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Detection of *Escherichia coli* Enterotoxins in Stools

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We determined whether enterotoxigenic *Escherichia coli* diarrhea could be diagnosed by direct examination of stools for heat-labile (LT) and heat-stable (ST) enterotoxins. The Y-1 adrenal cell and an enzyme-linked immunosorbent assay (ELISA) detected LT in 85 and 93%, respectively, of stool specimens obtained from adults with acute diarrhea from whom an LT- and ST-producing organism had been isolated. Furthermore, the ELISA assay detected LT in 8 of 35 stool specimens from which no LT-producing *E. coli* had been isolated. The infant mouse assay was utilized to detect ST in these stool specimens and was found to be an insensitive method, showing positive results in only 36% of the specimens from which an ST-producing organism was isolated. Further studies are warranted to determine the diagnostic value of direct detection of LT in stools, especially by the ELISA method.

Enterotoxigenic *Escherichia coli* (ETEC) are now a well-recognized cause of acute dehydrating diarrhea worldwide (23). These organisms produce diarrhea by elaborating heat-labile (LT) and heat-stable (ST) enterotoxins. Some strains produce both LT and ST, while others elaborate only one of the enterotoxins. LT is believed to have a molecular weight of ca. 72,000 (16) and is immunologically and structurally similar to cholera enterotoxin (25). A number of STs have been described with a much smaller molecular weight ranging from 1,800 to 5,100 (1, 11).

ETEC cannot be differentiated from non-ETEC by conventional enteric isolation procedures, and their identification has usually involved the selection of *E. coli* colonies or pools of these colonies from MacConkey agar plates and the testing of these colonies for LT and ST production. Production of LT has been usually measured either by bioassay systems such as rabbit ileal loop preparations (23) or by tissue culture (13, 22) or, more recently, by immunological assays such as passive immune hemolysis (10), staphylococcal coagglutination (3), radioimmunoassay (12), and an enzyme-linked immunosorbent assay (ELISA) (27). The major test that has been used for detection of ST has been the infant mouse assay (7). All of these procedures require that *E. coli* be isolated before toxin testing is done, thus causing a delay in diagnosis. Also, these procedures are restricted by the number of colonies that can be conveniently tested so that an ETEC present in stools may not be detected if the appropriate colony is not selected for testing.

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Because of these constraints in the ability to diagnose ETEC diarrhea, we examined the possibility of detecting LT and ST directly in stool specimens obtained from adults with acute diarrhea. For LT detection we examined the capabilities of two assay systems, the Y-1 adrenal cell (YAC) and ELISA, and for ST detection we assessed the appropriateness of the infant mouse assay.

MATERIALS AND METHODS

Specimens. Stool specimens were obtained by rectal catheter from two groups of patients admitted to the Cholera Research Laboratory Hospital, Dacca, Bangladesh (now the International Centre for Diarrhoeal Disease Research, Bangladesh) who had clinical signs of significant dehydration, (i.e., loss of ≥5% of their body weight) due to acute watery diarrhea of less than 48 h duration. Group I cases consisted of 117 adult males (over age 10) who were included in a study in 1976 of tetracycline as treatment for ETEC diarrhea (19). Group II cases included 64 male and female patients who ranged in age from 4 months to 75 years and who were part of a 1-year clinical study of acute diarrhea causing significant dehydration in 1977-1978. A portion of each stool specimen was promptly studied microbiologically, and the remainder was frozen immediately and stored at −20°C until testing.

Microbiological studies. All stools were examined for *Shigella*, *Salmonella*, *Vibrio*, and rotavirus as previously described (18). Lactose-positive colonies with typical *E. coli* morphology were selected for

testing for LT production by the Chinese hamster ovary (CHO) cell assay and for ST production by the infant mouse assay as previously described (18). For specimens from group I patients, 10 individual colonies and pools were tested. For specimens from group II patients, only two individual colonies were tested, as this was found to detect 95% of this type of patient identified by the testing of 10 colonies and pools (18). Enterotoxin-producing organisms were confirmed as *E. coli* (17).

