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Adaptation of the Staphylococcal Coagglutination Technique for Detection of Heat-Labile Enterotoxin of *Escherichia coli*

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Protein A-containing staphylococci coated with specific antiserum were tested for heat-labile enterotoxin of *Escherichia coli*. The immunological cross-reactivity of *E. coli* heat-labile enterotoxin with *Vibrio cholerae* toxin (choleragen) was the basis for sensitizing stabilized suspensions of the Cowan I strain of *Staphylococcus aureus* with anticholeragen. Unconcentrated culture supernatant fluid containing *E. coli* heat-labile enterotoxin produced macroscopic agglutination when mixed with sensitized staphylococci in capillary tubes. A total of 15 toxigenic and 61 nontoxigenic isolates were tested by the staphylococcal coagglutination technique in a coded fashion and found to be in agreement with previous results of the Chinese hamster ovary cell assay and the passive immune hemolysis test. The staphylococcal coagglutination technique is simple, relatively inexpensive to perform, and requires the immunoglobulin fraction of anticholeragen as the only specific reagent. The staphylococcal coagglutination technique appears to have potential for routine use in diagnostic microbiology laboratories.

Enterotoxigenic *Escherichia coli* have been implicated as the causative agents of diarrheal illness in all age groups (19, 28, 33). The severity of disease caused by these organisms can vary considerably, with symptoms ranging from uncomplicated mild diarrhea to potentially life-threatening dehydration and electrolyte imbalance due to major fluid loss (28, 32).

Toxigenic strains of *E. coli* are capable of producing an antigenic, heat-labile toxin (LT), a nonantigenic, heat-stable toxin, or both (28, 30). Toxigenicity is mediated by a plasmid termed Ent and is transmissible to other strains of *E. coli* and other members of the family *Enterobacteriaceae* (21, 34, 35). The exact mode of action of the heat-stable toxin is unknown at this time. LT has been shown to share biological activity with toxin produced by *Vibrio cholerae* (choleragen). These toxins cause activation of adenyl cyclase in intestinal mucosal cells of the small intestine. As a result, cyclic adenosine monophosphate levels increase, and a net fluid loss into the intestinal lumen follows (12, 22).

Antiserum prepared against LT from any given *E. coli* will react with LT from a variety of isolates (6, 31). Besides sharing a common mechanism of action, *E. coli* LT and *V. cholerae* choleragen are immunologically cross-reactive; each toxin is neutralized by heterologous as well as homologous antisera (6, 36).

Recent evidence suggests that pili-like structures that are responsible for adherence to the intestinal epithelium are necessary for enterotoxigenic strains to express their full pathogenicity (10). This colonization factor antigen has been shown to be plasmid controlled (11) and found to be present on a variety of strains recovered from outbreaks of diarrheal illness (9).

Investigation into the actual prevalence of toxigenic strains of *E. coli* and their significance in clinical diseases has been hampered by lack of a simple means of detecting such strains. A variety of test systems with proven effectiveness in detecting enterotoxigenic *E. coli* are currently available including animal models, tissue culture lines, and in vitro assays (6, 8, 14, 16-18, 38). Despite their usefulness in research-oriented facilities, none of these systems is readily adaptable for routine use in diagnostic laboratories.

The introduction of the staphylococcal coagglutination technique (staph-coA) for detecting the presence of capsular polysaccharides from a variety of bacteria (4, 5, 7, 24, 37) provided a procedure which could aid in the study of *E. coli* enteritis and ultimately prove useful in clinical microbiology laboratories.

MATERIALS AND METHODS

Stock organisms. The Cowan I strain of *Staphylococcus aureus* (ATCC 12598), particularly rich in protein A, was used as the indicator particle in the test system. *E. coli* strain H-10407, originally isolated from a diarrheal patient in Dacca, Bangladesh, and *E. coli* strain 334, originally recovered from a patient in Cal-

cutta, India, served as the prototype toxigenic strains. *E. coli* CDC 009 was used as the negative control. Stock cultures were maintained on slants of nutrient agar (Baltimore Biological Laboratory) stored at room temperature and subcultured monthly.

