

## Test for Enterotoxigenic *Escherichia coli* Using Y1 Adrenal Cells in Miniculture

DAVID A. SACK\* AND R. BRADLEY SACK

*Departments of Medicine, Baltimore City Hospitals\* and the Johns Hopkins Hospital, Baltimore, Maryland 21224*

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A rapid, potentially clinically useful test for detection of enterotoxigenic *Escherichia coli* is described. Whole bacterial cultures of enterotoxigenic *E. coli*, when briefly exposed to Y1 adrenal cells in tissue miniculture, effect a rounding response in the tissue culture that can be discerned at 18 to 24 h. The tissue culture technique agreed with the rabbit ileal loop in all 58 enterotoxigenic and 52 non-enterotoxigenic *E. coli* strains tested.

Although serological methods for identifying enteropathogenic *Escherichia coli* have been available for many years, these serotypes at the present time correlate poorly with enterotoxin production (6, 11). Indeed, it is likely that any serotype may acquire the ability to produce enterotoxin since plasmid-mediated enterotoxin production has been shown (12, 13). Several animal models, including the rabbit ileal loop (10), infant rabbit (6), rabbit skin (5), and others (1, 9), have been used to test for the enterotoxigenicity of strains of *E. coli*. Recently, the Y1 mouse adrenal cell tissue culture has been found useful since it increases its production of  $\Delta^4,3$ -ketosteroids and changes its cellular morphology from a flat cell to a round cell in response to cholera toxin and heat-labile *E. coli* enterotoxin (LT) (3). The effects of both of these toxins are mediated through adenyl cyclase stimulation (7, 8). Filtrates of strains which are either invasive or which produce heat-stable enterotoxin, or which are nonpathogenic, do not produce any change in cell morphology (3).

With the aim of developing a rapid, clinically useful screening tool, we describe here a mini-adaptation of the adrenal cell tissue culture assay which makes possible the testing of several hundred *E. coli* cultures per day for LT production.

### MATERIALS AND METHODS

**Tissue culture.** Y1 adrenal cells were maintained in Ham F10 media supplemented with 12.5% horse serum and 2.5% fetal calf serum and gentamicin (40  $\mu$ g/ml). The tissue culture was subcultured into either 60-mm petri dishes (Falcon) or 96-well miniculture plates (Cooke). The tissue culture was available for assay when a monolayer was formed, usually 1 to 3 days.

**Tissue culture assay using bacterial supernatants.** Isolates from stock nutrient agar slants were grown in peptone water (1.0% peptone; 0.5% NaCl) overnight, and 0.01 ml was inoculated into syncase media (glucose substituted for sucrose) (10) (10 ml in a 50-ml Erlenmeyer flask). Flasks were shaken (200/min) at 37 C overnight, then the culture was centrifuged at 25,000  $\times$  g for 30 min, and the supernatant was removed for assay. Two-tenths milliliter of the supernatant was added to a tissue culture growing in a 60-mm petri dish with 1.8 ml of media. The cells were observed after 18 to 24 h with a phase contrast inverted microscope (Olympus) for the presence of rounding. Known positive and negative strains of *E. coli* tested with each group served as controls.

**Tissue miniculture assay using whole bacterial cultures.** Isolates from stock nutrient agar slants were inoculated into syncase-glucose media (0.5 ml in a flat bottomed 8-ml screw-capped vial) and incubated at 37 C without shaking. Whole live culture (0.05 ml) was added to each well of the tissue culture growing in a 96-well tissue culture plate (0.01 ml of media per well) utilizing a transfer plate (Cooke). After 5 min, the tissue culture media containing bacteria was removed by using a micropipette. The tissue culture wells were washed once with phosphate-buffered saline (PBS) and fresh media were replaced. The cells were observed in 18 to 24 h for typical rounding. Known positive and negative control strains were tested with each 96-well plate.

**Rabbit ileal loop.** Bacterial cultures were made as described above for supernatants. One milliliter of the whole live culture was inoculated into 5-cm rabbit ileal loops. Positive (a known enterotoxigenic *E. coli*) and negative PBS controls were included in each rabbit. At least three rabbits were inoculated with each test strain, and the results were accepted only from rabbits with appropriate control responses. The rabbits were sacrificed 18 h later and the loops were examined for fluid accumulation. A positive reaction was defined as a mean volume:length (milliliters to centimeters) of 1.0 or greater. A ratio of 0.3 or less was considered negative. Strains giving intermediate ratios were retested.

## RESULTS

A total of 110 strains of *E. coli* from 12 stool cultures, obtained from 11 patients with acute diarrhea admitted to the Indian Health Service Hospital in White River, Ariz. (R. B. Sack, N. Hirschhorn, I. Brownlee, R. A. Cash, W. E. Woodward, and D. A. Sack, manuscript in preparation), were tested simultaneously for enterotoxin production using the three methods described. Fifty-eight strains were positive in all three systems and 52 strains were negative in all three systems.

The tissue miniculture assay was also done on 6-, 24-, and 48-h whole cultures of the 110 strains. By 6 h, 13 strains were positive; by 24 h, 58 strains were positive; there was no further change when testing 48-h cultures.

A semiquantitative assay for LT was performed using twofold dilutions in PBS of 11 enterotoxigenic live cultures of *E. coli* grown for 8, 16, 24, and 48 h. Nine strains were isolated from nine patients with diarrhea at White River Indian Hospital, one strain was isolated from a patient with diarrhea from Calcutta, India (10), and one strain was isolated from river water near White River, Ariz. The results are shown in Table 1. All strains were positive by 16 h at a mean titer of 1:64 (1:10 to 1:160). The titer continued to increase at 48 h to a mean titer of 1:256.

