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Evaluation of Immuno-Dot-Blot Assay for Detection of Cholera-Related Enterotoxin Antigen in *Salmonella typhimurium*

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Twenty-five strains of *Salmonella typhimurium* isolated in India were examined for the presence of cholera/coli-related enterotoxin antigen by a previously described latex particle agglutination test and by a newly developed immuno-dot-blot test using immunopurified goat antibody against the cholera-related enterotoxin isolated from an *Escherichia coli* strain of human origin. The immuno-dot-blot assay could detect 0.02 ng of purified enterotoxin. The amount of toxin antigen detected varied widely from strain to strain. Fourteen of the 25 polymyxin B-treated extracts of bacteria harvested from 6-h Casamino Acids-yeast extract broth cultures gave positive results in both serologic assays as well as in rabbit skin tests for delayed permeability factor. An additional strain was positive only in the immuno-dot-blot. Five of six stool isolates and six of seven blood isolates tested gave positive reactions. Two isolates of *Salmonella enteritidis* tested were also positive. The immuno-dot-blot test appears to be a simple, rapid, and reliable method for detection of cholera-related enterotoxin antigen in *S. typhimurium*. The demonstration of a cholera-related enterotoxin, even in small amounts, in a facultative intracellular pathogen raises interesting questions regarding its potential role in pathogenesis both of diarrheal disease and systemic infections caused by salmonellae.

Salmonellae are widely distributed in nature, infecting and causing disease in humans and other animals (17, 29). Nontyphoidal salmonellae are major causes of diarrheal disease. Of these, *Salmonella typhimurium* is the most prevalent organism. The pathogenesis of *S. typhimurium* infections has recently been reviewed in detail by Stephen et al. (28) and by Peterson (22). As suggested by many workers, salmonella-induced diarrhea seems to be a complex phenomenon involving several pathogenic mechanisms (28) including a heat-labile enterotoxin (S-LT) which is structurally, functionally, and immunologically related to enterotoxins elaborated by *Vibrio cholerae* and diarrheagenic heat-labile enterotoxin elaborated by strains of *Escherichia coli* (6, 15, 20, 22, 28).

Many groups of investigators working on clinical isolates of *S. typhimurium* have demonstrated enterotoxic activity in various kinds of preparations including culture filtrates (1, 2, 24–26), cell extracts (16, 18, 23, 30), and bacterial sonicates or mitomycin C lysates (14, 21). The enterotoxic activity has been demonstrated in a variety of test systems including rabbit ileal loops (3, 24, 27, 30), skin permeability tests (15, 16, 23, 25), and cell cultures (1, 15, 21, 25, 30). The isolation of small amounts of S-LT to apparent homogeneity has been reported (6). However, there is no convenient, rapid, reliable, and economical method for the detection of S-LT in the clinical laboratory.

Peterson et al. (21) were able to detect S-LT in an enzyme-linked immunosorbent assay (ELISA) using anticholera toxin; more recently, Hariharan et al. (13) described a competitive ELISA for S-LT using immunopurified antibody against H-LT, a cholera toxin-related, heat-labile enterotoxin from an *E. coli* strain of human origin. Although the latter assay was somewhat more sensitive than the latex particle agglutination test (LPAT) examined in parallel, it is not easy to perform and requires an expensive microplate optical density reader to detect optical density differences.

In the present communication, 25 clinical isolates of *S. typhimurium* from India have been studied for production of enterotoxin antigen immunologically related to cholera toxin and H-LT by using a newly developed, rapid immuno-dot-blot test in comparison with the previously described LPAT (7, 8). In both tests, immuno-affinity purified goat antibody against H-LT was employed.

