

## Passive Immune Hemolysis for Detection of Heat-Labile Enterotoxin Produced by *Escherichia coli* Isolated from Different Sources

M. B. SERAFIM,<sup>1</sup> A. F. PESTANA DE CASTRO,<sup>1\*</sup> M. H. LEMOS DOS REIS,<sup>2</sup> AND L. R. TRABULSI<sup>2</sup>

*Department of Microbiology and Immunology, University of Campinas, Campinas, São Paulo,<sup>1</sup> and Department of Microbiology, Immunology and Parasitology, Escola Paulista de Medicina, São Paulo, São Paulo,<sup>2</sup> Brazil*

Received for publication 5 February 1979

Fifty-one strains of *Escherichia coli* isolated from humans, swine, food, and water and identified as enterotoxinogenic by the Y-1 adrenal cell assay, were examined for heat-labile enterotoxin (LT) production by the passive immune hemolysis test. Cholera antitoxin, anti-cholera toxin and anti-LT were used as antisera. Cholera antitoxin was much more potent than anti-cholera toxin and LT antiserum in the detection of LT-positive strains. All strains isolated from pigs and sausage were negative in tests made with LT antiserum. A few strains isolated from humans, food, and water also gave negative results. These data showed that the passive immune hemolysis test is not as efficient as the Y-1 adrenal cell assay in the detection of enterotoxinogenic *E. coli* strains.

It has been shown that heat-labile enterotoxin (LT) produced by *Escherichia coli* strains can be detected by a passive immune hemolysis (PIH) test (5). The principle of the test rests on the addition of LT antitoxin and complement to sheep erythrocytes previously treated with a polymyxin extract of the *E. coli* strain under study. Good correlation between this method and the Y-1 adrenal cell assay has been demonstrated with enterotoxinogenic *E. coli* isolated from human feces (5).

During a study on the occurrence of enterotoxinogenic *E. coli* strains we noticed that the PIH test frequently failed to detect LT, mainly in strains of porcine origin. The purpose of this paper is to report results obtained with the PIH test with 51 *E. coli* strains isolated from different sources, all of which produced LT as detected by the Y-1 adrenal cell assay. It also presents an evaluation of the use of cholera antitoxin and anti-cholera toxin sera in the PIH test.

### MATERIALS AND METHODS

**Strains.** The sources and designations of the 51 *E. coli* strains are shown in Table 1.

The toxin was demonstrated by Y-1 adrenal cell assay (1) just before and after performing the PIH test.

*E. coli* H-10407 (LT positive) and K-12, kindly provided by D. J. Evans, Jr., were used in the preparation of the respective positive and negative polymyxin extracts, included as controls in the PIH test. *E. coli* 40T, an LT-producing strain isolated by us, was also included in this investigation for the prepa-

ration of a known LT-positive polymyxin extract to be used in the standardization of the PIH test.

Stock cultures were maintained on peptone-agar slants.

**Immune sera.** *E. coli* LT antitoxin was kindly provided by D. J. Evans, Jr. Cholera antitoxin prepared by the Swiss Serum and Vaccine Institute and anti-cholera toxin serum prepared by R. Finkelstein were generous gifts from Carl E. Miller, Enteric Diseases Program Officer, National Institutes of Health, Bethesda, Md.

**Polymyxin extract preparation.** *E. coli* LT was prepared by the polymyxin-release technique (3). Organisms were inoculated in CYE medium (2), incubated at 37°C in a rotatory incubator-shaker at 150 rpm for 18 h. A 1.0-ml amount of polymyxin containing 2.2 mg/ml in 0.04 M phosphate-buffered saline (PBS), pH 6.7, was then added to each flask. Shaking was continued at 37°C for an additional 15 min. Supernatants recovered after centrifugation of the polymyxin-treated cultures were considered as the extract preparations to be assayed.