Direct toxin studies. Stools from group I cases were assayed directly for LT by the YAC assay and for ST by the infant mouse assay. Stools from group II cases were assayed directly only for LT by using ELISA.

YAC assay. Frozen stool specimens from group I cases were thawed rapidly in cold water and centrifuged at 6,000 × *g* for 30 min at 4°C. The supernatants were placed immediately in an ice bath where they remained for 15 to 30 min while being inoculated in triplicate into the standard YAC assay used for identification of LT (22). Aliquots of supernatants were also preincubated with equal volumes of a 1/10 dilution of cholera antitoxin (Swiss Serum Vaccine Institute) and tested in triplicate as described above. The supernatants were in contact with the adrenal cells for 10 to 15 min and then removed. The cells were then rinsed with phosphate-buffered saline (pH 7.4), and fresh media were added. Morphological changes in the adrenal cells were read at 6 h. The few specimens in which cytotoxicity was observed were considered negative.

ELISA. (i) Reagents. Burro anti-cholera toxin was obtained from John Robbins, Bureau of Biologics, Food and Drug Administration, Bethesda, Md. Guinea pig anti-cholera toxin was prepared by immunization with purified cholera toxin (Schwartz/Mann), as previously described (12, 27), and further purified by affinity isolation (5). Five milligrams of purified cholera toxin diluted in 0.1 M carbonated 0.5 M NaCl buffer (pH 8.5), was coupled to 1 g of CNBr-activated Sepharose 4B (Pharmacia) which had been packed into a column (7 by 200 ml). After an overnight incubation at 4°C, the remaining active groups were blocked with 1 M glycine, and the column was washed extensively with acetate buffer (pH 4) and borate buffer (pH 8.5). One milliliter of guinea pig anti-cholera toxin was added to the column and absorbed for 15 min at room temperature. The column was then washed extensively with phosphate-buffered saline (pH 7.4). The anti-cholera toxin was eluted with glycine-hydrochloride buffer (pH 2.8). The eluted antibody was neutralized in this buffer before it was used for ELISA.

Fetal calf serum and adult horse serum were obtained from commercial sources (GIBCO Laboratories, Grand Island, N.Y.). Alkaline phosphatase-labeled goat anti-guinea pig immunoglobulin G (IgG) was prepared as previously described (27).

(ii) Assay. Frozen specimens from group II cases were thawed and assayed for LT by an ELISA system which utilized antibodies to immunologically related cholera toxin as previously described (6, 27). The optimal dilutions of reagents were determined by checkerboard titration. Approximately 0.5 ml of stool specimen was diluted with 4.5 ml of phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20, 1% fetal calf serum, and 1% horse serum (PBS-TFH). The suspension was clarified by centrifugation at 110 × *g* for 30 min, and 100 μl of supernatant was added to triplicate wells of polyvinyl microtitration plate (Dynatech 220-24) which had been previously coated with burro anti-cholera toxin. The specimens were incubated for 2 h at 37°C and washed with PBS-TFH. Optimally diluted guinea pig anti-cholera toxin was added, and the plate was incubated for 1 h at 37°C. After another washing with PBS-TFH, optimally diluted alkaline-phosphate-labeled goat anti-guinea pig IgG was added and incubated for 1 h at 37°C. After another washing a solution of *p*-nitrophenyl phosphate (Sigma 104) diluted in 1 mg/ml in 1 M diethanolamine buffer (pH 9.8) was added, and the intensity of yellow caused by the reaction of alkaline phosphatase bound in the previous step with the substrate was measured by a spectrophotometer capable of determining optical density directly in the wells of a microtiter plate.

