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Modified Elek Test for Detection of Heat-Labile Enterotoxin of Enterotoxigenic *Escherichia coli*

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The Elek test was modified for detection of the heat-labile enterotoxin of enterotoxigenic *Escherichia coli*. A total of 164 strains of *E. coli* were tested by the modified Elek test, and the results correlated well with those of the Chinese hamster ovary cell assay and passive immune hemolysis. It is concluded that the modified Elek test is a simple and reproducible assay method for identification of *E. coli* which produce heat-labile enterotoxin, and is suitable for use in clinical laboratories.

Enterotoxigenic *Escherichia coli* produce two distinct enterotoxins: one is heat labile (LT), of high molecular weight, and antigenic, and the other is heat stable, of low molecular weight, and nonantigenic. Both have been considered to be responsible for diarrhea in humans and cattle (24, 27, 28). For detection of the LT of enterotoxigenic *E. coli*, various assay methods have been developed, including the ileal loop test (20), vascular permeability test (11), Chinese hamster ovary (CHO) cell assay (13), Y-1 adrenal cell assay (7, 23), passive immune hemolysis (10, 25, 30), reversed passive hemagglutination (18), staphylococcal coagglutination method (1), solid-phase radioimmunoassay (12), and ganglioside G_{M1} enzyme-linked immunosorbent assay (29). However, many of these assay methods are unsuitable for routine clinical purposes because they need special material or techniques or both, such as a large number of animals, stocks of special tissue culture cells, and radioisotopes. Thus, to develop a simple and reproducible assay method which can be widely used in clinical laboratories, we modified the classical Elek test for detection of LT produced by enterotoxigenic *E. coli*.

MATERIALS AND METHODS

Bacterial strains. All of the *E. coli* strains used in this study were isolated at Osaka International Airport Quarantine Station from patients with diarrhea who had just come back from cholera-infected areas of South East Asian countries.

Purified antiserum against cholera enterotoxin. Purified cholera enterotoxin (22), kindly supplied by N. Ohtomo, Chemo-Sero-Therapeutic Research Institute, Japan, was used as an immunizing antigen. Twenty micrograms of the purified cholera enterotoxin in 1 ml of phosphate-buffered saline (pH 7.0) was emulsified with an equal volume of Freund incomplete adjuvant (Difco). The emulsion was inoculated intramuscularly into young rabbits weighing about 2 kg. Two booster injections were given on days 25 and 41, and antiserum was obtained on day 50. This antiserum gave one major and a few minor precipitin lines against crude concentrated culture supernatant fluid of LT-producing *E. coli* in an Ouchterlony gel diffusion plate (26). Monospecific antiserum against the purified cholera enterotoxin was obtained by immunoaffinity chromatography essentially by the method of Cuatrecasas and Anfinsen (5). In brief, cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was coupled with the purified cholera enterotoxin. The immunoglobulin against the cholera enterotoxin applied to the column was eluted with 0.2 M glycine-hydrochloride buffer (pH 2.7) containing 0.5 M NaCl. The purified antiserum (specific immunoglobulin) against the cholera enterotoxin formed a single precipitin line against the purified cholera enterotoxin and against crude concentrated culture supernatant fluid of LT-producing *E. coli* in an Ouchterlony gel diffusion plate, and the two precipitin lines gave spur formation as reported by Clements and Finkelstein (2, 3).

Medium. The standard medium used was CAYE-2 medium as reported by Mundell et al. (21), supplemented with 1.5% Noble agar (Difco) to make solidified plates. This medium consists of 2% Casamino Acids (Difco), 0.6% yeast extract (Difco), 0.25% NaCl, 0.871% K_2HPO_4, 0.25% glucose, 0.1% (vol/vol) of a trace salt solution (5% $MgSO_4$, 0.5% $MnCl_2$, and 0.5% $FeCl_3$), and 1.5% Noble agar (Difco), pH 8.5. The indicated amount of lincomycin (Japan Upjohn Co., Tokyo) was added to the medium.

Modified Elek test. In tests for production of LT by *E. coli* strains, the classical Elek test (8) was modified as described previously (16). The strains to be examined were inoculated onto agar plates and incubated for about 40 h at 37°C. Then a paper disk (6 mm in diameter), soaked in 25 μl of polymyxin B (Taitoh Pfizer Co., Tokyo) solution (20,000 IU/ml), was placed on a colony formed on the plate and incubated for several hours at 37°C. After incubation, 30-μl volumes of purified (immunoabsorbed) antiserum against the cholera enterotoxin, containing 1%

NaN_3, were placed in 4-mm wells made about 5 mm from the colony to be tested. The antiserum used was diluted to give a titer of 1:4, which means that a fourfold dilution of the antiserum gave a precipitin line against 2.5 μg of purified cholera enterotoxin in an Ouchterlony gel diffusion plate. The plate was kept at 37°C overnight and then examined for a precipitin line.

