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Microtiter Ganglioside Enzyme-Linked Immunosorbent Assay for Vibrio and *Escherichia coli* Heat-Labile Enterotoxins and Antitoxin

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We have developed a microtiter enzyme-linked immunosorbent assay method for detecting the heat-labile enterotoxins of *Vibrio cholerae* and *Escherichia coli* using G_{M1} ganglioside as the base coat. This method compares favorably with a similar assay using anticholera toxin as the base coat, and with the Y_1 adrenal cell assay. The assay should be useful in detecting enterotoxin production in *E. coli* and vibrios (including non-agglutinating *Vibrio*), in quantitating the toxin, and in determining binding properties of enterotoxins to ganglioside. The assay can also be used to quantitate antibodies which block the attachment of the toxin to the ganglioside.

Several methods are available to detect the heat-labile enterotoxin of *Escherichia coli* or *Vibrio cholerae*. These can broadly be grouped into assays which depend on toxin activity (i.e., rabbit intestinal loop [11], permeability in rabbit skin [2], Y_1 adrenal cell [9], Chinese hamster ovary [CHO] cell [7], and other tissue culture assays [4]) and immunological assays (e.g., staphylococcal coagglutination [1], passive immune hemolysis [3], radioimmunoassay [6], and enzyme-linked immunosorbent assays [ELISAs; 12, 14]). The most widely used assays for screening for *E. coli* heat-labile toxin (LT) production have been the Y_1 adrenal (9) and CHO cell culture assays (7). Cell culture facilities are not widely available, however, and this has limited the application of these two assays to research laboratories. Recently Yolken et al. described an ELISA for detecting *E. coli* LT which made use of cross-reacting antibody between *E. coli* LT and cholera toxin (CT) and which compared favorably in sensitivity and specificity with the Y_1 adrenal assay (14). Similarly, Svennerholm and Holmgren reported an ELISA for LT using a ganglioside in the precoat layer (12). We have combined what we feel are the best features of these two methods and have now established this new method at the International Centre for Diarrhoeal Disease Research, Bangladesh (formerly the Cholera Research Laboratory) as a screening procedure in detecting and quantitating the heat-labile toxins of *E. coli* and *V. cholerae*. We have extended the method to quantitating antibodies to these toxins.

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MATERIALS AND METHODS

Polyvinyl microtiter "U" plates (Cooke catalog no. 1-220-24) were used for all ELISA procedures. G_{M1} ganglioside (Supelco catalog no. 4-6033, lot 631), in a concentration of 1 μg/ml diluted in phosphate-buffered saline, was used. (G_{M1} ganglioside is supplied in a chloroform-methanol suspension; when it is to be diluted in the aqueous solution, the chloroform must be evaporated before dilution.) Guinea pig anti-CT was harvested from guinea pigs immunized with CT. Guinea pigs were immunized subcutaneously with 10 μg of CT in Freund complete adjuvant on day 0, with 10 μg of CT in Freund incomplete adjuvant on day 21, and with 10 μg of CT in saline on day 36; blood was collected on day 50. Goat anti-guinea pig globulin (Antibodies Incorporated, Davis, Calif.) was conjugated with alkaline phosphatase (Sigma type VII) (13). *p*-Nitrophenyl phosphate (Sigma 104 phosphate substrate) was used as the substrate in the reaction.

The standard toxin preparations were purified CT (Schwarz/Mann) and a crude dialyzed lyophilized culture filtrate of *E. coli* strain 408-3 obtained from R. B. Sack. This strain produces both LT and heat-stable enterotoxin.

Strains of *E. coli*, *V. cholerae* O group I, and *V. cholerae* non-O group I (NAG vibrios) were clinical isolates from patients at the International Centre for Diarrhoeal Disease Research, Bangladesh. Isolates to be tested in the ELISA assay were grown either in Casamino Acids-glucose medium with yeast extract (Casamino Acids [Difco], 30 g/liter; yeast extract, 3.0 g/liter; K_2HPO_4, 0.5 g/liter; glucose, 2.0 g/liter; pH adjusted to 8.0 with NaOH) or Trypticase soy broth (BBL Microbiology Systems) with 0.6% yeast extract

under conditions as described. To screen *E. coli* isolates for toxin production, we used whole cultures grown in Trypticase soy broth with yeast extract in microtiter plates (0.2 ml per well) for 48 h at 37°C. These whole cultures could then be transferred either to the microtiter ELISA plate or to the Y_1 adrenal plate (9) for assay. We used two standard antisera against CT: Swiss Serum Vaccine Institute anticholera serum [lot no. EL3(A-2/67)B] and the U.S. standard anticholera serum (NIH lot 1) (5). Both of these were obtained from Carl Miller at the National Institutes of Health. R. B. Sack provided the anti-*E. coli* LT serum (10).

