Blinded, Two-Laboratory Comparative Analysis of *Escherichia coli* Heat-Stable Enterotoxin Production by Using Monoclonal Antibody Enzyme-Linked Immunosorbent Assay, Radioimmunoassay, Suckling Mouse Assay, and Gene Probes

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Heat-stable enterotoxin (ST)-producing enterotoxigenic *Escherichia coli* (ETEC) can be identified by a variety of assays, including the suckling mouse assay (SMA), radioimmunoassay (RIA), polyclonal or monoclonal antibody enzyme-linked immunosorbent assay (ELISA), and DNA hybridization with STH and STp gene probes. To compare the sensitivity and reliability of these assays, 100 coded ETEC and non-ETEC isolates were blindly tested in two independent laboratories. SMA, RIA, and monoclonal ELISA were performed in Cincinnati, Ohio, while gene probe analysis was performed in Baltimore, Md. The method of storage of organisms had a profound effect on the stability of plasmids in certain strains. Hybridization experiments to determine the presence or absence of the enterotoxin gene showed that strains stored on Dorset egg medium at room temperature better retained their plasmids than strains stored frozen in skim milk. Forty-four of the 100 organisms obtained from the skim milk stock were found to produce STa in liquid culture by the RIA, SMA, and monoclonal ELISA (100% agreement). However, 50 of 54 of the strains stored on Dorset egg medium which were originally classified as STa⁺ or ST⁺ LT⁺ (positive for both heat-stable and heat-labile [LT] enterotoxins) were found to produce STa and retain the plasmid by each of these assays. Three additional strains were found which harbored the plasmid but did not elaborate STa by any of the assays (3% discrepancy). The monoclonal antibody ELISA appears to be highly reliable for determination of STa production by ETEC and can be easily scored visually even by untrained personnel. Furthermore, when this STa assay is coupled with a polyclonal antibody assay, it is possible to predict the genotype of STH- and STp-producing organisms.

Enterotoxigenic *Escherichia coli* (ETEC) can elaborate a variety of virulence factors which have been implicated in the diarrheal process. One of these is a family of heat-stable enterotoxins, commonly designated as STa, which consists of 18- or 19-amino-acid peptide toxins (1, 10, 12, 18, 19, 26). These peptides share highly conserved primary sequences (19, 26) and common antigenic epitopes (2, 9, 25). Recently, monoclonal antibodies have been raised against one member of the STa family, an 18-amino-acid toxin (STp) purified from a human isolate (2, 25). These monoclonal antibodies recognize common epitopes on both 18- and 19-amino-acid toxins produced by human, bovine, and porcine isolates and have been incorporated into an enzyme-linked immunosorbent assay (ELISA) (25) for *E. coli* STa. In our initial communication, we demonstrated that the assay could detect STa in culture supernatants and that the assay correlated well with our previously developed radioimmunoassay (RIA) (8).

There exist a variety of assays for the *E. coli* STa's: these include gene probe analysis (3, 6, 11, 15, 20), suckling mouse bioassay (SMA) (7), ELISA (9, 13, 17, 25), and RIA (5, 8). The ELISA format alone shows promise for actual field use, since the other assays require sophisticated laboratory equipment, isotopes, or a cumbersome bioassay procedure.

The screening procedures commonly used are the SMA and gene probe analysis.

In this paper, we report the results of a two-laboratory cooperative project comparing the monoclonal antibody ELISA, the SMA, and a polyclonal antibody RIA (Cincinnati, Ohio) with gene probe analysis (Baltimore, Md.) of STa produced in culture supernatants from 100 coded *E. coli* isolates. Thus, the characterization of STa production by these strains encompasses all forms of the assays currently at our disposal. Assays were performed in a blind fashion. The RIA, ELISA, and SMA were performed in physically separated laboratories. Complete agreement was observed among these assays, thereby demonstrating the efficacy of the monoclonal antibody ELISA to accurately detect toxigenic strains of *E. coli*. While the ST gene probe assays identified all strains demonstrating STa in the above assays, an additional three strains were identified as STa producers by DNA hybridization.

