Search for Cytolethal Distending Toxin Production among Fecal *Escherichia Coli* Isolates from Brazilian Children with Diarrhea and without Diarrhea

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The enteropathogenic role of cytolethal distending toxin-producing *Escherichia coli* was investigated by searching sequences homologous to the *cdt* genes of an O86 strain among 2,074 isolates from 200 children with acute diarrhea and 200 controls in Brazil. Only one (0.5%) diarrheic child and two (1.0%) nondiarrheic controls harbored *cdt*-positive isolates.

Cytolethal distending toxin (CDT) induces enlargement and death of some cultured eukaryotic cell lines by causing an irreversible blockage of the cell division cycle at the stage immediately before mitosis (6, 21). A broad spectrum of gram-negative bacterial species has been shown to produce CDT (6), and three closely linked genes (*cdtA*, *cdtB*, and *cdtC*) are required for toxin expression (6, 21). The deduced amino acid sequences of the A, B, and C genes from an *Escherichia coli* strain of serogroup O86 (E6468-62) and from a strain of serogroup O128 (strain 9142-88) are 38, 56, and 37% homologous, respectively (22, 24), and the corresponding toxins are called CDT-I and CDT-II (20). The predicted products of the genes from strain S5 of serogroup O15 (CDT-III) are >90% homologous to CDT-II and 55 to 69% homologous to CDT-I (20). Unlike the other genes, the *cdt*-III genes were cloned from a plasmid that also carries the genes of cytotoxic necrotizing factor (CNF), a toxin that induces enlargement and multinucleation of cultured eukaryotic cells (20).

Studies conducted in children up to 5 years of age and DNA probes for CDT (I and/or II) in Bangladesh (1) and Nigeria (16) failed to demonstrate a role for CDT-producing *E. coli* strains in the development of acute diarrhea. However, suckling mice injected intragastrically with culture supernatants from a recombinant *E. coli* strain bearing the *cdt* genes from *Shigella dysenteriae* presented with diarrhea more often than did mice inoculated with supernatants from an isogenic CDT-negative strain, and a partially purified preparation of CDT induced profuse watery diarrhea in this animal model (17).

In the present study, 2,074 fecal *E. coli* isolates from 200 children (1 to 4 years old) with bloody or nonbloody acute diarrhea (1,030 isolates) and 200 age-matched nondiarrheic children (1,044 isolates) were examined for the presence of *cdt*-I genes. These children were selected randomly from 500

case-control pairs enrolled in a previous study of bacterial and viral etiologies of acute diarrhea conducted at the emergency room of a child hospital in São Paulo City, Brazil, from April 1989 to March 1990 (9). CDT-producing *E. coli* strains of serogroups O86 (10) and O128 (12), as well the CDT-III-producing strain S5 (20), were used as positive controls, whereas K-12 strains C600 and HB101 were used as negative controls. All isolates and strains were stored in 15% glycerol at -70°C .

Bacterial colony blots prepared on Whatman 541 filter papers (Whatman, Inc., Clifton, N.J.) were processed and hybridized under stringent conditions (15) with the 1,375-bp *AccI* radioactively labeled DNA fragment derived from pCVD448 (1). To search for toxin activity, all probe-positive isolates were grown statically in 2 ml of brain heart infusion (Difco) broth at 37°C for 48 h, and filtered culture supernatants (diluted 1:5) were subjected to HeLa cell cytotoxicity assays as previously described (10). Filtered bacterial lysates, obtained by sonication of whole cultures (two 30-s bursts at 4°C in an MSE ultrasonic disintegrator; MSE, Crawley, United Kingdom), were subjected to neutralization assays with rabbit antiserum specific for CDT or CNF type 1 (CNF1) and CNF2. Equal volumes of antiserum (single or combined) properly diluted were added to lysates diluted serially (twofold) and incubated at 37°C for 2 h prior to the cytotoxicity assays.

E. coli isolates carrying *cdt*-I were identified in the feces of only one (0.5%) child with diarrhea (case 1) and two (1.0%) controls. As shown in Table 1, culture filtrates of most isolates studied from this case and all isolates from one control child (control 1) induced both cell distention and multinucleation, and these activities were neutralized by either by a mixture of antiserum to CDT and CNF1 or anti-CNF1 serum alone. The culture filtrate of one isolate from another control child (control 2) induced only cell distention, an effect completely abolished by preincubation with CDT antiserum. Only *E. coli* control strain O86 hybridized with the *cdt*-I probe and expressed a distending activity that was completely neutralized by the CDT antiserum used. The toxic titers of culture lysates from control strain S5 were reduced by preincubation with anti-CNF2, but

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TABLE 1. Characteristics of all fecal *E. coli* isolates studied from children identified as carriers of CDT-probe-positive *E. coli*

Child ^a	No. of isolates	Serotype ^b	HeLa cell activity of culture filtrates		Neutralization of cell activity by antiserum	Hybridization with CDT probe	Other DNA sequence(s) ^d
			Distension	Distension and multinucleation			
Case 1	5	O2:NM	—	+	CDT + CNF1	+	<i>cnf</i> , <i>hly</i> , <i>pap</i> , <i>sfa</i>
	1	O4:HND	—	+	CNF1	—	<i>cnf</i> , <i>hly</i> , <i>pap</i> , <i>sfa</i>
	1	ND	—	—	NT ^c	—	<i>inv</i>
Control 1	5	O6:HND	—	+	CNF1	+	<i>cnf</i> , <i>hly</i> , <i>pap</i> , <i>sfa</i>
Control 2	1	O177:NM	+	—	CDT	+	<i>eae</i>
	5	NT	—	—	NT	—	None

^a Case, child with diarrhea; control, child without diarrhea.

