Value of Passive Immune Hemolysis for Detection of Heat-Labile Enterotoxin Produced by Enterotoxigenic Escherichia coli

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The method of passive immune hemolysis of Evans and Evans (Infect. Immun. 16:604–609, 1977) for detection of heat-labile enterotoxin produced by enterotoxigenic Escherichia coli was modified. A total of 373 strains of E. coli were tested by this method using materials obtained by treating the cells with polymyxin B and rabbit antiserum against cholera enterotoxin, purified by affinity gel column coupled with purified cholera enterotoxin, in N-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer (pH 6.7). The results correlated very well with those obtained in an assay with Chinese hamster ovary cells. It is concluded that passive immune hemolysis is useful as a routine clinical method for identifying E. coli strains that produce heat-labile enterotoxin.

Heat-labile enterotoxin (LT) of enterotoxigenic Escherichia coli has been assayed by various methods, such as the ileal loop test (9), vascular permeability test (4), Chinese hamster ovary (CHO) cell assay (6), Y1 adrenal cell assay (2, 12), passive immune hemolysis (3), solid-phase radioimmunoassay (5), ganglioside GM1 enzyme-linked immunoassay (14), and reverse passive hemagglutination (8). Of these methods, those using tissue culture cells (2, 6, 12) have been used most frequently to identify LT-producing enterotoxigenic E. coli, but these methods are unsuitable for routine clinical purposes because they require stocks of special tissue culture cells and, thus, are rather complicated. Therefore, we tried to develop a simple, reproducible assay method for detecting LT and found that a modification of the method of passive immune hemolysis originally reported by Evans and Evans (3) was useful for identifying E. coli that produce LT.

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 Southeast Asian countries.

Antiserum against cholera enterotoxin. Purified cholera enterotoxin (11), kindly supplied by N. Ohtomo, Chemo-Sero-Therapeutic Research Institute, was used as an immunizing antigen. Rabbit antiserum against the purified cholera enterotoxin was further purified by affinity column chromatography, in which BrCN-activated Sepharose 4B (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) was coupled with the purified cholera enterotoxin.

Preparation of LT samples. E. coli cells were cultured at 37°C for 24 h with vigorous shaking in the CAYE medium described by Mundell et al. (10). The medium consisted of 2% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.6% yeast extract (Difco), 0.25% NaCl, 0.871% K2HPO4, 0.25% glucose, and 0.1% (vol/vol) of a trace salt solution (5% MgSO4, 0.5% MnCl2, 0.5% FeCl3, and 0.001% H2SO4). The pH of the medium was 8.5. After incubation, the culture was centrifuged at 12,000 × g for 30 min, and the supernatant was collected for tests. In addition, polymyxin B solution (10,000 IU/ml of the indicated buffer solution containing 0.9% NaCl) was added to the precipitate, and the mixture was incubated at 37°C for 30 min with gentle shaking. Then the mixture was centrifuged at 12,000 × g for 30 min, and the supernatant was used as the sample of LT.

Procedure for passive immune hemolysis. The procedure used for passive immune hemolysis was essentially as described by Evans and Evans (3). A sample of 0.1 ml was mixed with 0.1 ml of 2% sheep erythrocyte suspension in the indicated buffer solution containing 0.9% NaCl and incubated at 37°C for 30 min. This solution showed an absorbance at 420 nm of 1.5 after complete hemolysis. 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 the same buffer was added, and the mixture was centrifuged at 480 × g 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 solution. Some E. coli
strains are hemolytic. For these strains it was necessary to include control experiments in which no antiserum or complement was added. Thus, each sample was examined with a control experiment in which neither antiserum nor complement was added.

CHO cell assay. The CHO cell assay of LT was carried out as described previously (7). Eagle minimal essential medium was used in place of the F12 medium originally described by Guerrant et al. (6), since this modification decreased the extent of elongation of CHO cells in the absence of LT.

Buffer. N-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (0.02 M, pH 6.7) was prepared by mixing 0.02 M HEPES solution with 0.02 M NaOH solution to obtain the indicated pH value.

RESULTS

Passive immune hemolysis in the presence of various buffer solutions. The LT activities of 16 strains of LT-producing E. coli identified by CHO cell assay were examined by passive immune hemolysis. When phosphate buffer was used, as originally described by Evans and Evans (3), none of the 16 culture supernatants gave a positive reaction. On the other hand, most of the samples obtained by treating the cells with polymyxin B gave a positive reaction (Fig. 1A). In the presence of either tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 6.7) or HEPES buffer (pH 6.7), the same materials generally gave higher absorbances at 420 nm, owing to hemolysis, than those obtained in the presence of phosphate buffer (Fig. 1B and C). This was especially true when HEPES buffer was used.

