Rapid Screening Method for Enterotoxigenic *Escherichia coli*

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A rapid screening method for detection of *Escherichia coli* producing heat-labile enterotoxin is described. Single colonies are transferred directly from primary culture plates into 96-well microculture plates containing 0.3 ml of brain heart infusion broth in each well. After 24 h at 37°C, each brain heart infusion broth culture is assayed by the miniculture method in the corresponding well of a microculture plate in which Y-1 mouse adrenal tumor cells have been grown. All enterotoxigenic isolates detected by this method were confirmed in the same assay but with culture supernatants.

A simple assay method for detecting *Escherichia coli* producing heat-labile enterotoxin, using mouse adrenal tumor cells in miniculture, was described by Sack and Sack in 1975 (3). This method represented an improvement, for the purposes of rapid screening, over previous methods calling for 35- or 60-mm petri dishes for growing the adrenal cells and methods requiring centrifuged and/or filtered supernatants from broth cultures of the *E. coli* to be tested (2). Considerable time was saved by growing the organisms in 0.5 ml of broth in small screw-capped vials and exposing the broth culture without centrifugation or filtration to the adrenal cells in 96-well microculture plates. Even though these cultures contained living bacteria, damage to the adrenal cell monolayer was usually prevented through limited exposure (5 min) and the use of gentamicin in tissue culture media. There was also significant savings in the amount of broth used for bacterial culturing, as well as tissue culture medium.

Recently, I have initiated two types of studies requiring screening of many colonies of *E. coli* for heat-labile enterotoxin production. One of these is a series of "mating" experiments where a minimum of 90 separate colonies of potential transconjugants (colonies that may or may not have received the plasmid coding for enterotoxin production) are screened for enterotoxin production in each experiment. The other is a prospective family study of healthy adults and children, with one of the goals being to determine the asymptomatic carriage rate for enterotoxigenic *E. coli*. Both of these studies require the screening of many colonies for entero-

toxin production. The miniculture method (3) still is somewhat cumbersome and time consuming for this purpose. The limiting factor appears to be the use of individual screw-capped vials for growing *E. coli* in broth prior to the exposure of the whole bacterial culture to the adrenal cells. Since the method requires the use of individual vials, which then must be individually capped, filled with nutrient medium, inoculated with bacterial colonies to be tested, labeled, and finally washed and autoclaved, this becomes a time-consuming procedure when screening large numbers of colonies. A method for testing *E. coli* that eliminates the use of separate vials was devised: the *E. coli* were instead grown in 96-well ethylene oxide-sterilized, disposable microculture plates; each plate replaces 96 vials. This minor modification saved considerable time and permitted screening of many more colonies.

Individual colonies were picked with a thin, straight wire and inoculated directly into individual wells of a 96-well microculture plate (LinBro), containing 0.3 ml of brain heart infusion (BHI) (Baltimore Biological Laboratories) broth in each well. The colonies were picked either from a MacConkey agar isolation plate of a fecal culture or from plates containing presumed transconjugants of mating experiments. At least two positive controls were included in each microculture plate. The microculture plates were then incubated overnight at 37°C without covering individual wells. To prevent drying, the plates were placed inside a large container (such as a metal cannister) with water at the bottom of the container. The container was then partially covered with a metal

lid angulated such that the drops of condensation fall outside the container rather than onto the microculture plates. After overnight incubation at 37°C, each culture containing broth and bacteria was individually transferred, by a 0.05-ml loop, directly to the corresponding well of another microculture plate containing Y-1 mouse adrenal tumor cells (ATCC 79) in confluent monolayers. The miniculture method was then followed with minor modifications (3). After 5 min, the tissue culture medium containing bacteria was removed with a micropipette. The tissue culture wells were washed once with phosphate-buffered saline (NaCl, 7.5 g; KCl, 0.2 g; KH₂PO₄, 0.2 g; Na₂HPO₄, 1.15 g; phenol red, 0.01 g; and water to make up 1 liter; the mixture was autoclaved at a pressure of 15 lb/in² for 15 min). The adrenal cells were then incubated overnight with fresh tissue culture medium (modified minimal essential medium; Flow Laboratories, no. 1A-020) supplemented with L-glutamine and 2% fetal calf serum (Flow Laboratories) containing 40 µg of gentamicin sulfate per ml and examined with an inverted microscope (Olympus) the next day. The number of rounded cells in a lower-power field was estimated, and greater than 50% of rounding of cells was considered a positive response. We have found that visual estimation is as good for distinguishing positives from negatives as a precise count based on counting 200 to 400 cells in fixed and stained specimens (M. Gurwith and C. Langston, unpublished observations).

