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# Controlled Study of Cytolethal Distending Toxin-Producing *Escherichia coli* Infections in Bangladeshi Children

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**The role of cytolethal distending toxin (CDT)-producing *Escherichia coli*, a newly described category of *E. coli*, in the causation of diarrhea was studied by screening *E. coli* isolates from 546 children <5 years of age with diarrhea and 215 matched controls without diarrhea by using a specific DNA probe. Although CDT-positive *E. coli* strains were isolated from more children with diarrhea than from healthy controls (3.1 versus 0.93%), this difference did not reach statistical significance ($P = 0.082$). All CDT-positive strains also possessed the virulence factors of enteropathogenic *E. coli* or enteroaggregative *E. coli* isolates.**

Some strains of *Escherichia coli* produce a novel toxin called cytolethal distending toxin (CDT). The toxin is so named because of the characteristic morphological changes that it produces on a variety of cell lines such as Chinese hamster ovary, HeLa, HEp-2, and Vero cells. However, the toxin does not have an effect on the Y1 adrenal tumor cell line (12). The toxin causes elongation of cells at 24 h; this is followed by progressive cellular distention and cytotoxicity for up to 120 h. This toxin is distinctly different from the other toxins of enteric bacteria described so far (12). It is a trypsin-sensitive, heat-labile (destroyed by heating at 70°C for 15 min) toxin and appears to have a molecular mass of >30,000 Da (11). CDT has also been reported to be produced by strains of *Shigella* spp. (10) and *Campylobacter* spp. (11). No study in which the epidemiological association of CDT-positive ($CDT^+$) *E. coli* with acute diarrhea was assessed has yet been reported. The lack of such data is due to the cumbersome method of the CDT assay, which requires the incubation of a tissue culture cell line with the toxin preparation for up to 120 h. Recently, however, two groups of workers have cloned and sequenced the gene encoding CDT (*cdt*) from *E. coli* (19, 23). It was found that *cdt* consists of three open reading frames (*cdtA*, *cdtB*, and *cdtC*) with 4-bp overlaps between them (19, 23). In the present study, a DNA probe constructed from cloned *cdt* was used to screen fecal *E. coli* isolates from a group of children with acute diarrhea and matched healthy controls in Bangladesh to assess the role of $CDT^+$ *E. coli* in the causation of diarrhea. This probe (see below) has previously been found to be 100% sensitive and specific for $CDT^+$ *E. coli* because all of several well-characterized, laboratory stock $CDT^+$ bacteria (identified by tissue culture assay) hybridized with the probe, whereas CDT-negative ($CDT^-$) bacteria did not (13). The further utility of an almost identical probe was demonstrated in a recent study with several enteric bacteria (18).

Children <5 years of age with acute diarrhea seen at the Clinical Research and Service Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), located in Dhaka, Bangladesh, during July 1993 to May 1994 were studied. They were part of the routine 4% surveillance sampling of all patients with diarrhea seen at the Clinical Research and Service Center (24). Controls were healthy children from the same neighborhood as the case patients. Stool samples from controls were collected within 2 weeks of the collection of samples from patients. Control children had not taken antibiotics within the previous 2 weeks.