Five stool specimens negative for LT obtained from patients living in Bangladesh were included in each assay as negative controls. A positive-to-negative (P/N) ratio was computed for each specimen by dividing the mean optical density of the test specimen by the mean optical density of the negative controls. A specimen was considered to be positive if it yielded a P/N ratio of greater than 2.0.

Positive specimens were confirmed by a blocking assay with acute- and convalescent-phase antisera directed against LT. A 50-μl portion of stool suspension was incubated in triplicate for 2 h at 37°C with an equal volume of 1:10 dilution of preinfection and convalescent-phase sera obtained from a human volunteer with LT-induced gastroenteritis. The stool antiserum mixture was transferred to a coated well of microtiter plate and processed as described above.

A specimen was considered to contain LT if the mean value after neutralization with the convalescent-phase serum was 2 standard deviations less than the mean value after preincubation of the same specimen with preinfection-phase serum.

Infant mouse assay. A total of 101 stool supernatants from group I cases prepared for the YAC assay were tested for ST production by the standard infant mouse assay used for detecting ST in culture supernatants (7). Each specimen was tested in three mice; an average ratio of intestinal weight to remaining body weight of ≧0.083 was considered positive.

To determine the heat stability of the toxin activity, we heated stool supernatants from 19 specimens for 15 min at 100°C before inoculation into the mice.

RESULTS

LT testing. The YAC assay was positive for LT in stools from 85% of group I cases from which LT- and ST-producing *E. coli* had been isolated, 77% of stools from which *Vibrio cholerae* had been isolated, and 1 of 5 (20%) stools from which LT *E. coli* had been isolated (Table 1). The positivity rate in the LT- and ST-producing *E. coli* cases was significantly greater than in the LT-producing *E. coli* cases ($P <$

0.001). All the positive tests were confirmed by blocking with anti-cholera toxin sera. The assay was not positive in stools from which either ST-producing *E. coli* or no pathogens were isolated. The results of the YAC assay and microbiological tests were concordant for 95% of the specimens.

The ELISA was positive for LT in stools from 93% of group II cases from which an LT- and ST-producing *E. coli* had been isolated, 29% from which an ST-producing *E. coli* had been isolated, and 19% from which no pathogen had been isolated. All the positive tests were confirmed by blocking with anti-LT serum. The results of the ELISA and the microbiological tests were concordant for 84% of the specimens. There were two specimens that were positive for LT by microbiological testing only and eight that were only positive by the direct examination of stools by ELISA (Fig. 1).

The probability of detecting LT by direct assay of stools was not significantly different than that of isolating an LT- and ST-producing *E. coli* from either group of subjects by matched sample analysis (4) of the subjects harboring LT- and ST-producing *E. coli* and ST-producing *E. coli*, and the subjects from whom no pathogen had been isolated.

ST testing. Positive results were found for 44% of the specimens from which LT- and ST-producing *E. coli* had been isolated and for 24% of the specimens from which ST-producing *E. coli* had been isolated (Table 2). Positive results were also found for two specimens from which both *Shigella flexneri* and rotavirus had been found and for four specimens (20%) in which no pathogen had been found.

TABLE 1. *Detection of LT by YAC assay and ELISA in stools of adults with acute diarrhea*

Microbiological diagnosis[a]	Group I No. tested	Group I Positive YAC No.	Group I Positive YAC %	Group II No. tested	Group II Positive ELISA No.	Group II Positive ELISA %
ETEC[b]						
LT and ST	33	28	85[c]	28	26	93
ST	21	0	0	14	4	29
LT	5	1	20[c]	0	—[d]	—
V. cholerae	13	11	77	0	—	—
Others[e]	16	0	0	0	—	—
No pathogen	29	0	0	21	5	19

[a] In group I, 10 colonies plus a pool of 10 were examined from each subject; in group II, 2 colonies only were examined.

[b] Seven group I and one group II ETEC subjects were also infected with a second pathogen: four were infected with rotavirus, three were infected with *V. cholerae* non-O1, and one was infected with *Shigella*.

