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Increased *Escherichia coli* Enterotoxin Detection After Concentrating Culture Supernatants: Possible New Enterotoxin Detectable in Dogs but Not in Infant Mice

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The heat-stable enterotoxin (ST) of *Escherichia coli* can be detected by infant mouse or dog intestinal loop tests. These tests differ in that the dog assay uses concentrated culture supernatants and is based on measurements of net intestinal absorption, whereas the mouse test uses unconcentrated supernatants and depends on gross fluid accumulation. To compare the relative sensitivities of these assays, culture supernatants of randomly selected *E. coli* isolates from 34 Bangalee diarrhea patients were tested for ST in dog loops and infant mice. Supernatants were also tested for heat-labile enterotoxin (LT) in dog loops, Y-1 adrenal cells, and Chinese hamster ovary cells. *E. coli* supernatants that produced positive responses for both ST and LT in the dog loop assay (ST+/LT+) also produced positive responses when tested for ST in infant mice and for LT in cell lines. Supernatants of strains negative for ST and LT in dog loop (ST−/LT−) were also negative in other assays. Of 10 strains positive for just ST in the dog loop test (ST+/LT−), only 5 were ST positive in the standard infant mouse test. Supernatants of the other five strains (dog loop positive, mouse test negative) were then concentrated 100-fold and retested in mice. Three of these five gave consistently positive results after concentration, and two were only intermittently positive. Concentrated supernatants of negative control strains (ST−/LT−) were all negative in mice. The dog assay detects more strains producing ST than the infant mouse test. The infant mouse test, which detects only gross fluid accumulation, failed to detect approximately half of the 10 strains which produced ST alone (ST+/LT−; $P = 0.025$). Concentrating supernatants for the mouse assay increases sensitivity for detection of ST, but certain *E. coli* strains produce a variety of ST to which infant mice do not respond.

Escherichia coli which elaborate heat-labile (LT) enterotoxin and/or heat-stable (ST) enterotoxin are important agents of traveller's diarrhea and endemic diarrhea in certain areas of the world (3, 10). Though *E. coli* which produce only ST (ST+/LT−) can cause diarrheal disease (7), the relative frequency of ST+/LT− strains as pathogens is unclear. Nalin et al. (8-9) detected ST by measuring its effect on net fluxes in dog intestinal loops, and isolated ST+/LT− *E. coli* from 30% of Bangalee inpatients with idiopathic diarrhea. In contrast, Ryder et al. (11) and Sack et al. (12), using the more popular infant mouse assay for ST, found very few ST+/ST− strains in their patients with diarrhea in the same population. Although differences in length of study, seasonality, and sampling among the studies may account for the observed differences in frequency of isolation of ST+/LT− strains, it is also possible that the infant mouse and dog loop assays vary significantly in their sensitivity to detect ST. This study compared the sensitivity of dog loop and infant mouse assays for detection of ST; identification of LT by dog loop and cell assays (Chinese hamster ovary [CHO] and Y-1 adrenal cells) was also compared.

MATERIALS AND METHODS

Bacterial isolates. Thirty-four fecal *E. coli* isolates, randomly selected from a larger series of 546 fecal *E. coli* isolates which had been obtained from Bangalees with idiopathic diarrhea, (and five control strains) were examined. Methods of primary isolation and storage were previously reported (9-10), and, under the conditions employed, no change in toxigenicity has been observed in any isolate during 3 to 7 years of repeated preparation of supernatants from stock cultures (9). After initial dog loop tests, lyophilized stock

cultures were prepared; for mouse and cell tests, and confirmatory repeat dog tests, lyophilates of the isolates were dissolved in Trypticase soy broth and immediately plated on Trypticase soy agar with 5% sheep erythrocytes (blood agar plate, or BAP). After incubation, colonies from the BAP were dissolved in Trypticase soy broth and replated on trypticase soy agar; after incubation, Trypticase soy agar colonies were stored in milk at −70°C until preparation of cultures for harvesting of supernatants. For preparation of supernatants, organisms in milk were plated on BAP again, and also on brain heart infusion medium for biochemical tests, to reconfirm *E. coli* identity. For the infant mouse tests, media were inoculated directly from the BAP; for the dog tests, media were inoculated from the brain heart infusion plate.

E. coli strains H10407 and B7A served as positive (ST+/LT+) controls and strain HS as the negative (ST−/LT−) control in all infant mouse assays. Strains B7A or 10400 (ST+/LT+) and HS or 20902 (ST−/LT−) served as positive or negative controls, respectively, in the dog assay.