Preparation of staphylococci. Stabilization and sensitization of reagent staphylococci followed procedures described previously by Kronvall (24) with modifications made for convenience. The Cowan I strain of *S. aureus* was grown overnight in tryptic soy broth (TSB; Baltimore Biological Laboratory). A 5-ml amount of this growth was added to each of two 1-liter flasks containing 500 ml of TSB. After 24 h of incubation at 37°C with aeration, the broth was centrifuged, and the cells were washed three times in phosphate-buffered saline (PBS; 0.1 M phosphate, pH 8.5). A 10% (vol/vol) suspension of the cells in PBS containing 0.5% Formalin was allowed to stand at room temperature for 3 h with occasional agitation. The cells were washed three times in PBS, resuspended to a 10% suspension in PBS, and stored at 4°C until used. Such preparations were stable for up to 3 months.

A 1-ml amount of the 10% suspension of staphylococci was added to 0.1 ml of a 1:8 dilution of purified anticholeragen. The immunoglobulin G fraction of antisera prepared in rabbits by immunization with purified choleragen (2, 3, 27) was provided by S. H. Richardson. After 1 h of incubation at room temperature with occasional agitation, 4 ml of PBS was added, and the mixture was centrifuged. The sensitized cells were diluted to a final working suspension of 2% in PBS and stored at 4°C. Before actual use in the assay, each newly sensitized suspension was mixed thoroughly in a screw-cap glass tube and allowed to stand upright in a test tube rack for approximately 30 min. This allowed the particles that had failed to go back into suspension after centrifugation to settle to the bottom of the tube. The portion of the mixture containing these heavy particles was discarded, and only the smooth, homogeneous staphylococcal suspension was actually used in the assay.

The protein A in the cell wall of the staphylococci binds immunoglobulin molecules via the Fc portion, thus orienting the free, antigen-binding Fab sites outward. When mixed with the appropriate antigen, the sensitized staphylococci agglutinate. The principle of the staph-coA technique is represented diagrammatically in Fig. 1.

Conditions for enterotoxin production. The broth used for culture of toxigenic and nontoxigenic strains was essentially that described previously by Evans et al. (16) and modified by Mundell and his coworkers (29). Casamino Acids-yeast extract-salts medium (CAYE), containing 2.0% Casamino Acids (Difco Laboratories), 0.6% yeast extract (Difco), 0.25% NaCl, 0.871% K_2HPO_4, 0.25% glucose, and 0.1% (vol/vol) trace salts solution consisting of 5% $MgSO_4$, 0.5% $MnCl_2$, and 0.5% $FeCl_3$, was adjusted to a pH of 8.5 with 2.5 N NaOH and 1.0 N HCl and sterilized by autoclaving for 20 min at 120°C. The glucose was sterilized by filtration and added aseptically to the autoclaved broth.

Blind study. Toxigenic and nontoxigenic strains of *E. coli* and other members of the family *Enterobacteriaceae* were submitted for testing as part of a blind study. These organisms represent a collection of clinical isolates from around the world and laboratory stock strains maintained at this institution for study of a variety of characteristics. Included in the study were other *E. coli* isolates from Bangladesh (strains 10401, 9190, 9192, and 19210 among others), organisms donated by R. L. Guerrant at the University of Virginia, and isolates from North Carolina Baptist Hospital.

