JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1981, p. 473-478
0095-1137/81/110473-06$02.00/0

Vol. 14, No. 5

Single Radial Immune Hemolysis Test for Detection of *Escherichia coli* Thermolabile Enterotoxin

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Received 23 January 1981/Accepted 4 May 1981

A single radial immune hemolysis test for the detection of thermolabile enterotoxin has been developed for routine purposes. Stationary cultures from enterotoxigenic *Escherichia coli* in Casamino Acids-yeast extract medium may be used for the detection of this enterotoxin, and under the conditions of the experiment, the single radial immune hemolysis test was as sensitive as the passive immune hemolysis test. The results obtained in the single radial immune hemolysis test agreed entirely with those obtained in the passive immune hemolysis test, and no false-positive reactions were obtained when cholera antitoxin diluted 1:80 was used. The assay is easy to perform, inexpensive, and specially designed for less-equipped laboratories.

Production of heat-labile (LT) toxin from enterotoxigenic *Escherichia coli* (ETEC) has mainly been assayed by tissue culture techniques (4, 10, 14). Alternatively, LT toxin can also be detected by either radioimmunoassay or by enzyme-linked immunosorbent assay (1, 9, 15). These tests, although quite sensitive, are expensive and require special equipment, drugs, and reagents. In the case of tissue culture assays, special handling of cell lines as well as experience for interpretation of the results are necessary. As a consequence, these techniques are not available to all laboratories, especially those in developing countries.

Although a single radial immune hemolysis (SRIH) test has been previously described to detect hypotoxigenic mutants of *E. coli* and *Vibrio cholera* (2), so far it has not been used for clinical purposes. Taking this into consideration, our purpose was to report an adaptation of the SRIH test for the routine detection of ETEC.

MATERIALS AND METHODS

Strains. The sources and designations of the LT *E. coli* strains studied are as follows: strains 40T, 05/75, 13D, 38, 73/1, 82/2, 156/2, 269/7, 2161, and 19927 (supplied by L. R. Trabulsi, Escola Paulista de Medicina, Sao Paulo, Brazil) were isolated from human sources; strains 36 and 153 (supplied by L. R. Trabulsi) were isolated from river water; and strains A, 306, 339, 406, 2423, and 3406 were isolated by us from porcine sources. *E. coli* 40T (LT^+), kindly supplied by L. R. Trabulsi, was used as a standard positive control for the passive immune hemolysis (PIH) and SRIH tests and the Y-1 adrenal cell assay. *E. coli* K-12 (LT^-), supplied by D. J. Evans, was used as a standard negative control for these tests. Ten other LT^- strains (not numbered) were used to evaluate the specificity of the SRIH test.

Immune sera. Cholera antitoxin prepared by the Swiss Serum and Vaccine Institute was a generous gift from G. I. Curlin (Enteric Diseases Program Officer, National Institutes of Health, Bethsda, Md.). For all experiments, cholera antitoxin, after being reconstituted with distilled water as recommended, was diluted 1:10 with 0.15 M saline. After inactivation at 56°C for 30 min, the antitoxin was absorbed three times with sheep erythrocytes (SRBC).

Enterotoxin preparation. Enterotoxins to be tested by the PIH and SRIH tests were prepared by the polymyxin B-release technique (7). Organisms were inoculated in Casamino Acids-yeast extract medium (6) and incubated at 37°C in a rotary shaker at 150 rpm for 18 h. To each 10 ml of Casamino Acids-yeast extract cultures, 1.0 ml of polymyxin solution (2.2 mg/ml) in 0.04 M phosphate-buffered saline, pH 6.7, was added. Supernatants recovered after centrifugation at 12,000 × *g* for 30 min were considered as the polymyxin-extract preparations.

Y-1 adrenal cell assay and PIH test. For the investigation of LT toxin by the Y-1 adrenal cell assay, tests were performed with Casamino Acids-yeast extract supernatants from shaking cultures, according to the procedures recommended by Donta et al. (4). The performance of the PIH test was similar to that previously described (12), except that Veronal-buffered saline plus Ca^{2+} and Mg^{2+} was used instead of phosphate-buffered saline to improve the sensitivity of the test (3). This single modification rendered the test much more sensitive than that described by other authors (5, 12). Other conditions, such as concentration and volume of reagents, as well as technical procedures were the same as those reported formerly (12).