Dog loop assay. Isotonic supernatants of the *E. coli* strains were prepared from overnight (16 h) shaken, aerated Casamino Acid broth cultures by Evans method (2) at pH 8 (37°C) and were concentrated (73×) by precipitation with 80% ammonium sulfate followed by overnight dialysis against 0.85% NaCl. These precipitates were suspended to 1/73 of their original volume. The assay method, with freshly prepared cannulated dog intestinal loops, is described in detail elsewhere (8–10). In this assay, after a lag period of 4 to 6 h, supernatants containing LT cause a decrease in loop absorption followed by net secretion. ST, in contrast, is recognized in the dog assay as a significant diminution in absorption (≥50%) within 20 min after loop exposure to supernatants (8, 9). Whereas it is not known whether a fraction of the ST present in a given supernatant may be lost during concentration, supernatants prepared in this manner from control ST+ strains give uniformly positive results for ST.

CHO cell, adrenal cell, and infant mouse assays. Unconcentrated supernatants were tested for LT in CHO and adrenal cell assays (4, 13) and for ST in the infant mouse assay (1). Supernatants for infant mouse tests were prepared from overnight shaken, aerated trypticase soy broth cultures with 0.6% yeast extract and were membrane filtered (0.22 μm; Millipore Corp., Bedford, Mass.) and tested immediately, or within 7 days if stored (−4°C). Supernatants giving results in the mouse assay discordant with those obtained in dog loops (along with supernatants of five ST−/LT− strains) were concentrated and retested at least twice in the infant mouse assay. Concentrated supernatants were prepared by precipitation with 80% saturated ammonium sulfate, dialysis against 0.85% NaCl, and volume adjustment to 1/100 original volume.

Comparative sensitivity of dog and mouse for ST. The relative sensitivity of the dog and mouse ST assays had been previously determined by comparison of the response to concentrated and unconcentrated culture supernatants in dogs and mice (9). With strain CRL 10400 (our positive control in dog studies), the minimum dose per 20-cm loop of dog intestine that invariably gave a positive ST response (in 52 loops of 49 dogs) was 4 ml of 73×-concentrated supernatant. Lower doses gave variable responses. Titration of supernatants of the same strain in mice was carried out by courtesy of J. Craig and A. A. Andremont. The ST content of CRL 10400 supernatants was 800 ED/ml.

TABLE 1. *Detection of toxigenic E. coli in different assay systems*

Strain no.[a]	Test				
	LT			ST	
	Dog loop[b]	CHO[c]	Y-1[c]	Dog loop[b]	Mouse[c]
ST−/LT−					
19487	−	−	−	−	−
19521S+	−	−	−	−	−
19578	−	−	−	−	−
19632	−	−	−	−	−
20339S+	−	−	−	−	−
20483S−	−	−	−	−	−
20546S−	−	−	−	−	−
20902*	−	−	−	−	−
20913S−	−	−	−	−	−
22141S+	−	−	−	−	−
22205S+	−	−	−	−	−
35733	−	−	−	−	−
36013	−	−	−	−	−
40835	−	−	−	−	−
41251	−	−	−	−	−
41451	−	−	−	−	−
41788	−	−	−	−	−
35622	−	−	−	−	−
HS*	−	−	−	−	−
ST+/LT+					
35639	+	+	+	+	+
35671	+	+	+	+	+
35678	+	+	+	+	+
35760	+	+	+	+	+
35875	+	+	+	+	+
35880	+	+	+	+	+
36004	+	+	+	+	+
10407*	+	+	+	+	+
10400*	+	+	+	+	+
B7A*	+	+	+	+	+
ST+/LT−					
20580	−	−	−	+	+
20590	−	−	−	+	+
35631	−	−	−	+	+
35647	−	−	−	+	+
41591	−	−	−	+	+
35642	−	−	−	+	−
36000	−	−	−	+	−
41736	−	−	−	+	−
20178S+	−	−	−	+	−
20546S+	−	−	−	+	−

[a] S+ or S−, Sucrose fermenters or nonfermenters (tested separately). *, control strains.

[b] 73× concentrated.

[c] Unconcentrated supernatants.

(LT was 400 to 500 BD4/ml.) Thus, dogs are many times less sensitive to "classical" ST than infant mice.

RESULTS

E. coli strains identified as LT+ in the dog loop were all positive in the Y-1 adrenal cell and CHO cell assays as well (Table 1). There was also complete agreement between these assays regarding LT− strains. Nine strains that were both LT+ and ST+ in the dog loop assay were positive for ST in the standard infant mouse assay (including controls, Table 1).