**PIH test.** The procedures used to perform the PIH test were similar to those described by Evans and Evans (5). The following modifications were introduced: (i) sheep erythrocyte (SRBC) suspensions were prepared in PBS and adjusted for each test so that after lysis at a 1:20 dilution in distilled water, the resulting hemoglobin solution gave a reading of 1.3 at 420 nm in a Coleman, Junior spectrophotometer. Preliminary tests indicated that this reading was usually obtained with suspensions containing  $2 \times 10^9$  cells per ml; (ii) before use, LT antitoxin was diluted twofold from 1:40 to 1:1,280 in 0.04 M PBS (pH 6.7) and tested against crude standardized extract prepared from *E. coli* H-10407 and polymyxin extracts prepared from *E. coli* 40T (LT positive) and *E. coli* K-12 (LT negative).

TABLE 1. Sources of enterotoxinogenic *E. coli* strains

| Source                                 | Strains                                                                                                                                                                                  |
|----------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Children with diarrhea <sup>a</sup>    | TR 146/2, TR 156/2, TR 60/1, TR 103/3, TR 13-D, TR 126/75, TR 05/75, TR 204/75, TR 113/3, TR 2161                                                                                        |
| Children with no diarrhea <sup>a</sup> | TR 150/2, TR 71/7, TR 81/8, TR 4/7, TR 73/1, TR 227/1, TR 82/2, TR 269/7                                                                                                                 |
| Human diarrhea <sup>b</sup>            | 9192, <sup>c</sup> 16932, <sup>c</sup> 19927, <sup>c</sup> TD 427, <sup>d</sup> FC 7-2, <sup>e</sup> FC 24-6, <sup>e</sup> FC 11-2, <sup>e</sup> B 16-4, <sup>e</sup> B 1-5 <sup>e</sup> |
| Swine with diarrhea <sup>a</sup>       | 17, 100, 306, 339, 406, 446, 2423, 3406, A, B                                                                                                                                            |
| River water <sup>a</sup>               | TR 36, TR 113, TR 151, TR 153, TR 170                                                                                                                                                    |
| Food                                   |                                                                                                                                                                                          |
| Sausage                                | TR 106, TR 107, TR 108                                                                                                                                                                   |
| "Keebe"                                | TR 104, TR 105                                                                                                                                                                           |
| Hamburger                              | TR 100, TR 101, TR 102, TR 103                                                                                                                                                           |

<sup>a</sup> Strains isolated by the authors.

<sup>b</sup> Strains supplied by other investigators.

<sup>c</sup> Supplied by W. K. Maas, New York, N.Y.

<sup>d</sup> Supplied by D. J. Evans, Jr., Houston, Texas.

<sup>e</sup> Supplied by R. L. Guerrant, Charlottesville, Va.

Similar procedures were adopted for cholera antitoxin and anti-cholera antigen; (iii) pooled sera collected from 30 guinea pigs were used as the source of complement. It was stored in 0.5-ml volumes and kept frozen at -70°C until use.

Based on the results of these tests, LT antitoxin and anti-cholera antigen were diluted, respectively, 1:60 and 1:80 in 0.04 M PBS (pH 6.7). Cholera antitoxin was diluted 1:80 and 1:640 in the same buffer. In addition to known LT-positive and LT-negative polymyxin extracts, which were included as controls of the PIH test, tubes containing the following reagents were also examined to correct for non-immune hemolysis: (i) SRBC, extract under test, and PBS; (ii) SRBC, extract, PBS, and complement; (iii) SRBC, PBS, complement, and antiserum (either LT antitoxin, cholera antitoxin, or anti-cholera antigen). All other conditions, such as the concentrations and volumes of reagents, as well as the technical procedures, were the same as those reported previously (5).

## RESULTS

The results of the PIH test carried out with standardized SRBC sensitized with polymyxin-released LT, prepared from strain 40T and standard crude LT, tested against different dilutions of LT antiserum, anti-cholera antigen, and cholera antitoxin are shown in Fig. 1. According to D. J. Evans, Jr. (personal communication), LT antiserum diluted 1:60 when tested with crude standard LT diluted 1:8 in 0.04 M PBS (pH 6.7) gave an absorbance of 0.50 at 420 nm. The anti-cholera antigen and cholera antitoxin dilutions which showed similar readings with the standard

LT were, respectively, 1:80 and 1:640 (Fig. 1, line A). The absorbance values obtained with SRBC sensitized with polymyxin-released LT prepared from strain 40T were lower but still equivalent (Fig. 1, line B). Polymyxin-released extracts prepared from *E. coli* K-12 (LT negative) gave negative results with all antisera (Fig. 1, line C), even when cholera antitoxin was diluted 1:80.