SRIH test. (i) Preparation of slides. Common microscopic slides were boiled in a solution of trisodium phosphate (5 g/liter) for 30 min. Afterwards, the slides were washed with tap water (10 times) and then placed into a solution of hydrochloric acid (250 ml per 20 liters of distilled water). Washing with tap water was repeated, followed by washing with distilled water. After drying in an incubator at 56°C, the slides were covered with a pellicle of 1% agarose and left at room temperature for solidification of the gel.

(ii) Agarose preparation. Agarose low (Sigma Chemical Co., St. Louis, Mo.) was diluted in Veronal-buffered saline plus Ca^{2+} and Mg^{2+} ions at pH 7.4 (10) in a final concentration of 1%. The gel was melted by boiling, distributed into 5-ml glass tubes (2.7 ml per tube), and kept at 45°C in a water bath.

(iii) Test. Performance of the SRIH test was similar to that described for the detection of rubella antibody (8).

SRBC were washed three times in Veronal-buffered saline and then diluted in the same buffer at a concentration of 10%. For sensitization, 1 ml of polymyxin-released extracts from Casamino Acids-yeast extract cultures was added to an equal volume of SRBC suspensions. Mixtures were incubated in a water bath at 37°C for 30 min and then centrifuged. Pellets of sensitized SRBC were resuspended in the same buffer at a concentration of 5%. Other concentrations (0.25 and 0.125%) of sensitized SRBC were also tested to verify whether there was any increase in the sensitivity of the reaction. Volumes of 0.3 ml of each sensitized SRBC preparation were added to each tube of molten agarose. The contents of the tubes were mixed vigorously, poured on the slides, and then cooled. Two wells, 2.5 cm apart and 5 mm in diameter, were punched in the SRBC-agarose. Special care was taken to make these wells on the midline of the longitudinal length of the microscopic slide. A 25-μl amount of cholera antitoxin in different dilutions (1:10 to 1:160) was added to each well. The slides were kept at 4°C for 24 h, after which 2 ml of 1:10 guinea-pig sera (source of complement) in Veronal electrendosmosis-buffered saline was pipetted over the surface of the gel. The slides were incubated in a wet chamber at 37°C for 6 h. The excess of complement was then removed, and the diameters (in millimeters) of the hemolytic zones (in two dimensions) were measured to the nearest 0.5 mm.

RESULTS

Concentration of SRBC. We found no differences in the diameters of the hemolytic zones as a function of the SRBC concentrations used (0.5, 0.25, and 0.125%). However, the clarity of the zones changed noticeably; as the red background decreased, it became increasingly difficult to discern hemolysis. Therefore, for all standardization procedures concerning the SRIH test, the final SRBC concentration of 0.5% was chosen.

Determination of enterotoxigenic potency of ETEC by the PIH test. Supernatants from ETEC were positive in the Y-1 adrenal cell assay, with titers ranging from 1:2 to 1:16, calculated from the 50% rounding effect. The degree of hemolysis in the PIH test is read spectrophotometrically, therefore detecting minor differences regarding enterotoxigenicity. Because of this and on account of its sensitivity when compared with the Y-1 adrenal cell assay (Table 1), the PIH test was chosen to determine the enterotoxigenic potency of some ETEC to be studied by the SRIH test.

According to the highest reciprocal dilution of the polymyxin-release extract, which gave absorbancy at 420 nm (A_{420}) readings of >0.18 (>32 μg of hemoglobin release), the strains were classified as strong, intermediate, and weak producers of LT toxin (Table 1).

