

Competitive Enzyme-Linked Immunosorbent Assay for Cholera-Related Enterotoxins in *Salmonella typhimurium*

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We report a rapid competitive enzyme-linked immunosorbent assay to screen *Salmonella typhimurium* strains for cholera-related enterotoxin antigens. Polymyxin B extracts of bacterial cells from syncase-glucose broth cultures of 7 of 15 strains gave positive results. The specificity of the test was confirmed with known heat-labile-enterotoxin-positive and -negative *Escherichia coli* strains which gave significantly different values.

Evidence has been accumulating for the involvement of an enterotoxin produced by *Salmonella* species in the pathogenesis of diarrhea (4, 16, 23). Enterotoxin or related factors have been demonstrated in culture filtrates (2, 10, 19-22), in bacterial extracts (11, 12, 23), and in mitomycin C lysates (9, 15, 17). A heat-labile enterotoxin (LT) of *Salmonella* origin (S-LT) has been shown to be antigenically related to enterotoxins from *Escherichia coli* of human origin (H-LT) and from *Vibrio cholerae* (CT) (6, 9, 16, 18, 21). Because biological assays such as the rabbit ileal loop test are not practicable for routine testing, a specific in vitro test is needed. Only one test, a direct enzyme-linked immunosorbent assay (ELISA) that uses a purified antibody to CT, has been reported (9). This test takes at least 18 h to obtain results. The present study was undertaken to develop a rapid and specific ELISA for the detection of S-LT. For this purpose, we used immunopurified antibody (anti-H-LT) instead of anti-CT, because previous results suggested a closer immunologic relationship between H-LT and S-LT than between S-LT and CT (6). The 15 strains of *Salmonella typhimurium* used in this study included 11 that were provided by G. K. Morris, K. Wachsmuth, and J. Wells of the Centers for Disease Control, Atlanta, Ga.; 3 that were provided by J. Peterson; and 1 that was provided by S. B. Formal of the Walter Reed Army Institute of Research, Washington, D.C. Strain B2245 from the Centers for Disease Control originated from the recent milk-borne outbreak in Chicago (3). Of 22 *E. coli* strains tested, 21 were from a previous study (7). *E. coli* HB101 was used as an LT-negative control.

All the strains were grown in broth and agar versions of modified syncase with glucose (SG) instead of sucrose (5), tryptic soy (TS; Difco Laboratories), and Casamino Acids-yeast extract (CYE; Difco) (14). Broth cultures (25 ml) in 125-ml Erlenmeyer flasks were incubated at 37°C for 18 h in a shaking water bath (100 oscillations per min) and centrifuged at 4°C. Pellets were suspended in a 2-ml solution of polymyxin B (Sigma Chemical Co.) (20,000 U/ml) in phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-T). The suspensions were incubated at 37°C for 30 min and centrifuged, and extracts were collected. For agar plates, an 18-h surface growth was scraped off, 100 mg of growth was suspended in 1 ml of polymyxin-PBS-T, and extraction was done as above. Whole-cell lysates were prepared by sonicating cell suspensions in polymyxin solu-

tion. Cell debris was removed by centrifugation. A standard ELISA was performed by a previously described method (9), except that immunopurified goat anti-H-LT (13) was substituted for anti-CT. For a competitive ELISA (C-ELISA), H-LT (8) was diluted in carbonate-bicarbonate buffer (pH 9.6), and 10 ng of the protein in 100- μ l volumes was added to the wells of microtiter plates (Falcon; Becton Dickinson Labware). After a 16-h incubation at 22°C, the wells were washed with PBS-T and blotted, and 3% (wt/vol) bovine serum albumin in PBS-T was added to block nonspecific binding sites. After 1 h at 22°C, the plates were frozen. For the test, the precoated plates were thawed, washed, and blotted. Samples to be assayed were added in 100- μ l quantities in duplicate wells, and 50 μ l of a 1:5,000 dilution of immunopurified goat anti-H-LT was added immediately. After a 1-h incubation at 37°C and a subsequent washing, 100 μ l of a 1:1,000 dilution of peroxidase-labeled rabbit anti-goat immunoglobulin G (Cooper Biomedical, Inc.) was added to each well. After a 1-h incubation, the plates were washed, and 100 μ l of substrate solution [11 mg of 2,2'-azino-bis-(3-ethylbenz-thiazolinesulfonic acid) (Sigma) plus 46 μ l of 30% H₂O₂ in 50 ml of citric acid-phosphate buffer (pH 4.0)] was added to each well. The plates were further incubated for 15 min. The reaction was stopped with 20 μ l of 10% sodium dodecyl sulfate per well, and optical densities (ODs) were read in a microplate reader (MR 600; Dynatech Laboratories, Inc.) at 450 nm. Controls included 100 ng of H-LT per well, PBS-T with and without antibodies, and extracts from enterotoxin-positive and -negative *E. coli* strains.

