J Diar Dis Res 1984 Mar;2(1):33-36
0253-8768/84 $1.00+0.10

DETECTION OF HEAT-LABILE ENTEROTOXIN-PRODUCING ESCHERICHIA COLI STRAINS BY A STAPHYLOCOCCAL COAGGLUTINATION TECHNIQUE

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Received: 16 Jun 1983

Abstract

Culture filtrates of 50 strains of *Escherichia coli* isolated from children with diarrhoea were tested for heat-labile enterotoxin (LT), by the staphylococcal coagglutination technique in a slide and capillary tube. The results were compared with those obtained in bio-assays of rabbit ileal-loop and skin permeability factor. Coagglutination in a capillary tube was found to be far superior to that on a slide; and the technique detected LT in 38 of the culture filtrates. The results obtained in capillary tube coagglutination were identical to those observed in a skin permeability factor assay. The ileal-loop test detected LT in 36 of the culture filtrates and ST in 5, results which were confirmed by suckling mice assay. Thus, the staphylococcal coagglutination test is as sensitive and specific as are bio-assays. This test, being very simple and inexpensive, can be used in routine diagnostic laboratories, to assess the role of LT-producing *E. coli* strains in diarrhoeal diseases.

Key words: Escherichia coli; Enterotoxins; Laboratory Diagnosis; Coagglutination; Diarrhoea.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains elaborate an antigenic heat-labile toxin (LT) and non-antigenic heat-stable toxin (ST), and are responsible for a large proportion of acute diarrhoeal episodes in man and domestic animals. (1) As there is no reliable and easy biochemical or other marker to distinguish ETEC from non-ETEC strains, their recognition depends upon the demonstration of enterotoxin production. Animal models, such as adult rabbit ileal-loop (2) and infant rabbit (3); increase in capillary permeability (PF) of rabbits' skin (4); and tissue culture assays in Chinese hamster ovarian (CHO) (5), mouse adrenal (Y-1) (6), and Vero cell cultures (7), usually are used to detect heat-labile enterotoxin. These methods are expensive, time-consuming and inconvenient for routine diagnostic laboratories. Immunological techniques, such as radio-immunoassay (RIA) (8) and enzyme-linked immunosorbent assay (ELISA) (9), are more sensitive and specific, but require special costly equipment. The Biken test (10), although easy to perform and specific, takes 4-5 days. Therefore, there is an urgent need to develop a simple, rapid inexpensive method of identifying ETEC strains. Recently, staphylococcal coagglutination has been tried for detection of *E. coli* LT (11, 12). The present study was undertaken to (i) determine the ability of the coagglutination technique to detect *E. coli* LT, and to correlate the results with those of rabbit ileal loop and skin PF tests; and (ii) compare the efficiency of the slide and capillary tube methods of the coagglutination technique.

Materials and methods

Organisms

Fifty strains of *E. coli*, isolated from infants and children with diarrhoea attending the Outpatients' Department of the Institute Hospital, were used in this study. The strains were maintained in peptone agar stab cultures at room temperature, and none had undergone more than two sub-cultures before being tested for enterotoxin. The toxigenic *V. cholerae* strain 569B was used as a positive control for ileal loop and skin PF assays. The *E. coli* strains 36004 (ST producer) and 36000 (non-toxic) served as positive and negative controls in a suckling mice assay. *Staphylococcus aureus* 4972 (Copenhagen strain), a protein A-rich organism, was used in the coagglutination test.

Ileal-loop, skin PF and suckling mice assays with culture filtrates of Escherichia coli strains

The culture filtrates of the test and control *E. coli* strains and of *V. cholerae* 569B were prepared following the method described elsewhere (13). Briefly, 5 or 6 colonies from an overnight grown culture in trypticase soy agar (TSA, Difco) were inoculated into 10 ml of Richardson's medium (14), contained in 50 ml conical flasks, incubated overnight at 37°C in a shaking water bath with 120 oscillations/min. Centrifuged in the cold at 22,000 xg, the supernates passed through millipore membranes of 0.22 μm average pore diameter, and were preserved at –20°C until further use. The rabbit ileal loop (2) and skin PF assays (4) were performed in duplicate with all the culture filtrates of test strains. The culture filtrates of *V. cholerae* strain 569B and Richardson's medium served, respectively, as positive and negative controls. All the cell-free culture supernates also were tested in the suckling mice assay (15). The known positive and negative controls always were included in each set of experiments.