Standardization of the SRIH test in relation to the PIH test. Strain 40T (human origin), because of its stronger enterotoxigenicity, was selected for the standardization of the SRIH test in relation to the PIH test. Therefore, reactions with different dilutions of antitoxin and polymyxin-release extract from strain 40T were performed in parallel by the PIH and SRIH tests. A_{420} readings and the respective amount of hemoglobin release were compared with the diameters obtained in the SRIH test (Table 2). These diameters were then plotted against the A_{420} values (Fig. 1), and micrograms of hemoglo-

TABLE 1. *Determination of enterotoxigenic potency of some ETEC[a] strains by the PIH test and Y-1 adrenal cell assay*

Strain	PIH test: Reciprocal dilution of the end titer[b]	PIH test: A_{420} readings at the end titer	Y-1 assay[c]	Classification of enterotoxigenicity according to PIH test[d]
40T	256	0.37	8	Strong
156/2	16	0.46	2	Intermediate
05/75	4	0.25	2	Weak
3406	16	0.22	8	Intermediate
2423	32	0.29	2	Intermediate
339	8	0.25	2	Weak
36	16	0.28	2	Intermediate

[a] Supernatants from shaking cultures (150 rpm) of ET *E. coli* in Casamino Acids-yeast extract medium were diluted twofold and then assayed by the PIH test and the Y-1 adrenal cell assay.

[b] Highest dilution of toxin which gave A_{420} readings of >0.18 (32 μg of hemoglobin release).

[c] Numbers represent the reciprocal dilution which gave 50% of rounding effect.

[d] Strong, Positive tests (A_{420} > 0.18) with reciprocal dilutions of enterotoxin higher than 32; Intermediate, positive tests with reciprocal dilutions of enterotoxin between 8 and 32; Weak, positive tests with reciprocal dilutions of enterotoxin less than 8.

TABLE 2. *Comparative results of the PIH and SRIH tests with polymyxin-release extract from strain E. coli 40T*

Reciprocal of dilution of:		Test result		
		PIH		SRIH (mean diameter [±1 SEM] of hemolysis [mm])
Toxin	Antitoxin	A_{420} ± 1 SEM[a]	μg of hemoglobin release ± 1 SEM	
Net	10	1.34 ± 0.0057	241.44 ± 1.0416	17.50 ± 0.2886
	20	1.33 ± 0.0050	240.08 ± 1.1234	16.68 ± 0.1875
	40	1.33 ± 0.0048	240.53 ± 1.1636	15.81 ± 0.2362
	80	1.34 ± 0.0111	241.88 ± 1.9977	14.81 ± 0.1870
	160	1.32 ± 0.0110	239.63 ± 1.7834	14.00 ± 0.2041
1:2	10	1.33 ± 0.0081	239.63 ± 1.4711	17.50 ± 0.3535
	20	1.31 ± 0.0266	237.38 ± 4.7879	16.31 ± 0.2362
	40	1.34 ± 0.005	242.79 ± 4.7879	15.93 ± 0.2140
	80	1.34 ± 0.0028	241.89 ± 0.4490	14.62 ± 0.3752
	160	1.24 ± 0.0521	223.41 ± 9.3921	13.5 ± 0.4203
1:4	10	1.31 ± 0.0239	237.38 ± 4.3128	17.31 ± 0.1190
	20	1.28 ± 0.0058	231.52 ± 10.6201	16.06 ± 0.0645
	40	1.27 ± 0.0047	228.82 ± 8.6409	15.37 ± 0.2160
	80	1.21 ± 0.031	218.46 ± 9.2276	14.25 ± 0.3947
	160	1.15 ± 0.067	207.65 ± 12.2046	13.37 ± 0.2397
1:8	10	1.08 ± 0.040	195.94 ± 7.2575	16.87 ± 0.2886
	20	0.94 ± 0.027	170.71 ± 4.9684	15.62 ± 0.2972
	40	0.88 ± 0.015	160.13 ± 2.8808	15.00 ± 0.2041
	80	0.86 ± 0.014	156.52 ± 2.5571	13.5 ± 0.5000
	160	0.83 ± 0.019	149.54 ± 3.5072	12.93 ± 0.0645
1:16	10	0.74 ± 0.029	134.22 ± 5.3290	15.5 ± 0.2886
	20	0.71 ± 0.013	129.27 ± 2.4532	14.5 ± 0.2886
	40	0.67 ± 0.026	121.16 ± 4.8303	14.06 ± 0.1581
	80	0.59 ± 0.029	107.20 ± 5.3934	13.12 ± 0.1258
	160	0.49 ± 0.046	89.41 ± 8.3668	11.2 ± 0.00
1:32	10	0.410 ± 0.025	75.44 ± 4.6560	14.5 ± 0.02886
	20	0.34 ± 0.037	62.15 ± 6.8011	11.25 ± 0.4787
	40	0.28 ± 0.019	52.02 ± 3.5341	12.56 ± 0.6487
	80	0.23 ± 0.008	41.43 ± 1.6038	11.00 ± 0.4082
	160	0.16 ± 0.016	29.42 ± 3.0479	10.66 ± 0.6668
1:64	10	0.14 ± 0.020	26.12 ± 3.7148	12.00 ± 0.00
	20	0.11 ± 0.021	20.94 ± 3.8824	11.00 ± 1.0
	40			
	80			
	160			
E. coli K-12 (net)	10	0.14 ± 0.0020	24.32 ± 0.3668	6.00 ± 0.048