MATERIALS AND METHODS

Strains. Twenty-five human clinical isolates of *S. typhimurium* were kindly supplied by K. C. Agarwal, Department of Medical Microbiology, Postgraduate Medical Institute, Chandigarh, India. Of these, six strains were stool isolates, seven were from blood, five were from cerebrospinal fluid, six were from urine, and one was isolated from arthritic pus. Upon receipt, the strains were streaked on petri plates of McConkey agar (Difco Laboratories, Detroit, Mich.) for confirming purity and on meat extract agar (Difco) for confluent growth. After overnight incubation at 37°C, the growth from the confluent plates was harvested in 5 ml of tryptic soy broth (Difco) with glycerol (4:1) and stored at −70°C in 0.5-ml volumes until used. Ten strains which had been evaluated previously by the competitive ELISA (13) were also included. These were A9705, A9707, SSU-2050, SSU-2051, SSU-2633, SSU-2635, and W118-2, which previously gave positive results under one or more conditions of growth, and A9706, B1265, and SSU-2049 which were negative in previous assays. Two isolates of *Salmonella enteritidis*, kindly provided by Felipe Cabello, New York Medical College, from a patient with choleralike secretory diarrhea, one (which was ampicillin susceptible) from blood and the other (which was ampicillin resistant) from stool, were also tested.

Toxin extraction. Toxin was extracted from each of the strains essentially as described by Wallis et al. (30). The bacterial cells, harvested by centrifugation from 250 ml of a 6-h culture in CYE broth (30) incubated at 37°C in a 1-liter

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Erlenmeyer flask with longitudinal shaking (100 oscillations per min), were extracted with 20 ml of polymyxin B (Sigma Chemical Co., St. Louis, Mo.) solution (6 g per liter of phosphate-buffered saline [pH 7.2] containing 0.05% Tween-20 [Sigma] [PBS-T] instead of Tris hydrochloride buffer). All extracts were then concentrated 20× using B15 Minicon concentrators (Amicon Corp., Danvers, Mass.).

LPAT. The LPAT was performed as described previously (7, 8). A 5-μl sample of immunopurified anti-H-LT-sensitized latex particles were mixed with 10 μl of toxin extracts in a well of a Boerner slide (American Scientific Products, St. Louis, Mo.). The mixtures were gently shaken for 5 min and observed for agglutination by using transmitted oblique illumination and a hand lens. Purified H-LT (30 ng/ml) (9, 19) and polymyxin B solution were used as positive and negative controls, respectively, with each test procedure.

Immuno-dot-blot assay. Each extract was spotted in 2-μl amounts onto nitrocellulose sheets (type HA; pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). Polymyxin B solution and purified H-LT were used as negative and positive controls, respectively. The sheets were transferred to a petri dish containing PBS-T and 3% bovine serum albumin (Sigma) and incubated at room temperature (20 to 22°C) for 1 h to saturate nonspecific binding sites. The sheets were then removed, washed twice with agitation in PBS-T, then placed in primary antibody solution, and incubated at 37°C for 1 h with gentle agitation. The primary antibody solution consisted of immunoaffinity-purified goat anti-H-LT (19), 1 mg/ml, diluted 1:50 in PBS-T–1% fetal calf serum. Higher dilutions of primary antibody up through 1:1,000 are also satisfactory. Sheets were removed and washed thrice at room temperature with PBS-T with gentle agitation; the last wash was for 5 min. The sheets were then placed in secondary antibody, peroxidase-labeled, rabbit anti-goat immunoglobulin G (Cooper Biomedical, Malvern, Pa.) diluted 1:1,000 in PBS-T–1% fetal calf serum, and incubated at 37°C with gentle agitation for 1 h. The sheets were removed and washed three times as above. Substrate stock solution, 10 mg of *O*-dianisidine (Sigma) dissolved in 1 ml of methanol, was prepared immediately before use. It was added to 100 ml of Tris hydrochloride (pH 7.4), to which 100 μl of H_2O_2-H_2O mixture was added (100 μl of H_2O_2 [30%] plus 200 μl of H_2O). The nitrocellulose sheets were then placed in the substrate solution for about 10 min until spots developed. The reaction was stopped by rinsing the sheets in distilled water. The sheets were then dried by blotting and kept at −70°C overnight, which enhances the intensity of the spots.

Delayed skin permeability factor. One-tenth milliliter of each of the extracts of the 25 strains of *S. typhimurium* were injected intradermally in the shaved backs of New Zealand White rabbits as described by Peterson and Sandefur (23). Positive controls included purified H-LT and cholera toxin at doses of 1, 10, and 100 ng, and negative controls included the polymyxin B solution and CYE broth. The intensity of reactions was graded from + to 4+.