The ELISA assay involves the consecutive addition of ganglioside, antigen, and antibody as illustrated in Table 1. After each such addition, the plate was washed three times with phosphate-buffered saline-Tween to remove unbound material. The appropriate dilution and concentrations of reagents were determined by checkerboard titrations. The optical density of the color reaction was measured in a colorimeter with a micro, flow-through cuvette capable of reading optical density (OD) of a volume of 80 μl or more (Elisa Reader, Dynatech). For detection of toxin, a P/N ratio was calculated by dividing the OD of the unknown sample by the OD of the negative control. A known positive strain of *E. coli* and a media control were included on each plate for positive and negative controls, respectively. Negative strains gave OD readings not significantly different from the media control (OD, 0.08 to 0.15), and samples with a P/N ratio ≤2.0 were considered negative.

The blocking antibody assay was determined by incubating serum diluted with 1% fetal calf serum, 75 μl, in wells of an uncoated microtiter plate, with 75 μl of toxin on a rotating board for 1 h. A 100-μl sample of this mixture was then added at step 3 (Table 1), and the assay was performed as for toxin. The concentration of toxin used in the blocking test was four times the dose of the respective toxin that gave an OD of 1.00 ($ED_{OD_{1.00}}$). This dose was 10 ng/ml for CT and 568 μg/ml for the crude lyophilized *E. coli* toxin. (Since the toxin was diluted with an equal volume of serum, the final concentration of toxin in the ELISA plate, assuming no neutralization, was two times the $ED_{OD_{1.00}}$.) The Y_1 adrenal cell assay was performed as described (9).

RESULTS

The titration curves for CT and *E. coli* toxin are shown on Fig. 1, and those for non-O group I *V. cholerae* are given in Fig. 2. The sensitivity of the G_{M1} ELISA was 265 pg/ml for CT and 285 μg/ml for the crude *E. coli* toxin. This is somewhat less sensitive than the morphological Y_1 adrenal cell assay, which will detect 100 pg of CT. The lowest detectable concentration of toxin was taken as that concentration which resulted in the optical density twice that of the buffer control.

E. coli isolates grown using the miniplate culture method gave comparable results when tested in the G_{M1} ELISA and Y_1 adrenal cell assays (Table 2). Two strains gave results that did not agree in the two assays. One strain was consistently strongly positive in the ELISA but was negative in adrenal cells. One strain gave consistently negative ELISA results but was strongly and repeatedly positive in the adrenal cells (and in the rabbit loop), and this activity was neutralized by Swiss Serum Institute anticholera serum.

Twenty-seven strains of *E. coli* (excluding the two disparate strains) were tested in the ganglioside and antiserum ELISA simultaneously using the same culture broth samples and the same guinea pig anti-CT (step 5, Table 1). Of 20 strains that were positive in the adrenal cell assay, all

TABLE 1. *ELISA methods: procedure for determination of E. coli LT or CT*

Step	Diluent[a]	Concn	Vol per well (μl)	Incubation time	Incubation temp
1. Precoat plate with G_{M1} ganglioside	PBS	1 μg/ml	100	Overnight	Room temp
2. Wash 3 × with PBS-Tween			200	3 min	Room temp
3. Add test sample	Undiluted or diluted PBS-Tween with 1% FCS		100	Overnight	Room temp
4. Wash 3 × with PBS-Tween			200	3 min	Room temp
5. Add guinea pig anti-CT	PBS-Tween 1% FCS	1:2,500[b]	100	1 h	37°C
6. Wash 3 × with PBS-Tween			200	3 min	Room temp
7. Add enzyme-labeled goat anti-guinea pig globulin	PBS-Tween with 1% FCS	1:1,000[b]	100	1 h	37°C
8. Wash 3 × with PBS-Tween			200	3 min	Room temp
9. Add substrate	10% DEA buffer	1 mg/ml	100	45 min	37°C
10. Stop reaction with NaOH		3 M	25		
11. Read OD at 405 nm in colorimeter					

[a] PBS, Phosphate-buffered saline; FCS, fetal calf serum; DEA, diethanolamine (13).
[b] Variable.

