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Evaluation of a Competitive Enzyme-Linked Immunosorbent Assay for Porcine *Escherichia coli* Heat-Stable Enterotoxin

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A competitive enzyme-linked immunosorbent assay (ELISA) was compared with the conventional suckling mouse assay for the identification of heat-stable enterotoxin (ST_a) in samples from piglets suffering from diarrhea. A total of 110 *Escherichia coli* isolates, 22 primary cultures, and 26 fecal samples from piglets up to 8 weeks of age with diarrhea were compared in parallel by both assays. Of the 110 isolates tested, all gave consistent results by the ELISA and the suckling mouse assay; 60 strains were negative and 50 strains produced ST_a by both tests. Identical results were obtained when 22 primary agar cultures were screened for ST_a production by both methods; 6 were found to produce ST_a, while 16 did not. When 26 fecal samples were tested for the presence of ST_a, 10 were negative and 12 were positive by both assays. One of the remaining four samples gave questionable positive results by both the suckling mouse assay and the ELISA, but *E. coli* isolated from this sample gave positive results by both tests. The remaining three samples were negative by the suckling mouse assay, but gave questionable positive results by the ELISA. *E. coli* isolates from these three samples were always negative by both assays. The ELISA used in this study provides a reliable and convenient method for diagnosing ST_a-producing enterotoxigenic *E. coli* of porcine origin.

Production of heat-stable enterotoxin (ST_a) by *Escherichia coli* mainly has been assayed in suckling mice (5). This method is laborious and needs trained laboratory personnel, which makes the assay less suitable for routine laboratory use. Recently, radioimmunoassays have been developed for the detection of ST_a (4, 6). Although radioimmunoassays are very sensitive, such tests require radioactive reagents and are therefore not suitable for the routine diagnosis of ST_a. DNA hybridization probes, including ST_a-coding genes (12), have proven to be highly sensitive in detecting ST_a-producing *E. coli* strains, but the complexity of this test, which requires radiolabeled gene probes, makes its use less suitable for routine testing of ST_a. We have recently developed a competitive enzyme-linked immunosorbent assay (ELISA) for the detection of ST active in suckling mice (ST_a) (14) using an antibody against ST_a purified from a human strain of *E. coli*.

In this study, ELISA was compared with the suckling mouse assay for the detection of *E. coli* ST_a, using culture supernatants from *E. coli* isolates, primary cultures, and fecal samples of porcine origin with the aim of evaluating the usefulness of ELISA for routine testing of ST_a.

MATERIALS AND METHODS

Materials and isolation of *E. coli* strains. Fecal samples or contents from the proximal part of the small intestine of piglets, obtained within 24 h after death, were collected from different herds with diarrhea as the clinical diagnosis during 1976, 1977, 1978, and 1984. The age of the piglets varied from 1 day to 8 weeks.

Specimens were plated on beef extract agar with lactose and bromocresol purple indicator (E. Merck AG, Darmstadt, Federal Republic of Germany). Lactose-positive colonies from the primary cultures were subcultured and examined biochemically by the following tests at 37°C: gas from glucose, fermentation of adonitol and raffinose, and the indole test. Identified *E. coli* strains were stored in meat extract broth with 5% horse serum at −20°C until use.

Cultivation of *E. coli* strains for the enterotoxin assay. The strains were transferred from blood agar to tubes containing 10 ml of Casamino Acids yeast extract (CAYE; Difco Laboratories, Detroit, Mich.) medium (pH 8.5) (3) and incubated for 18 h at 37°C. After centrifugation at $3,000 \times g$ for 20 min the supernatants were stored at −20°C until they were tested for ST_a activity by the suckling mouse assay (15) and ELISA (14).

Cultivation of primary cultures for the enterotoxin assay. When tests were performed directly from the primary culture, a loop from the dense growth on the agar plate was inoculated in CAYE medium and incubated for 18 h at 37°C. After centrifugation, supernatants (referred to as primary cultures in the text) were tested for ST_a activity.

Cultivation of fecal samples for enterotoxin assay. Fecal samples were directly inoculated in CAYE medium and incubated for 18 h at 37°C. Supernatants (referred to as fecal samples in the text) after centrifugation were tested for ST_a activity.

Preparation of enterotoxin and antienterotoxin serum. The ST_a produced by a human strain (C57/26C2) of *E. coli* was prepared and purified as described previously (15). Antiserum to purified ST_a was produced by immunizing rabbits with ST_a coupled to human thyroglobulin (14). The same antiserum was used throughout the study.