To make comparative comments on the interpretation of our PIH tests, the criterion recommended by Evans and Evans (5) was followed: those strains of *E. coli* whose extracts after correction for non-immune hemolysis gave an absorbance lower than 0.16, which corresponded to less than 30 µg of hemoglobin released from SRBC, were considered negative in the PIH test. Strains which released more than 30 µg of hemoglobin were recorded as LT positive.

Twenty-four (88.8%) of 27 strains of enterotoxinogenic *E. coli* of human origin gave positive results in the PIH test with cholera antitoxin diluted 1:80. When antisera used in the reaction were cholera antitoxin, anti-cholera antigen, and anti-LT diluted 1:640, 1:80, and 1:60, respectively, three other strains also released less than 30 µg of hemoglobin in the hemolytic test. Consequently the number of positive strains in the PIH test was reduced to 21 (77.7%). All the negative strains were isolated by the authors (TR 2161, TR 269/7, TR 82/2, TR 150/2, TR 103/3, and TR 05/75; Table 1).

Four out of nine (44.4%) enterotoxinogenic *E. coli* strains isolated from food were positive in the PIH test with all antisera used. Among the negative strains two (TR 100 and TR 102) were isolated from hamburger and the remaining (TR 106, TR 107, and TR 108) were isolated from sausage, both of which contain pork. Among five enterotoxinogenic *E. coli* strains isolated from river water, one was negative (TR 151) when cholera antitoxin diluted 1:80 was used. When the antisera were cholera antitoxin diluted 1:640, anti-cholera antigen, or anti-LT, another strain (TR 113) gave a negative PIH test.

All 10 enterotoxinogenic *E. coli* strains isolated from pigs with diarrhea were negative in the PIH test made with cholera antitoxin, anti-cholera antigen, and anti-LT diluted 1:640, 1:80, and 1:60, respectively. When cholera antitoxin was diluted 1:80, one strain (2423) gave a positive result in the PIH test. All these results are shown in Table 2, and strain numbers can be seen in Table 1.

## DISCUSSION

In this study we have demonstrated that cholera antitoxin when diluted 1:80 is more efficient than either LT antiserum or anti-cholera antigen for the detection of *E. coli* LT-positive strains