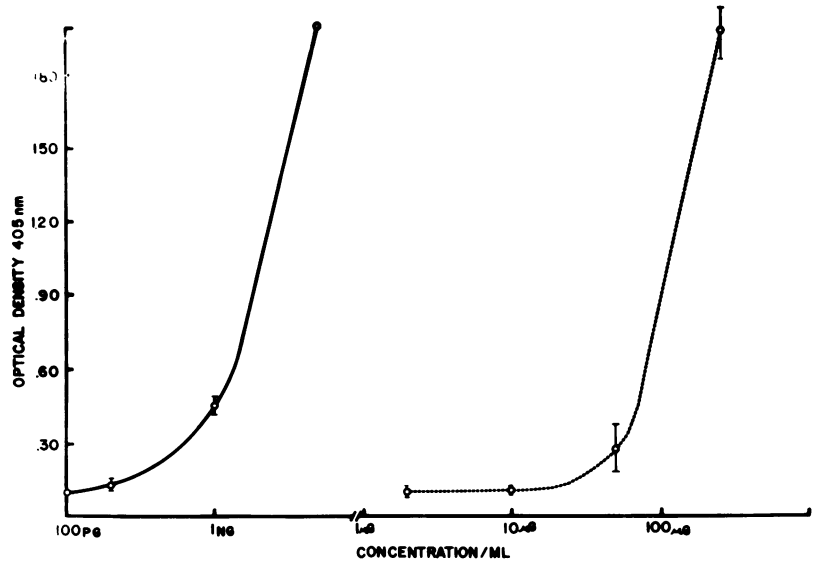

FIG. 1. *Titration curves for CT and E. coli enterotoxin in G_{M1} ganglioside micro-ELISA. Points and bars represent means and 2 standard errors of 10 determinations.* (- - -) *E. coli toxin;* (——) *CT.*

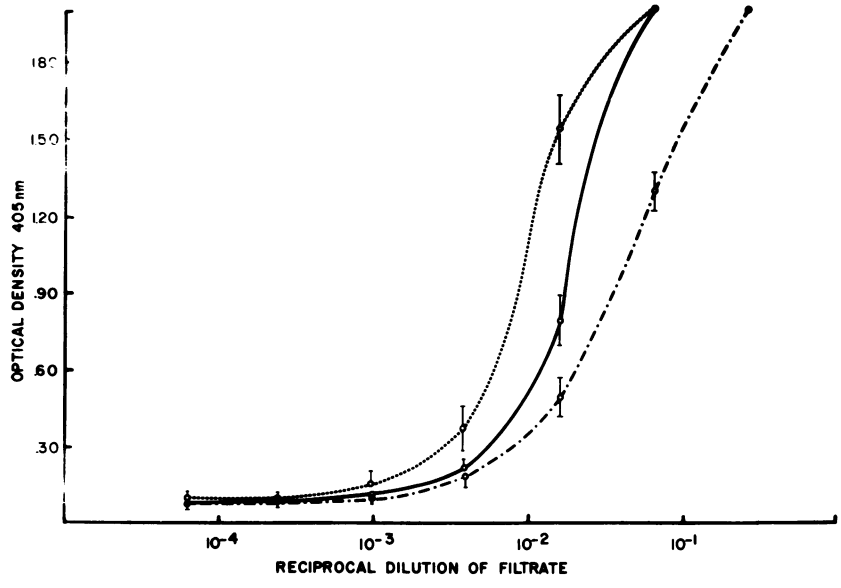

FIG. 2. *Titration curves for non-O group I V. cholerae enterotoxin in G_{M1} glanglioside micro-ELISA. Fourfold dilutions of culture filtrates from three strains of enterotoxigenic non-O group I V. cholerae were used including strain 178* (·····), *188* (——), *and 16* (-·-·-). *Points and bars represent mean and 2 standard errors of four determinations.*

were positive in both ELISA assays; however, 19 of 20 gave a higher OD with the ganglioside assay. The mean P/N ratio for the positive strains as measured in the ganglioside ELISA was 11.4 ± 4.9 (standard deviation) and that for antiserum ELISA was 3.4 ± 0.9. Of seven adrenal cell negative strains, all were negative in both ELISA assays. The P/N ratios were 1.4 ± 0.1 and 1.1 ± 0.1, respectively, for the negative strains as measured in the ganglioside and antiserum ELISA.