CHO cell assay. CHO cell assay of LT was carried out as described previously (17). Eagle minimal essential medium was used in place of the F12 medium originally described by Guerrant et al. (13), since this modification decreased the extent of elongation of CHO cells in the absence of LT.

Procedure for passive immune hemolysis. The procedure for passive immune hemolysis was the modification of the method of Evans and Evans (10) described previously (30). *E. coli* cells were cultured at 37°C for 24 h with vigorous shaking in 5 ml of CAYE-2 medium. Then the culture was centrifuged at 10,000 rpm for 30 min, and the precipitate was mixed with 1 ml of polymyxin B solution (10,000 IU of drug per ml of 0.02 M *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] buffer, pH 6.7, containing 0.9% NaCl) and incubated at 37°C for 30 min with gentle shaking. Then the mixture was centrifuged at 10,000 rpm for 30 min, and the supernatant fluid was used as the LT preparation. A sample of 0.1 ml was mixed with 0.1 ml of 2% sheep blood cell suspension in 0.02 M HEPES buffer (pH 6.7) containing 0.9% NaCl and incubated at 37°C for 30 min. Purified antiserum against cholera enterotoxin (0.1 ml) was added, and incubation was continued at 37°C for 30 min. Then 0.1 ml of guinea pig complement solution was added, and incubation at 37°C was continued for 60 min. Finally, 1.6 ml of 0.02 M HEPES buffer (pH 6.7) containing 0.9% NaCl was added, and the mixture was centrifuged at 2,000 rpm for 5 min. All incubations were carried out with gentle shaking. LT activity was then measured by reading the absorbance at 420 nm of the supernatant fluid. Samples which gave an absorbance at 420 nm of greater than 0.3 were considered to be positive for LT.

RESULTS

Culture conditions for maximal production of LT on agar plates. To find the optimal conditions for production of LT by enterotoxigenic *E. coli*, agar plates with various concentrations of the components of CAYE-2 medium and some other components were examined. The production of LT was assayed by testing for formation of a precipitin line on agar as described above. As seen in Table 1, the optimal concentrations of Casamino Acids, yeast extract, NaCl, K_2HPO_4, glucose, and trace salts solution no. 2 were 1, 1, 0.25, 1.5, 0.5, and 0.05%, respectively. Addition of either meat extract, tryptone, peptone, or tryptic soy broth did not give a better yield. Trace salts solution no. 2, which contains 5% $MgSO_4$, 0.5% $FeCl_3$, and 2% $CoCl_2 \cdot 6H_2O$, gave a better yield than solution no. 1, which contains 5% $MgSO_4$, 0.5% $MnCl_2$, and 0.5% $FeCl_3$. The optimal pH was 7.5. Addition of lincomycin to the medium was important for formation of a precipitin line (Tables 1 and 2). The optimal concentration of lincomycin was 90 μg/ml, and with this concentration all the *E. coli* strains tested grew on agar plates. Some strains did not grow when the concentration of linco-

TABLE 1. *Effects of various concentrations of medium components on precipitin line formation (Elek test) on agar plates*[a]

Component	Concn (%) or condition tested	Optimal concn or condition giving best results
Casamino Acids	1, 2, 4	2
Yeast extract	0, 0.6, 1, 2	1
NaCl	0, 0.25, 1	0.25
K_2HPO_4	0, 0.871, 1.5, 3	1.5
Glucose	0, 0.25, 0.5, 0.75	0.5
Trace salts		
no. 1[b]	0, 0.1, 0.3	—[c]
no. 2[d]	0.05, 0.1	0.05
Meat extract	1	—
Tryptone	0.5, 1.5	—
Peptone	0.5, 1	—
Tryptic soy broth	0.5, 1	—
Lincomycin (μg/ml)	30, 90, 150[e]	90
pH	7.0, 7.5, 8.0, 8.5, 9.0	7.5

[a] The concentration of each component of CAYE-2 medium was varied independently as indicated. The components which are not included in CAYE-2 medium were added to the medium. Six strains of LT-producing *E. coli* were tested in each experiment. When the best result was obtained with several concentrations of a component, the lowest concentration giving the best result was taken as the optimal concentration.

[b] Trace salts solution no. 1: 5% $MgSO_4$, 0.5% $MnCl_2$, 0.5% $FeCl_3$.