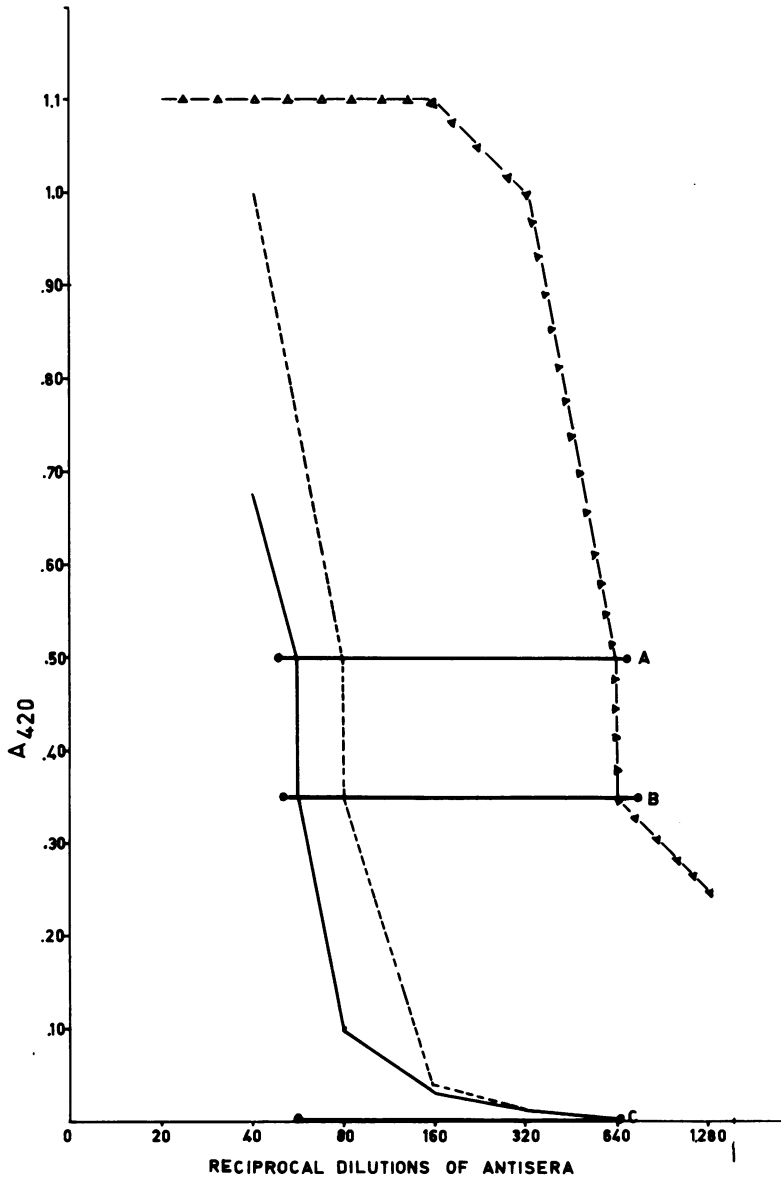


FIG. 1. Results of the PIH test made with polymyxin extracts from strains 40T and H-10407 (standard LT) using LT antiserum, cholera antitoxin, and anti-choleraegenoid. (line A) polymyxin-released extract from strain H-10407; (line B) extract from strain 40T; (line C) extract from *E. coli* K-12. (—) LT antiserum; (---) anti-choleraegenoid; ( $-\Delta-\Delta-$ ) cholera antitoxin.  $A_{420}$  represents hemoglobin release from hemolysed cells.

by the PIH test. Cholera antitoxin, anti-choleraegenoid, and LT antiserum, diluted 1:640, 1:80, and 1:60, respectively caused the release of approximately the same amount of hemoglobin, i.e., ca. 90  $\mu\text{g}$  (absorbance at 420 nm [ $A_{420}$ ] = 0.5).

Different findings were reported by Evans and Evans (5), who showed that LT antiserum diluted 1:320 or less gave higher  $A_{420}$  readings than

did cholera antitoxin. Since the cholera antitoxin we employed was from another source, it is possible that the differences observed between their data and ours may have been due to different potencies of the cholera antitoxins used or the relative proportion of anti-A and anti-B in both antisera.

A comparison of the results of the PIH test with those obtained in the Y-1 adrenal cell assay

TABLE 2. Results of the PIH test with enterotoxinogenic *E. coli* strains isolated from humans, food, river water, and swine<sup>a</sup>

| Sources of strains        | Antisera                   |                      |                      |                      |
|---------------------------|----------------------------|----------------------|----------------------|----------------------|
|                           | Cholera                    |                      | Choleraegenoid, 1:80 | LT, 1:60             |
|                           | 1:80                       | 1:640                |                      |                      |
| <b>Humans</b>             |                            |                      |                      |                      |
| Adults with diarrhea      | 7/7                        | 7/7                  | 7/7                  | 7/7                  |
| Children with diarrhea    | 11/12                      | 9/12                 | 9/12                 | 9/12                 |
| Children with no diarrhea | 6/8                        | 5/8                  | 5/8                  | 5/8                  |
| Subtotal, %               | 24/27 (88.8%) <sup>b</sup> | 21/27 (77.7%)        | 21/27 (77.7%)        | 21/27 (77.7%)        |
| <b>Food</b>               |                            |                      |                      |                      |
| Hamburger                 | 2/4                        | 2/4                  | 2/4                  | 2/4                  |
| "Keebe"                   | 2/2                        | 2/2                  | 2/2                  | 2/2                  |
| Sausage                   | 0/3                        | 0/3                  | 0/3                  | 0/3                  |
| Subtotal, %               | 4/9 (44.4%)                | 4/9 (44.4%)          | 4/9 (44.4%)          | 4/9 (44.4%)          |
| River water               | 4/5 (80%)                  | 3/5 (60%)            | 3/5 (60%)            | 3/5 (60%)            |
| Swine with diarrhea       | 1/10 (10%)                 | 0/10 (0%)            | 0/10 (0%)            | 0/10 (0%)            |
| <b>Total, %</b>           | <b>33/51 (64.7%)</b>       | <b>28/51 (54.9%)</b> | <b>28/51 (54.9%)</b> | <b>28/51 (54.9%)</b> |

<sup>a</sup> Results are expressed as number of positive strains/total examined.