MATERIALS AND METHODS

Overall study design. One hundred coded ETEC and non-ETEC isolates were kindly provided by Y. Takeda, Institute of Medical Science, University of Tokyo, Tokyo, Japan. These strains were inoculated on Dorset egg medium slants and shipped by air mail to Cincinnati. When the

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primary slants arrived in Cincinnati, organisms were inoculated onto tryptic soy agar plates (Difco Laboratories, Detroit, Mich.), grown overnight, and then frozen in 10% skim milk and stored at -70°C . The primary slants were stored at room temperature. Subsequently, strains stored either in frozen skim milk or on the original Dorset egg medium slants were sent from Cincinnati to Baltimore for gene probe analysis on either tryptic soy agar slants or fresh Dorset egg medium slants (Difco Laboratories). Retesting was performed with organisms obtained directly from the original slants.

Cultures for toxin production were inoculated by loop from the skim milk stocks. Each strain was grown in Casamino Acids-yeast extract medium in loosely capped roller tubes for 22 h as previously described (7, 8). Organisms were removed by centrifugation at $12,000 \times g$, and culture supernatants were frozen at -20°C in aliquots before being assayed. All assays were performed within 90 days on these frozen samples. RIA and ELISA determinations were done in separate laboratories on identical aliquots. The SMA was performed on frozen culture supernatant as mice became available. All assays were performed on coded samples.

STa standard. An STa was purified from *E. coli* 18D as previously reported (24, 27). This toxin has been sequenced and is a member of the 18-amino-acid class of STa, commonly called STp. The STa, purified by high-pressure liquid chromatography, was quantitated by amino acid analysis and aliquoted for use as a reference standard.

ELISA reagents and procedure. STa-bovine serum albumin (BSA) conjugate and monoclonal antibody were prepared as previously described (25).

The affinity-purified 20c1B8 monoclonal antibody was obtained from Genetic Diagnostics Corp., Great Neck, N.Y. (2).

The overnight competition ELISA format (standard assay) originally described (25) was used in this study. Briefly, Immulon II microtiter plates were sensitized with 50 μl of STa-BSA conjugate diluted to 1 μg of total protein per ml in 20 mM carbonate-bicarbonate buffer (pH 9.6). Plates were sealed and incubated for at least 18 h at 4°C . All plates were stored in sensitization solution at 4°C for up to 60 days before being assayed. On the day of assay, the STa-BSA conjugate was removed, the plates were rinsed twice in wash buffer (0.02% Tween 20, 10 mM sodium phosphate [pH 7.2], 0.15 M NaCl). The wells were filled with 1% BSA-phosphate-buffered saline to reduce nonspecific binding, and plates were then incubated for 1 h at 37°C . Either dilutions of pure toxin (STp) or 22-h culture supernatants were mixed with monoclonal antibody 20c1B8 diluted in 0.1% BSA-phosphate-buffered saline. Samples (50 μl) of these mixtures were added to sensitized and unsensitized wells, and then plates were incubated at 4°C for 18 h. After incubation, plates were rinsed five times with wash buffer. The second antibody (50 μl of 1:1,000 affinity-purified horseradish peroxidase-labeled goat anti-mouse immunoglobulin G [H+L; Kirkegaard and Perry, Gaithersburg, Md.]) was added to the wells, which were then incubated for 1 h at room temperature (22°C). The wells were washed five times, and then 100 μl of freshly prepared substrate solution [2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) in 0.1 M citrate buffer (pH 4.0) with 0.03% H_2O_2] was added to each well. Color development was terminated after 20 min at room temperature by the addition of 25 μl of 0.5% sodium azide in water to each well. Plates were interpreted visually for toxin estimation and were in addition read in an automatic plate

reader at 405 nm (Artek). The concentration of STa in the sample was read from the standard competition curve provided on each plate. Standard curves were included on each plate and were plotted on semilog graph paper and analyzed by least-squares regression with a standard program.

Culture supernatants were assayed initially in an overnight screening assay to determine whether toxin was present or absent. Each supernatant was assayed four different times to ensure consistency in the assay.

For screening purposes, a strain was considered STa positive when a concentration of 20 ng of STa per ml of culture supernatant was measured in the ELISA. This concentration corresponds to 100 pg of toxin in a well, with 5 μl of culture supernatant per well. The ratio of absorbance developed in the presence of 100 pg of STa to the absorbance developed in the absence of added toxin was calculated (usually approximately 0.65 to 0.80). The absorbance ratio for each unknown was likewise calculated and compared with that found for the 100-pg standard. Strains were considered STa positive when the absorbance ratio was less than that of the standard and negative when the ratio was equal to or greater than that of the standard.

