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Comparison of Methods to Detect *Escherichia coli* Heat-Labile Enterotoxin in Stool and Cell-Free Culture Supernatants

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We compared the standard Y-1 adrenal cell (YAC) assay for heat-labile enterotoxin (LT) with newer rapid and economical immunological methods of detection. Stool samples were collected from 164 acutely ill American students in Guadalajara, Mexico. Supernatants were prepared from each stool. Stools were cultured for *Escherichia coli* by standard techniques. Individual *E. coli*-like colonies were examined for LT production by the Biken assay. Culture supernatants and stool supernatants were assayed for the presence of LT by the YAC assay, counterimmunoelectrophoresis, and enzyme-linked immunosorbent assay (ELISA). Standard YAC assays of culture supernatants revealed that 40 of the 164 specimens (24%) were LT positive. Counterimmunoelectrophoresis detected 60% and ELISA detected 65% of the 40 known positives when culture supernatants were used. The Biken assay detected 35% of the 40 known positives. With stool supernatants, the YAC assay detected only 18% of the known positives, counterimmunoelectrophoresis detected 60%, and ELISA detected 90%. In addition, ELISA detected 13 LT-positive stool supernatants not detected by the YAC assay of culture supernatants. The ELISA in which stool supernatants are used may be a useful method to detect LT.

Enterotoxigenic (ET) *Escherichia coli* is a well-recognized etiological agent in acute gastroenteritis (6, 11, 18). ET *E. coli* has been shown to be responsible for approximately one-half of the cases of travelers' diarrhea (7, 15) and produces a noninvasive, highly secretory diarrhea by elaboration of a heat-labile enterotoxin (LT) or heat-stable enterotoxin or both. LT, a large protein with an approximate molecular weight of 91,000 (2), is a homogeneous, highly immunogenic molecule which closely resembles cholera toxin in structure and activity and which binds to the monosialoganglioside residues of intestinal epithelial cells and causes fluid secretion by stimulating adenyl cyclase activity.

Enterotoxin activity is classically detected by fluid accumulation in ligated rabbit ileal loops (3, 4). A more convenient way of detecting LT is the Y-1 adrenal cell (YAC) assay (5). Although the YAC assay is an improvement over the expensive and time-consuming rabbit loop assays, it is not ideal. The immunogenic nature of LT makes it detectable by methods such as agar diffusion, counterimmunoelectrophoresis (CIE), and enzyme-linked immunosorbent assay (ELISA). We compared these methods of LT detection, using both culture supernatants and stool supernatants.

MATERIALS AND METHODS

Specimens. Stool samples were collected in Guadalajara, Mexico, from 164 American students with acute gastroenteritis. Criteria for acceptance of a patient into the study were four or more unformed stools within a 24-h period or three or more unformed stools within an 8-h period with an additional symptom of gastroenteritis (nausea, vomiting, cramps, or fever). Samples were collected within 48 h of the onset of symptoms. To be eligible for participation in our study, students could not have received antibiotics within the previous week. Portions of each sample were used for microbiological screening (Fig. 1) and frozen at −70°C for storage. The remainder was diluted 1:10 with sterile phosphate-buffered saline (PBS) (pH 7.4; Difco Laboratories, Detroit, Mich.).

Preparation of stool supernatants. The diluted stool sample was mixed well and then centrifuged at 400 × *g* for 15 min. All stool supernatants were collected, frozen, and then used in the CIE and ELISA assays (Fig. 1). Supernatants were sterilized by passage through a 0.45-μm (pore size) Acrodisc filter (Gelman Sciences, Ann Arbor, Mich.) and used in the YAC assay (Fig. 1). Where indicated, sterile supernatants were concentrated 10 times with B15 Minicon concentrators (Amicon Corp., Lexington, Mass.). All assays were run simultaneously and in triplicate on each of the 164 prepared stool supernatants.

