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Sensitivity, Precision, and Accuracy of the Y1 Adrenal Cell Enterotoxin Assay

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The Y1 adrenal cell assay for heat-labile enterotoxin (LT) was found to be relatively insensitive for filtrates of toxigenic *Escherichia coli* H10407. When observations were made blindly and subjected to rigorous controls, reliable detection of LT occured only at filtrate dilutions from 1:4 to 1:10. Detection of LT was unreliable when *E. coli* H10407 was mixed with another enteric organism. Overall, 7.1% of observers' reports from a single assay were imprecise, and 2.6% would have resulted in errors in detection of LT; however, triplicate assays prevented false positive reports of LT detection. Routine testing for LT production in the clinical diagnostic laboratory awaits a simpler, more sensitive, and more direct method of testing stool specimens.

During a study of the etiology of diarrhea in pediatric patients in Rochester, Minn., no strains of *Escherichia coli* producing heat-labile enterotoxin (LT) were found in 31 stool specimens submitted for examination (S. P. Taraska, K. H. Rhodes, T. F. Smith, and J. A. Washington II, Mayo Clin. Proc., in press). These negative findings stimulated an investigation of the sensitivity of the Y1 adrenal cell assay (1) as performed in our laboratory. With a view to introducing the assay as a diagnostic test offered by clinical laboratories, it seemed that the detection of LT-producing strains of *E. coli* would be simpler and less costly if the adrenal cell assay detected enterotoxin when mixtures of toxin-positive and toxin-negative organisms were incubated together.

MATERIALS AND METHODS

Test organisms. We selected 10 enteric isolates (7 *Escherichia coli*, 2 *Klebsiella pneumoniae*, and 1 *Citrobacter freundii*), from stool specimens submitted to our diagnostic laboratory, and two toxigenic organisms, kindly supplied by S. T. Donta, University of Iowa, Iowa City: *E. coli* H10407 (produces LT and heat-stable enterotoxin) and *E. coli* B41 (711) (produces heat-stable enterotoxin only.)

Filtrates. Colonies were subcultured from 18-h cultures on slants or plates into 10 ml of Trypticase soy broth, which was incubated aerobically on a shaker at 37°C for 18 h. The broth was centrifuged (700 × *g*) for 10 min, the supernatant fraction was passed through a 0.45-um Swinnex filter (Millipore Corp., Bedford, Mass.) and the filtrate was diluted as required in Eagle minimum essential medium.

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Assay for LT. Y1 mouse adrenal cells were seeded into glass tubes (16.4 by 125 mm) closed with screw caps so that discrete clones of cells were present after 24 h (5). For the test, the Eagle minimum essential medium was removed from the cell cultures and replaced with 1 ml of diluted bacterial filtrate. The cultures were slanted at an angle of 5 degrees and incubated for 24 h at 36°C. After 24 h, 1 ml of Eagle minimum essential medium was added to each assay tube, and the cultures were reincubated for an additional 24 h.

Cell cultures were examined with a light microscope (×125) after 24 and 48 h of incubation. Morphological changes produced by LT (cell rounding as described and photographed by Donta et al. [1]) were recorded using the following scoring system: 0, <5% rounded cells; 1+, between 5 and 25%; 2+, between 25 and 50%; 3+, between 50 and 75%; 4+, between 75 and 95%; 5+, ≥95%. Scores at 48 h were more consistent and are used in this report.

Experimental design and recording of results. We performed triplicate assays for all experimental filtrates and included triplicate assays of a positive control (1:10 dilution of filtrate of *E. coli* H10407) and a negative control [1:10 filtrate of *E. coli* B41 (711)] for each experiment. In a blind fashion, several observers scored the experimental and control assay tubes, which had been arranged in random order. For each observer in each experiment a median score was determined for each blind control assay. Then for each experiment an observer's individual reference score was determined. The reference score was equal to the median score for the positive control if the difference between the median scores of the positive and negative controls was 1 or 2. The reference score was 1 less than the median score for the positive control if the above difference was greater than 2. A difference between median scores for positive and negative controls of less than 1 never occurred but would have indicated

inconsistent results requiring a repeat experiment. For each experimental filtrate, the observer's median score was compared with his individual reference score. A score greater than or equal to the reference score indicated the presence of LT.

