Enzyme-Linked Immunosorbent Assay for *Escherichia coli* Heat-Stable Enterotoxin

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The sensitivity of an enzyme-linked immunosorbent assay (ELISA) to detect pure native *Escherichia coli* heat-stable toxin (ST) and to identify ST-producing strains among clinical isolates was determined. Two synthetically produced ST preparations were used to raise hyperimmune antisera in rabbits and goats: ST(S), which has the same antigenicity as native ST; and ST(C), which is 15-fold more immunogenic. These antisera were used in the double-sandwich technique as either crude double-species antisera or pure single-species antibody. The sensitivity of the assay was increased by using either a purer antibody preparation or the antiserum to the more potent immunogen; the assay in which pure antibody to ST(C) was used was 2,857-fold more sensitive in detecting ST than the assay in which crude antiserum to ST(S) was used. The minimum amount of ST detectable by the ST(C) ELISA was 140 pg/ml, which was an amount 285-fold smaller than that detectable by the suckling mouse assay. Among 50 human *E. coli* isolates examined by both the ST(C) ELISA and an ELISA for heat-labile toxin (LT), which had a sensitivity of 290 pg/ml for LT, the respective toxins were consistently identified in broth cultures of 10 LT⁺ and ST⁻, 15 LT⁺ and ST⁺, and 10 LT⁻ and ST⁺ strains, and there were no false-positive responses. The ST(C) ELISA also detected ST in all of seven ST-producing *E. coli* strains tested of human origin, which had been shown elsewhere by DNA hybridization probes to have ST-coding genes of either human or porcine origin, and in all of three ST-producing *E. coli* strains tested of porcine origin. These results indicate that the sensitivity of the ST(C) ELISA is the same as that of previously described LT ELISAs. The concomitant use of both ST and LT ELISAs provides a rapid, simple, and sensitive method for identifying among clinical isolates enterotoxigenic strains of *E. coli* which produce either toxin.

Enterotoxigenic strains of *Escherichia coli* (ETEC) are a common worldwide cause of acute diarrhea, particularly among travellers to developing countries (18), children living in these areas (40), and neonatal herd animals (28). Since these strains produce heat-labile toxins (LT) and heat-stable toxins (ST), either singly or together (18, 36, 40, 44), laboratory tests to establish their presence must be able to detect both of these toxins. The first generation of such tests employed clinically consisted of tissue culture assays for LT and the suckling mouse assay for ST in broth cultures of isolates (36). Other techniques were subsequently introduced for assaying LT in broth cultures; some, such as passive immune hemolysis (12) and radioimmunoassay (5, 17), proved too complex for adoption in general use. More recently, simple, sensitive enzyme-linked immunosorbent assays (ELISAs) for LT, based on the use of ganglioside GM1 as the solid phase and hyperimmune antiserum to LT as the second antibody (GM1-LT), have been developed (3, 9, 19, 35, 42, 51, 52). A number of techniques have now been applied to the detection of LT in primary isolates; these include ELISA (9), immunoprecipitation on agar plates as in the Biken test (20, 21), various immunoagglutination assays (13, 22, 43), and DNA hybridization probes with LT-coding genes (37, 38, 39). The necessity for primary culture itself can even be circumvented by direct assay of stool samples for LT by ELISA (33, 35).

Advances in techniques for detecting ST have not kept pace with those for LT, and assay of broth cultures by the

suckling mouse assay remains the standard test for ST, even though the cumbersome nature of this test precludes its widespread use in general clinical laboratories. Although sensitive radioimmunoassays for ST have been developed (11, 14, 16), their applicability to clinical isolates has not been described and they have the practical disadvantage of requiring short-lived, radioisotopically labeled ST and special equipment. DNA hybridization probes which have included both the human (H) and porcine (P) ST-coding genes have proven to be highly sensitive in detecting ST-producing human ETEC strains either in secondary broth cultures (15, 47) or during primary culture of stool samples (37, 38), but the complexity of this test, which requires radiolabeled gene probes and several days to develop autoradiograms, makes it unlikely that its use will be extended beyond research laboratories concerned with the epidemiology of ETEC strains.