[c] —, Its addition did not improve the result.

[d] Trace salts solution no. 2: 5% $MgSO_4$, 0.5% $FeCl_3$, 2% $CoCl_2 \cdot 6H_2O$.

[e] Some strains tested did not grow on addition of 150 μg of lincomycin per ml.

TABLE 2. *Comparison of various conditions for precipitin line formation*

Expt	Medium	Addition of lincomycin (90 μg/ml)	Polymyxin B treatment	No. of positive strains/no. of strains examined
1	CAYE-2	+[a]	+	59/73
2	Biken agar 2	+	+	73/73
3	Biken agar 2	+		67/73
4	Biken agar 2		+	6/73

[a] +, The condition was applied in the experiment.

mycin was increased to 150 μg/ml, although results were clearer when strains could grow at this concentration.

As shown in Table 2, some strains did not give a precipitin line in CAYE-2 medium even with lincomycin and polymyxin B treatment (experiment 1), whereas all 73 LT-producing strains gave a precipitine line in Biken agar 2 with lincomycin and polymyxin B treatment (experiment 2). Some strains grown on Biken agar 2 containing 90 μg of lincomycin per ml did not give a precipitin line when they were not treated with polymyxin B (experiment 3). Only 6 of 73 strains formed a precipitin line in Biken agar 2 without lincomycin even when they were treated with polymyxin B (experiment 4).

Thus, it was concluded that the best conditions for formation of the precipitin line are growth on agar plates consisting of 2% Casamino Acids, 1% yeast extract, 0.25% NaCl, 1.5% K_2HPO_4, 0.5% glucose, 0.05% (vol/vol) of trace salt solution no. 2, 90 μg of lincomycin per ml, and 1.5% Noble agar, pH 7.5, and then treatment of the colonies with polymyxin B. The medium used was provisionally named Biken agar 2.

Modified Elek test for LT on Biken agar 2. Typical results of the modified Elek test for LT are shown in Fig. 1. Colonies of *E. coli* producing LT formed a precipitin line against purified anti-cholera enterotoxin which showed complete identity with the precipitin line formed between the culture supernatant of the LT-producing strain and the anti-cholera enterotoxin, but formed a spur with the line between the purified cholera enterotoxin and anti-cholera enterotoxin.

In all, 164 strains of *E. coli*, including both LT-producing and LT-nonproducing strains, were tested by the modified Elek test on Biken agar 2. As shown in Table 3, the results of the modified Elek test on Biken agar 2 correlated well with those of CHO cell assay and passive immune hemolysis. A total of 161 strains gave consistent results in all three assays. Of these, 73 were positive and 88 were negative. Only 3 of the 164 strains gave discrepant results in the three assays: 2 gave positive results only in the Elek test, and 1 gave a positive result only by passive immune hemolysis.

DISCUSSION

It bas been reported that *E. coli* LT shows immunological cross-reactivity with cholera enterotoxin (2, 3, 6, 9, 14, 15). Gyles (14) carried out immunodiffusion tests using anti-cholera enterotoxin antiserum and found that a precipitin line between LT and anti-cholera enterotoxin showed partial identity with a precipitin line

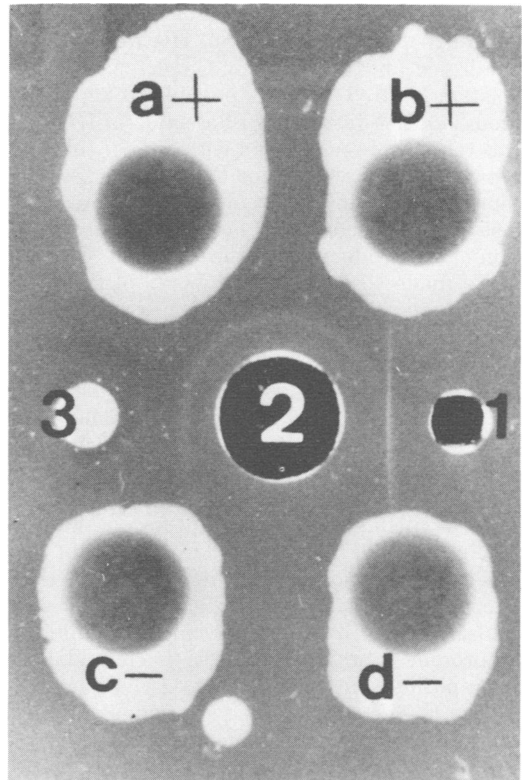

FIG. 1. *Results of the modified Elek test. Strains of E. coli were grown in Biken agar 2, and the modified Elek test was carried out as described in the text. Wells 1, 2, and 3 contain purified cholera enterotoxin, purified antiserum against cholera enterotoxin, and concentrated culture supernatant of LT-producing E. coli, respectively. a+ and b+ are colonies of E. coli producing LT, and c− and d− are colonies of E. coli not producing LT.*

