactions with one or the other probe (R. Maas, personal communication). All three LT-ST strains hybridized with the LT probe in the colony assay, but only two hybridized with the Southern hybridization technique (50); one of these strains hybridized with the porcine ST Ia probe, one strain hybridized with the human ST Ib probe, and the third did not hybridize with either of the ST probes. Of these two LT strains, as determined by Y1 adrenal tissue culture and colony hybridization assays, only one hybridized in the Southern blotting technique. Previous investigators have pointed out that false-positive hybridization results can occur in the colony assay (23). We do not believe that this is the case here, because the strains were repeatedly positive in the colony hybridization and biological assays. Analysis of the plasmids containing the DNA sequences encoding for enterotoxins in these strains revealed that these sequences were most frequently located in plasmids ranging from 52 to 75 MDa, particularly in those of approximately 57 to 59 MDa (Tables 1 and 2). Interestingly, we found an LT-ST strain that did not hybridize with either of the ST probes, which could indicate that there is a DNA sequence which encodes for ST in that geographical area and which differs from those previously reported.

CFA. CFA, which allow the attachment of bacteria to the intestinal mucosa, are important to the ability of ETEC to produce disease. CFA I and II were investigated by hemagglutination of human group A and bovine erythrocytes, respectively, in the presence of mannose. It was found that 13 of the 14 ST strains (92.8%) expressed CFA I and two of the LT-ST strains possessed CFA II (Table 2). CFA I was also found in 28 of 76 *E. coli* strains (36.8%) which did not produce enterotoxins (data not shown). The presence of other adhesion antigens described in ETEC (9, 52) was not investigated in strains which did not exhibit CFA I or II. Such an investigation will be the subject of future studies.

TABLE 1. Analysis of the characteristics of the *E. coli* ST strains[a]

Strain denomination	Plasmid[b] sizes (MDa)	Antibiotic[c] resistance pattern	O:H antigens
2	**61**,[d] 45, 32	Cb^r	O49:H^-
98	**78**, 6.1, 3.1, 2.7		O115:H40
11	**71**, 59, 52	Tc^r Cb^r Kn^r Sm^r	O153:H45
12	**61**, 52	Tc^r Cb^r Kn^r Sm^r	O153:H45
29	61, **54**[d]	Tc^r Cb^r Kn^r Sm^r	O153:H45
34	66, 59, **52**[d]	Tc^r Cb^r Kn^r Sm^r	O153:H45
39	71, **57**, 51, 48	Tc^r Cb^r Kn^r Sm^r	O153:H45
40	71, 58, **52**	Tc^r Cb^r Kn^r Sm^r	O153:H45
42	71, 59, **52**	Tc^r Cb^r Kn^r Sm^r	O153:H45
52	80, **70**, 57, 1.8	Tc^r Cb^r Kn^r Sm^r	O153:H45
53	71, **54**, 43	Tc^r Cb^r Kn^r Sm^r	O153:H45
100	70, 62, **59**	Tc^r Cb^r Kn^r Sm^r	O153:H45
38	78, **71**, 57	Tc^r Cb^r Kn^r Sm^r	O sp.agg:H45
73	70, **54**,[e] 50	Tc^r Cb^r Sm^r	O155:H45

[a] All but strain 38 expressed CFA I. All strains hybridized with both human ST Ib and ST A2 probes exept as indicated for footnotes *d* and *e*.
[b] Plasmids containing enterotoxin genes are in boldface.
[c] Abbreviations: Cb, carbenicillin; Tc, tetracycline; Kn, kanamycin; Sm, streptomycin.
[d] Only hybridized with the ST A2 probe.
[e] Only hybridized with the ST Ib probe.