RESULTS

Sensitivity of assay. In a series of three experiments, filtrates of *E. coli* H10407 were assayed at twofold dilutions from 1:2 to 1:8 and 1:10 to 1:320. The 10 enteric isolates were assayed at twofold dilutions from 1:2 to 1:8 and at 1:10. Five observers (three technologists and two investigators) participated in recording observations during the course of these experiments.

All observers consistently reported the presence of toxin for filtrates of *E. coli* H10407 at dilutions from 1:2 to 1:10. At dilutions of 1:20 and 1:40, observers' results varied. Three of 12 median scores indicated the presence of toxin. At dilutions from 1:80 to 1:320 no observer reported the presence of toxin (Table 1). When diluted 1:10, all filtrates from the 10 enteric isolates were reported negative for LT production by all observers in all experiments. When diluted 1:2, the filtrate of one isolate of *E. coli* produced abnormal cell death in all three tubes of triplicate assay.

Mixtures of two isolates. *E. coli* H10407 and the 10 enteric isolates were grown on MacConkey agar plates. A colony of *E. coli* H10407 was picked to 10 ml of Trypticase soy broth; immediately thereafter, a colony of one of the enteric isolates was picked and inoculated into the same broth. Using separate tubes of broth, the same procedure was followed for each of the 10 enteric isolates, producing 10 mixtures of two isolates. Incubation and assay for LT were as described in Materials and Methods. The presence of *E. coli* H10407 was verified in broths subcultured prior to filtration. The experiment was done in duplicate with two investigators recording observations for two filtrates of each of the 10 mixtures.

Each of the 10 mixtures was categorized according to the results for toxin detection (Table 2). For 7 of the 10 mixtures, one filtrate was toxin positive, and the other was toxin negative, despite the fact that each mixture contained an organism producing LT (*E. coli* H10407). In no case did both observers report both filtrates of any mixture as positive for enterotoxin.

Observer variation. Reference scores for LT detection varied from 2 to 4 depending on the observers; however, reference scores for any one observer over the series of experiments did not differ by more than one score rank. The data indicated that different observers perceived the morphological changes produced by LT differently but that each observer had a relatively consistent perception of these changes over a series of experiments. Absolute criteria for the presence of LT could not be established, but use of reference scores relative to the observer and the experiment produced consistent experimental results.

Individual scores reported by an observer were considered imprecise if they differed by more than 1 from his median reported score. Of experimental scores at risk, 7.1% were imprecise. Technologists made significantly more precise observations than the investigators (Table 3).

All observers classified 12 experimental filtrates as toxin positive and 55 as toxin negative. From these data we estimated the accuracy of a single tube assay. Of single-tube scores, 2.6% represented errors in detection of toxin (2.7% false positive, 2% false negative). Investigators tended to make more inaccurate observations than technologists (Table 3).

DISCUSSION

The adrenal cell assay for LT enterotoxin was disappointingly insensitive in our hands. We found that reliable results could be expected only over a twofold dilution range (1:4 to 1:10). At higher dilutions, toxin produced by *E. coli* H10407 could not be detected reproducibly. At a lower dilution, nonspecific adrenal cell death occurred with one filtrate of nontoxigenic enteric isolate, possibly as a result of accumulation of

TABLE 1. *Detection of LT in dilutions of filtrates of a toxigenic E. coli strain (H10407)*

Filtrate dilution	Enterotoxin detected	
	No.[a]	%
1:2–1:10	24/24	100
1:20, 1:40	3/12	25
1:80–1:320	0/12	0

[a] Number of dilutions with enterotoxin detected/number of observations.