Results of the standard ELISA (Table 1) indicate that although purified H-LT, partially purified S-LT, whole-cell lysates, and polymyxin extracts from *S. typhimurium* A9705 gave significant OD values, similar preparations from an

TABLE 1. Results of standard ELISA and C-ELISA

Prepn	Standard ELISA OD	C-ELISA OD inhibition
H-LT (100 ng)	1.899	0.627
S-LT ^a (100 ng)	0.306	0.076
Whole-cell lysate		
<i>S. typhimurium</i> A9705	0.293	0.233
<i>E. coli</i> HB101 (LT -)	0.348	0.094
Polymyxin extract		
<i>S. typhimurium</i> A9705	0.284	0.097
<i>E. coli</i> HB101 (LT -)	0.330	0.016

^a Partially purified S-LT was derived from strain SL1027 as previously described (6).

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TABLE 2. C-ELISA results for polymyxin B extracts

Strain (no. tested)	OD inhibition range					
	SG broth	SG agar	CYE broth	CYE agar	TS broth	TS agar
<i>E. coli</i> LT ⁺ (11)	0.215–0.362	0.248–0.324	0.121–0.409	0.100–0.314	0.279 ^a	0.163–0.193
<i>E. coli</i> LT ⁻ (11)	0.000–0.083	0.000–0.019	0.000–0.041	0.000–0.090	0.000	0.000–0.006
<i>S. typhimurium</i>	0.000–0.135 (7/15) ^b	0.000–0.118 (5/13)	0.000–0.124 (2/13)	0.000–0.103 (4/15)	0.000–0.100 (3/15)	0.000–0.087 (0/14)

^a Only two strains were tested.

^b The number of strains giving OD inhibition >0.090/number of strains tested.

H-LT⁻ strain, HB101, gave false-positive results. When a C-ELISA was performed, preparations from H-LT⁻ *E. coli* gave much lower OD inhibitions compared with the values given by *S. typhimurium*. After preliminary tests to determine optimum conditions, a comparison was made between the reactivity of partially purified S-LT from two different strains of *S. typhimurium*, A9705 and SL1027, and purified H-LT. These extracts were assayed in parallel by the C-ELISA on two separate occasions (Fig. 1). The results of these quantitative comparisons indicate that the C-ELISA was capable of detecting H-LT at levels as low as 0.3 to 0.5 ng and that the OD differences were dose related. The partially purified S-LTs were less active. It is not clear whether this difference was due to the smaller amount of S-LT in the preparations, the differences in sensitivity attributable to the use of cross-reactive enterotoxins, or to both factors. However, the results for S-LT activity were in reasonable accord with those reported previously by Houston et al. (9) and by Duebber and Peterson (4) in comparisons of partially purified S-LT with purified cholera toxin.

To evaluate further the specificity of the C-ELISA, the reactivities of polymyxin extracts of 22 *E. coli* strains cultivated in various media were tested (Table 2). The 11 LT-positive *E. coli* strains showed OD inhibitions ranging from 0.100 to 0.409 (mean, 0.249; 95% confidence limits, 0.121 to 0.377) under different cultivation conditions. All of these extracts were positive for toxin antigen by the latex particle agglutination test described previously (7). Eleven LT-negative strains, on the other hand, never gave C-ELISA values higher than 0.090 (mean, 0.025; 95% confidence limits, 0.000 to 0.083), and all of these strains were also negative by the latex particle agglutination test. Of 15 strains

of *S. typhimurium* tested, 11 gave positive results, depending on the growth medium. With SG broth, seven strains gave OD inhibition values above 0.090, which was considered a positive reading. These were A9705, B2245, SSU2635, SSU2051, SSU2633, W118-2, and 986. When SG agar was used, four of these strains gave positive results, as did one additional strain, SR11. In CYE broth only two strains gave positive results; in CYE agar four strains, including two strains which were negative in other tests (A9707 and SSU2050), gave positive results. Strain SL1027 and two other strains were positive in TS broth. No strains were positive in TS agar. None of the *Salmonella* polymyxin extracts showed strong latex particle agglutination test reactions, but reactions varying from doubtful positive to 1⁺ were shown by 12 of 21 (60%) of the C-ELISA-positive extracts.