Stool specimens were examined microscopically, assayed for diarrhea-causing viruses, and cultured for recognized enteric bacterial pathogens by standard methods practiced at ICDDR,B (1, 20, 22, 25, 26). The pathogens sought were rotavirus, *Vibrio cholerae* O1 and O139, non-O1 vibrios, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Aeromonas* spp., *Plesiomonas* spp., and enterotoxigenic *Bacteroides fragilis*. *Clostridium difficile* toxin from stool was sought by a standard tissue culture technique with Vero cells (16). The specificity of the cytopathic effect was verified by neutralization with *Clostridium sordelli* antitoxin (Wellcome Research Laboratories, Beckenham, United Kingdom). Diarrheagenic strains of *E. coli* (enterotoxigenic, enteropathogenic, enteroaggregative, enteroinvasive, enterohemorrhagic, and diffuse adherent) were identified with DNA probes (2). As part of the identification of diarrheagenic *E. coli* isolates, three different *E. coli* colonies from MacConkey plates inoculated with each stool specimen were tested. One set of colony blots on Hybond-N filters (Amersham International plc, Aylesbury, United Kingdom) were hybridized with a DNA probe specific for the *cdt* gene. The DNA probe was derived from the plasmid pCVD448, which contains the complete cloned *cdt* gene (2,305 bp) from the wild-type strain *E. coli* E6468/62 (serotype O86:H34) (23). Digestion of the plasmid with the restriction enzyme *Acc*I yielded a 1,375-bp probe which contained the 3′ half of *cdtA*, all of *cdtB*, the 5′ end of *cdtC*, and no vector sequences (23). The probe was labelled by random priming (8) with [$\alpha$-$^{32}$P]dCTP (3,000 Ci per mmol; Amersham International) and a random primer labelling kit (Bethesda Research Laboratories, Bethesda, Md.). Colony blots were processed and hybridized under stringent conditions as described previously (2). *E. coli* E6468/62 was used as a positive control for the *cdt* gene. To study CDT production, bacteria were grown in the medium of Evans et al. (7) as stationary cultures at 37°C for 48 h. The supernatants obtained after filtration through 0.22-$\mu$m-pore-size membrane filters (Sartorius, Gotengen, Germany) were tested in HEp-2

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TABLE 1. Isolation of CDT$^+$ *E. coli* isolates from children with diarrhea and healthy controls

| Age (mo) | No. of children with CDT$^+$ *E. coli*/ total no. (%) | | *P* value |
|---|---|---|---|
| | Patients | Controls | |
| 0–12 | 15/334 (4.5) | 1/98 (1.0) | 0.136 |
| 13–24 | 1/124 (0.8) | 1/83 (1.2) | 1.00 |
| 25–26 | 1/39 (2.6) | 0/14 (0) | 1.00 |
| 37–48 | 0/36 (0) | 0/13 (0) | NA[a] |
| 49–60 | 0/13 (0) | 0/7 (0) | NA |
| All | 17/546 (3.1) | 2/215 (0.93) | 0.082 |

[a] NA, statistical test not applicable.

cell monolayers. The changes in the morphologies of the cells were recorded every 24 h for 5 days as described previously (12). The pattern of adherence of the *E. coli* cells to the HEp-2 cell monolayer was tested by the method of Nataro et al. (17). The fluorescent actin staining assay for the attaching-and-effacing lesion of *E. coli* was carried out with HEp-2 cells by the method of Knutton et al. (14). All assays were carried out with appropriate positive and negative controls. *E. coli* isolates were serotyped with antisera to all of the accepted O antigens from O1 to O173 and all of the accepted H antigens from H1 to H56 as described previously (3, 6). Chi-square and Fisher's exact tests were used to compare the differences between the groups.

The rates of isolation of *cdt* probe-positive *E. coli* from patients and controls are given in Table 1. Overall, CDT$^+$ *E. coli* isolates were isolated from 17 (3.1%) patients and 2 (0.93%) controls. Although CDT$^+$ isolates were frequently isolated from patients, the difference did not reach statistical significance ($P = 0.082$). Analysis of data by age stratum also did not show significant differences. Most of the CDT$^+$ *E. coli* isolates were isolated from individuals in their first year of life. Of the 15 patients in their first year of life positive for CDT$^+$ *E. coli*, copathogens were isolated from 7 of them (5 had a *Campylobacter* sp. coinfection and 2 had a rotavirus coinfection). Both of the patients beyond 12 months of age positive for CDT$^+$ *E. coli* had copathogens (one was infected with *Shigella flexneri* isolate and the other was infected with an enterotoxigenic *Bacteroides fragilis* isolate).

The clinical symptoms of eight patients who had CDT$^+$ *E. coli* as the only pathogen are listed in Table 2. All patients had watery diarrhea, and half of them also had vomiting. In a previous study (5), when CDT$^+$ *E. coli* isolates were inoculated into adult rabbit ileal loops, histopathology showed lesions typical of an enterotoxin-like effect, with some inflammatory infiltrates in the submucosa and proliferation of the epithelium, although no fluid accumulation in the loops was noticed. However, in another study (12) in which stored *E. coli* isolates were screened for CDT production, the CDT$^+$ isolates originated from patients with a variety of syndromes including watery diarrhea, invasive diarrhea, persistent diarrhea, septicemia, and hemolytic-uremic syndrome. On the basis of the observations presented above and since established diarrheagenic bacteria such as *Shigella* spp. and *Campylobacter* spp. also product CDT, it is believed that CDT is diarrheagenic. It is not known whether CDT is produced in vivo in sufficient quantities. Future studies should aim at assaying for CDT in intestinal secretions or stool specimens.