For the past several years, we have employed an ST ELISA to quantitate the antigenicity of the ST component of a vaccine, composed of synthetic ST cross-linked to the LT B subunit, that is under development in this laboratory for use against ETEC strains (24, 25, 27). This ELISA uses the double-sandwich technique with crude hyperimmune antisera raised in two animal species to a synthetic ST preparation that has the same antigenic properties as native ST (26). Although the sensitivity of this assay is adequate for monitoring the antigenicity of toxoid modifications of native or synthetic ST preparations (23-25, 27), it is insufficient to serve as a reliable test for the detection of ST-producing strains among clinical isolates (F. A. Klipstein and R. F. Engert, unpublished observations). We now describe the

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development of an ST ELISA that uses affinity column-purified antibody derived from hyperimmune antiserum raised to a hyperimmunogenic synthetic preparation of ST. This approach has increased the sensitivity of the ELISA to detect ST by >2,800-fold, to the picogram range. The new ELISA was found to be capable of detecting amounts of ST that are several hundredfold smaller than those detected by the suckling mouse assay and to be consistently reliable in identifying ST-producing ETEC strains among human and porcine clinical isolates.

MATERIALS AND METHODS

Bacterial strains. Three groups of *E. coli* strains were tested: (i) 35 ETEC and 15 nontoxigenic human strains were provided by B. Rowe; (ii) 7 human ETEC strains which had previously been analyzed by DNA colony hybridization probe with both the ST(H)- and ST(P)-coding genes (37) were kindly provided by S. Moseley, National Animal Disease Center, Ames, Iowa; and (iii) 4 porcine ETEC strains were kindly provided by H. Moon, National Animal Disease Center, Ames, Iowa. The enterotoxigenic properties of each of these strains were confirmed by tissue culture assays for LT and the suckling mouse assay for ST. For assay, these strains were grown under agitated conditions at 37°C for 18 h in brain heart infusion medium (Difco Laboratories, Detroit, Mich.) and then sterile filtered.

Enterotoxins. LT was prepared in purified form by published chromatographic techniques (7) from *E. coli* PDF82, a transformed K-12 derivative bearing LT gene(s) of the Ent plasmid from human *E. coli* H10407 (8). Native ST was purified to homogeneity from *E. coli* 18 D (kindly provided by R. Giannella, University of Cincinnati Medical Center, Cincinnati, Ohio) by the methods described by Staples et al. (49). Two forms of synthetically prepared ST were used. The methods used for their preparation have been described previously (25, 27; R. A. Houghten, J. M. Ostrech, and F. A. Klipstein, submitted for publication). Both synthetic ST preparations have the same 18-amino-acid sequence as that described by Chan and Gianella (6) for human *E. coli* 18D, but they differ in the configurations of their disulfide linkages. Preparation ST(S) has the same secretory potency and antigenic properties as pure native ST (26), whereas preparation ST(C) has one-third the secretory potency but 15 times the antigenicity of either native ST or ST(S) (27). The amounts of LT and of the synthetic ST preparations used were based on their protein concentration as determined by the method of Lowry et al. (32).

Hyperimmune antisera. (i) **Immunogens used.** Both of the synthetic ST preparations were conjugated to porcine immunoglobulin G (PIG) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and glutaraldehyde as the conjugating agents for ST(S) and ST(C), respectively, by previously described methods (26, 27). In each instance, conditions resulting in an optimal conjugate were determined by experimental manipulations of the molar ratio of ST added to the carrier and of the ratio of the conjugating reagent to the total protein value of the mixture as described previously (24, 27). Optimal conditions were defined as an ST(S)/PIG molar ratio of 100/1 with a carbodiimide/total protein ratio of 2/1 by weight, which yielded a conjugate containing 42% ST(S) by weight and antigenicity; and an ST(C)/PIG molar ratio of 100/1 with a glutaraldehyde/total protein ratio of 2/1 by weight, which yielded a conjugate that contained 50% ST(C) by weight and antigenicity.