[a] SEM, Standard error of the mean.

bin release (Fig. 2) were detected in the PIH test. The *r* values for 25 determinations were 0.7489 and 0.7557, respectively.

The 95% confidence interval for *y* (diameter) values of the hemolytic zones in the SRIH test were 11.8867 ± 0.5481 mm; i.e., diameters less than 11.33 were negative, and those higher than 12.43 were positive (Fig. 3). Intermediate values were considered doubtful. Except at low dilutions (1:10) of cholera antitoxin, no zones of hemolysis were obtained when *E. coli* K-12 (Fig. 4) was used as the standard negative control for all reactions. The results of the SRIH tests carried out with polymyxin-release extracts from enterotoxigenic strains are shown in Table 3. It can be verified that our cholera antitoxin diluted 1:160 gave some low readings, within the range of the doubtful results. Thus, 1:40 and 1:80 di-

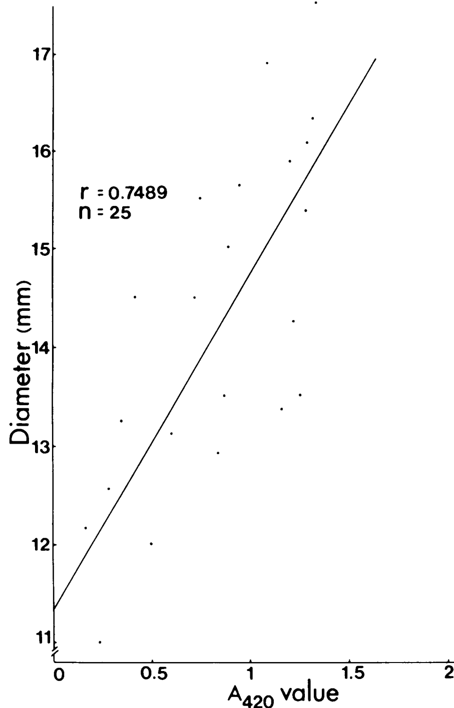

FIG. 1. *Correlation between the A_{420} obtained in the examination of polymyxin extracts from cultures of E. coli 40T by the PIH test and the diameters (in millimeters) obtained in the SRIH test.*

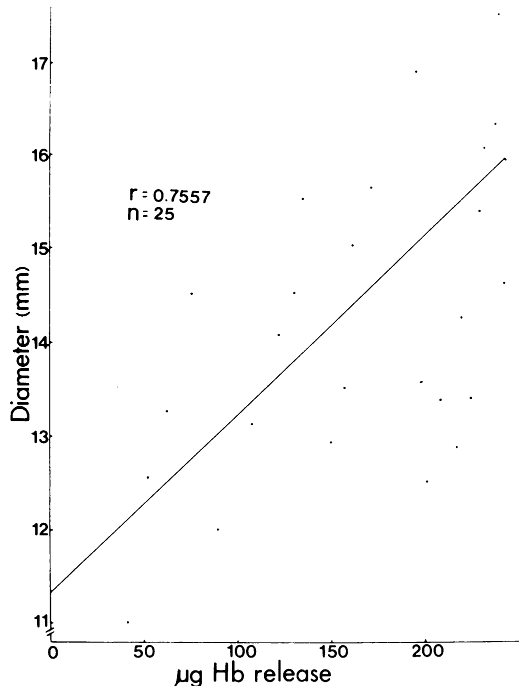

FIG. 2. *Correlation between the amount (in micrograms) of hemoglobin (Hb) released in the examination of polymyxin extracts from E. coli 40T by the PIH test and the diameters (in millimeters) obtained in the SRIH test.*