Microbiological studies. Five lactose-positive *E. coli*-like colonies were selected from primary MacConkey plates from each patient's stool specimen. The colo-

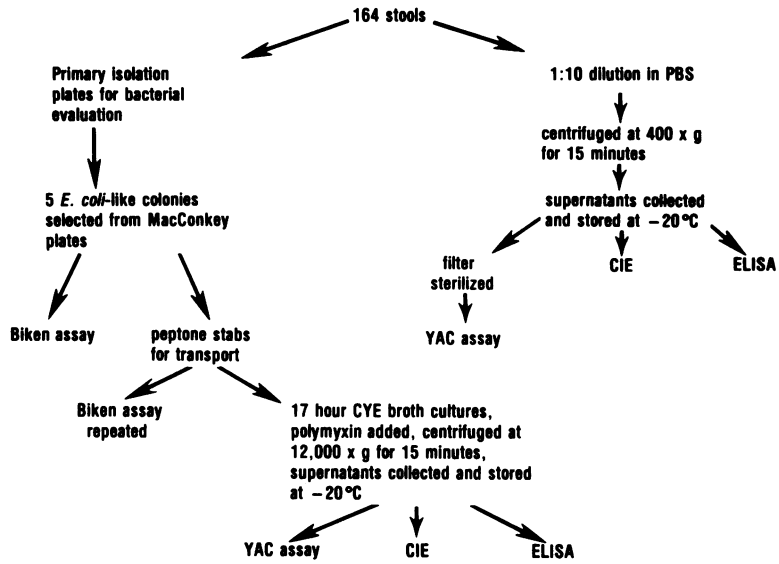

FIG. 1. Diagramatic scheme of processing of stool samples, including preparation of culture supernatants and stool supernatants and the assays performed with each.

nies were subcultured onto Biken agar (14) for detection of LT and into peptone stabs for transport to our laboratory in Houston, Tex. (Fig. 1).

Preparation of cell-free culture supernatants. Cell-free culture supernatants were prepared as described by Evans et al. (8, 9). The individual peptone stabs from each patient were cultured separately in 10 ml of Casamino Acids-yeast extract broth (8) for 17 h at 37°C in a shaker incubator. Aeration was provided by shaking at 200 rpm. Polymyxin B sulfate (Burroughs Wellcome Co., Greenville, N.C.), diluted in PBS (pH 6.6) to a final concentration of 10,000 U/ml, was added to each flask in 1-ml volumes and allowed to incubate for an additional 10 min. Culture supernatants were collected after centrifugation at 12,000 × *g* for 15 min (Fig. 1). The individual culture supernatants from each patient were pooled and tested as pools in all assays. Simultaneously, the peptone stab cultures were inoculated onto Biken agar for detection of LT. Culture supernatants prepared from strains 47A (LT producing) and HS (nontoxigenic), kindly supplied by Doyle Evans, University of Texas Medical School, Houston, were used as positive and negative controls in all assays.

Antiserum preparation. Human LT, purified as described by Clements and Finkelstein (1), was kindly supplied by Richard Finkelstein, University of Missouri Medical School, Columbia. Female albino rabbits were inoculated intramuscularly with 2 ml of 50-μg/ml LT in complete Freund adjuvant (Difco). After 14 days, the animals were inoculated with 2 ml of 50-μg/ml LT in incomplete Freund adjuvant (Difco). Two weeks later, the rabbits were inoculated with 2 ml of 50-μg/ml LT in PBS. After an additional 2 weeks, the rabbits were exsanguinated. The sera were pooled and frozen at −20°C after heat inactivation of complement (56°C for 30 min). Antitoxin activity was determined by neutralization of LT activity in the YAC assay. Preimmune (normal) rabbit serum showed no neutralizing activity at any concentration. Pooled hyperimmune rabbit serum showed neutralizing activity at a dilution of 1:500.

Standard toxin assays. Culture supernatants and sterile stool supernatants were used for detection of LT by the accepted YAC assay (5). A 100-μl volume of culture supernatants or sterile stool supernatants was added to YAC monolayers. After an 18-h incubation period, the monolayers were examined for percent rounding of cells (>20% was considered positive). Neutralization of LT activity was performed to confirm all LT positives. In these assays, 100 μl of positive culture supernatant or sterile stool supernatants was allowed to incubate with YAC monolayers for 15 min. Culture medium was removed and replaced by a 1:200 dilution of anti-LT serum. Neutralization of the cytopathic rounding effect was read at 18 h.

Biken assay. Detection of LT was performed by an Elek test first described by Honda et al. (13, 14). The five *E. coli*-like colonies selected from primary MacConkey plates in Guadalajara and from peptone stabs in Houston were inoculated onto Biken agar plates. After 48 h of incubation at 37°C, polymyxin disks (PB50; BBL Microbiology Systems, Cockeysville, Md.) were added to each colony. After an additional 4 h of incubation, 20 μl of anti-LT serum (diluted 1:2) was added to a center well cut into the agar. The plates were incubated for 24 h and examined for visible precipitin lines with an illuminator. Negatives were stored overnight at 4°C and reexamined for precipitin lines.