Dosages of the ST(S) and ST(C) conjugates were based on ST(S) antigen units (AU) which are equivalent to those of

native ST (26). One AU is defined as the weight of conjugate that contains the same amount of ST(S) antigenicity as 1 µg of ST(S), as determined by ELISA (24). The ST(S)-PIG conjugate contained 420 ST(S) AU per mg and the ST(C)-PIG conjugate contained 7,100 ST(S) AU per mg.

(ii) **Immunization of animals.** Rabbits and goats were given primary immunization in four quadrants (two subcutaneously and two intramuscularly) with Freund complete adjuvant and two booster immunizations at monthly intervals with Freund incomplete adjuvant. Respective dosages for primary and booster immunizations in rabbits were as follows: LT, 1,000 and 1,000 µg; ST(S), 300 and 500 ST(S) AU; and ST(C), 5,500 and 7,000 ST(S) AU. In goats the respective doses were as follows: LT, 1,000 and 1,000 µg; ST(S), 1,100 and 2,300 ST(S) AU; and ST(C), 6,200 and 12,400 ST(S) AU.

Reciprocal titers of antisera obtained after the second booster immunization were determined by ELISAs with 10 µg of LT or 50 µg of an ST(S)-human serum albumin conjugate per ml as the solid-phase materials and the appropriate alkaline phosphatase-conjugated species-specific antiserum. Titers for LT were 32,768 in rabbits and 131,072 in goats; for ST(S) they were 8,192 in rabbits and 16,382 in goats; and for ST(C) they were 32,768 in rabbits and 65,536 in goats.

Affinity purification of the antisera. The immunoglobulin fractions of goat antisera to ST(S) and ST(C) were prepared by the passage of the antisera through a CM Affi-Gel Blue column (Bio-Rad Laboratories, Richmond, Calif.), followed by ammonium sulfate precipitation of the optically identified unbound elution fraction by procedures defined by the manufacturer (Bio-Rad bulletin 1092). ST(S) and ST(C) were separately coupled to epoxy-activated Sepharose 6B gel (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) by published methods (50). The immunoglobulin fractions were applied to these specific gels in phosphate-buffered saline, eluted in phosphate-buffered 3 M sodium chloride (pH 7.0), dialyzed immediately against phosphate-buffered saline to remove excess salt, and concentrated on a YM-10 ultrafiltration membrane (Amicon Corp., Danvers, Mass.) to a convenient volume. The material obtained is referred to as pure antibody.

Alkaline phosphatase conjugation. A portion of the affinity column-purified antibodies was conjugated to alkaline phosphatase by the method of Voller et al. (54).

ELISAs. These assays were performed with reagents and methods that have been described previously by this laboratory for LT and ST ELISAs (23, 24). All samples were tested in duplicate, working volumes were 200 µl, and all dilutions of antigens and antisera were made with phosphate-buffered saline-Tween buffer. Optical densities were determined spectrophotometrically with a model EL 307 ELISA reader (Bio-Tek Instruments, Burlington, Vt.).