Of the 10 LT− *E. coli* strains positive for ST in the dog loop assay, only 5 strains gave positive results in the standard infant mouse assay; the other 5 strains were repeatedly ST− in this assay. When 100-fold concentrations of supernatants of the five discordant strains were retested in the infant mouse assay, three of the five strains gave positive results in two consecutive runs (four mice per run); the remaining two concentrates gave positive results in some runs but not in others (Table 2). Hundred-fold concentrations of supernatants of strains negative for ST in dog loops were used as controls and were consistently negative in the mouse assay. The two strains intermittently positive in the mouse assay were retested in dog loops after completing mouse tests and remained clearly ST+ in dogs.

DISCUSSION

This study was undertaken to determine if differences between the dog intestinal loop assay and the infant mouse assay in their relative sensitivity for the detection of ST could account for the divergent results reported by three groups in the frequency of isolation of ST+/ST− strains from individuals with diarrhea in Bangladesh. Five of 10 *E. coli* strains identified as ST+/LT− in the dog loop assay were corroborated by the infant mouse assay. When supernatants of the five discordant strains were concentrated 100-fold, three of five gave consistently positive results in the infant mouse assay, and the other two were intermittently positive. Strains negative for ST in the dog remained negative in the mouse even after concentration. These observations demonstrate that there are quantitative differences in the production of ST by certain ST+/LT− strains and that the assays differ considerably in their ability to detect ST. Though the amount of concentrated supernatant needed to give consistently positive ST results in dog tests contains far more ST than needed to give positive mouse tests using the control CRL 10400 strain, strains producing less toxin appear to require concentration even for detection by the mouse test. Sensitivity of the infant mouse test for ST+/LT− supernatants was increased by 60% in our small series when supernatants were concentrated 100-fold before testing. For detecting strains of low toxigenicity, the dog test may be advantageous in that it detects ST by measuring fluxes and net absorption; it is not dependent on gross fluid accumulations as is the infant mouse assay. Thus, it

TABLE 2. *Comparison of dog results with results of infant mouse tests before and after concentration*

Strain	Results		
	Dog test (% change in absorption) 73× concentrated	Mouse test	
		Unconcentrated	100× concentrated
ST+/LT− in dog			
35642	Positive (−157%)	3/3 negative[a]	2/2 positive
36000	Positive (−100%)	2/2 negative	2/2 positive
41736	Positive (−86%)	3/3 negative	2/2 positive
20178S+	Positive (−123%)	3/3 negative	2/5 positive
20546S+	Positive (−100%)	3/3 negative	1/7 positive[b]
ST−/LT− in dog (one run each)			
35622	Negative (−12%)	Negative	Negative
20546S−	Negative (+100%)	Negative	Negative
19487	Negative (+40%)	Negative	Negative
19578	Negative (+12%)	Negative	Negative
19632	Negative (+19%)	Negative	Negative
Controls			
ST+ (10407, B7A)	Positive (−122, −125%)	5/5 positive	5/5 positive
ST− (HS)	Negative (+27%)	6/6 negative	6/6 negative

[a] Number of positive runs/total runs (four mice per run).
[b] Positive only when prepared with medium for dog assay.

resembles the method of Klipstein et al. (5) in rats, which also has revealed marked strain-to-strain variation in toxin production under given conditions in vitro.

The fact that some *E. coli* supernatants produce ST-like responses in dog loops but not in infant mice suggests the additional possibility of a distinct type of *E. coli* enterotoxin to which dogs, but not mice, respond. Whereas the role of possible mouse test inhibitors in supernatants of some *E. coli* ST+/LT− strains needs to be ruled out, we have recently demonstrated that other *E. coli* strains, epidemiologically incriminated in outbreaks of infantile diarrhea, are negative in the Y-1 adrenal cell test for LT and in the infant mouse test for ST (even after 100-fold concentration) but are positive in the dog loop and cause diarrhea when fed to human volunteers (6). These supernatants, like those from strains 20546S+ and 20178S+, have an effect which begins within 20 min in dog loops and is heat stable.

The standard infant mouse assay for "classical" ST is the simplest available test for ST and can be used to screen large numbers of strains but will fail to detect a significant number of the ST+/LT− strains identifiable by the more cumbersome dog loop test. Concentration of supernatants 100-fold before testing in the infant mouse assay offers a compromise between sensitivity and simplicity. This modification of the infant mouse assay allows detection of significantly more *E. coli* which produce ST alone (as defined by dog loop assay) but are ST− in the standard infant mouse assay. However, some *E. coli* strains which can produce ST-like activity in dog loops remain negative in infant mice even after concentration. Further chemical and immunological characterization of these different enterotoxic activities will determine whether or not they are related.

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