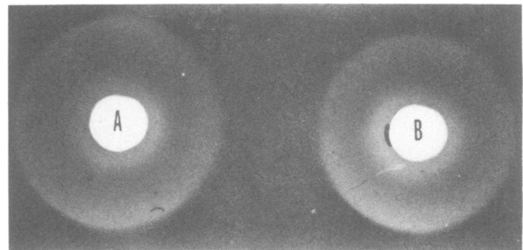

FIG. 3. *SRIH test with polymyxin B-release extract from an enterotoxigenic strain of E. coli (40T). (A) Diameter of hemolysis obtained with cholera antitoxin diluted 1:40; (B) diameter of hemolysis obtained with cholera antitoxin diluted 1:80.*

lutions of this antitoxin are likely to give more reliable results for routine purposes and are now recommended for the test.

Under these conditions, the results obtained in the SRIH test with 27 strains of colibacilli (17 ETEC strains [Table 1] and 10 non-ETEC strains) revealed that none of the non-ETEC strains gave any hemolytic area around the wells punched in the agarose gel. Also, there was total agreement between positive results obtained in the PIH and SRIH tests (Table 4).

DISCUSSION

The SRIH test described herein for the detection of ETEC is simple, very sensitive, and permits several strains of *E. coli* to be examined per day. Any junior technician can carry out this test, which does not require any special equipment. Standardization of SRBC suspensions is not done spectrophotometrically, and cholera antitoxin, which reacts serologically with LT toxin (13), may be obtained commercially. Alternatively, this antitoxin may be prepared by the immunization of animals such as sheep and rabbits with cholera toxin. Complement is used in excess and consists of a 1:10 dilution of pooled sera from at least 25 guinea pigs.

The amount of reagents used for the test is very low; i.e., 50 μl of cholera antitoxin diluted 1: 80 or more, depending on the standardization of the test, is enough to examine each strain of ETEC, in duplicate. In addition, 500 g of agarose, the most expensive material needed for this assay, should be enough for a laboratory to carry out 50 tests in duplicate daily for almost 2 years. With a single reading one can identify an ETEC strain, and, used as described, the test does not

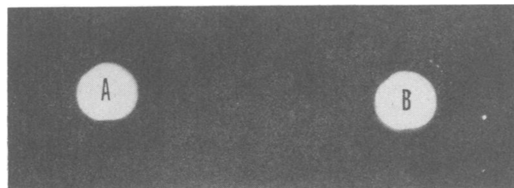

FIG. 4. *SRIH test with polymyxin B-release extract from E. coli K-12 (non-ET). A and B correspond to reactions carried out with cholera antitoxin diluted 1:40 and 1:80, respectively. No hemolysis was obtained with either dilution of cholera antitoxin.*

TABLE 3. *Results of the SRIH test with ETEC of different enterotoxigenic potencies, using decreasing amounts of cholera antitoxin*

Strain	Enterotoxigenicity	Mean diam (mm) of hemolysis with cholera antitoxin dilution of:			
		20	40	80	160
40T	Strong	16	15	14.8	14.0
156/2	Intermediate	16	15	14.0	12.0
05/75	Weak	16.5	14.5	14.0	12.5
3406	Intermediate	16.5	15.5	13.0	13.0
2423	Intermediate	16.5	14.5	14.0	12.0
339	Weak	15	14.0	14.0	13.0
36	Intermediate	17	16.5	15.5	14.0

show any false-positive reactions, showing total agreement with the PIH test and being more sensitive than the Y-1 adrenal cell assay.