The working dilution of each antiserum was determined by checkerboard titration against the appropriate antigen. LT was assayed with either 50 µg of GM ganglioside (Type III; Sigma Chemical Co., St. Louis, Mo.) per ml or a 1:50 dilution of crude rabbit antiserum to LT as the solid phase, a 1:500 dilution of crude goat antiserum to LT as the second antibody, and a 1:400 dilution of alkaline phosphatase-conjugated rabbit anti-goat antiserum (Miles Laboratories, Inc., Elkhart, Ind.). In the ST assays, crude rabbit antiserum to either ST(S) or ST(C) at 1:10 and 1:25 dilutions, respectively, was used as the solid phase; the second antibody was crude goat antiserum to these ST preparations at a 1:100 dilution or 5 µg of either the immunoglobulin fraction or pure antibody per ml; and the conjugate was a 1:400 dilution of

alkaline phosphatase-conjugated rabbit anti-goat antiserum. Additionally, pure goat antibody was used as the solid phase with alkaline phosphatase-conjugated pure goat antibody as the second antibody, both at a concentration of 5 µg/ml.

The responses of LT and either pure biological ST or ST(S) in the respective ELISAs were determined by assaying twofold serial dilutions of these toxins, starting at 500 ng of LT per ml and 50 µg of ST or ST(S) per ml in assays with crude antisera and 50 ng of ST or ST(S) per ml in assays in which pure antisera were used. That amount of toxin which yielded an optical density of ≥ 0.200 was considered to be the minimal amount detectable in that particular assay.

RESULTS

Sensitivity of the ELISAs. The minimal amount of LT detectable in the LT ELISAs was 185 pg/ml when crude rabbit antiserum to LT was used as the solid phase and 290 pg/ml when GM was used as the solid phase (Fig. 1). Antisera to the ST(S) and ST(C) preparations were each tested in four different ELISAs which used progressively more purified antibodies (Table 1). The responses of pure native ST and ST(S) were identical in all of these assays. Antiserum to ST(S) could detect 400 ng/ml when used as crude antiserum; the sensitivity was increased 286-fold to 1.4 ng/ml when pure antibody was used. The sensitivity of crude antiserum to ST(C), 9.0 ng/ml, was in the same range as that of the pure antibody to ST(S); it was increased a further 64-fold to 140 pg/ml when pure antibody was used. The most sensitive ST(C) ELISA was capable of detecting an amount of ST that was 2,857-fold smaller than that detectable by the ELISA in which crude antiserum to ST(S) was used, which we have employed in the past (24, 25).

The minimal detectable amount of ST in the suckling mouse assay in our laboratory is 40 ng/ml (4 ng in 100 µl per animal) (26); this value is in the same range as that found in other laboratories (2, 30, 49, 53). The sensitivity of the ELISA in which crude antiserum to ST(S) was used was thus 10-fold less than that of the suckling mouse assay, the sensitivities of ELISAs in which either pure antibody to ST(S) or crude antiserum to ST(C) was used were in the

FIG. 1. Dose response of pure native ST or LT in respective ELISAs. LT was assayed by the GM-LT ELISA. AB, Antibody; asterisk indicates that the second antibody was conjugated with alkaline phosphatase.

TABLE 1. Sensitivity of ELISAs with progressively more purified antisera (α) to either ST(S) or ST(C)

Hyperimmune antiserum used ^a		ST detected with ^b	
Solid phase	Second antibody	α ST(S)	α ST(C)
Rabbit, crude	Goat, crude	400.0	9.00
Rabbit, crude	Goat, Ig	130.0	1.60
Rabbit, crude	Goat, pure	40.0	0.95
Goat, pure	Goat, pure ^c	1.4	0.14

^a Ig, Immunoglobulin fraction of the serum; pure, antibody obtained after affinity column purification of the immunoglobulin fraction.

^b ST tested was purified biological ST. Values are the minimal amount of ST, in nanograms per milliliter, that yielded an optical density of ≥ 0.200 .

^c The second antibody was conjugated to alkaline phosphatase.

same range as that of the suckling mouse assay, and the sensitivity of the ELISA in which pure antibody to ST(C) was used was 285-fold greater than the suckling mouse assay.