CIE. Culture supernatants and stool supernatants were subjected to CIE for LT detection. Wells in CIE plates (Hyland Diagnostics, Detroit, Mich.) were filled with 10 μl of culture supernatant or stool supernatant and 10 μl of anti-LT serum (diluted 1:2, 1:4, and 1:8), according to the manufacturer's instructions. Gels were run with a barbitol buffer (pH 8.6; Hyland Diagnostics), at 40 mA for 60 min on a Hyland

electrophoresis unit. Plates were examined for visible precipitin lines with an illuminator. All plates were stored overnight at 4°C and reexamined for precipitin lines.

ELISA. Culture supernatants and stool supernatants were examined for the presence of LT by an ELISA modified from Gustafsson and Möllby (12). Immulon II microtiter plates (Dynatech Laboratories, Alexandria, Va.) were coated overnight at 22°C with 200 μl of 1.5 mM monosialoganglioside (GM1; Supelco, Inc., Houston, Tex.) in PBS. Remaining binding sites were blocked by incubation at 37°C for 60 min with 200 μl of 1% bovine serum albumin in PBS. Culture supernatants or stool supernatants were added in 100-μl volumes and allowed to bind overnight at 4°C. Anti-LT serum was then added in 100-μl volumes. Binding was allowed to occur at 22°C for 2 h. Goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was added in 100-μl volumes. After 60 min of incubation at 37°C, 200 μl of *p*-nitrophenyl phosphate (5 mg/ml; Sigma) diluted in a carbonate-bicarbonate buffer (pH 9.6) was added to visualize the reaction that occurred. Plates which contained culture supernatants were incubated for 90 min at 37°C. The reaction occurred more quickly with stool supernatants. These plates were incubated for an average of 45 min at 37°C. The reaction was stopped by the addition of 40 μl of 3 N NaOH. The optical density was read with a Mini-ELISA reader (Dynatech) with a 410 filter. Positive and negative culture supernatant controls were run each time. A positive/negative optical density ratio of >1.5 was considered to be positive.

RESULTS

LT detection by YAC assay. The presence of LT in sterile culture supernatants and stool supernatants was determined with YAC monolayers. Pooled culture supernatants from 40 of 164 (24%) stool samples produced LT (see Tables 1 and 2). These results were then compared with those obtained with the corresponding stool supernatants in the other assays. LT was detected in only 7 of the 40 (18%) stool supernatants from patients with known ET *E. coli* by YAC assay of culture supernatants. Of the stool supernatants from patients with positive pooled culture supernatants (but negative stool supernatants) in the YAC assay, 16 were concentrated 10 times. The concentrated stool supernatants were also negative in the YAC assay, indicating that concentration did not improve the sensitivity of the YAC assay of stool supernatants.

TABLE 1. Detection of LT by Biken assay of individual *E. coli* colonies and by CIE and ELISA of culture supernatants compared with YAC assay of culture supernatants

Result by YAC assay of culture supernatants (no. of specimens)	No. (%) positive for LT by:		
	Biken assay	CIE of culture supernatants	ELISA of culture supernatants
LT positive (40)	14 (35)	24 (60)	26 (65)
LT negative (124)	0	0	0

TABLE 2. Direct detection of LT in stool supernatants by YAC assay, CIE, and ELISA compared with YAC assay of culture supernatants

Result by YAC assay of culture supernatants (no. of specimens)	No. (%) positive for LT by:		
	YAC assay of stool supernatants	CIE of stool supernatants	ELISA of stool supernatants
LT positive (40)	7 (18)	24 (60)	36 (90)
LT negative (124)	0	0	13[a] (10)

[a] These 13 samples included 8 in which no other etiological agent could be found. From the remaining five samples, two *Entamoeba histolytica* strains, two *Salmonella enteritidis* strains, and one *Shigella sonnei* strain were identified.

LT detection by Biken assay. The Biken assay was performed when the individual five stabs for each patient were inoculated. This assay was performed a second time when the peptone stabs were inoculated into the Casamino Acids-yeast extract broth to prepare culture supernatants. In both cases, 14 of the 40 (35%) known YAC-positive specimens were found to produce LT by the Biken assay (Table 1).

LT detection by CIE. Culture and stool supernatants were examined for LT by CIE. It was necessary to use all three concentrations of antiserum to detect LT production. Of the 40 YAC-positive specimens, 24 (60%) were determined to produce LT by this method (see Tables 1 and 2); pooled culture and stool supernatants gave identical results.