In our hands, no negative strain gave any zone of hemolysis when the antitoxin dilution was higher than 1:20. However, in some tests, during standardization procedures of the SRIH, some narrow zones of hemolysis were seen around the wells when the cholera antitoxin used was diluted 1:10. The same phenomenon was observed from time to time with the PIH test when cholera antitoxin was used at lower dilutions.

Although we do not have any explanation for these results, we believe that they may be due to the presence in the antitoxin of antibodies against extracellular components of *E. coli* K-12 used as the negative control for the SRIH test.

Quantitative studies of the SRIH test showed that strains of ETEC that gave zones of hemolysis whose mean diameters ranged from 11.33 to 12.43 could be considered as doubtful positive, according to the 95% confidence limits calculated from our data. In fact, results in the PIH test were not different. In other words, LT^- strains gave A_{420} readings of up to 0.17, which corresponds to 30 μg of hemoglobin release. Weak LT^+ strains gave A_{420} values within the range of 0.18 to 0.25, which corresponds to 32 and 45 μg of hemoglobin release, respectively, and readings over 0.26 (more than 46 μg of hemoglobin release) are definitely positive.

TABLE 4. *Comparative results of the PIH and SRIH tests with polymyxin-release extracts of ETEC and non-ETEC strains*

Strain	Test Result	
	PIH	SRIH
ETEC		
05/75	1.10 ± 0.0035[a]	15.00 ± 0.1580[b]
13D	1.21 ± 0.0050	16.00 ± 0.2887
38	1.32 ± 0.0029	15.25 ± 0.2500
73/1	1.31 ± 0.0014	16.00 ± 0.2887
82/2	1.10 ± 0.0029	15.50 ± 0.2887
156/2	1.27 ± 0.0228	16.00 ± 0.2980
269/7	1.10 ± 0.0152	14.75 ± 0.1443
2161	1.22 ± 0.0202	15.25 ± 0.1443
19927	1.32 ± 0.0043	16.00 ± 0.0000
36	1.32 ± 0.0048	16.00 ± 0.2112
153	1.14 ± 0.0050	15.75 ± 0.1443
A	1.05 ± 0.0043	14.50 ± 0.2887
306	0.20 ± 0.0022	11.38 ± 0.0306
339	1.13 ± 0.0039	14.50 ± 0.2898
406	0.22 ± 0.0202	12.00 ± 0.0000
2423	1.19 ± 0.0030	14.37 ± 0.1230
3406	1.26 ± 0.0224	15.00 ± 0.1488
Non-ETEC		
10 strains (not numbered)	0.09 ± 0.0029[c]	—[d]

[a] Arithmetic mean of the A_{420} values in four PIH tests carried out with each preparation of ETEC strains ± 1 standard error of the mean.

[b] Arithmetic mean of the diameters of hemolysis obtained in four SRIH tests carried out with each preparation of ETEC strains ± 1 standard error of the mean.

[c] Arithmetic mean of the A_{420} values obtained in the PIH test carried out with non-ETEC strains ± 1 standard error of the mean.

[d] —, No zone of hemolysis in the SRIH tests carried out as described in the text.

In the case of questionable results, it is advisable to repeat the PIH test, and the same is true for those strains which in the SRIH test give mean diameter values between 11.33 and 12.43. Therefore, based on our results, only strains which give mean diameters higher than 12.43 should be considered positive. However, we would like to stress the fact that none of the LT^- strains showed any hemolytic zone around the wells in the gel when proper dilutions (1:40 to 1:80) of cholera antitoxin were used. Concerning the diameter of the zones of hemolysis observed in the SRIH tests, it is important to point out that the size of the hemolytic areas is related to the rate of diffusion of the cholera antitoxin, which is a function of the antitoxin concentration. Therefore, though very clear-cut reactions were observed with strains of intermediate and strong enterotoxigenicity, a wider zone of he-

molysis cannot be taken as an indication of a stronger production of LT toxin. However, since in some reactions involving a constant dilution of antitoxin (1:80) against different dilutions of toxin a variation in the size of the hemolytic areas was observed (Table 2), we believe that further studies should be conducted on the influence of toxin concentration to be assayed by this test. As described for the PIH test (3), stationary cultures may be used to cultivate ETEC strains to be examined by the SRIH test. The addition of mitomycin (0.5 μg/ml) to the lag phase of stationary cultures rendered the test more sensitive (unpublished data).