Assay of clinical isolates. Fifty human isolates were tested. Of these strains, 15 were nontoxigenic, 10 were LT⁺ and ST⁻, 15 were LT⁺ and ST⁺, and 10 were LT⁻ and ST⁺. The GM-LT ELISA detected all 25 LT-producing strains, and the ST(C) ELISA detected all 25 ST-producing strains; in no instance was there a false identification (Fig. 2). These strains were tested by four ST ELISAs. The ELISA in which crude antiserum to ST(S) was used failed to detect ST-producing strains in all instances, whereas ELISAs in which pure antibody to ST(S) and crude antiserum or pure antibody to ST(C) were used all consistently detected the ST-producing strains. The reactions of the broth cultures were progressively stronger as more sensitive ELISAs were used (Fig. 3).

Assay of strains identified by genetic probes. Seven additional human isolates were assayed. All of these strains had been identified as ST producers by means of DNA hybridization probes (37), but four of the strains were positive only when ST(H) genes were used, and three were positive only when tested with ST(P) genes. All seven strains were identified as ST producers when tested by ST ELISA with pure antibody to ST(C).

Assay of porcine strains. Four ETEC strains were tested; one was LT⁺ and ST⁻, one was LT⁺ and ST⁺, and two were LT⁻ and ST⁺. ELISAs with GM-LT and ST with pure antibody to ST(C) detected LT- and ST-producing strains in all instances.

DISCUSSION

The optimal laboratory test to detect ETEC strains should be rapid and simple, use stable reagents, and require no elaborate equipment. It must also detect strains which produce either LT or ST. A variety of tests that fulfill the first criterion are available to detect LT. The most sensitive GM1-LT ELISAs can detect between 4 and 10 ng of LT per ml (9, 42, 52), and different types of LT ELISAs with various degrees of sensitivity all show excellent correlation with the conventional tissue culture assays in their abilities to detect LT in broth cultures of clinical isolates (3, 19, 35, 42, 45, 52).

FIG. 2. Assay of broth cultures of 50 *E. coli* clinical isolates by the GM-LT ELISA (●) and ST ELISA (○), in which pure antibody to ST(C) was used. NTG, Nontoxigenic.

The GM1-LT ELISA can also be applied to primary growth; it can detect nanogram quantities of LT within 24 h (9), which compares favorably with the immunoprecipitation technique of the Biken test that has a sensitivity limited to microgram quantities of LT and requires 4 to 5 days (20, 21). The difficulty has been that none of these simpler assays can detect ETEC strains that produce ST only, which are recognized to be common among both travellers to and residents of developing areas (18, 40, 44, 46). For this reason, the suckling mouse assay has continued to be the standard test used to detect ST-producing strains, despite the fact that, even when applied to primary growth in conjunction with the Biken test for LT (21), it is too cumbersome for use in general clinical laboratories. Although radioimmunoassays capable of detecting 50 pg of ST per assay tube have been described (11, 14, 16) and DNA hybridization probes have been shown to be reliable for detecting ST-producing strains (15, 27, 28, 47), the complex nature of these assays and their requirements for short-lived isotopes and, in the latter test, for radioautography also make them unadoptable for use in general clinical laboratories.

The ST ELISA described in the present report provides a sensitive, simple, and rapid method for assaying ST that does not require the use of either isotopes or elaborate equipment. We found that the sensitivity of this ELISA depends on both the immunogenicity of the ST preparation used to raise antiserum and the purity of the antibodies used. The ELISAs in which crude antiserum to the ST(S) preparation, which has the same antigenicity as pure native ST, was used were less sensitive than the suckling mouse assay and were unable to identify ST-producing strains among clinical isolates. ELISAs in which either pure antibody to ST(S) or crude antiserum to the hyperimmunogenic ST(C) preparation was used had sensitivities within the range of the suckling mouse assay and consistently identified ST-producing strains. The most sensitive ST ELISA, in which pure

antibody to the hyperimmunogenic ST(C) preparation was used, had a sensitivity of 140 pg of ST per ml, which represented a 2,857-fold increase over that of the least-sensitive ELISA. The sensitivity of this ELISA for ST was in the same range as that for LT in the LT ELISAs and 285-fold greater than that of the suckling mouse assay for ST.