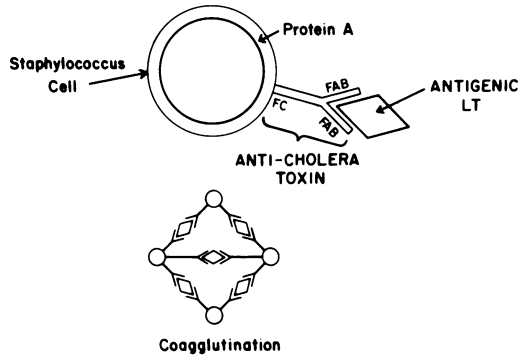

FIG. 1. *Diagrammatic representation of the principle of the staph-coA technique. The antigen-antibody system illustrated is that of anticholeragen reacting with the antigenic LT.*

Organisms submitted for testing were first thawed from ampules of 40% glycerol in Columbia broth (Baltimore Biological Laboratory) which had been stored at −20°C for several months, and they were streaked onto plates of Columbia agar base (Baltimore Biological Laboratory) containing 5% sheep blood. Coded isolates were received for testing after overnight growth on plates of Columbia agar base containing 5% sheep blood. A generous loopful of this growth was added to 5 ml of TSB and incubated in a static waterbath at 37°C. A 0.1 ml sample of overnight growth in TSB was added to 5.0 ml of CAYE containing approximately 20 μg of the antibiotic lincomycin per ml in 125-ml Erlenmeyer flasks. Lincomycin, an inhibitor of protein synthesis, has been shown to be an inducer of toxin production in both *E. coli* and *V. cholerae* (26). Flasks were incubated at 35°C for 22 to 24 h on a mechanical shaker (horizontal displacement, 4 cm; 174 rpm). *E. coli* strains H-10407 and CDC 009 served as positive and negative controls with each set of unknowns tested.

Testing for toxigenicity. Culture supernatant fluids from test isolates were assayed for the presence of LT. Cultures were centrifuged at moderate speeds without any subsequent filtration or concentration. Approximately 45 μl of supernatant fluid was drawn up into capillary tubes (7 cm; length, OD, 2 mm) by capillary action, and the exterior was wiped clean of excess supernatant fluid with tissue paper. The sensitized staphylococcal suspension was mixed thoroughly, and a portion was placed in a disposable Pasteur pipette by using suction from a rubber bulb. The capillary tubes already containing the test solution were held in a horizontal position, slanted slightly to force the test solution against one end. To this same

end approximately 40 μl of the staphylococcal suspension was added with a Pasteur pipette. The pipette was held at a slight angle and off-center from the end of the capillary tube to ensure that air trapped within the pipette tip was allowed to escape and that only sensitized staphylococci would be dispensed into the capillary tube. The influx of sensitized staphylococci displaced the test solution to the other end of the tube. The capillary tube was then manipulated in such a way as to create gaps of air between the solution and the ends of the tube. The ends of the capillary tubes were plugged with clay and oriented in a manner that allowed the staphylococci to settle through the test solution. The capillary tubes were incubated vertically at 4°C overnight and examined the next day with the aid of a high intensity lamp. Most positive results were visible within 1 h. The staphylococcal suspension remained homogeneous in the negative control, giving the tube a milky appearance. The solutions containing LT caused macroscopic agglutination of the staphylococci and appeared granular in contrast to the smooth negative control. Some actual test results are shown in Fig. 2.

Determination of sensitivity level. Partially purified LT, provided by S. H. Richardson, was diluted in a serial twofold manner in sterile CAYE broth. Each dilution was tested in the fashion described above. The highest dilution causing agglutination of