Although we are not informed about the conditions of less-equipped laboratories in well-developed countries, we believe that the test described in this paper will undoubtedly be very useful because it is relatively cheap, efficient, and specific, and it does not need any elaborate equipment for its performance. Another potential application of the SRIH test which has already been used for some viral diseases (8) might be its use for measuring serological conversion among individuals from which the bacteriological diagnosis of diarrhea caused by ETEC strains is not possible.

Finally, although the report of inactive LT enterotoxin produced by colibacilli isolated from clinical specimens is rare (5), one must be aware of this possibility since, in this case, the SRIH and the PIH tests may give false-positive results without agreement with the Y-1 adrenal cell assay or other similar tests which detect active LT enterotoxin.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Animal Health Program sponsored by the CNPq (Brazilian National Research Council) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Project 204/78. We gratefully acknowledge the U.S.-Japan Cooperative Medical Sciences Program of the National Institute of Allergy and Infectious Disease, Bethesda, Md., for the supply of anticholeragenoid and cholera antitoxin.

LITERATURE CITED

1. **Bäck, E., A. M. Svennerholm, J. Holmgren, and R. Möllby.** 1979. Evaluation of a ganglioside immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. J. Clin. Microbiol. **10**:791–795.
2. **Bramucci, M. G., and R. K. Holmes.** 1978. Radial passive immune hemolysis assay for detection of heat-labile enterotoxin produced by individual colonies of *Escherichia coli* or *Vibrio cholerae*. J. Clin. Microbiol. **8**:252–255.
3. **de Castro, A. F. P., M. B. Serafim, J. A. Gomes, and M. S. V. Gatti.** 1980. Improvements in the passive immune hemolysis test for assaying enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **12**:714–717.
4. **Donta, S. T., H. W. Moon, and S. C. Whipp.** 1974. Detection of heat-labile *E. coli* enterotoxin with the use of adrenal cells in tissue culture. Science **183**:334–336.
5. **Evans, D. J., Jr., 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.
6. **Evans, D. J., Jr., 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.
7. **Evans, D. J., Jr., D. G. Evans, and S. L. Gorbach.** 1974. Polymyxin B-induced release of low-molecular-weight, heat-labile enterotoxin from *Escherichia coli*. Infect. Immun. **10**:1010–1017.
8. **Forger, J. M., III, and R. F. Gilfillan.** 1979. Single-radial hemolysis as a cost-effective determinant of *Rubella* antibody status. J. Clin. Microbiol. **9**:115–119.
9. **Greenberg, H. B., D. A. Sack, W. Rodriguez, R. B. Sack, R. G. Wyatt, A. R. Kalica, R. L. Horswood, R. M. Chanock, and A. Z. Kapikian.** 1977. Microtiter solid-phase radioimmunoassay for detection of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **17**:541–545.
10. **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.
11. **Mayer, M. M., A. G. Osler, O. G. Bier, and M. Heidelberger.** 1946. The activating effect of magnesium and other cations on the hemolytic function of complement. J. Exp. Med. **84**:535–548.
12. **Serafim, M. B., A. F. P. de Castro, M. H. Lemos dos Reis, and L. R. Trabulsi.** 1979. Passive immune hemolysis for detection of heat-labile enterotoxin produced by *Escherichia coli* isolated from different sources. Infect. Immun. **24**:606–610.
13. **Smith, N. W., and R. B. Sack.** 1973. Immunologic cross reaction of enterotoxins from *Escherichia coli* and *Vibrio cholerae*. J. Infect. Dis. **127**:164–170.
14. **Speirs, J. I., S. Stavric, and J. Konowalchuk.** 1977. Assay of *Escherichia coli* heat-labile enterotoxin with Vero cells. Infect. Immun. **16**:617–622.
15. **Yolken, R. H., H. B. Greenberg, M. H. Merson, R. B. Sack, and A. Z. Zapikian.** 1977. Enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. J. Clin. Microbiol. **6**:439–444.