No nonspecific cytotoxic effects were noted when whole bacterial cultures were inoculated into the adrenal cell tissue culture.

## DISCUSSION

Animal assay methods for detecting enterotoxin, though valuable research tools, have had limited clinical usefulness because they are time consuming, cumbersome, and expensive. The adrenal tissue culture method, however, does have the versatility and reproducibility to serve as a useful clinical tool. Using the method described here, a reliable result can be obtained within 2 to 3 days of stool collection, while testing several hundred strains per day. Since the adrenal cell assay will detect very minute amounts of toxin (0.2  $\mu$ g of crude *E. coli* toxin

per ml or 5 ng of purified cholera toxin per ml) (2), it is unlikely that any enterotoxigenic strain which produces LT under these in vitro conditions will produce a subdetectable amount of toxin. Of the positive strains tested all gave positive results even when diluted to 1:40 at 24 h.

A 16- to 24-h bacterial culture is useful for the test since this identifies all of the positive strains. Shorter culture times may give false negative results as seen with three of 11 strains when tested at 8 h. Toxin titer continues to increase up to 48 h; this longer incubation period could be useful in testing doubtful strains.

This technique makes use of the rapid tight binding of the toxin to the cell membrane (7). Since the toxin is bound tightly, the live bacteria can be removed without displacing the toxin or inhibiting the rounding. The small numbers of bacteria that remain in the tissue culture after washing with PBS are inhibited by the gentamicin in the fresh media. Failure to change the tissue culture media after adding the bacteriological culture results in destruction of the monolayer in spite of the gentamicin. Using this short enterotoxin exposure time, this method should be useful in testing other fluids (e.g., stool supernatants or jejunal aspirates) for the presence of enterotoxin, since this minimizes the cytotoxic effects of these fluids.

False negative adrenal cell results may be obtained if the tissue culture is used when it is too sparse, i.e., before a monolayer is formed. To avoid this, the tissue cultures should be examined prior to the addition of the bacterial culture. False positive results are possible if nonspecific cytotoxic rounding is mistaken for specific enterotoxic rounding. With experience, however, these can easily be differentiated. This nonspecific cytotoxic rounding is minimized by changing the media after adding the test substance.

The adaptation of adrenal cell to a miniculture plate capable of assaying large numbers of whole live cultures should prove useful in screening *E. coli* strains for heat-labile enterotoxin production.

TABLE 1. Enterotoxin titers in cultures of *E. coli* measured after different incubation times

Determinants	Duration of bacterial incubation			
	8 h	16 h	24 h	48 h
No. of strains tested	11	11	11	11
No. of positive strains	8	11	11	11
Mean titer of positive strains	1:35	1:64	1:96	1:256
Range of positive strains	1:10 to 1:320	1:10 to 1:320	1:40 to 1:320	1:80 to 1:640

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## LITERATURE CITED

1. Dean, A. G., Y. Ching, R. G. Williams, and L. B. Harden. 1972. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. *J. Infect. Dis.* **125**:407-411.
2. Donta, S. T., M. King, and K. Sloper. 1973. Induction of steroidogenesis in tissue culture by cholera enterotoxin. *Nature (London) New Biol.* **243**:246-247.
3. Donta, S. T., H. W. Moon, and S. C. Whipp. 1974. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. *Science* **183**:334-336.
4. Donta, S. T., and D. M. Smith. 1974. Stimulation of steroidogenesis in tissue culture by enterotoxigenic *Escherichia coli* and its neutralization by specific antisera. *Infect. Immun.* **9**:500-505.
5. Evans, D. J., D. G. Evans, and S. L. Gorbach. 1973. Production of vascular permeability factor by enterotoxigenic *Escherichia coli* isolated from man. *Infect. Immun.* **8**:725-730.
6. Gorbach, S. L., and C. M. Khurana. 1972. Toxigenic *Escherichia coli*: a cause of infantile diarrhea in Chicago. *N. Engl. J. Med.* **287**:791-795.
7. Kantor, H. S., P. Tao, and S. L. Gorbach. 1974. Stimulation of intestinal adenyl cyclase by *Escherichia coli* enterotoxin: comparison of strains from an infant and an adult with diarrhea. *J. Infect. Dis.* **129**:1-9.
8. Pierce, N. F., W. B. Greenough, and C. C. J. Carpenter. 1971. *Vibrio cholerae* enterotoxin and its mode of action. *Bacteriol. Rev.* **35**:1-13.
9. Punjashthiti, K., and R. A. Finkelstein. 1971. Enteropathogenicity of *Escherichia coli*. I. Evaluation of mouse intestinal loops. *Infect. Immun.* **4**:473-478.
10. Sack, R. B., S. L. Gorbach, J. G. Banwell, B. Jacobs, B. D. Chatterjee, and R. C. Mitra. 1971. Enterotoxigenic *Escherichia coli* isolated from patients with severe cholera-like disease. *J. Infect. Dis.* **123**:378-385.
11. Sack, R. B., B. Jacobs, and R. Mitra. 1974. Antitoxin responses to infection with enterotoxigenic *Escherichia coli*. *J. Infect. Dis.* **129**:330-335.
12. Skerman, F. J., S. B. Formal, and S. Falkow. 1972. Plasmid-associated enterotoxin production in a strain of *Escherichia coli* isolated from humans. *Infect. Immun.* **5**:622-624.
13. Smith, H. W., and S. Halls. 1968. The transmissible nature of the genetic factor in *Escherichia coli* that controls enterotoxin production. *J. Gen. Microbiol.* **52**:319-334.