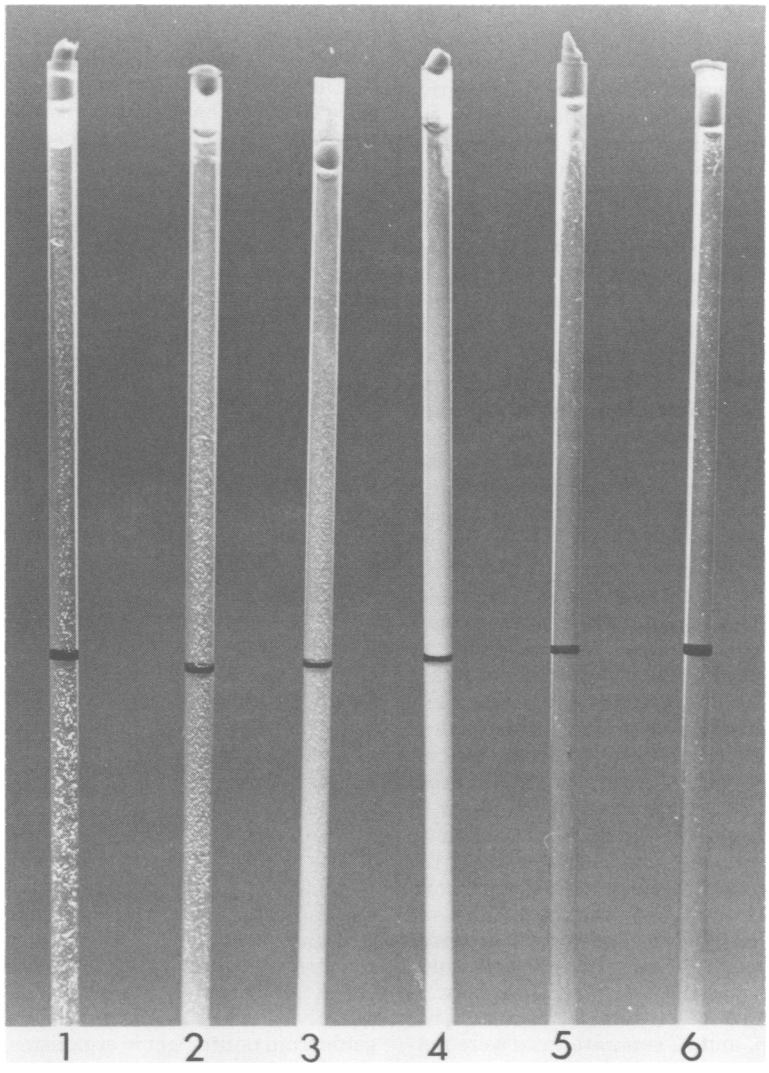

FIG. 2. *Test results of staph-coA technique. Tube 1, undiluted LT; tube 2, LT diluted 1:100; tube 3, LT diluted 1:1000; tube 4, negative control; tube 5, supernatant fluid from E. coli 334; tube 6, supernatant fluid from E. coli 10407. Note that particles of staphylococci have settled to the bottom of the positive control tubes, giving them the appearance of clearing.*

the staphylococci was determined on at least four separate occasions, and the sensitivity of the assay was calculated from the protein concentration of undiluted material. Purified choleragen was tested in a similar manner to provide another means of comparison with other assay methods.

RESULTS

Sensitivity level. Serial twofold dilutions of partially purified LT made in sterile CAYE broth, when tested by the staph-coA technique, showed diminishing degrees of agglutination, ranging from a solid core of agglutinated staphylococci in the more concentrated samples to a granular agglutination in greatly diluted samples. The endpoint of 1:2,048 was finely granular, but clearly distinguishable from the homogeneous suspension of staphylococci in the negative control. Based upon an undiluted protein concentration of 68 μg of protein per ml, the staph-coA technique was sensitive down to 32 ng of protein per ml. A purified choleragen preparation containing 11.6 mg of protein per ml was diluted 1:1,000 and then in a serial twofold manner. Testing of this material indicated that 708 pg of choleragen per ml could be detected in the assay. A second determination was made with the choleragen preparation diluted 1:1,000,000 before making serial twofold dilutions. In this test the final positive dilution contained 725 pg of toxin per ml.