Each supernatant demonstrating STa in the screening assay was further tested at multiple dilutions for the purpose of quantitating toxin. A calculated concentration of toxin was obtained from the average value for three dilutions of supernatant yielding absorbance values within the linear portion of the standard curve.

Visual interpretation of ELISA plates. The 100 culture supernatants were assayed by using five microELISA plates. Each plate contained a purified toxin standard curve and up to 24 distinct culture supernatants. After the plates were developed, they were given to eight laboratory and secretarial personnel, only one of whom was familiar with the assay. These individuals were asked to compare the color developed with each isolate to the color developed with 100 pg of STa (one of the pure STa dilutions in the standard curve). They were asked to assign a positive, negative, or inconclusive score to each determination. A negative score was recorded for isolates developing more color than the 100-pg standard, and a positive score was recorded for isolates yielding less color than the standard. Individuals were at liberty to give an inconclusive score when they were uncertain. In the event of complete disagreement between the ELISA score and the SMA score (e.g., ELISA positive and SMA negative or ELISA negative and SMA positive), a percentage of zero was assigned to each determination. All inconclusive ELISA determinations were assigned a value of 50%. Whenever there was complete agreement between the two assays (e.g., both positive or both negative), a value of 100% was assigned to reflect 100% agreement. The agreement between the SMA and the ELISA was calculated for each isolate. The tabulated results from all individuals were statistically analyzed and agreement correlated with the results of the SMA.

RIA of *E. coli* culture supernatants. The detailed description of the RIA has been previously published (8). In the RIA, polyclonal antibodies, raised in goats to STa conjugated to bovine immunoglobulin, were used. Each culture supernatant was tested at three or more dilutions, and each supernatant was analyzed on at least two different occasions. The same purified STa standard was used for the RIA and the ELISA.

SMA. The SMA has been described in detail elsewhere (7). Briefly, 100 μl of culture supernatant was injected per mouse. An STa-positive response was defined as a gut-to-

carcass ratio of 0.083 or greater. Each assay was performed in triplicate.

Hybridization with enterotoxin gene probes. All strains stored in frozen skim milk were inoculated onto tryptic soy slants and sent to Maryland for gene probe analysis. After the code was broken, organisms from the original slants were inoculated onto Dorset egg medium slants and probe as well. The strains were directly plated from Niese slants onto culture plates and assayed for the toxin gene by using STp and STh probes as described by Lanata et al. (11) and by Moseley et al. (15). To eliminate possible contamination of the probe fragments with pBR322 vector sequences, the probes originally described by Moseley et al. (15) were recloned by using synthetic oligonucleotide linkers. The recombinant plasmid pCVD402 contains the STh probe on *EcoRI* linkers cloned into pBR325 and pCVD404 contains the STp probe on *PstI* linkers cloned in the same vector (J. B. Kaper and S. Trinker, unpublished results). Digestion of these plasmids with the appropriate restriction enzyme generates only two widely separated fragments (the probe and the vector) which are easily purified away from each other.

RESULTS

Initial Results. Initial assays were performed on culture supernatants from organisms previously frozen in skim milk. In the blind screening of these 100 isolates (Table 1), 44 were found to produce STa by the ELISA, suckling mouse assay, and RIA (100% correlation). Gene probe analysis of the isolates showed excellent (97%), albeit not 100%, correlation with the phenotypic assays. Three strains which were STa⁺ by all assays in Cincinnati were found to be probe negative (one was ST⁺ LT⁻; two were ST⁺ LT⁺ [LT, heat-labile enterotoxin]), while an additional STa-negative strain was gene probe positive. These results were obtained from organisms which had been stored for approximately 30 to 60 days frozen at -70°C in skim milk. After the code was broken, we found that 54 of the strains were originally classified as STa or STa and LT positive and that 43 of our 44 STa-positive strains were in this group. One STa false-positive was identified in our original phenotype screening (Table 1); this was later found to be a result of contamination or operator error, since when the strain was sent from Japan a second time it was found to be consistently STa negative. This strain was consistently probe negative as well. Two strains which were originally designated as STa⁻ were found to be positive for the STa gene. Thus, 11 of the strains which were originally classified as STa⁺ or STa⁺ LT⁺ by Y. Takeda did not produce toxin measurable by the suckling mouse assay, RIA, or ELISA in our initial screening. Each corresponded to a strain which was originally shown to be STa positive in Japan.