In control experiments in which non-enterotoxigenic E. coli were tested, absorbances at 420 nm were not more than those observed with buffers alone (data not shown).

Correlation between the CHO assay and passive immune hemolysis. As described above, passive immune hemolysis in the presence of HEPES buffer with samples obtained by treating the cells with polymyxin B gave the highest values. Therefore, these conditions were used with various clinical isolates to see whether the values obtained by passive immune hemolysis correlated with the activities determined by the CHO cell assay. In this experiment, 373 strains were each cultured in 5 ml of CA YE medium, and samples were prepared as described in the text. Passive immune hemolysis (PIH) with 0.1 ml of supernatant (A) or polymyxin B-treated material (C) was carried out as described in the text (A) in the presence of 0.02 M phosphate buffer (pH 6.7), (B) in the presence of 0.02 M Tris-hydrochloride buffer (pH 6.7), or (C) in the presence of 0.02 M HEPES buffer (pH 6.7).

Fig. 1. Effects of various buffers on passive immune hemolysis of culture supernatants and polymyxin B-treated materials of various LT-producing strains; 16 LT-producing strains were each cultured in 1 ml of CAYE medium, and samples were prepared as described in the text. Passive immune hemolysis (PIH) with 0.1 ml of supernatant (A) or polymyxin B-treated material (C) was carried out as described in the text (A) in the presence of 0.02 M phosphate buffer (pH 6.7), (B) in the presence of 0.02 M Tris-hydrochloride buffer (pH 6.7), or (C) in the presence of 0.02 M HEPES buffer (pH 6.7).

gave a negative reaction in passive immune hemolysis. On the other hand, 166 strains that gave a positive reaction in the CHO cell assay (more than 30% of the CHO cells were elongated) gave a positive reaction in passive immune hemolysis, namely, the absorbance at 420 nm was more than 0.3. There were two exceptional strains; one gave a positive reaction in the CHO cell assay (69.1% of the CHO cells were elongated) but a negative reaction in the passive immune hemolysis (absorbance at 420 nm, 0.15), and the other gave a positive reaction in passive immune hemolysis (absorbance at 420 nm, 1.04) but a negative reaction in the CHO cell assay (0.8% of the CHO cells were elongated). The correlation
coefficient (r) between the results in the CHO cell assay and in passive immune hemolysis was 0.930.

**DISCUSSION**

In this work we modified the method of passive immune hemolysis for detection of LT reported by Evans and Evans (3) to make it more sensitive and reproducible. Application of the modified method to 373 strains of *E. coli* isolated from patients with diarrhea showed that results correlated very well with those obtained by the CHO cell assay (Fig. 2). There were only two exceptional strains; the reason(s) for this has not been clarified. From these results, it is concluded that the method of passive immune hemolysis is useful for identifying enterotoxigenic *E. coli* strains producing LT. Since this method does not require the special equipment and techniques necessary in methods using tissue culture cells, it should be useful as a routine clinical assay.

As shown in Fig. 1, individual samples gave different values of hemolysis in the presence of different buffer solutions; the highest values among the various buffers tested were obtained with HEPES buffer. Possibly, the phosphate buffer somehow inhibits passive immune hemolysis, whereas HEPES buffer does not; or possibly HEPES buffer inhibits some unknown factor(s) that interferes with passive immune hemolysis.

Figure 1 also shows that more LT was detected in samples obtained by treating the cells with polymyxin B than was detected in the culture supernatant. This is consistent with the finding of Clements and Finkelstein (1) that a whole-cell lysate was the richest source of LT.

Serafim et al. (13) reported that antiserum against cholera enterotoxin was much more potent than that against LT in passive immune hemolysis for detection of LT. Thus, in this study we used antiserum against cholera enterotoxin, which is more easily obtained than anti-LT antiserum. We found that with some *E. coli* strains it was necessary to purify the antiserum by affinity column chromatography coupled with purified cholera enterotoxin. This was probably because crude rabbit antiserum contains antibodies against some components of *E. coli*, thus causing nonspecific immune hemolysis.

The appropriate amount of antiserum and complement to use in the assay system can be determined by using purified cholera enterotoxin as the antigen sample. As little as 2.5 to 5 ng of purified cholera enterotoxin gave a maximal value in the assay under the conditions employed. We found that addition of excess antiserum to the assay system was necessary to obtain reproducible results. Also, it was essential to use fresh sheep erythrocytes to avoid nonspecific hemolysis.

One major disadvantage of this method is that false-positive reactions are observed with *E. coli* strains that produce hemolysins(s). This disadvantage can be overcome by carrying out control experiments in which no antiserum or complement is added. In this study, we found only 1 hemolytic strain out of 374 strains tested. This strain was omitted from the experiments and the results with 373 strains were presented.

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**LITERATURE CITED**


