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Rapid Detection by a Coagglutination Test of Heat-Labile Enterotoxin in Cell Lysates from Blood Agar-Grown *Escherichia coli*

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Colonies of *Escherichia coli* from blood agar plates were suspended and lysed in saline containing polymyxin B and a detergent (Triton X-100). The lysates were assayed for heat-labile enterotoxin (LT) by a coagglutination test (coa-test). The coa-test reagent consisted of Formalin-treated and heat-killed cells of *Staphylococcus aureus*, strain Cowan 1, sensitized with a high-titer rabbit anti-LT serum. Purified LT was detected in the coa-test at the nanogram level (2 to 5 ng), whereas larger amounts of cholera toxin (50 ng) were required to give a positive test. The coa-test was compared with the CHO cell test for detection of LT among *E. coli* strains isolated from human and animal stool cultures. The results of the coa-test and the CHO cell test correlated with 63 of 67 strains of human origin. Six of nine animal strains, defined as LT positive by the CHO cell test, gave positive results in the coa-test. We conclude that the coa-test is probably accurate and sensitive enough to be used in routine diagnosis of LT-producing *E. coli* strains isolated from human stool cultures. No special laboratory equipment is required, which makes the coa-test suited for diagnosis of enterotoxigenic *E. coli* in small hospital laboratories and in developing countries.

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of acute diarrheal disease in developing countries as well as among travelers in such areas (4, 14). ETEC organisms can cause a severe diarrhea indistinguishable from clinical cholera (4) elaborating one or two enterotoxins, heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST).

The intestinal loop and the rabbit skin tests were the first methods described for assaying cholera toxin (CT) and *E. coli* LT, these being followed by a second generation of tests based on the detection of enterotoxin activity in tissue culture cells such as the CHO cell test (8). After the cell culture assays, the first of the immunoassays (radioimmunoassay and enzyme-linked immunosorbent assay [ELISA]) were developed, giving higher sensitivity but requiring expensive equipment and trained laboratory personnel, and thus not suited for most hospital or public health laboratories. More recently, a simple gel immunodiffusion test (the Biken test) was developed that is reported to be more suited for laboratories in need of a simple test for CT and *E. coli* LT for routine diagnostic purposes. The main disadvantage of the Biken test (9) is that results are obtained after 3 to 4 days—too late to be of practical importance in clinical medicine.

The coagglutination test (coa-test) presented here is a rapid and simple immunoassay, needing minimum laboratory equipment and test reagents and no special education of laboratory personnel. Brill et al. (2) developed a capillary modification of the coa-test usually performed on glass slides, using antisera against cholera toxin (CT) to detect *E. coli* LT. We found that the immunological cross-reaction of CT antibodies from rabbit antisera was too weak to give a coa-test accurate and sensitive enough to be used as a standard laboratory method for the detection of "weak" LT-producing strains (B. Rönnberg and T. Wadström, in press).

The aim of this study was to develop a coa-test from *E. coli* LT, using *Staphylococcus aureus* coated with antiserum against highly purified LT as the reagent used to detect LT in cell lysates of suspended bacterial colonies from cultures grown on agar. The whole test procedure can be performed in 90 min and the test result can be obtained within 2 min after mixing the cell lysates and the coa-test reagent on a glass slide or cellulose strip.

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by the International Association of Biological Standardization and the World Health Organization.)

MATERIALS AND METHODS

Bacterial strains. The human and animal strains used in this study have previously been studied and defined as LT-positive and LT-negative strains as determined by the rabbit intestinal loop test, rabbit skin test, and cell culture asays. Strains of animal origin were kindly put at our disposal by H. U. Betschinger, Faculty of Veterinary Medicine, Zurich, Switzerland, and by O. Söderlind, National Veterinary Institute, Uppsala, Sweden. The O:H:K serotypes of the ETEC strains in this study have also been classified (1).