TABLE 1. Initial findings: comparison of various STa assays with ST gene probe analysis

Original designation (n) ^a	No. of strains positive for production of STa by:			No. of strains positive by ST gene probe
	Bioassay	ELISA	RIA	
ST ⁻ LT ⁺ or LT ⁻ (46)	1	1	1	1
ST ⁺ LT ⁻ (32)	26	26	26	26
ST ⁺ LT ⁺ (22)	17	17	17	15

^a Designation of phenotypic toxin profile by Y. Takeda, University of Tokyo.

TABLE 2. Final results: comparison of various STa assays with ST gene probe analysis

Original designation (n) ^a	No. of strains positive for toxin production by:			No. of strains positive by ST gene probe
	Bioassay	ELISA	RIA	
ST ⁻ LT ⁺ or LT ⁻ (46)	0	0	0	1
ST ⁺ LT ⁻ (32)	31	31	31	32
ST ⁺ LT ⁺ (22)	19	19	19	20

^a See footnote a of Table 1.

Reconciled results. To investigate the source of this assay discrepancy, strains were retested directly from the original Dorset egg slants by STh and STp gene probe analysis. From the retesting, we found that 7 of these discrepant 11 strains had apparently lost their plasmids in the transfer or during storage in frozen skim milk or both, since the probes hybridized with DNA in the organisms from the original slants but did not do so with the organisms stored in skim milk. When these seven organisms were then subcultured directly from the original slants and then grown under the identical conditions in Casamino Acids-yeast extract medium, each of these strains demonstrated toxin production in all three assays (100% concordance).

The four remaining strains originally categorized as ST⁺ were found to consist of the following. (i) Two strains were gene probe negative on the original slants; these organisms presumably lost their plasmids on the Dorset egg medium slants before receipt in Cincinnati and did not demonstrate toxin production. (ii) Another two strains (ST⁺ LT⁺) were found to contain the gene when organisms were obtained from the original slants, but these organisms failed to demonstrate significant STa production in culture supernatants by any of the assays. An additional strain, originally classified by Takeda as LT⁺ ST⁻, was found to contain the gene for ST but did not produce detectable levels of toxin in any of the assays (Table 2). Thus, a total of 53 of the 100 strains were found to contain the plasmid, and 50 of these were found to produce biologically and immunologically recognizable STa (Table 2).

Toxin quantitation by RIA and ELISA. The quantity of toxin produced by each isolate in culture was measured with both the monoclonal antibody ELISA and the polyclonal RIA. Interestingly, two populations of strains emerge when the concentration of toxin measured in the RIA is compared with that measured (in the same culture supernatant) in the ELISA. When the gene probe data are used to assign each strain as STh or STp, the differential binding of polyclonal and monoclonal antibody to these toxins demonstrates that these two populations are in fact the 18-amino-acid (STp) and 19-amino-acid (STh) toxins (Fig. 1). The 19-amino-acid toxins appeared to be more poorly recognized by the 20c1 monoclonal antibody, in comparison with the polyclonal antibody used in the RIA. In this group of organisms, the highest apparent concentration of STh measured in the ELISA was less than 150 ng/ml, while the same isolate culture supernatant, when analyzed by the RIA, showed an apparent toxin concentration of almost 600 ng/ml. The concentration of STa measured in the RIA was consistently greater than that measured in the ELISA with each of the strains that produce the 19-amino-acid toxin. In contrast with this behavior, 18-amino-acid toxins are essentially recognized equally well in the RIA and the ELISA. Furthermore, the apparent concentrations of ST in these STp

FIG. 1. Correlation of measured STa in culture broths by RIA and ELISA. Each point represents the mean concentration of STa determined from three dilutions of culture broth in the linear range of the standard curves from three separate assays (total of nine determinations). Symbols: ●, STh gene probe-positive isolates; ○, STp gene probe-positive isolates.

culture supernatants are considerably higher than those in STh supernatants when measured in the ELISA, ranging up to approximately 1.3 µg/ml. This differential behavior shows that when the RIA and ELISA apparent concentrations are calculated for an unknown toxin, it may be possible to predict the genotype of the organism with a phenotypic recognition assay.