<sup>b</sup> Data in parentheses are percentages of LT-positive strains detected by PIH test.

showed discrepant results regarding the origin of the strains. Thus, from 27 strains of human origin, 24 (88.8%) were positive by the PIH test. Conversely, the test was positive with only one strain (7.7%) from 13 isolated from swine and sausage containing pork. A good agreement between both assays was better when cholera antitoxin was used diluted 1:80 (Table 2).

The reasons for these unexpected results with the PIH test are not known. One may think that the porcine strains as a rule produce less LT than human strains, that their toxin is not suitably extracted by the polymyxin technique, or that LTs from porcine and human strains of *E. coli* are not immunologically identical. Further studies must be conducted to prove these hypotheses. In relationship to immunological differences between LT, there are few studies, and these are somewhat controversial. Sack (9) mentions that LT from porcine and human strains are neutralized to about the same degree by an antitoxin produced against one of the LT preparations. However, other authors (7, 8), using neutralization tests, have observed partial neutralization of the specific biological effects of the LTs prepared from *E. coli* isolated from humans and animals. Also, partial or no cross-reactions have been observed between LT from porcine and human strains in diffusion tests by Finkelstein et al. (6) and Schenkein et al. (10).

In practical terms it should be stressed that the PIH test can not be used to look for LTs in

*E. coli* strains without taking in account the source of the strains.

Immunological studies on these enterotoxins are in progress in our laboratories.

#### ACKNOWLEDGMENTS

This investigation was supported by grants 204/78 from Fundação de Amparo à Pesquisa do Estado de São Paulo, 22220734/75 from the CNPq (Brazilian National Research Council), and the U.S. National Science Foundation. Financiadora de Estudos e Projetos supported the technical assistance of Mary Nilce Clemente.

We gratefully acknowledge the U.S.-Japan Cooperative Medical Sciences Program of the National Institutes of Allergy and Infectious Diseases, Bethesda, Md., for the supply of anticholeraegenoid and cholera antitoxin. We are also indebted to D. J. Evans, Jr., for the supply of standard crude polymyxin-release LT and LT antiserum. We thank J. P. Craig for reviewing the manuscript.

#### LITERATURE CITED

1. Donta, S. T., H. W. Monn, 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.
2. Evans, D. J., Jr., D. G. Evans, and S. L. Gorbach. 1973. Production of vascular permeability factor by enterotoxinogenic *Escherichia coli* isolated from man. *Infect. Immun.* **8**:725-730.
3. 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.
4. Evans, D. J., Jr., D. G. Evans, S. H. Richardson, and S. L. Gorbach. 1976. Purification of the polymyxin-released, heat-labile enterotoxin of *Escherichia coli*. *J. Infect. Dis.* **133**:S97-S102.
5. Evans, D. J., Jr., and D. G. Evans. 1977. Direct sero-

- logical assay for the heat-labile enterotoxin of *Escherichia coli*, using passive immune hemolysis. *Infect. Immun.* **16**:604-609.
6. Finkelstein, R. A., M. K. LaRue, D. W. Johnston, M. L. Vasil, G. J. Cho, and J. R. Jones. 1976. Isolation properties of heat-labile enterotoxin from enterotoxigenic *Escherichia coli*. *J. Infect. Dis.* **133**:S120-S137.
  7. Giugliano, L. G., M. A. M. Meneguetti, and L. R. Trabulsi. 1978. LT enterotoxin production by *Escherichia coli*. *Rev. Microbiol.* **9**:11-17.
  8. Gyles, C. L. 1974. Relationship among heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae*. *J. Infect. Dis.* **129**:277-283.
  9. Sack, R. B. 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. *Annu. Rev. Microbiol.* **29**:333-353.
  10. Schenkein, I., R. F. Green, D. S. Santos, and W. K. Maas. 1976. Partial purification and characterization of a heat-labile enterotoxin of *Escherichia coli*. *Infect. Immun.* **13**:1710-1720.