TABLE 2. *Detection of LT in paired filtrates of 10 mixtures of two organisms, E. coli H10407 and a nontoxigenic enteric isolate*

Categories of results reported by two observers[a]	No. of mixtures in each category
(+/+):(+/+)	0
(+/+):(+/−)	2
(+/+):(−/−)	7
(+/−):(−/−)	1
(−/−):(−/−)	0

[a] +, Observer detected toxin; −, observer did not detect toxin; (/), two observers' results for one filtrate; (/):(/), two observers' results for two filtrates of one mixture.

TABLE 3. *Adrenal cell assay for enterotoxin: imprecision and inaccuracy of single observations*

Observers	Imprecise scores[a]		Inaccurate scores	
	No.[b]	%	No.[c]	%
Total	31/438	7.1	16/606	2.6
Investigators	30/292[d]	10	14/402[e]	3.5
Technologists	1/146[d]	0.7	2/204[e]	1
False positive			13/477	2.7
False negative			3/129	2

[a] For each filtrate assayed and scored in triplicate, two observations are at risk of being imprecise.
[b] Imprecise scores/total observations.
[c] Inaccurate scores/total observations.
[d] $P < 0.001$ (Fisher's exact test).
[e] $P \simeq 0.05$ (Fisher's exact test).

metabolic products. Gurwith (4) has reported that his adrenal cell assay detected toxin from *E. coli* (known toxigenic) at dilutions of 1:30 to 1:100. In nonrandom unblinded pilot experiments that did not include nontoxigenic control organisms, we also detected morphological changes in filtrates of *E. coli* H10407 diluted as much as 1:80. Unfortunately, these results did not withstand more rigorous experimental conditions.

The highly variable results from filtrates of mixtures of two organisms including *E. coli* H10407 may possibly be explained by the poor sensitivity of the assay. If production of LT by *E. coli* H10407 is reduced in the presence of a nontoxigenic organism, then one might expect variable results analogous to those seen at the 1:20 and 1:40 dilutions of *E. coli* H10407 incubated alone. In this regard, our experimental results do not agree with the clinical experience of Donta et al. (2), who processed nine isolated colonies from each specimen obtained from patients in their study and, in addition, processed an "isolate" taken from the thick portion of the agar plate as a composite of multiple colonies of *E. coli*. Of eight patients with stools positive for LT in their study, only four (50%) were detected by the processing of individual colonies for assay. Nevertheless, three of eight patients' specimens (38%) were not positive by processing the composite isolate alone. Therefore, based upon our results and those of Donta et al. (2), we cannot recommend mixing enteric organisms prior to testing in the adrenal cell assay as a replacement for processing single colonies.

Our results helped resolve several practical issues important to diagnostic laboratory protocol for performing the adrenal cell assay to detect LT: (i) to obtain consistent results, the assay score indicative of the presence of toxin must be determined for each observer in each experiment; (ii) to eliminate bias and prevent overestimation of assay sensitivity, blind positive and negative controls should be used to determine this score, and all experimental assays should be read randomly and blindly. Under these conditions, using triplicate assays, our data demonstrated observer disagreement about the presence of toxin only when levels of toxin present apparently approximated the sensitivity limit of the assay.

About 1 in 40 single tube assays resulted in a toxin detection error. Triplicate testing by technologists with daily experience with the morphological behavior of Y1 adrenal cells and the assay will reduce the rate of these errors. Error rates may be expected to vary from laboratory to laboratory and are important to determine before reporting diagnostic results. A false positive rate of 1 in 40 may seem acceptable. However, if we used a single tube assay, and only 1 of every 200 stool organisms tested in our laboratory actually produced enterotoxin, then on the average four of five positive reports would be false positives.

Routine testing for enterotoxin production in diagnostic laboratories awaits epidemiological studies that define the clinical significance of an enterotoxin-producing organism in the stool of an individual patient with a sporadic case of diarrhea (2, 3) and technology that allows simpler, more sensitive, and more direct testing of stool specimens.

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