LT detection by ELISA. Pooled culture and stool supernatants were examined for LT by ELISA. With positive stool supernatant specimens, the enzyme-substrate reaction occurred more quickly than when positive culture supernatant samples were used. The reactions were stopped before any yellow color appeared in the negative control wells. Of the 164 culture supernatants tested, 26 (65% of the positive pooled culture supernatants as determined by YAC assay) were positive for LT by ELISA (Table 1). Study of the stool supernatants by ELISA was of greater interest. Of the 164 stool supernatants tested by ELISA, 49 (30%) were positive for LT (Table 2), including 36 stool supernatants of the 40 specimens (90%) found to have LT-producing *E. coli* when pooled culture supernatants were tested by the YAC assay. In addition, 13 of the 124 stool supernatants (10%) which had previously been determined to be negative by the YAC assay of pooled culture supernatants were positive by ELISA.

DISCUSSION

The standard assays for enterotoxin are overdue for improvements. Although several investigators have shown other methods to be feasible (10, 12, 14, 16, 17), the YAC assay remains the standard assay. Pooling of culture supernatants derived from isolated *E. coli* has been the standard method for screening samples for enterotoxin production. A limited number of samples can be assayed by this technique. This procedure requires initial isolation of *E. coli* and special animal and tissue culture facilities. In many countries where ET *E. coli* is a major problem, these assays are rarely performed.

This study was designed to evaluate three newer immunological methods for detection of LT in comparison with the currently accepted YAC assay of pooled culture supernatants from *E. coli* colonies. The goal was to find a rapid, simple, economical method to detect LT. Direct examination of stools seemed to be a better approach to the problem, since subculturing of *E. coli* could be avoided. Comparisons were also made with culture supernatants from the patient isolates.

It is not surprising that the YAC assay did not detect a larger percentage of the known positives even when concentrated stool supernatants were used. This result may be due to the low sensitivity of the test as well as a possible enterotoxin-binding effect of free gangliosides found in stools. Gustafsson and Möllby (12) have found that such free gangliosides in culture media inhibited the binding of LT to the cultured cells. In addition, the toxin may be destroyed or inactivated by passage through the 0.45-μm (pore size) filter, a necessary step when contaminated specimens are used in tissue culture.

This study demonstrates that ELISA of nonsterile stool supernatants is a good method for LT detection. ELISA plates can be precoated and stored at 4°C. Results can then be obtained within 24 h from addition of stool supernatants. We did not attempt to quantitate the levels of toxin present in stool supernatants, however. ELISA techniques, in other systems, are known to detect nanogram levels of antigen. It is understandable that ELISA should surpass Biken and CIE assays in sensitivity. These latter techniques are generally only sensitive to microgram levels of antigen. The Biken assay takes up to 4 days to obtain results. In our hands, this assay was less than satisfactory. These results are in direct contrast to the excellent correlation with tissue culture assays reported by Honda et al. (13, 14). The differences may be attributed to the differences in antiserum. Honda et al. stress the importance of proper concentration of antiserum. Preliminary trials in our laboratory in Houston led us to believe that a 1:2 dilution of our antiserum was optimal. The CIE, although results are obtained in 1 h, requires that three dilutions of antisera are run for each sample. One dilution of serum was not shown to be superior in all cases.

It may be noteworthy that the ELISA was positive in 10% of the stool supernatants not shown to be positive when *E. coli* was assayed by the YAC assay. Further studies of patient stools will be undertaken to attempt to isolate ET *E. coli*. In addition, the ELISA of stool supernatants detected more samples known to be positive (by YAC assay of pooled culture supernatants) than did the ELISA of culture supernatants, possibly owing to the incubation periods employed during the assay. Perhaps the use of a longer conjugate incubation period would increase the sensitivity of the assay for culture supernatants.

The ELISA is convenient to use for both large and small numbers of specimens. It can be readily adapted for reading larger volumes of sample on a standard Spec 20 spectrophotometer instead of a microtiter ELISA reader. In some cases, the ELISA can be read visually. Good positives are bright yellow in color, whereas negatives are generally pale to colorless. The ELISA may soon replace the YAC assay, since it is a rapid, economical, and sensitive assay that is easy to perform and interpret, even in the field setting. Further studies, currently under way in our laboratory, must be conducted to quantitate the levels of enterotoxin which can be detected in stool. Perhaps then a correlation can be made between the amount of toxin in a patient's stool and the predominance of ET *E. coli* colonies isolated from a stool.

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