Reliability and reproducibility of visual scoring of the ST ELISA. We wished to determine whether the ELISA screening assay could be accurately scored visually. Interestingly, the mean agreement between the visual interpretation of ELISA plate color development by the eight individuals and the results of the suckling mouse assay was 93.3%. This is extraordinary agreement, considering the diverse group of individuals that participated in the test.

DISCUSSION

The monoclonal ELISA for *E. coli* STa has been compared with other assays for STa to validate the use of the assay as a screening procedure for detecting STa produced by ETEC. There was 100% agreement between the ELISA, SMA, and RIA, identical to the results of our previous publication (25). Thus, the monoclonal ELISA screening procedure is a good method for detecting ETEC. The assay is convenient and rapid and requires no isotope.

The assay can be visually interpreted with a high degree of reliability even by untrained personnel. The overall correlation of individual ELISA interpretations with the suckling mouse bioassay was remarkably high, i.e., 93%. In the ELISA, only 2 of the 100 isolates tested yielded a color of which three or more of the individuals reading were uncertain, i.e., gave an inconclusive score. Since the chromagen chosen yields a dark blue-green color in the presence of antibody, the plates are easy to read. Thus, the assay can be used with a high degree of accuracy even without sophisticated plate-reading equipment.

However, the quantity estimated by either the monoclonal antibody ELISA or the RIA may significantly differ from the

true concentration. We have exploited this effect to show that 18- and 19-amino-acid toxins react differently in these two assays. It is interesting that the phenotypic expression of the STa's, as reflected in the differential binding of a monoclonal antibody and a population of polyclonal antibodies, can apparently be used to predict the genotype of the toxin expressed. The monoclonal antibody 20c1B8 apparently recognizes a region of the STa which is substantially different between the 18- and 19-amino-acid toxins. Since the primary sequence and structure of these toxins are virtually identical at the carboxy terminus of the molecule (1, 9, 12, 13, 17), it seems likely that the antibody may bind preferentially to the amino terminus, wherein the greatest difference between the two toxins exists.

Thus, the ability to determine the STa genotype by immunological means has great potential. Although this work was performed with an RIA, the polyclonal antisera can just as easily be used in an ELISA format, as has been previously reported (13, 17).

The particular conditions used for storage and transport of strains to be tested are critical to the results obtained. Our results demonstrate the loss of toxin production by a significant number of organisms, presumably from the loss of the STa plasmid, as shown by gene probe analysis. During the course of this project, a total of nine strains appeared to lose the STa plasmid when stored in skim milk at -70°C and two appeared to lose it during storage on Dorset egg medium at room temperature ($P = 0.056$; Fisher two-tailed exact test). Similar findings have been reported by a number of investigators (4, 14, 16, 21-23). It is clear that the medium of transport and the time and method of storage of ETEC may have a profound influence on the apparent prevalence of STa-producing organisms.

In the group of 100 isolates tested, a 3% discrepancy was seen between the genotypic and phenotypic analysis, which was reflected in gene-positive, toxin-negative assignments. The gene for toxin production was present, but the product was not measurable in either the biological or the immuno-

logical assays. It is possible that these strains may contain inactive genes or that strains may require different growth conditions for expression of the toxin. The ST probes can clearly be used to screen large numbers of isolates, but the probe-positive strains may need to be further tested by ELISA, SMA, or RIA to confirm production of the toxin. Our rate of discrepancy (which involved both STp and STh probes) is much lower than that recently reported by Echeverria et al. (3). These investigators found a 28% false-positive rate due to contaminating pBR322 sequences. To avoid such contamination and to ease probe preparation, we recloned the probes by using synthetic oligonucleotide linkers, a procedure which greatly reduces the possibility of contamination.