Purification of LT. Human *E. coli* T63, kindly supplied by R. Holmes, Uniformed Services University of the Health Sciences, Bethesda, Md., was cultured in a tryptic yeast extract medium (Difco Laboratories, Detroit, Mich.) in a 10-liter fermentor (Biotec-LKB Produkter, Bromma, Sweden) with control of cultivation, temperature, pH, and agitation (11). LT was purified essentially as described by Clements and Finkelstein (3). The homogeneity of purified LT was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis and by crossed immunoelectrophoresis.

Production of LT and CT antisera. Rabbits were injected subcutaneously at multiple sites along the back with purified LT mixed with an equal amount of Freund complete adjuvant from Difco (equivalent to 80 μg of LT each). These rabbits received subcutaneous booster doses (equivalent to 80 μg of LT each) suspended in Freund incomplete adjuvant at 2- to 4-week intervals for 5 months. Sera were collected before immunization, starting 4 weeks after the first immunization. Sera were stored at −20°C.

The immunization scheme was the same for both LT and CT. Purified immunoglobulin G (IgG) was obtained by chromatography on protein A-Sepharose CL-4B according the manufacturer, Pharmacia Fine Chemicals, Uppsala, Sweden.

Production of the coagglutination reagent. A 1-ml amount of a 10% heat-killed and Formalin-treated suspension of *S. aureus*, strain Cowan 1 (10), provided by Pharmacia Diagnostics, Uppsala, Sweden, was washed in phosphate-buffered saline (PBS; 0.01 M sodium phosphate buffer [pH 7.4] in 0.14 M NaCl) and sensitized with 0.1 ml of an optimal dilution of anti-LT or anti-CT sera in PBS. The sensitization mixtures were incubated for 1 h at room temperature, after which the bacteria were washed in PBS, mixed with a 4% solution of methylene blue in PBS, and further incubated for 1 h at room temperature. After being washed in PBS, the reagents were diluted to a final working suspension of 2% in PBS and stored at 4°C.

Other tests. The details concerning the test conditions to detect LT and CT in the CHO test have been described elsewhere (8).

Chemicals. Polymyxin B and Triton X-100 were purchased from Sigma Chemical Co., St Louis, Mo. Liquid culture media were obtained from Difco.

RESULTS

Development of a standard coa-test for LT. Comparative studies with staphylococcal cells coated with the anti-CT or anti-LT sera showed that purified LT was detected at the 5-ng level in the standard coa-test with anti-LT antibodies, whereas 50 ng or more of CT was necessary to obtain a positive test. Tests with staphylococci coated with the anti-CT antibodies were found to detect 1 ng of CT, whereas 10 ng of LT was necessary to give a positive test. Staphylococcal cells coated with whole anti-LT serum and cells coated with purified anti-LT IgG of the corresponding titer gave identical test results. ETEC strains in our collection have previously been defined as "strong" or "weak" LT producers (1).

A number of these strains were cultured on blood agar, and a loopful of bacteria was suspended in 0.1 ml of saline containing polymyxin B (2 mg/ml) and incubated at 37°C for 1 h. A nonionic detergent (Triton X-100, final concentration 0.1%) was added after 1 h, and the mixture was further incubated for 10 min to obtain cell lysates. The lysates were then centrifuged (3,000 × *g*, 20 min. 20°C), and equal volumes of cell lysate (25 μl) and coa-test reagent were mixed on a glass slide. The result should be read within 2 min. Tests performed on cellulose papers supplied by the manufacturer for Phadebact (Pharmacia Diagnostics, Uppsala, Sweden) gave identical results that were easier to interpret because of the greater contrast between the white paper and the blue reagent, as compared with the glass slide procedure (Fig. 1).