Staphylococcal coagglutination technique for detection of Escherichia coli LT

The coagglutination test was done following the method of Kronvall (16), with certain modifications. *S. aureus* 4972 (Copenhagen strain) rich in protein A (17) was used for the test. Five or six smooth colonies from an overnight grown culture of the strains on TSA were inoculated into trypticase soy broth (TSB, Difco), and were incubated overnight at 37°C. A 5 ml amount of this growth was added to each of two 1 L flasks, containing 500 ml of TSB and incubated at 37°C for 24 h with aeration. The broth was centrifuged, and the cells were washed three times in phosphate-buffered saline (PBS, 0.15 M, pH 7.2). A 10 per cent (v/v) suspension of the cells was treated with 0.5 percent formalin, and was allowed to stand at room temperature for 3 h with occasional stirring. The formalin-treated cells were washed three times in PBS, were resuspended to a concentration of 10 percent, and were stored at 4°C until further use.

Sensitized *Staphylococci* were prepared by mixing 1 ml of a 10 percent formalin-treated suspension with 0.1 ml of a specific antitoxin against *E. coli* LT (obtained through the courtesy of Prof. F. Dorner of Sandoz Forchungsinstitut, Wein). On incubation at room temperature for 3 h, the antitoxin-treated staphylococcal suspension was washed three times in PBS, was diluted in the same buffer to a final concentration of 2 percent (v/v). and was stored at 4°C until further use.

Just before use in the test, the sensitized suspension was mixed thoroughly in a screw-capped tube, and was kept for 30 min in an upright position in a test tube rack, to allow settling of tube particles that failed to go back into suspension. The smooth homogenous staphylococcal suspension was used.

The culture filtrates prepared with *E. coli* strains were tested for the presence of enterotoxin, by slide and capillary tube methods. In the first method, a drop of culture filtrate of the test strain was mixed with an equal amount of sensitized staphylococcal suspension on a clean microscopic glass slide; and was rocked gently for 30 s before being checked for agglutination. Macroscopic agglutination within one minute was taken as positive. In the second method, culture filtrates of the test strain first were drawn into a tube (7 cm long and 2 mm in outer diameter) by capillary action. Then the exterior was wiped off using tissue paper, and the sensitized staphylococcal suspension was drawn in and mixed by inverting the tube several times. One end was sealed with plasticin, and was allowed to stand vertically. The tube was incubated overnight at 4°C, and was examined the next day. The result was considered positive when the tube showed macroscopic agglutination of *Staphylococci* and the suspension appeared granular, whereas a homogenous suspension with a milky appearance was taken as negative. Diluted purified LT (100 μg/ml) and TSB served, respectively, as positive and negative controls.

Results

Ileal-loop, skin PF and suckling mice assays

Forty-one out of the 50 culture filtrates of *E. coli* strains tested caused accumulation of fluid in the range of 0.6-1.7 ml/cm, as compared to 0.8-1.6 ml/cm by the *V. cholerae*-positive control in rabbit gut loop. Strain-to-strain and loop-to-loop variations were noted in the amounts of fluid accumulated (13). No fluid was observed in negative control loops. An increase in skin permeability showing a blueing zone of 4 mm or more was seen with 38 culture filtrates. The intensity and diameter of this reaction were found to vary from strain-to-strain and rabbit-to-rabbit. Five of the 50 culture supernates showed gut weight/remaining body weight ratios of more than 0.09, and the others of less than 0.07.

Detection of LT by slide and capillary coagglutination techniques

Twenty-one out of a total of 50 culture filtrates tested showed positive coagglutination in a slide and 38 in a capillary tube (Table I). Seventeen culture filtrates that were positive in a capillary tube showed no coagglutination on a slide. The capillary tube method was more sensitive than was the slide test ($P < 0.001$).