Blind study. The toxigenicity of coded isolates determined by testing unconcentrated culture supernatant fluids by the staph-coA technique was compared with previous results obtained from the Chinese hamster ovary (CHO) cell assay (18) and the passive immune hemolysis (PIH) test (14). A total of 76 isolates were tested. Of these, 15 isolates were toxigenic by the staph-coA technique, which was consonant with previous results from the CHO cell assay and the PIH test. The remaining 61 isolates were determined to be nontoxigenic by the staph-coA technique and were in accord with results from the standard assay procedures. A summary of results from the blind study is contained in Table 1. The table also shows that most of the toxigenic organisms and some of the nontoxigenic organisms were tested on more than one occasion. The isolates retested under different code numbers gave entirely consistent results, indicating the reproducibility of the assay. A total of 26 separate tests were performed on the toxigenic group, and 67 separate tests were performed on the nontoxigenic group.

An unexpected finding was the discovery that 12 strains had lost their toxigenicity during storage at −20°C for a period of about 7 months. All 12 strains were negative when tested by the staph-coA technique, whereas positive results had been found previously with the CHO cell and PIH tests. When frozen culture supernatant fluids from the staph-coA procedure were submitted to the source laboratory for repeat testing by the CHO cell assay and the PIH test, they were found to be negative. To confirm the fact that these strains were indeed no longer toxigenic, the organisms were cultured and processed under the conditions used originally to determine their toxigenicity. The CHO cell assay and PIH test results from these preparations were also negative, corroborating the staph-coA technique negative results.

TABLE 1. *Summary of blind study results*

Type of isolate	No. of isolates tested by:		
	Staph-coA technique	CHO cell assay	PIH test
Toxigenic	15 (26)[a]	15	15
Nontoxigenic	61 (67)	61	61

[a] Numbers in parentheses indicate actual number of tests performed, because many isolates were tested repeatedly under different code numbers.

Some difficulty was encountered with an isolate of *Klebsiella pneumoniae*. Initial testing of this organism with the staph-coA technique yielded positive results where negative results were expected. The frozen culture supernatant fluid was negative in the CHO cell assay and PIH test. Repeating the staph-coA procedure gave unequivocally negative results, and the strain was then regarded as nontoxigenic.

One nontoxigenic organism not included in the above results failed to grow in the test CAYE broth on two attempts despite obvious viability in TSB. Lack of significance to this particular study precluded any further investigation into the cause of this observation, although susceptibility to lincomycin or inhibition by the alkaline broth, a factor previously studied by Evans and Evans (13), are the most likely explanations.

DISCUSSION

The Cowan I strain of *S. aureus* prepared and stabilized in the laboratory serves as a convenient indicator particle when sensitized with specific antiserum. In this study the staphylococcal suspension sensitized with the immunoglobulin G fraction of anticholeragen possessed adequate sensitivity and specificity to differentiate toxigenic from nontoxigenic organisms. Results from the staph-coA procedure showed complete agreement with previous results obtained from a standard tissue culture assay, the CHO cell assay, and an in vitro system, the PIH test. The enterotoxigenic group of isolates tested in this

study would have included 12 more organisms. However, these particular isolates no longer expressed toxigenicity after storage, quite possibly due to the loss of the Ent plasmid.

Previous work utilizing the phenomenon of protein A-immunoglobulin interaction has centered around the detection of capsular polysaccharides of various bacteria (4, 5, 7, 24, 37). Although some of these procedures have been modified by applying sensitized staphylococci directly to bacterial colonies, most are performed by mixing the reagent staphylococci with test material on a glass slide. Early work in this study employed the classical slide test method in testing for the LT of *E. coli*. Prolific toxin producers such as the *E. coli* strains H-10407 and 334 yielded culture supernatant fluids that were positive in the staph-coA test at a maximum dilution of 1:4. These results indicated a disappointing level of sensitivity by that method, and some means of enhancing the reactivity of the reagent staphylococci was sought. Capillary tubes have been used for years in clinical laboratories for test systems such as C-reactive protein and grouping of beta-hemolytic streptococci (1, 25). These tests involve precipitation reactions, but such a modification of the staph-coA test was thought to have promise. Culture supernatant fluids that were positive by the slide test at a maximum dilution of 1:4 gave positive results at dilutions of 1:64 within 1 h when tested in capillary tubes. Incubation at refrigerator temperatures overnight increased the positive dilutions to 1:256. Prolonged contact between the antibody-coated staphylococci and the antigenic LT without dehydration and the clarity of the reaction appear to be responsible for the success of this modification.