Figure 1a shows the coagglutination occurring after the cell lysate of an LT-positive strain was

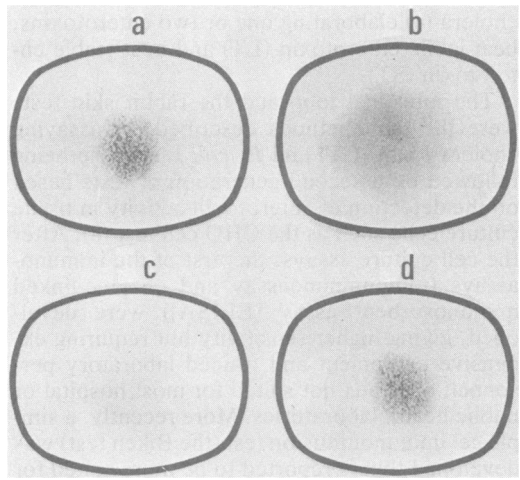

FIG. 1. The coa-test with staphylococci coated with anti-LT antibodies performed with an LT-producing strain (a) and an LT-negative control strain (b). Panel c shows the coa-test performed with PBS, and 50 ng of purified LT was applied in panel d.

mixed with the reagent containing staphylococci coated with anti-LT antibodies on a cellulose paper. In Fig. 1b, the cell lysate of an LT-negative strain was mixed with the reagent, giving a negative result in the coa-test. PBS was added to the reagent in Fig. 1c, but there was no autoaggregation of the coated staphylococci. In Fig. 1d, 50 ng of purified LT was added to the reagent, giving an instant coagglutination.

Sensitivity of the standard coa-test. Bacterial cell lysates were serially diluted in PBS and tested in the standard coa-test, using staphylococci coated with anti-LT or anti-CT antibodies. Cell lysates gave a positive test with staphylococcal cells coated with anti-LT antibodies at higher dilutions than with cells coated with anti-CT antibodies (Table 1).

LT in extracellular fluids and cell lysates. Five human LT-producing ETEC strains were grown in Casamino Acids yeast extract medium. Relative quantities of extracellular and cell-associated LT in these cultures were determined in the CHO cell test, showing that the culture fluid gave positive tests at lower dilutions than in cell lysates (Table 2). The yield of LT in these lysates corresponded to LT titers in standard cell lysates obtained by suspending ETEC grown on blood agar.

Conditions for culturing. Three human and one animal ETEC strain were grown on a variety of solid media used in diagnostic procedures for bacterial intestinal pathogens. Besides blood agar, deoxycholate citrate agar, ethylene blue agar, endoagar, and XLD agar were used, and all gave reproducible test results, whereas cell lysates obtained from colonies grown on MacConkey agar often gave negative LT coa-test results.

Detection of LT-producing human and animal ETEC strains by the standard coa-test. Cell lysates from human and animal ETEC strains grown on blood agar were compared for LT production by the coa-test, with staphylococci coated with anti-LT antibodies, and by the CHO cell test (Table 3).

Ninety-four percent of the strains of human origin gave similar results in the coa-test and the CHO cell test. However, four strains were positive in the CHO cell test but negative in the coa-test. One of these four strains was a low LT producer (cell lysate diluted 1:10), and the three other coa-test-negative human strains were high LT producers, according to the CHO cell test (cell lysates diluted ≥1:100). Among the animal strains, we found three strains that were negative in the coa-test but positive in the CHO cell test. These three animal strains, together with the six other coa-test- and CHO cell test-positive strains, were defined as high LT producers by the CHO cell test (cell lysates diluted ≥1:100). No strains defined as coa-test positive were found to be negative in the CHO cell test.

TABLE 1. Titers of LT obtained in cell lysates from blood agar-grown human ETEC strains

ETEC strain	LT titer	
	Anti-LT serum	Anti-CT serum
T63	1/128	1/32
1628-15	1/32	1/16
33426	1/16	1/4
E80a	1/16	1/8

TABLE 2. Comparative titers of extracellular and cell-associated LT in the CHO cell test

ETEC strain[a]	Titer	
	Extracellular LT	Cell-associated LT[b]
T63	1/100	1/1,000
1628-15	1/20	1/1,000
E80a	1/20	1/1,000
C922b	ND[c]	1/100
C922c	ND	1/100

[a] ETEC strains were grown on Casamino Acids yeast extract medium.

[b] Idential results were obtained with strains grown on blood agar or in Casamino Acids yeast extract medium.

[c] ND, Not detectable.