[c] $P < 0.001$, Fisher's two-tailed exact test.

[d] —, No patients in this category.

[e] Includes subjects infected with *Shigella* (5), *V. cholerae* non-O1 (4), *V. parahaemolyticus* (3), *Shigella* and *V. cholerae* non-O1 (2), and *Shigella* and rotavirus (2).

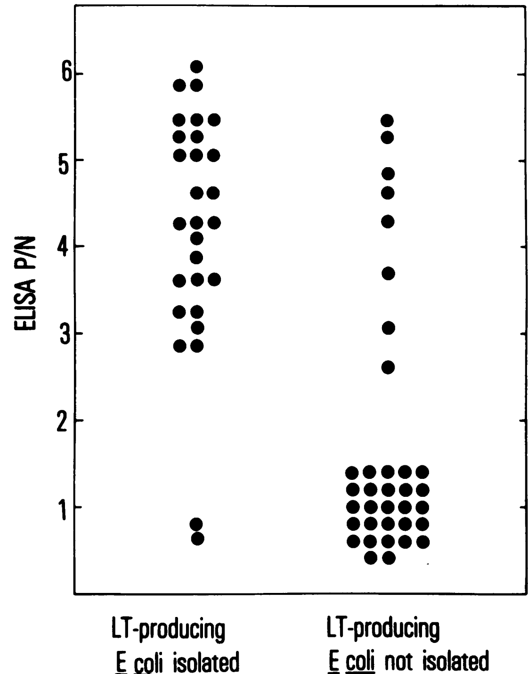

FIG. 1. *Detection of LT in stool specimens by ELISA.*

In all 19 of the ST-positive specimens tested for heat stability of the ST activity, complete loss of enterotoxicity was observed after heating at 100°C for 15 min (Table 3).

DISCUSSION

Previous studies in animals (8, 26) and humans (9) that involved a small number of specimens demonstrated that LT and ST could be detected in feces, thus suggesting that both toxins are produced in vivo. Our particular interest in this study was to assess whether direct examination of stools for LT and ST was a feasible means to diagnose ETEC diarrhea. We thus thought it best to initially study specimens from adults with significant dehydration who would be presumably more heavily colonized with ETEC in their small intestine and perhaps have more toxin in their stools.

The YAC was used by Echeverria et al. (9) to detect LT in the stools of two of three humans infected with LT-producing ETEC. In the specimens from our group I cases, the YAC assay was positive for LT for 85% of the specimens

TABLE 2. *Detection of ST in stools of group I cases*

Microbiological diagnosis[a]	No. tested	Positive	
		No.	%
ETEC[b]			
LT and ST	32	14	44[c]
ST	21	5	24[c]
LT	5	0	0
V. cholerae	13	0	0
Others[d]	10	2	20
No pathogen	20	4	20

[a] See text for microbiological methods used.

[b] Five ETEC cases were infected with a second pathogen: three were infected with rotavirus and two were infected with *V. cholera* non-O1.

[c] $\chi^2 = 2.19$; P = not significant.

[d] Includes cases infected with *V. parahaemolyticus* (3), *V. cholerae* non-O1 (2), *Shigella* (2), *Shigella* and rotavirus (2), and *V. cholerae* non-O1 (1). The two positive specimens were those containing *Shigella* and rotavirus.

TABLE 3. *Effect of heating[a] on enterotoxic activity of ST in stool supernatants from group 1 patients*

Microbiological diagnosis	Specimen no.	GW/BW[b]	
		Before heating	After heating
LT- and ST-producing *E. coli*	1	0.150	0.069
	2	0.105	0.053
	3	0.141	0.060
	4	0.120	0.052
	5	0.124	0.063
	6	0.098	0.057
	7	0.107	0.050
	8	0.103	0.048
	9	0.094	0.046
	10	0.107	0.047
	11	0.102	0.047
	12	0.116	0.058
ST-producing *E. coli*	1	0.102	0.053
	2	0.090	0.055
	3	0.106	0.064
	4	0.109	0.066
No diagnosis	1	0.110	0.059
	2	0.128	0.054
Shigella and rotavirus	1	0.132	0.062

[a] Specimens were heated for 15 min at 100°C.