Although visual readings were usually reliable, occasional strains were weakly positive and were barely detectable with the eye although

they had a P/N ratio greater than 2. The OD of the negative controls varied between 0.08 and 0.15 from day to day, and well OD of less than 0.30 did not appear to the eye to be yellow.

Table 3 shows the results with five bacterial strains grown under different cultural conditions. Strain *V. cholerae* TC26909 was used because it was recently isolated from a patient with cholera. *E. coli* 61103C1 gave a weak positive (P/N 2.8) reaction in the screening ELISA, and strain 61237C3 gave a strong positive (P/N > 18) reaction in the screening ELISA. Tenfold dilutions were made of the whole culture or supernatant fluids. The titer given is the highest dilution giving a P/N ratio of greater than 2. Several cultural conditions can be used for *V. cholerae* and *E. coli* to screen for toxin production, though the optimal conditions vary with the individual strains.

Figure 3 shows the toxin blocking curves for *E. coli* LT toxin by three antisera. The antisera give similar curves, though the two anticholera sera gave higher titers than the *E. coli* LT antiserum. Figure 4 shows the toxin blocking curves for CT by two antisera. Again the curves are similar in shape, though the anticholera serum is much higher in titer than the anti-*E. coli* LT. The titers of the sera could be determined by using the $ED_{OD_{1.00}}$ as the endpoint and comparing the endpoints of the test sera to the Swiss Serum Vaccine Institute serum which has been assigned 4,470 anticholera units and 1,000 anti-*E. coli* LT units (10). With this standard, the U.S. standard anti-CT was determined to have 2,970 anti-CT units (curve not shown) and 1,088 anti-*E. coli* LT units; the anti-*E. coli* serum was determined to have 4.4 anti-CT units and 268 anti-*E. coli* LT units. This anti-*E. coli* LT serum was previously found to have 280 anti-LT units when measured in rabbit loops (10).

TABLE 2. *Comparison of G_{M1} ELISA with Y_1 adrenal assay for detection of E. coli LT in whole cultures of bacteria*[a]

G_{M1} ELISA	Y_1 adrenal cells	
	Positive	Negative
Positive	37	1
Negative	1	41

[a] Cultures are grown in Trypticase soy broth with 0.6% yeast extract in miniplates for 48 h, 0.2 ml per well.

DISCUSSION

The ELISA technique is a very useful procedure to detect many antigens and antibodies. While retaining the sensitivity and specificity of a radioimmunoassay, it uses stable and less expensive reagents and simple equipment and is especially useful in developing countries where these factors are crucial. After proper conditions are set, it is quite simple to perform.

In comparing the G_{M1} ELISA with the previously described antiserum ELISA (14) and the Y_1 adrenal cells (9), all seem to be acceptable methods for screening bacterial cultures for toxin and for quantitating toxin production. Where tissue culture facilities are available, the Y_1 adrenal cell method is still more efficient in screening large numbers of *E. coli* for LT production. The ELISA does not have the restriction of tissue culture facilities, is not affected by

TABLE 3. *Toxin production by bacterial strains grown under different cultural conditions, as measured by the G_{M1} ganglioside ELISA*[a]

Strain	TBS-YE				CA-YE/flask		
	Miniplate/37°C/still		Flask/37°C/shake/18 h		37°C		30°C/shake/18 h/supernatant
	24 h/whole culture	48 h/whole culture	Whole culture	Supernatant	Shake/18 h/supernatant	Still/18 h/supernatant	
569 Classical *V. cholerae*	100	100	>1,000	>1,000	>1,000	100	>1,000
TC26909 El Tor *V. cholerae*	1	10	1	1	1	1	1
61103Cl *E. coli* O6:K5:H16 (LT and ST)	Neg	1	1	1	10	ND	Neg
61237C3 *E. coli* O59:H − (LT only)	1	10	1	1	100	ND	10
61151C1 *E. coli* O9:K8:H2 (ST only)	Neg	Neg	Neg	Neg	Neg	ND	Neg

[a] Headings give cultural conditions in order: medium (TSB-YE, Trypticase soy broth-yeast extract; CA-YE, Casamino Acids-yeast extract), culture (flask, 50-ml flask with 5 ml of medium), temperature, agitation (still or shaken), incubation time, and culture fraction tested. Numbers indicate reciprocal of highest dilution giving positive result. Tenfold dilutions were used. Neg, Negative. ND, Not done. ST, Heat-stable toxin.