DISCUSSION

Several immunoassays for detecting LT-producing ETEC strains have been reported recently, designed to replace conventional bioassays such as intestinal and skin tests in rabbits and tissue culture cell assays. Radioimmunoassays, ELISAs, and several variations of such tests, like the GM_1-ELISA, are tests which need expensive equipment, reagents, and trained personnel and are therefore less suited for use in large hospital laboratories in industrialized countries or public health laboratories in developing nations where such tests are needed for epidemiological surveys, as well as in the future,

TABLE 3. Comparison of the results of the coa-test with anti-LT antibodies and the CHO cell test for detection of LT in cell lysates of 67 human and 11 animal strains of *E. coli* grown on blood agar

LT activity		No. of human strains	No. of animal strains
Coa-test	CHO cell test		
+	+	39	6
−	−	24	2
+	−	0	0
−	+	4	3

to follow and evaluate new vaccination programs for enterotoxigenic pathogens (14).

The simple gel immunodiffusion test (Biken test) recently developed to detect LT in crude cell lysates (9) is a simple test to perform, needing a minimum of laboratory equipment and training of personnel. The main drawback with the Biken test is that it takes 4 days to obtain the test results, and large amounts of anti-cholera or anti-LT antibodies are needed for each test.

Brill et al. (2) first reported a staphylococcal coagglutination test for the rapid detection of LT, and a coagglutination test was recently evaluated in an epidemiological study in India (12).

Besides the rapid, simple procedure, the requirement for highly purified test reagents like LT or CT antibodies is much less than in a gel immunodiffusion test. Moreover, we have found that there is no need to purify the specific LT antibodies for coating the bacteria, because heat-killed staphylococci preferentially bind the Fc part of the IgG antibodies to cell surface protein A (10), and such antibodies seem to be very stable upon storage at 4°C and even at room temperature (20 to 30°C), which is an additional advantage for the shipping and storage of reagents.

There are only a few reports on the relative amount of cell-associated and extracellular LT in ETEC strains grown under different conditions. However, Wadström et al. (17) found that most LT activity was detected in cell lysates, and only about 10% of total activity in cultures was generally found in the culture fluid. A similar amount of toxin was released from the cell envelope by osmotic shock or polymyxin treatment. We have taken advantage of the fact that ETEC do not seem to actively secrete LT in the same way as recently reported for another toxin—i.e., the hemolysin (6)—by using agar cultures to develop the standard assay in this report. This simplifies the whole test procedure, as we believe that in future studies using this test in diagnostic laboratories on primary stool cultures, it would be an advantage to avoid the cumbersome extra step of growing each isolate from stool cultures in liquid culture before performing the immunoassays as described in radioimmunoassays, ELISA, and GM_1-ELISA test procedures (7, 15, 18).

Recent reports on the molecular heterogeneity of the heat-labile enterotoxin from human (LT_h) and porcine (LT_p) origins (5, 16) explain the discrepancy between the coa-test and the CHO cell test results of the animal isolates (Table 3). The coa-test reagent used in this study contained staphylococci coated with anti-LT_h antibodies, and although nine of the animal strains were high LT producers according to the CHO cell test, three of these isolates gave negative results in the coa-test. An explanation is that LT_h and LT_p share a common antigenic determinant(s), but they also have a unique antigenic determinant(s). Moreover, recent studies with monoclonal antibodies to LT_h have shown immunological heterogeneity of both the A and B subunits of LT_h (R. Holmes, personal communication). These findings probably explain the discrepancy between the coa-test and the CHO cell test results obtained for three of the human strains that were high LT producers according to the CHO cell test. The fourth human strain that was coa-test negative but CHO cell test positive, probably also has a unique antigenic determinant(s) or produces LT in an amount too low to be detectable in the coa-test.

We believe that these results are so promising that the test should be evaluated in developing countries and in hospital laboratories in industrialized countries as a new test procedure in studies of travelers' diarrhea and in diarrhea of unknown origin, especially in young children in conjunction with serotyping in larger outbreaks of enteropathogenic *E. coli* of the classical EPEC serotypes (13).

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