A total of 1,135 separate colonies from 232 fecal cultures were tested. Of these, none was positive for enterotoxin production in the modified adrenal cell assay. All fecal cultures were obtained from healthy adults and children in a prospective family study of rotavirus infections. In 15 mating experiments, using known enterotoxigenic *E. coli* isolates from Manitoba (M. Gurwith, C. Langston, R. Gross, and B. Rowe, submitted for publication) or known enterotoxigenic controls (kindly provided by R. B. Sack, Johns Hopkins University, Baltimore, Md., and S. Gorbach, Tufts University Medical School, Boston, Mass.), total of 1,389 potential transconjugants were examined. Of these, 52 were positive for enterotoxin production and had the antibiotic and biochemical characteristics of the recipient organism, usually a lactose-negative *E. coli* K-12 (kindly provided by S. Scotland, Central Public Health Laboratory, London, England). All enterotoxigenic *E. coli* detected in this way were reconfirmed either by repeating the rapid miniculture assay or by retesting a supernatant of a new broth culture in the adrenal cell assay. In addition,

four transconjugants detected in this system, which were mated with organisms capable of producing heat-stable toxin as well as heat-labile toxin, were confirmed as having received the enterotoxin plasmid by demonstration of heat-stable toxin in the suckling infant mouse assay (1). Eight isolates gave questionably positive results. On the initial reading, the number of rounded cells exceeded that seen in negative controls (less than 20% rounding, M. Gurwith et al., submitted for publication) but were less than the 50 to 90% of the rounding seen with positive controls. All eight, when repeated, were unquestionably negative by the same method and/or by testing supernatants.

A semiquantitative comparison of three methods of enterotoxin testing was attempted. Two enterotoxin-positive controls were used, *E. coli* O?:H40 and O78:H12 (Table 1). The organisms were grown in three ways: in 8 ml of BHI broth in 50-ml Erlenmeyer flasks and incubated with agitation for 24 h at 37°C; in 2 ml of BHI broth in 25-ml screw-capped vials and incubated without shaking for 24 h; and direct inoculation into 0.3 ml of BHI broth in microculture plates and incubated without shaking. Serial half-log₁₀ dilutions of the resultant cultures were made in phosphate-buffered saline (Table 1) (half-log₁₀ dilutions were made by adding 0.9 ml of enterotoxin preparation to 2 ml of diluent, removing 0.9 ml, and adding it to the next tube containing 2 ml of diluent, etc.). The serial dilutions from the microculture plates or screw-capped vials were then exposed to the adrenal cells for 5 min as whole bacterial cultures (3), and the dilutions from Erlenmeyer flasks were in contact with the cells as sterile, centrifuged supernatants for 24 h (2). It does not appear that any one of the three methods was appreciably more sensitive; there was no more than a one-tube difference in the highest dilution giving a positive result (Table 1).

TABLE 1. Comparison of three types of enterotoxin assays

Assay method	Highest dilution ^a giving a positive response	
	<i>E. coli</i> O?:H40	<i>E. coli</i> O78:H12
Filtered supernatant of broth culture	10 ^{-1.5}	10 ^{-2.0}
Whole bacterial culture grown in screw-capped vials	10 ^{-2.0}	10 ^{-1.5}
Whole bacterial culture grown in microculture plate	10 ^{-2.0}	10 ^{-1.5}

^a Half-log₁₀ dilutions made in phosphate-buffered saline.

Inoculating organisms directly into BHI broth in microculture plates shortened considerably the time required to test large numbers of organisms. No vials or bottles need be filled, capped, sterilized, labeled, and washed. Instead, 1 microculture plate replaces 96 of these containers. BHI broth was easily and rapidly dispensed into wells with a Cornwall syringe (Becton-Dickinson & Co.), and the broth was inoculated with a thin straight wire. Bacterial cultures for testing were then transferred with a 0.05-ml loop to the corresponding adrenal cell culture of a 96-well microculture plate. As long as the adrenal cell monolayers were confluent, there did not seem to be a problem with false negatives. At least, the known positive controls were positive in all assays. In a relatively short time, one technician was able to screen 2,524 colonies for enterotoxin production, finding 52 "new" positive isolates; as well, 78 enterotoxigenic controls were also included and were positive by this method. Ninety-six organisms, including controls, can be assayed in less than 2 h, spread out over 3 days. Occasionally, some organisms gave a result that appeared to be an

intermediate between positive and negative. It was easiest simply to repeat the assay and, if the result was still questionable, to use centrifuged culture supernatants. Also, occasionally the individual tissue culture wells became contaminated; it was simplest to repeat these toxin assays as well.

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