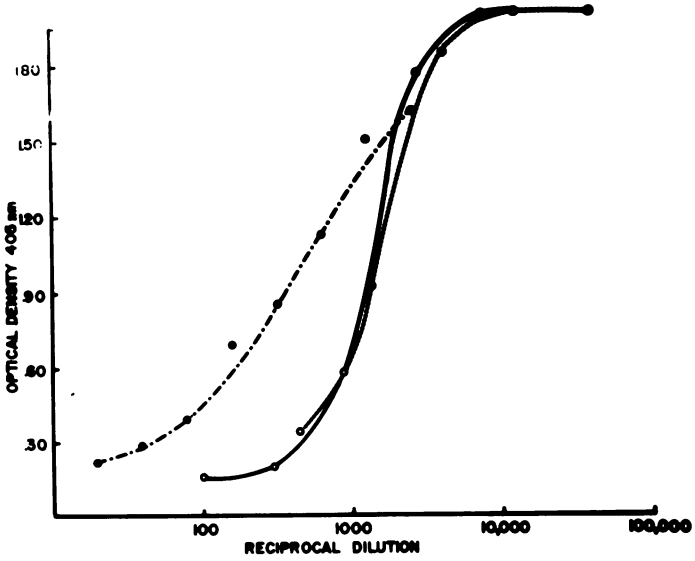

FIG. 3. *Blocking of E. coli LT binding by three antisera. Sera tested were: anti-408-3 (an E. coli strain that produces LT and heat-stable toxin)* (-·-·-); *U.S. Standard anti-CT serum, lot 1* (——); *Swiss Serum Vaccine Institute CT serum* (·····). *Points represent mean of two determinations.*

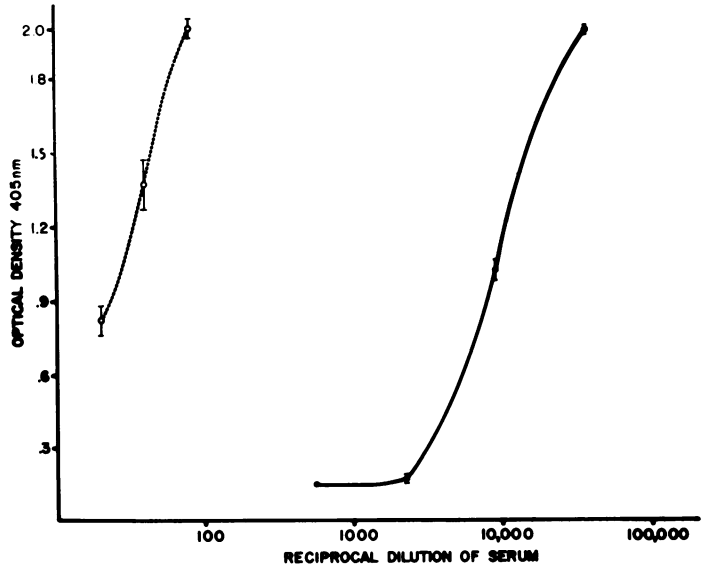

FIG. 4. *Blocking of CT binding by two antisera. Sera tested were anti-408-3 (an E. coli that produces LT and heat-stable toxin)* (·····), *and SSVI anticholera serum* (——).

cytotoxic materials which may also be present in bacterial culture broth, and may be more suitable for clinical laboratories. The G_{M1} ELISA has some theoretical advantages over the antiserum ELISA including: (i) occasional cross-reactions between species proteins do not occur when a purified chemical is used in the precoat layer. (ii) The use of a biological receptor G_{M1} ganglioside in the precoat offers an opportunity to study receptor binding by the toxin. (iii) The use of a purified chemical in the precoat obviates problems of a lab-to-lab variation caused by the use of different reagents at this step. In practice both the antiserum ELISA and G_{M1} ganglioside ELISA can be used effectively.

Since the G_{M1} ELISA depends on both the

attachment of the unknown toxin to the ganglioside as well as the binding of the anti-CT to the toxin, toxin preparations positive in this assay are likely to have both antigenic and binding properties similar to CT. This would suggest that the toxin produced by non-O group I *V. cholerae* (NAG vibrio) has these properties. Previously this toxin has been shown to be similar to CT in several respects: it is heat labile, it gives positive reactions in rabbit skin, rabbit loop, CHO cells, and adrenal cells similar to CT, and it is neutralized by anti-CT serum in the adrenal cell system.