TABLE 3. *Results of assays of 164 strains of E. coli*

Group[a]	Assay method[b]			No. of strains
	Elek[c]	CHO[d]	PIH[e]	
I	+	+	+	73
II	+	+	−	0
III	+	−	+	0
IV	−	+	+	0
V	+	−	−	2
VI	−	+	−	0
VII	−	−	+	1
VIII	−	−	−	88

[a] Grouping was based on the results of the assays.
[b] Symbols + and − indicate positive and negative results, respectively, in the indicated assay.
[c] Modified Elek test.
[d] Chinese hamster ovary cell assay.
[e] Passive immune hemolysis.

between pure subunit B of cholera enterotoxin and anti-cholera enterotoxin. His results were confirmed and extended by Clements and Finkelstein (2, 3). Thus, it is possible to apply the modified Elek test (16) for detection of LT, and this test can be performed with *E. coli* colonies grown on agar plates. For this purpose, we attempted to modify the conditions of the Elek test and obtained suitable conditions for detection of LT produced by enterotoxigenic *E. coli*.

We made several improvements of the Elek test to obtain better precipitin lines on the agar plates. First we examined all the constituents of the agar medium to determine the optimal concentrations for a good precipitin line between LT produced by the *E. coli* colonies and anticholera enterotoxin. The optimal pH of the medium was found to be 7.5. Moreover, we found that addition of 90 μg of linocomycin per ml to the agar medium was important for obtaining a precipitin line. This important improvement was based on the finding of Levner et al. (19) that lincomycin induces the production of LT and cholera enterotoxin. Lincomycin is an inhibitor of protein synthesis which is bactericidal to gram-positive but not to gram-negative bacteria. All the *E. coli* strains tested grew on agar plates containing 90 μg of lincomycin per ml.

The agar medium giving the best precipitin line was provisionally named Biken agar 2. It consists of 2% Casamino Acids (Difco), 1% yeast extract (Difco), 0.25% NaCl, 1.5% K_2HPO_4, 0.5% glucose, and 0.05% (vol/vol) of trace salts solution no. 2 (5% $MgSO_4$, 0.5% $FeCl_3$, and 2% $CoCl_2 \cdot 6H_2O$).

Another major modification was the use of polymyxin B to release LT from the cells. Clements and Finkelstein (4) reported that the richest source of LT is not the culture supernatant but the cell lysate. This was true of the LT-producing *E. coli* that we examined (data not shown). Therefore, a paper disk soaked in polymyxin B was placed on the colony formed on the agar plate. Several strains gave clear precipitin lines only after polymyxin B treatment (Table 2).

The precipitatin line was clear enough to be read although it was not strong in some cases. Thus, the reading was performed in a blind fashion: that is, the reader did not have any knowledge of the results of the other assay systems. Moreover, we asked several unbiased observers to read the results, and they reported consistent observations. Several investigators who are not familiar with the assay system tried the modified Elek test and also obtained consistent results.

With some *E. coli* strains, rabbit antiserum against purified cholera enterotoxin gave nonspecific precipitin lines in addition to the major line between cholera enterotoxin and its antibody. This was probable because normal (preimmune) serum contains antibodies against some components of *E. coli*. Therefore, the antiserum was purified by affinity column chromatography coupled with purified cholera enterotoxin.

There were three discrepant results in 164 strains tested in the three assays. As described in Materials and Methods, polymyxin B-treated materials (intracellular LT) were used for passive immune hemolysis, whereas both intracellular and extracellular LT were tested in the modified Elek test. This may explain the case of the positive Elek test and negative passive immune hemolysis. Positive passive immune hemolysis and negative Elek test may be explained by hemolysin production by the tested strain. The case of a positive Elek test and negative CHO cell assay may indicate the possibility of production of toxin molecules without biological activity.

We previously reported a modification of passive immune hemolysis for use as a simple method for detection of LT (30). The modified passive immune hemolysis method is not as useful as the modified Elek test because it requires several centrifugation steps and various reagents, such as fresh sheep blood cells, complement, and buffer. The modified Elek test is simple and reproducible and is easy to carry out in clinical laboratories. One slight disadvantage of the method, however, is that it takes about 3 days to obtain results.

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