^b NM, nonmotile; HND, H antigen not determined.

^c NT, not tested.

^d *cnf*, CNF; *hly*, alpha-hemolysin; *pap*, PAP; *sfa*, SFA; *inv*, plasmid of enteroinvasive *E. coli*; *eae*, *E. coli* attaching and effacing.

the activity in the lowest dilutions was not neutralized by the combination of this antiserum to anti-CDT.

Since CNF expression is frequently found in extraintestinal pathogenic *E. coli* (ExPEC), all isolates from the three children were assayed with the following ExPEC DNA probes: a 335-bp *Pst*I-*Cla*I DNA fragment of pEOSW1 (18) that recognizes both types of CNF; the ~6-kb *Ava*I-A fragment of pSF4000 that identifies the alpha-hemolysin (Hly) operon (25); and the 328-, the 410-, and the 750-bp fragments amplified, respectively, from the pyelonephritis-associated pili (PAP), the S-fimbrial adhesin (SFA), and the afimbrial adhesin (AFA) genes as described by Le Bouguenec et al. (14). *E. coli* J96 (3) was included as the control for the *cnf*1, *hly*, and *pap* genes, and strain S5 (20) was the control for *cnf*2. Strains HB101(pANN801-13) and KS52 were used as controls for *sfa* and *afa*, respectively (13, 19). All isolates that induced multinucleation in HeLa cells hybridized with the *cnf* probe and all *cdt*-positive, *cnf*-positive isolates hybridized with *hly*, *pap*, and *sfa* probes (Table 1).

Although CDT-producing *E. coli* strains have been isolated since the late 1980s from children with diarrhea (11, 12), the enteropathogenic role of these bacteria has not been established. In the Bangladeshi case-control study (1), CDT probe-positive strains were isolated more often from children with acute diarrhea (3.1%) than from healthy control children (0.93%), but this difference was not statistically significant. Our study also showed that such strains are not associated with acute diarrhea. In fact, the single child with diarrhea carrying CDT probe-positive isolates was also infected with an enteroinvasive *E. coli* strain, a finding of the first study after the enrollment of the children (9).

Almost all *cdt*-positive *E. coli* isolates identified in the present study also harbored *cnf* gene homologous sequences and expressed CNF1. *E. coli* strains producing this type of CNF are associated mainly with human extraintestinal infections, and *cnf*1 has been shown to be closely linked to *hly* and *pap* in the chromosome of several strains (5). All *cdt*-positive, *cnf*-positive isolates identified here also hybridized with DNA probes specific for other virulence factors (Hly, PAP, and SFA) of ExPEC (2, 14, 23) and may, therefore, be considered as potential ExPEC strains that compose, at least transitorily, the human normal intestinal flora.

Recently, Clark et al. (4) also reported the detection of both

cdt and *cnf* in *E. coli* isolates. By performing PCR with primers specific for each type of CDT genes, these authors showed that 12 (50%) of 24 *cdt*-I-positive isolates, but only 1 of 5 *cdt*-II-positive isolates, harbored sequences homologous to *cnf*1. These findings and the results obtained here with CDT-producing control strains belonging to the same serogroups of CDT-I (O86) or CDT-II (O128) prototype *E. coli* strains suggest that our *cdt*-positive, *cnf*-positive isolates may produce CDT-I. Moreover, neutralization assays performed with preparations from these control strains showed that there are antigenically distinct types of CDT.

In the Bangladeshi study (1), most CDT probe-positive strains further characterized belonged to serogroup O127 and had all of the virulence factors of enteropathogenic *E. coli* (EPEC). CDT production has also been shown in EPEC strains of serotype O86:H34 isolated in Brazil and other countries (8, 10). As shown in Table 1, none of the *cdt*-positive isolates identified in the present survey belonged to the O86, O127, or O128 serogroup or carried all of the EPEC DNA sequences, whereas all of the *cdt*-positive, *cnf*-positive isolates were of serogroups (O2 and O6) commonly identified as ExPEC (7). One single *cdt*-positive isolate (O177:NM) harbored DNA sequences of the genes encoding intimin (*eae*), an outer membrane protein involved in the attaching-effacing lesions produced by EPEC and some Shiga-toxin producing *E. coli* (STEC), a finding also reported by Clark et al. (4).

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