Two of the strains of *E. coli* gave discrepant results when tested in both the Y_1 adrenal cell assay and ELISA. The lesser sensitivity of the ELISA when compared to the Y_1 adrenal cell assay may explain the one false-negative result seen. The false-positive strain (the strain positive in ELISA but negative in Y_1 adrenal cells) gave consistently elevated ODs and may represent the presence of material which is similar to CT in its binding and antigenic characteristics but is not active in the cell system. Further work is planned to characterize this material. The simultaneous use of an antigenic assay (e.g., ELISA) and an activity assay (e.g., adrenal cell) should be useful in detecting strains in which there is a discordance between these two properties of a bacterial product, and identification of these strains may be important in vaccine development.

The ELISA assay should also be helpful in measuring antibodies to these bacterial enterotoxins. The method for determining blocking antibodies in the ELISA is different from the immunoglobulin-specific anticholera antibody assay previously described (8) and is more analogous to a neutralization assay since it measures the ability of the antibody to block the attachment of the toxin to the G_{M1} ganglioside. Since this is the current notion of the way in which antitoxin is able to neutralize the activity of the toxin, the results obtained should be similar to those obtained using a neutralization assay. Using this assay, which has a sensitivity of about 0.2 anti-CT units, we have detected rises in antitoxin antibody titer in serum, milk, and intestinal specimens from patients convalescent from cholera.

The G_{M1} ELISA described here is a practical, simple, and inexpensive assay using commercially available reagents (the guinea pig anti-CT serum must be prepared by immunizing guinea pigs with CT); it is also a potential research tool which may be used to detect bacterial mutants, investigate binding properties of bacterial enterotoxins, and quantitate blocking antibodies to the enterotoxins.

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LITERATURE CITED

1. **Brill, B. M., B. L. Wasilauskas, and S. H. Richardson.** 1979. Adaptation of staphylococcal-coagglutination technique for detection of heat-labile enterotoxin of *Escherichia coli*. J. Clin. Microbiol. **9**:49–55.
2. **Craig, J. P.** 1966. Preparation of the vascular permeability factor of *Vibrio cholerae*. J. Bacteriol. **92**:793–795.
3. **Evans, D. J., and D. G. Evans.** 1977. Direct serological assay for the heat-labile enterotoxin of *Escherichia coli*, using passive immune hemolysis. Infect. Immun. **16**: 604–609.
4. **Farkas-Himsley, H., J. Jessop, and P. Corey.** 1977. Development and standardization of a cytotoxic microassay for detection of enterotoxins: survey of enterotoxins from *Escherichia coli* of infant origin. Microbios **18**:195–212.
5. **Feeley, J., E. S. Beck, J. P. Craig, C. Hardegree, C. E. Miller, R. Northrup, and E. Seligmann.** 1976. Cholera antitoxin: report of an ad hoc committee, U.S. Cholera Panel. Proceedings of the 12th Joint Conference U.S.-Japan Cooperative Medical Science Program, Cholera Panel, Sapporo.
6. **Greenberg, H. B., D. A. Sack, W. Rodriquez, R. B. Sack, R. G., Wyatt, A. R. Kalica, R. L. Horswood, R. M. Chanock, and A. Z. Kapikian.** 1977. A microtiter solid phase radioimmunoassay for detection of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **17**:541–545.
7. **Guerrant, R. L., L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, and A. G. Gilman.** 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **10**:320–327.
8. **Holmgren, J., and A.-M. Svennerholm.** 1973. Enzyme-linked immunosorbent assay for cholera serology. Infect. Immun. **7**:759–763.
9. **Sack, D. A., and R. B. Sack.** 1975. Test for enterotoxigenic *Escherichia coli* using Y_1 adrenal cells in miniculture. Infect. Immun. **11**:334–336.
10. **Sack, R. B.** 1973. Immunization with *Escherichia coli* enterotoxin protects against homologous enterotoxin challenge. Infect. Immun. **8**:641–644.
11. **Sack, R. B., S. L. Gorbach, and J. G. Banwell.** 1971. Enterotoxigenic *E. coli* isolated from patients with severe cholera-like disease. J. Infect. Dis. **123**:378–385.
12. **Svennerholm, A.-M., and J. Holmgren.** 1978. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (G_{M1} ELISA) procedure. Curr. Microbiol. **1**:19–24.
13. **Voller, A., D. Bidwell, and A. Bartlett.** 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p. 506–512. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. American Society for Microbiology, Washington, D.C.
14. **Yolken, R. H., H. B. Greenberg, M. H. Merson, R. B. Sack, and A. Z. Kapikian.** 1977. Enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. J. Clin. Microbiol. **6**:439–444.