No definitive statement of the sensitivity level of the assay is possible because complete purification of LT has not been accomplished. However, the detection of 32 ng of protein per ml in partially purified preparations of LT and positive results with culture supernatant fluids from toxigenic strains diluted 1:256 provide at least an indirect means of comparing the sensitivity levels with other currently available assays. On such a basis the sensitivity of the staph-coA technique appears to be roughly comparable to sensitivities reported for other in vitro assays (14, 17, 38) and tissue culture methods (6, 18). The sensitivity of other currently available assays exceeds that of the staph-coA technique in terms of the detectable concentrations of choleragen. Those investigators interested in quantitation of minute concentrations of choleragen might prefer these other assays. However, to put these sensitivity levels in perspective, the reader should bear in mind that the staph-coA technique employs unaided macroscopic observation of test results rather than the sophisticated instrumentation of the other techniques.

Several investigators have reported the profound effect that culture conditions and media ingredients can have upon enterotoxin biosynthesis (16, 17, 23, 29, 32). The CAYE broth used for growth of test isolates in this study has been shown to be an excellent medium for toxin production (16, 29). Other workers have shown that miniculture techniques provide adequate toxin elaboration as tested by other systems (17, 20, 38). Such a simplification of culture methods would further accommodate the staph-coA technique for routine use in clinical laboratories. The comparable sensitivity of the staph-coA technique to those of other assays already shown to be suitable for testing miniculture preparations makes the success of such a modification for staph-coA testing appear likely.

Several trials were made to determine the optimal concentration of antibiotic to be incorporated into the medium for induction of toxin production. The 20 μg of lincomycin per ml used during the blind study was found to greatly enhance toxin production over that of antibiotic-free media. This antibiotic reduced cell densities in cultures to a small degree. Because of the discontinued therapeutic use of lincomycin, availability of the antibiotic might present a problem. For this reason a close analog of lincomycin, namely clindamycin, which is used clinically, was tested as a suitable substitute. Despite relatively high minimum inhibitory concentrations for the two prototype toxigenic strains, as little as 5 μg of clindamycin per ml greatly reduced the cell densities of these cultures. Although the induction of toxin production is independent of the cell density (24), because other strains to be tested in a blind fashion or wild-type strains might show a greater sensitivity to clindamycin, toxin production may be jeopardized by inclusion of this antibiotic. On this basis lincomycin was chosen for use in the assay broth. Further testing may show that even lower concentrations of clindamycin may be used safely.

Because of the effect that culture conditions can have on toxin production and the apparent instability of the Ent plasmid in some strains (15, 19; see above), any direct comparison of the various assays in terms of sensitivity levels or ability to differentiate toxigenic from nontoxigenic organisms must be conducted simultaneously with fresh isolates or strains and appropriate controls.

The necessary maintenance of viable cells is a detracting factor of the tissue culture assays, and results are often highly subjective. The PIH

test avoids many of these problems, but still requires a supply of sheep erythrocytes, antisera, and complement. The other currently available in vitro assays suffer from the tedious nature of performing the tests and the excessive demand upon technical time.

The advantage of the staph-coA technique is that it is not demanding in terms of reagents, equipment, or actual performance of the assay, and interpretation of results is straightforward. Further simplification may be possible through the use of commercially prepared staphylococci (Pansorbin; Calbiochem-Behring), although the suitability of that preparation has not been evaluated in our laboratory. We believe the staph-coA technique to be a potentially useful tool in routine clinical laboratories. Through large-scale screening for enterotoxin-producing organisms, the actual prevalence and clinical importance of these strains may be determined.

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