TABLE I – DETECTION OF *ESCHERICHIA COLI LT* BY SLIDE AND CAPILLARY COAGGLUTINATION TECHNIQUES

Slide Coagglutination	Capillary Coagglutination	Number of Strains*
+	+	21
–	+	17
+	–	0
–	–	12

*Capillary tube method is more sensitive than is slide ($p < 0.001$)

Comparison of results of ileal-loop, skin PF and capillary coagglutination

The culture filtrates of 36 strains were positive in all three tests: 2 were positive in skin PF and capillary coagglutination, and 5 caused only fluid accumulation in ileal loops. The remaining 7 culture filtrates were negative in all tests.

($p < 0.001$). A low level of sensitivity of the latter method was indicated in an early study, and prolonged contact between the antibody-coated *Staphylococci* and the antigenic LT was thought to be responsible for the increased sensitivity of the capillary tube technique (11). In a recent study, the same sensitivity level of the two methods was reported (12), but the methods' LT titers were not compared.

The ligated rabbit ileal-loop technique (2) is the classical bioassay for enterotoxins (18), and the results of any new method developed should be compared with those obtained in that model (19). The diarrhoeal isolates of *E. coli* had been shown to produce enterotoxins on the basis of ileal-loop tests (20-22), and both LT and ST were known to cause fluid accumulation in loops. In the present study, fluid accumulation in ileal-loops was observed with culture filtrates of 41 out of 50 strains tested. Five of these strains were positive in a suckling mice assay, indicating that they produced ST (23). Thirty-six of the 41 ileal-loop-positive strains were negative in a suckling mice assay, but caused an increase in skin PF, suggesting that they elaborated only LT. Two strains were negative in the ileal-loop test, but positive in a skin PF assay. This kind of discrepancy is known to occur because the skin PF assay is more sensitive than is the ileal-loop assay. The bioassays thus indicate that 38 of the strains were LT producers, 5 were ST producers, and the remaining 7 were non-toxic.

TABLE II – COMPARISON OF THE RESULTS OBTAINED IN ILEAL-LOOP, SKIN PERMEABILITY, CAPILLARY TUBE COAGGLUTINATION AND SUCKLING MICE ASSAYS

Ileal loop	Skin PF	Capillary Coagglutination	Suckling Mice assay	Number of strains
+	+	+	–	36
–	+	+	–	2
+	–	–	+	5
–	–	–	–	7

Discussion

Several immunological techniques for detection of LT-producing ETEC strains have been developed during recent years to replace the expensive and cumbersome bioassays. Staphylococcal coagglutination, a new technique with high potentiality for sensitivity and reproducibility, detected LT in 38 strains by capillary tube and in 21 by slide methods. The capillary tube technique thus proved to be much more sensitive than did the slide method

No strain was an LT/ST producer. It is usually presumed that LT/ST-producing strains of *E. coli* are common in this Subcontinent. However, as the present study was done for a limited period of six months during 1981, such strains, even if present, might not have been detected. Coagglutination in a capillary tube detected enterotoxin in all 38 culture filtrates, and all 38 were found in the bioassays to be LT-producers. This technique showed identical results with skin PF assays, and a higher sensitivity than did the ileal-loop test.

In conclusion, a staphylococcal coagglutination test performed in capillary tubes is more sensitive than is the slide technique for culture filtrates of *E. coli* strains, and the results agree with those obtained in classical bioassays. The technique is simple to perform and not demanding in terms of reagents or equipment. Moreover, interpretation of results is straightforward and sensitive. Such a technique may prove useful in routine clinical laboratories, to assess the role of ETEC strains in diarrhoeal diseases.

Acknowledgements

We extend our sincere thanks to Prof. F. Dorner, Sandoz Forschungsinstitut, Wein, for the supply of pure heat-labile enterotoxin of *E. coli* P 263 and its antitoxin; and to Prof. D.S. Agarwal of the M.A. Medical College, New Delhi, for providing the *S. aureus* strain 4972. We are grateful to Prof. P.C. Sen of this Department for his helpful suggestions during the course of the work.

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