We have found that polystyrene microtitration plates precoated with either GM or pure goat antibody to ST(C) are stable when maintained at 4°C. The single-species pure-antibody ST ELISA has an added advantage: the second antibody is conjugated with alkaline phosphatase, thereby circumventing the need for an additional conjugated antiserum, which can easily be done for antiserum to LT. Assay of a broth culture for ST and LT thus consists of placing it in adjacent wells precoated with either GM or pure anti-ST(C) antibody and adding the respective alkaline phosphatase-conjugated antibody to either ST(C) or LT and then the substrate. As noted previously by others for LT ELISAs (42, 52), we found that positive samples can reliably be identified visually in LT and ST ELISAs without spectrophotometry. Although the concomitant use of ST and LT ELISAs represents a greatly simplified approach over those available hitherto for the identification of all ETEC strains, we suspect that the direct application of these assays to stool supernatants will provide an even simpler and more rapid assay method by circumventing the time needed for primary isolation and secondary growth of broth cultures. Direct assay of stool specimens by LT ELISAs, whose sensitivities were not described, detected LT in more than 90% of samples shown to be positive by other means (33, 35). We are currently evaluating the ability of ST(C) and LT ELISAs with picogram sensitivities to identify ETEC strains by this approach.

The optimal assay for a toxin should also have a sufficiently broad spectrum of sensitivity to detect toxins produced by ETEC strains from different sources. The genes for both human and porcine LT are highly homologous within the toxin-coding DNA (10), and DNA hybridization probes with

FIG. 3. Response of broth cultures of 25 ST-producing *E. coli* clinical isolates (15 LT⁺ and ST⁺ and 10 LT⁻ and ST⁺ strains) in ST ELISAs with increasingly purified antisera to ST(S) and ST(C). Values are the means $\pm$ standard errors of the means of the optical densities.

either human or porcine LT genes have shown a close correlation with other assays in detecting human LT-producing ETEC strains (15, 37–39, 47). In the case of ST-producing ETEC strains of human origin, DNA hybridization probes have shown that they may carry more than one nucleotide sequence coding for ST (38, 48, 55). For this reason, probes with the genes that code for ST(H) and ST(P) are necessary to detect all ST-producing isolates of human origin, whose genetic properties may vary according to geographic location (15, 37, 38, 47). Despite their genetic differences, the compositions and properties of human and porcine STs are closely similar. Both nucleotide sequence coding and direct sequencing of purified toxins of human and porcine origin have shown that only slight variations exist in the compositions and sequences of the 18 or 19 amino acids of these toxins (1, 6, 29). Their chemical and immunological properties are the same (11); antiserum to either human or porcine ST cross-neutralizes the heterologous toxin in the suckling mouse assay and can detect the heterologous toxin equally well by radioimmunoassay (11, 14, 16). The ST ELISA evaluated in the present study, in which antiserum to synthetic ST based on the sequence of human ST was used, consistently detected ST-producing isolates of human origin, irrespective of the presence of ST(H) or ST(P) genes, and it identified all of the few ST-producing isolates of porcine origin tested.

The ST toxin studied and discussed in this report is also known as ST_a (or ST I); it is methanol soluble; has biological activity in suckling mice, rats, rabbits, and piglets; and is responsible for diarrhea in humans and neonatal farm animals (4, 11). Some porcine strains produce a second toxin referred to as ST_b (or ST II); it is methanol insoluble, and its biological activity is confined to rabbits and weaned pigs (4). Whether strains that produce only ST_b cause acute diarrheal disease in farm animals is uncertain (34). The structure of ST_b has been shown by nucleotide sequencing to be markedly different from that of ST_a (31, 41), and ST_a and ST_b are immunologically unrelated (14, 16).

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