TABLE 2. Analysis of characteristics of LT and LT-ST strains

Strain denomination	Type of enterotoxin	Plasmid[a] sizes (MDa)	CFA type	Serotype
58	LT	79, 68, 54, 44, 8.2, 5.1[b]		O159:K^-:NM[c]
77	LT	**68**, 44, 37, 3		O6:K15:H16
74	LT-ST	99, **57**,[d] 1.9, 1.6, 1.4		(O9):K?:H16
76	LT-ST	73, **52**,[e] 3.5	II	O6:K15:H16
78	LT-ST	63, **56**,[f] 42, 37, 16, 3	II	O6:K15:H16

[a] Plasmids containing enterotoxin genes are in boldface.
[b] LT strain which hybridizes in the colony assay only.
[c] Nonmotile serotype.
[d] Plasmid harbors LT and ST Ia DNA sequences.
[e] LT and ST strain that hybridizes only with the LT probe.
[f] Plasmid hybridizes with ST Ib probe.

Antibiotic resistance. It was found that 12 of 14 ST strains (85.6%) were resistant to two or more antibiotics. This pattern of resistance was similar for all of the ST strains (Table 1). One LT strain was multiply resistant, but none of the other LT strain nor the LT-ST strains exhibited resistance to any of the antibiotics tested. The frequency of resistance among the non-ETEC strains in our study was 39.4%, a value similar to the frequencies of resistance found in *E. coli* belonging to the intestinal flora (21).

Biotyping and serotyping. The fermentation of various sugars was determined so that the pattern of fermentation could be associated with certain serotypes, as has been previously reported (35, 40, 45). A common pattern of fermentation was found in almost all of the ST strains (Table 3). This pattern did not correspond with any of those previously reported as being associated with enterotoxigenic serotypes (35, 40, 45). Biotypes of LT and LT-ST strains varied slightly among themselves. The O:H serotypification confirmed the previous observation that 10 of 14 ST strains were O153:H45, a serotype not previously found among enterotoxigenic *E. coli*. The other four ST strains were of the O49:H− serotype; the O115:H40 serotype, which has been associated with enterotoxigenicity; the O155:H45 serotype; and another O sp. agg:H45 serotype. Two LT-ST strains and one LT strain belonged to the O6:K15:H16 serotype, which was already known to be associated with LT-ST production (Table 2). The fermentation pattern of our O6:K15:H16

TABLE 3. Biotypes of ETEC serotypes isolated from infants with diarrhea in Chile

Substrate	Fermentation pattern of serotypes			
	O6:H16 (2) (n = 2)	O6:H16 (1) (n = 1)	O115:H40 (1) (n = 1)	O153:H45 (10) O111:H45 (1) O155:H45 (1) (n = 10 and 1, respectively)
Adonitol	+	+	−	−
Dulcitol	−	−	−	−
Sucrose	+	+	+	+
Xylose	+	+	+	+
Raffinose	−	+	+	−
Maltose	+	+	+	+
Sorbitol	+	+	+	+
Toxigenic phenotypes	LT-ST	LT	ST	ST

strains differed from the pattern previously reported for this serotype only in the fermentation of sucrose (35, 40, 45).

DISCUSSION

Acute diarrheal syndrome in children less than 3 years old accounts for 18 to 20% of the cases seen in outpatient clinics during the warm summer period in Chile, and it is the primary cause of hospitalization in children under 1 year of age during this time of year (51; V. Prado, unpublished observations). Investigations into the etiology of diarrheal disease in children have indicated that ETEC and rotaviruses are frequent causes of diarrhea in this group (3, 4, 14, 26, 27). The prevalence of isolation is influenced by the age of the child and the time of year (3, 5, 19, 51). Similar investigations in Chile have supported the relevance of rotavirus as an etiologic agent of diarrhea and of the uncertain etiologic role played by the classic serotypes of enteropathogenic *E. coli* (22, 41, 55). A recent study done in Chile determined that ETEC strains were isolated from 23.6% of infants with diarrhea, but the incidence of only LT and LT-ST strains was studied (43). We decided to undertake the present study to definitely ascertain the presence of ETEC among infants with diarrhea in Chile and because there was no information regarding the molecular aspects of enterotoxigenicity in South American strains.