Each of the toxin detection assays relies upon the determination of the toxin concentration in a culture supernatant. After an organism is isolated, approximately 24 h is required for liquid culture before the assay can be initiated. The RIA is the most time consuming, requiring an additional 2 days for completion. The SMA is rapid, requiring approximately 3 to 4 h to perform, but it is expensive and very cumbersome. The ELISA, as described in this communication, was performed over a 2-day period, (20 h) but can be performed in its entirety 1 to 4 h after the culture supernatant is obtained (25). Realistically, by modifying the incubation conditions, the assay can be completed within 26 to 28 h after isolated colonies are obtained, including time for liquid culture. This time compares favorably with that of the gene probe analysis assay, which does not require liquid culture. This assay can be performed easily within 30 h of receipt of the isolated organism.

In summary, our findings demonstrate that our STa ELISA is suitable for the detection of STa's elaborated by diverse strains of *E. coli* from around the world and that it compares favorably with biological assays (SMA), our previously described RIA, and STa gene probe analysis. The STa ELISA may be preferable to some of the other assays since it can be completed in 1 h, requires no isotopes, and can be accurately scored visually (without the requirement for specialized equipment) even by untrained personnel.

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LITERATURE CITED

1. Aimoto, S., T. Takao, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani. 1982. Amino acid sequence of a heat-stable enterotoxin produced by human enterotoxigenic *Escherichia coli*. *Eur. J. Biochem.* **129**:257-263.
2. Brandwein, H., A. Deutsch, M. R. Thompson, and R. A. Giannella. 1985. Production of neutralizing monoclonal antibodies to *Escherichia coli* heat-stable enterotoxin. *Infect. Immun.* **47**:242-246.
3. Echeverria, P., D. N. Taylor, J. Seriwatana, A. Chatkaemorakot, V. Khungvalert, T. Sakuldaipera, and R. D. Smith. 1986. A comparative study of enterotoxin gene probes and tests for toxin production to detect enterotoxigenic *Escherichia coli*. *J. Infect. Dis.* **153**:255-260.
4. Evans, D. J., Jr., D. G. Evans, H. L. DuPont, F. Ørskov, and I. Ørskov. 1977. Patterns of loss of enterotoxigenicity by *Escherichia coli* isolated from adults with diarrhea: suggestive evidence for an interrelationship with serotype. *Infect. Immun.* **17**:105-111.
5. Frantz, J. C., and D. C. Robertson. 1981. Immunological properties of *Escherichia coli* heat-stable enterotoxins: development of a radioimmunoassay specific for heat-stable enterotoxins with suckling mouse activity. *Infect. Immun.* **33**:193-198.
6. Georges, M. C., I. K. Wachsmuth, K. A. Birkness, S. L. Moseley, and A. J. Georges. 1983. Genetic probes for enterotoxigenic *Escherichia coli* isolated from childhood diarrhea in the Central African Republic. *J. Clin. Microbiol.* **18**:199-202.
7. Giannella, R. A. 1976. Suckling mouse model for detection of heat-stable *Escherichia coli* enterotoxin: characteristics of the model. *Infect. Immun.* **14**:95-96.
8. Giannella, R. A., K. W. Drake, and M. Luttrell. 1979. Development of a radioimmunoassay for *Escherichia coli* heat-stable enterotoxin: comparison with the suckling mouse bioassay. *Infect. Immun.* **33**:186-192.
9. Klipstein, F. A., R. F. Engert, R. A. Houghten, and B. Rowe. 1984. Enzyme linked immunosorbent assay for *Escherichia coli* heat-stable enterotoxin. *J. Clin. Microbiol.* **19**:798-803.
10. Lallier, R., F. Bernard, M. Gendreau, C. Lazure, N. G. Seidah, M. Cretien, and S. A. St. Pierre. 1982. Isolation and purification of *Escherichia coli* heat stable enterotoxin of porcine origin. *Anal. Biochem.* **127**:267-275.
