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

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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-choleragenoid and anti-LT were used as antisera. Cholera antitoxin was much more potent than anti-choleragenoid 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-choleragenoid sera in the PIH test.

MATERIALS AND METHODS

Strains. The sources and designations of the 51 LT *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 preparation 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-choleragenoid 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 2×10^9 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[a]	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[a]	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[b]	9192,[c] 16932,[c] 19927,[c] TD 427,[d] FC 7-2,[e] FC 24-6,[e] FC 11-2,[e] B 16-4,[e] B 1-5[e]
Swine with diarrhea[a]	17, 100, 306, 339, 406, 446, 2423, 3406, A, B
River water[a]	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

[a] Strains isolated by the authors.
[b] Strains supplied by other investigators.
[c] Supplied by W. K. Maas, New York, N.Y.
[d] Supplied by D. J. Evans, Jr., Houston, Texas.
[e] Supplied by R. L. Guerrant, Charlottesville, Va.

Similar procedures were adopted for cholera antitoxin and anti-choleragenoid; (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-choleragenoid 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-choleragenoid). 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-choleragenoid, 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-choleragenoid 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-choleragenoid, 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-choleragenoid, 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-choleragenoid, 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-choleragenoid for the detection of *E. coli* LT-positive strains

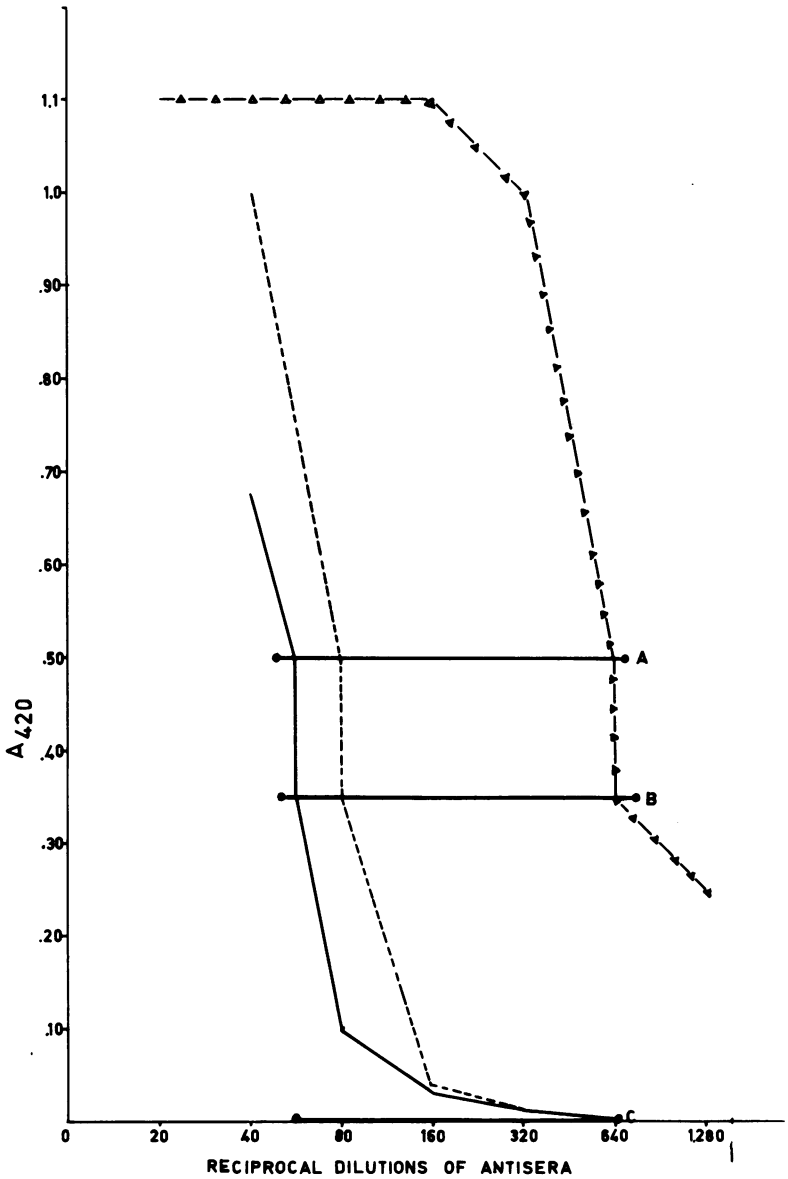

FIG. 1. *Results of the PIH test made with polymyxin extracts from strains 40T and H-10407 (standard LT) using LT antiserum, cholera antitoxin, and anti-choleragenoid. (line A) polymyxin-released extract from strain H-10407; (line B) extract from strain 40T; (line C) extract from E. coli K-12.* (——)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

by the PIH test. Cholera antitoxin, anti-choleragenoid, 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 μg (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*[a]

Sources of strains	Antisera			
	Cholera		Choleragenoid, 1:80	LT, 1:60
	1:80	1:640		
Humans				
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%)[b]	21/27 (77.7%)	21/27 (77.7%)	21/27 (77.7%)
Food				
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%)
Total, %	33/51 (64.7%)	28/51 (54.9%)	28/51 (54.9%)	28/51 (54.9%)

[a] Results are expressed as number of positive strains/total examined.
[b] 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.

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