We found that ETEC strains were identified in 31.2% of the patients and that 73.6% of these strains produced ST. Interestingly, most of these strains corresponded to the same bioserotype, expressed CFA I, and exhibited the same pattern of antibiotic resistance. They also hybridized with the human ST probes, and the plasmids containing those genes, with few exceptions, varied slightly in size. We can therefore postulate that there exists an O:H serotype, most commonly found in this geographical area, which differs from serotypes found in other regions of the world. The indiscriminate use of antibiotics has perhaps contributed to the selection and prevalence of these antibiotic-resistant enterotoxigenic clones. These results suggest that biotyping and serotyping with a few specific antisera would be helpful in identifying most of the ETEC in this area, as has been reported for other areas of the world (5).

The reported isolation of *E. coli* which produces LT or ST or both from patients with diarrhea varies in the published literature (3, 5, 12, 19, 26, 27, 31, 42), not only among different regions, but at different times of the year in the same geographical area (26, 27, 44). Moreover, it has been reported that strains which are phenotypically similar can vary genotypically in the same region at different times. This is the case with ST strains isolated in Bangladesh: ST strains isolated in 1980 hybridized primarily with the ST probe of porcine origin (38), but strains isolated in that area in 1982 were recognized in most cases by the human ST DNA probe (37). Our preliminary results do not rule out the possibility that the phenotype, serotype, and other characteristics of ETEC in Chile may change with different geographical locations and at different times. Nonetheless, previous studies (43) and the present results suggest that ETEC strains are frequently isolated from children with diarrhea in Chile.

Reports from other geographical areas indicate that LT-ST and ST strains that usually express CFA are isolated mainly from ill children, whereas LT strains can be isolated at comparable rates from diseased and control children (3, 5, 44). This seems to be the case in our study, in which 92.8% of the ST strains expressed CFA I and two of three LT-ST strains expressed CFA II. Therefore, on the basis of these findings and on the low incidence of rotavirus diarrhea in the warm season in Santiago, we suggest that the ETEC strains isolated in this study are most likely involved in the production of diarrhea (52). The isolation from the same patient of strains with different plasmid contents could reflect mixed infections, probably caused by the high prevalence of these agents in an environment with poor sanitary conditions (6). The in vivo or in vitro loss of some of the plasmids containing the enterotoxin genes could also explain these results.

The ETEC strains that we isolated frequently exhibited resistance to antibiotics. Similar studies have also reported that a high percentage of ETEC strains are multiply resistant and that this characteristic can be transferred jointly with enterotoxin production (15, 31, 48, 53). Studies are under way to detect the transferability of enterotoxigenicity associated with antibiotic resistance in our strains. This will be the first step in studies directed toward the physical and genetic characterization of some selected Ent plasmids from this ETEC population. It will be of special interest to study the LT-ST strain that does not hybridize with any of the ST probes used, which indicates the possible existence of an ST sequence that is characteristic of this geographical area. This information may be relevant to current efforts to produce vaccines which protect against ETEC infections. Similarly, more studies are needed to characterize further the CFA present in this group of strains.

In summary, our data suggest that ETEC strains may be an important cause of diarrhea among infants in Chile, findings which could be extended to the etiology of diarrhea in travellers to this area. Studies in progress with *E. coli* strains isolated from diseased and normal infants and from adults will determine their relevance in diarrheal disease in comparison with viral and parasitic agents which are also important causes of acute diarrhea in Chile (51, 55).

ACKNOWLEDGMENTS

This work was supported by the Fogarty International Center, National Institutes of Health Fellowship F05-TW03210 to M.E.A., and Public Health Service grant RO1 AI16078 from the National Institute of Allergy and Infections Diseases to F.C.C.

We thank Werner and Renata Maas for helpful discussions and for bacterial strains. We are greatly indebted to S. Falkow, S. Moseley, N. Harford, and D. Taylor for providing the strains containing the cloned enterotoxin genes. We also acknowledge the valuable advice of K. Wachsmuth and the help of G. Theis with the use of tissue culture techniques. We also thank Vicky Temponi for secretarial assistance.

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