[b] Gut-to-remaining body weight ratio of infant mouse 4 h after inoculation; each value represents mean of three mice.

from which an LT- and ST-producing *E. coli* was isolated and had 95% concordance with the microbiological results. However, it was only positive for one of five subjects from whom an LT-producing *E. coli* had been isolated. This latter observation may have been due to a lesser amount of LT from strains producing only LT compared to those producing both LT and ST; however, further studies using a large number of ETEC cases are needed to confirm and explain this observation. That the YAC assay was detecting LT was supported by the findings that all the LT-positive results were neutralized by cholera antitoxin and that an LT-producing *E. coli* was isolated from all the specimens shown to have LT activity. We have also found that much of the LT activity could be destroyed by exposure of the specimens to room temperature for 45 to 60 min (unpublished data). The assay was also positive for specimens from 11 of 13 cholera patients, which is similar to results reported by Echeverria et al. (9).

The ELISA was positive for LT in specimens from 93% of group II subjects from whom an LT-producing *E. coli* had been isolated and in an additional eight specimens from which no LT-producing *E. coli* had been isolated. It is unlikely that the discrepancy in the results of these eight specimens was due to differences in sensitivity of the procedures since the P/N ELISA values were identical for the specimens from which LT- and ST-producing *E. coli* were and were not isolated. It is similarly unlikely that false-positive ELISA reactions occurred due to the presence of an antigen unrelated to LT since the LT activity was neutralized by postinfectious serum from a volunteer with LT-induced disease. However, we cannot exclude the possibility that these positive reactions could be attributed to the presence of too few LT-producing *E. coli* to detect one microbiologically by the isolation procedures used; the production of an immunologically and biologically similar toxin by an organism other than ETEC; the production in vivo of an immunologically active, biologically inactive molecule; or the production of LT in vivo and not in vitro under culture conditions. This latter possibility might apply especially to the four specimens from which ST-producing *E. coli* were isolated. Further studies of enterotoxin production in vivo and in vitro are necessary to resolve these issues.

The failure to detect LT by ELISA in two specimens and by the YAC assay in five specimens from which an LT- and ST-producing *E. coli* had been isolated might be attributed to the production of toxin in vitro but not in vivo; the loss of toxin activity during freezing and storing; or the fact that *E. coli* may have been present in the stools for a longer period of time than was the toxin.

Testing for ST directly in stools was less successful. ST was present in only 44 and 24% of specimens, respectively, of group I cases from

which LT- and ST-producing *E. coli* and ST-producing *E. coli* had been isolated. This suggests that the infant mouse assay may be too insensitive for detection of ST in stools. ST-positive tests occurred for specimens containing *S. flexneri* and for specimens from which no pathogen was isolated; this may have been due in a few cases to the production of an ST by *S. flexneri* or by other pathogens we did not look for, such as *Yersinia enterocolitica* (20). Our finding that the ST-positive specimens lost their activity after 15 min of heating at 100°C confirmed the previous observations of Whipp et al. (26) and corroborated the finding of Jacks and Wu (14) that ST activity was inactivated by heating at 100°C when it was measured by the mouse bioassay, which is in contrast to results of studies in which ST appeared to be more heat stabile when measured in ligated intestinal segments of rabbit and pig small intestines (2, 21, 24).

In summary, we have found that LT can be readily detected in the stools of patients with relatively severe ETEC diarrhea by using the YAC assay or ELISA; the value of these tests as diagnostic and epidemiological tools, however, needs to be further assessed in persons with milder illnesses. In contrast, the detection of ST in stools of these persons by using the infant mouse assay was found to be a relatively insensitive diagnostic method.

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