A limited number of C-ELISA-positive and -negative strains were subjected to a delayed skin permeability factor test (18) and a rabbit ileal loop assay (1). For these tests, organisms were grown in SG broth. Live cells from two C-ELISA-positive SG broth cultures (A9705 and B2245) produced fluid accumulation (0.6 ml/cm) with inocula of 10⁹ to 10¹⁰ cells. Polymyxin B extracts of the same strains produced minimal reactions (0.1 to 0.3 ml/cm). In contrast, CT or H-LT, as controls at 1 µg, elicited 2.2 to 2.3 ml/cm of fluid. Of the two C-ELISA-negative cultures tested, extracts as well as live-cell suspensions of A9706 were negative in the rabbit ileal loop test. Polymyxin extracts of A9703 tended to be positive (0.8 ml/cm) in one of the two loops, whereas live-cell suspensions of A9703 were strongly positive. This indicates the possibility that *S. typhimurium* strains, such as A9703, produce enterotoxin not related to H-LT (or have other mechanisms for fluid production). In the delayed skin permeability factor test, polymyxin extracts from three C-ELISA-positive *S. typhimurium* strains (A9705, 986, and SSU2635) of 15 tested gave positive reactions.

Although several ELISA systems are available for the detection of H-LT, only one test has been reported (9) for the assay of S-LT. In this study, we found that a standard ELISA to detect *Salmonella* toxin antigen gave nonspecific reactions (false-positives) and that it took more than 18 h to obtain results. The advantages of the C-ELISA described in this paper were that it took less than 3 h after plating the samples to obtain results and that nonspecific reactions were minimal and could be eliminated. The sensitivity of the test was comparable to that of the ELISA reported by Houston et al. (9). The C-ELISA described here detected 0.3 ng of H-LT and gave significant OD inhibitions with partially purified S-LT preparations and crude extracts from *E. coli* and *S. typhimurium* strains.

In accord with earlier observations, these results indicate that the expression of H-LT-related toxin antigen by *S. typhimurium* is dependent on cultural conditions. Houston et al. (9) found that CYE broth gave the best results, but those

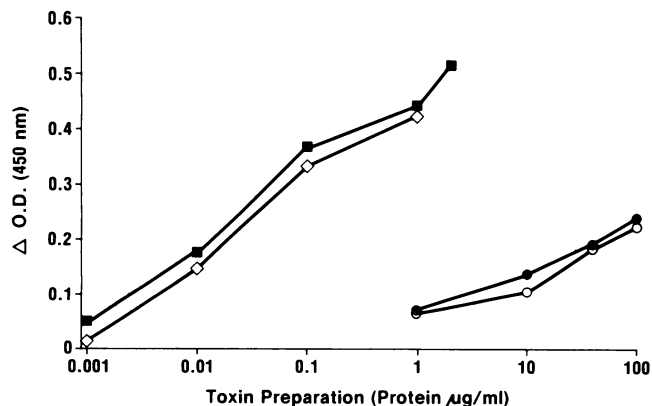


FIG. 1. Comparison of purified H-LT (■ and ◇ in two independent assays) with partially purified S-LT preparations from strain SL1027 (●) and strain A9705 (○) in C-ELISA. S-LT preparations were made as previously described (6). Δ OD, OD inhibition.

researchers were looking for toxin in culture supernatants rather than in cells. With strains SL1027 and W118-2, Stephen et al. (23) found that polymyxin extracts of cells grown in CYE broth were enterotoxic in some but not all tests in rabbit ileal loops. Sandefur and Peterson (18, 20) obtained variable results in the rabbit delayed permeability factor test with culture supernatants from known enteropathogenic strains of *S. typhimurium* under different culture conditions. Our results indicate the superiority of SG broth over other media for detecting cell-bound H-LT-related enterotoxin antigen in *S. typhimurium* strains.

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