RESULTS

Figure 1 illustrates the immuno-dot-blot results with the concentrated extracts from the 25 strains of *S. typhimurium*. The figure includes a titration of purified H-LT. The test was capable of detecting 20 pg of H-LT in 2 μl. Fifteen of the 25 strains tested (strains 1 through 5, 7 through 9, 11 through 13, 18, 21, 22, and 25) gave positive reactions which varied in intensity from barely detectable (e.g., equivalent to ca. 20 pg of H-LT) to strongly positive (as exemplified by strains 1, 4, 7, 13, and 25). With the exception of strain 9 (which gave one of the weakest reactions), all of the positive strains were also positive in the LPAT and in skin tests for delayed permeability factor activity. All strains which were negative in the immuno-dot-blot assay were also negative in LPAT and skin permeability assays. Five of the six stool isolates, six of the seven blood isolates (including strain 9), one of the five cerebrospinal fluid isolates, two of the six isolates from urine, and the single isolate from arthritic pus were positive in the immuno-dot-blot assay.

Seven strains which previously gave positive reactions in a competitive ELISA were also positive in the immuno-dot-blot assay in the present study. Of these, six were positive in the LPAT. Three strains which were previously negative in the competitive ELISA were likewise negative in both immuno-dot-blot tests and LPAT in the present study. The two isolates of *S. enteritidis* tested gave weakly positive reactions in both the immuno-dot-blot assay and LPAT.

From our results it is difficult to estimate with precision the amounts of S-LT which are produced by different strains because the immuno-dot-blot assay is based on the cross-reactivity of S-LT with antibody against H-LT and it is not clear whether S-LT reacts equivalently to H-LT. However, a sample of immunopurified S-LT, at 1 μg of protein per ml or 2 ng per 2-μl dot, gave a dot-blot reaction which was only slightly less intense than that of an equivalent sample of

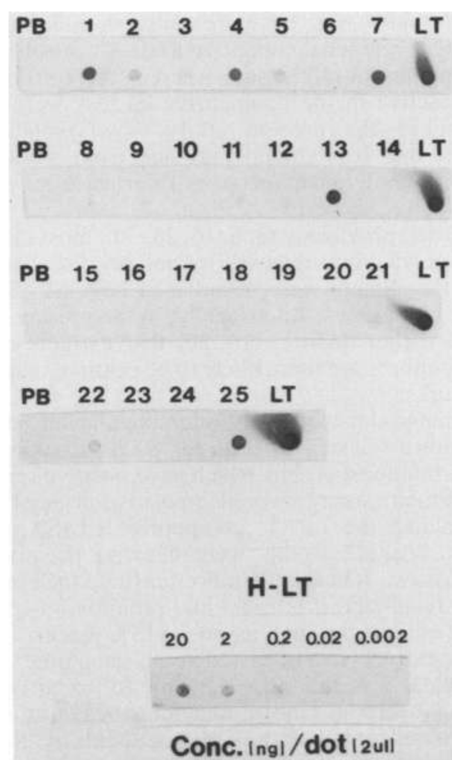

FIG. 1. Immuno-dot-blot assay of 25 concentrated polymyxin B extracts of *S. typhimurium*. Strains 1 through 6 were stool isolates, 7 through 13 were isolated from blood, 14 through 18 were from cerebrospinal fluid, 19 through 24 were from urine, and 25 was from arthritic pus. Positive results (visible to the naked eye before photography) were given by strains 1 through 5, 7 through 9, 11 through 13, 18, 21, 22, and 25. The bottom portion reveals that the assay could detect 20 pg of purified H-LT per 2 μl. PB, Polymyxin B control; LT, H-LT control.