TABLE 2. Clinical findings in eight children with only CDT$^+$ *E. coli* from stool

| Parameter | No. of children positive[a] |
|---|---|
| Watery diarrhea | 8 |
| Blood in stool | 0 |
| Leukocytes in stool[b] | 4 |
| Vomiting | 4 |
| Abdominal pain | 3 |
| Fever[c] | 1 |
| Dehydration[d] | 1 |

[a] The mean number of stools per day was 10 (range, 6 to 20). The mean duration of diarrhea before hospitalization was 8.1 days (range, 1 to 30 days).
[b] More than 10 leukocytes in a high-power field of a microscope.
[c] One child had a temperature of 38.8°C.
[d] One child had moderate dehydration by standard criteria (24).

Of the 17 patients positive for CDT$^+$ *E. coli*, three of three colonies tested from each of 13 patients were probe positive, two of three colonies from 2 patients were probe positive, and one of three colonies from 2 patients were probe positive. In the two controls positive for CDT$^+$ *E. coli*, all three colonies were probe positive.

*E. coli* colonies from 13 patients were available for serotyping, and single colonies from 11 patients were serotyped. With one exception, colonies from all of these patients belonged to serotype O90/127:H$^-$, which is essentially an enteropathogenic *E. coli* serotype; the *E. coli* colony from the other patient belonged to serotype OR:H$^-$. The dual serogroup designation of O90/127:H$^-$ meant that the strains reacted strongly to both antisera at the same titer. These two serogroups show very strong cross-reactivity because they are structurally related (21). Two colonies from each of two patients were serotyped. Both colonies from one patient belonged to serotype O33:H$^-$, and one colony from the other patient belonged to serotype O90/127:H$^-$ and the other colony belonged to serotype O106:H18. Single colonies from both of the controls positive for CDT$^+$ *E. coli* were serotyped and were found to belong to serotype O90/127:H$^-$. In previous studies (4, 5, 12), many *E. coli* isolates belonging to enteropathogenic *E. coli* serotypes or serogroups (including O127) were found to produce CDT. It has been reported that *E. coli* isolates with the H18 antigen belong to the enteroaggregative type of *E. coli* (EAggEC) (15). It is interesting that the *E. coli* serotype O106:H18 positive for CDT in our study hybridized with the EAggEC probe and adhered in an aggregative fashion in the HEp-2 cell adherence assay. Previous studies have reported CDT production by *E. coli* isolates that also produce heat-stable enterotoxin, heat-labile enterotoxin, or verotoxin (4, 12). This is the first report of CDT production by an EAggEC isolate. All 17 colonies from patients and controls that were serotyped were also tested for CDT production by the HEp-2 cell assay, and all 17 were positive, thereby confirming the DNA probe results. Also, all 17 colonies except 1, which was EAggEC, hybridized with the enteropathogenic *E. coli* adherence factor probe, which indicates positivity for localized adherence in the tissue culture adherence assay. As expected, these 16 colonies were positive for localized adherence and fluorescent actin staining by using HEp-2 cells. Therefore, these data suggest that the 16 colonies belonged to enteropathogenic *E. coli* (EPEC).

This is the first reported study that examined the association of CDT$^+$ *E. coli* with diarrhea. The results suggest that CDT$^+$ *E. coli* isolates are not associated with diarrhea, at least in Bangladesh. However, studies in other locations must be carried out to confirm or disprove our findings. We have found that CDT production is mostly confined to EPEC isolates of serotype O127:H$^-$. It may be that in certain strains of EPEC (and also of EAggEC), CDT may act as an additional virulence factor of diarrhea. The diarrheal syndrome in patients in whom CDT$^+$ *E. coli* was the sole pathogen (Table 2) was also similar

to that caused by EPEC (9). It is likely that CDT production per se in otherwise nonpathogenic strains may not confer a diarrheagenic property on the strains.

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