11. Lanata, C. F., J. B. Kaper, M. M. Baldini, R. E. Black, and M. M. Levine. 1985. Sensitivity and specificity of DNA probes with the stool blot technique for detection of *E. coli* enterotoxins. *J. Infect. Dis.* **152**:1087-1090.
12. Lazure, C., N. G. Seidah, M. Cretien, R. Lallier, and S. A. St. Pierre. 1983. Primary structure determination of *E. coli* heat-stable enterotoxin of porcine origin. *Can. J. Biochem. Cell Biol.* **61**:287-292.
13. Lockwood, D. E., and D. C. Robertson. 1984. Development of a competitive enzyme-linked immunosorbent assay (ELISA) for *E. coli* heat-stable enterotoxin. *J. Immun. Methods* **75**:295-307.
14. McConnell, M. M., L. V. Thomas, N. P. Day, and B. Rowe. 1985. Enzyme-linked immunosorbent assay for the detection of adhesion factor antigens of enterotoxigenic *E. coli*. *J. Infect. Dis.* **152**:1120-1127.
15. Moseley, S. L., P. Echeverria, J. Seriwatana, C. Tirapat, W. Chaicumpa, T. Sakuldaipera, and S. Falkow. 1982. Identification of enterotoxigenic *Escherichia coli* by colony hybridization using three enterotoxin gene probes. *J. Infect. Dis.* **145**:863-868.
16. Penaranda, M. E., M. B. Mann, D. G. Evans, and D. J. Evans. 1980. Transfer of an ST:LT:CFA/II plasmid into *E. coli* K12 strain RR1 by cotransformation with PSC301 plasmid DNA. *FEMS Lett.* **8**:251-254.
17. Ronnberg, B., O. Soderlind, and T. Wadstrom. 1985. Evaluation of a competitive enzyme-linked immunosorbent assay for porcine *Escherichia coli* heat-stable enterotoxin. *J. Clin. Microbiol.* **22**:893-896.
18. Ronnberg, B., T. Wadstrom, and H. Jernvall. 1983. Structure of a heat-stable enterotoxin produced by a human strain of *Escherichia coli*. *FEBS Lett.* **155**:183-186.
19. Saeed, A. M. K., N. S. Magnuson, N. Sriranganathan, D. Burger, and W. Cosand. 1984. Molecular homogeneity of heat-stable enterotoxins produced by bovine enterotoxigenic *Escherichia coli*. *Infect. Immun.* **45**:242-247.
20. Seriwatana, J., P. Echeverria, J. Escamilla, R. Glass, I. Huq, R. Rockhill, and B. J. Stoll. 1983. Identification of enterotoxigenic *Escherichia coli* in patients with diarrhea in Asia with three enterotoxin gene probes. *Infect. Immun.* **42**:152-155.
21. Smith, H. R., A. Cravioto, G. A. Willshaw, M. M. McConnell, S. M. Scotland, R. J. Gross, and B. Rowe. 1979. A plasmid coding for the production of colonization factor antigen and heat-stable enterotoxin in strains of *E. coli* of serogroup O78. *FEMS Lett.* **6**:255-260.
22. Smith, H. R., S. M. Scotland, and B. Rowe. 1983. Plasmids that code for production of colonization factor antigen II and enterotoxin production in strains of *E. coli*. *Infect. Immun.* **40**:1236-1239.
23. Smith, H. W., and M. A. Lingood. 1971. The transmissible nature of enterotoxin production in a human enteropathogenic

- strain of *E. coli*. J. Med. Microbiol. 4:301-305.
24. Staples, S. J., S. E. Asher, and R. A. Giannella. 1980. Purification and characterization of heat-stable enterotoxin produced by a strain of *E. coli* pathogenic for man. J. Biol. Chem. 255:4716-4721.
 25. Thompson, M. R., H. Brandwein, M. LaBine-Racke, and R. A. Giannella. 1984. Simple and reliable enzyme linked immunosorbent assay with monoclonal antibodies for detection of *Escherichia coli* heat stable enterotoxins. J. Clin. Microbiol. 20:59-64.
 26. Thompson, M. R., and R. A. Giannella. 1985. Revised amino acid sequence for a heat-stable enterotoxin produced by an *Escherichia coli* strain (18D) that is pathogenic for humans. Infect. Immun. 47:834-836.
 27. Thompson, M. R., M. Luttrell, G. Overmann, and R. A. Giannella. 1985. Biological and immunological characteristics of ¹²⁵I-4Tyr and -18Tyr *Escherichia coli* heat-stable enterotoxin species purified by high-performance liquid chromatography. Anal. Biochem. 148:26-36.