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H-LT. If we assume that the test is equally sensitive to both S-LT and H-LT, then minimally positive 20×-concentrated salmonella extracts, prepared as described above, contained approximately 10 ng of S-LT per ml. These extracts were prepared from the bacterial cells harvested from 250 ml of culture, i.e., approximately 250 × 10^9 bacteria which had been extracted in 20 ml of polymyxin B solution, so approximately 40 pg was extracted from 10^9 bacteria. Assuming a molecular weight of 10^5 and employing Avogadro's number, this calculates to less than one molecule per cell of the most weakly positive strains and, judging from the intensity of the dots, 1000- to 10,000-fold more for the most strongly positive strains such as strains 1, 4, 7, 13, and 25 (Fig. 1).

DISCUSSION

We report here a newly developed immuno-dot-blot assay for the detection of CT/LT-related enterotoxin in polymyxin-B extracts of *S. typhimurium* cells. The test, employing immunoaffinity-purified antibody against H-LT, is based upon the observed immunologic cross-reactivity of S-LT and H-LT. As performed, the minimal detectable amount of purified H-LT was approximately 20 pg per 2 μl. Fifteen of 25 isolates from India gave positive results in this assay. Fourteen of the reactive strains were also positive in the LPAT and in skin tests for delayed permeability factor activity. All strains which were negative in the immuno-dot-blot assay were also unreactive in LPAT and skin tests. An additional seven strains which had previously given positive reactions, under one or more cultural conditions, in a previously developed competitive ELISA were also reactive in the immuno-dot-blot assay, whereas three strains which were unreactive in the competitive ELISA were similarly unreactive in the present study. Two isolates of *S. enteritidis*, one from blood and one from stool, from a patient with choleralike secretory diarrhea were also positive.

As reported previously (3, 6, 16, 28, 30), most of the S-LT is cell associated and released by polymyxin; concentrated culture supernatants were negative in both LPAT and immuno-dot-blot assays. Additionally, in the present study we confirmed earlier findings (16, 30) that extracts of young, e.g., 6-h, cultures are more likely to be positive than those of older cultures.

The immuno-dot-blot assay offers significant advantages over previously described tests for S-LT. It appears to be reliable in that most strains which gave positive reactions in immuno-dot-blot assay were also positive in other tests for S-LT including the LPAT, competitive ELISA, and skin tests, and negative strains were likewise negative in the ancillary assays. It is slightly more sensitive than previously described tests, and it is rapid and requires no specialized expensive equipment such as an ELISA reader. Additionally, many extracts can be tested at the same time. Its major disadvantages are the requirements for relatively large amounts of bacterial culture and for concentration of the extracts—a reflection of the small amounts of S-LT produced by most strains.

Minimally reactive strains apparently produced relatively small amounts of S-LT. Assuming that S-LT reacts nearly equivalently to H-LT, extracts of weakly positive strains contained about 40 pg/10^9 bacteria, which calculates to less than one molecule per cell. Other extracts contained 1,000- to 10,000-fold more S-LT antigen. Although the number of strains tested is too small to permit any generalizations, it is interesting to note that five of six stool isolates gave positive reactions (two strongly positive), as did six of seven blood isolates (two strongly positive), one of five cerebrospinal fluid isolates, two of six urine isolates, and one isolate (strongly positive) from arthritic pus.

The results of this study raise some interesting questions. As indicated in the Introduction, the pathogenesis of salmonella-induced diarrheal disease is complex and may involve the interaction of several mechanisms including the cholera toxin–H-LT-like enterotoxin of the present study, cytotoxic factor(s) (22), and mucosal invasiveness and the host inflammatory response, which have been regarded as essential for the production of enteritis and diarrhea (10–12). Is it possible, as suggested by Peterson (22), that inside intestinal epithelial cells the presence of even trace amounts of S-LT and cytotoxin(s) can exert effects equivalent to larger amounts of these factors delivered to the cell surface by noninvasive enteric pathogens? What is the role (if any) of a choleralike enterotoxin, with profound immunomodulatory potential and other metabolic effects (4, 5), in disseminated *Salmonella* infections? Are there differences in the clinical manifestations of disease caused by S-LT-producing strains as compared with nonproducers? Do patients with salmonellosis produce antibody against S-LT? It is hoped that further studies will provide some answers.

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