ELISA procedure. ELISA was performed as reported previously (14) with minor modifications. Flat-bottom microtiter plates (M129A; Dynatech, Plochingen, Federal Republic of Germany) were coated overnight at 37°C with 0.2 ml of ST_a (0.2 μg/ml) diluted in 0.1 M sodium carbonate–bicarbonate buffer (pH 9.6). After the wells were rinsed with a

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phosphate-buffered saline solution containing 0.05% (vol/vol) Tween 20 (PBS-Tween; pH 7.4), the remaining binding sites were blocked by incubating the wells with 0.2 ml of 1% (wt/vol) bovine serum albumin (BSA) in PBS-Tween at 37°C for 1 h. This solution was then decanted and replaced by 0.1 ml of test sample and 0.05 ml of anti-ST_a serum diluted in PBS-Tween, and the plates were incubated at 37°C for 1 h. The contents were then decanted again, and the wells were rinsed three times with PBS-Tween. Anti-ST_a antibodies bound to the ST_a sorbent were demonstrated after incubation for 1 h at 37°C with 0.1 ml of anti-rabbit immunoglobulin G (IgG) conjugated with β-galactosidase. After the wells were rinsed three times with PBS-Tween, 0.1 ml of the enzyme substrate *o*-nitrophenyl-D-β-galactoside was added and incubated at 37°C for 1 h. The absorbance of the resulting *o*-nitrophenolate was read at 405 nm. All samples were measured in duplicate and as coded samples. Supernatants from four ST_a-positive and four ST_a-negative *E. coli* strains were used as positive and negative controls, respectively. Percent inhibition was calculated as follows: 100 [1 − (antibody binding in the presence of ST_a)/(antibody binding in the absence of ST_a)]. Samples with an inhibition of more than 10% above that of negative controls were considered positive. An inhibition of less than or equal to 10% above that of negative controls was considered questionably positive.

Chemicals. Human thyroglobulin was purchased from UCB Bioproducts, Brussels, Belgium. IgG–β-galactosidase conjugate and *o*-nitrophenyl-D-β-galactoside were kindly supplied by Pharmacia Diagnostics, Uppsala, Sweden. All media components were obtained from Difco Laboratories, unless otherwise indicated.

RESULTS

A total of 110 *E. coli* isolates, 22 primary cultures, and 26 fecal samples from neonatal pigs with diarrhea were screened for ST_a activity by the suckling mouse assay and ELISA (Table 1). All the 110 *E. coli* strains tested for ST_a production gave consistent results by both tests. By both assays 50 strains were shown to produce ST_a, while 60 strains did not.

TABLE 1. Comparison of the ELISA and the suckling mouse assays for detection of ST_a in *E. coli* strains, primary cultures, and fecal samples of porcine origin

No.	Suckling mouse assay	ELISA
Strains		
50	+	+
60	−	−
0	+	−
0	−	+
Primary cultures		
6	+	+
16	−	−
0	+	−
0	−	+
Fecal samples		
12	+	+
10	−	−
0	+	−
0	−	+
1	±[a]	±[b]
0	±	−
3	−	±

[a] A gut-to-body ratio of between 0.080 and 0.090 (15).
[b] An inhibition of less than or equal to 10% above that of negative controls.

Again, when 22 supernatants from primary cultures were assayed in parallel by both methods, all gave consistent results by ELISA and the suckling mouse assay; 6 were positive and 16 were negative.

Supernatants from 26 fecal samples tested for ST_a activity gave the following results: 22 of 26 samples gave consistent results by both tests; 12 were positive and 10 were negative. Three of the remaining four samples gave questionably positive results by ELISA, but were negative by the suckling mouse assay. *E. coli* isolates from these three samples were negative when tested by both methods. The last fecal sample gave questionably positive results by both the suckling mouse assay and ELISA, but isolated *E. coli* strains from this sample gave positive results by both tests.

Ten primary cultures already investigated (seven ST_a positive and three ST_a negative by both methods) were inoculated in CAYE medium and incubated at 37°C for 3 h instead of 18 h. None of these 10 primary cultures showed any ST_a activity by either the suckling mouse assay or ELISA (data not shown).

DISCUSSION

The usefulness of an ELISA for the detection of ST_a-producing *E. coli* strains of porcine origin was demonstrated. Although the antibodies used in this study were raised against ST_a produced by an *E. coli* strain of human origin, these antibodies never failed to detect ST_a's produced by porcine isolates. Amino acid sequences of ST_a's from calf, porcine, and human origins are very similar or identical (1, 2, 9, 11, 16–18). Thus, there is no need to raise antibodies against porcine ST_a to detect ST_a produced by porcine *E. coli* strains.

A total of 110 isolates, 22 primary cultures, and 26 fecal samples were screened for ST_a activity by the suckling mouse assay and ELISA. There was a 100% correlation between both assays when isolated *E. coli* strains or primary cultures were tested (Table 1). Fecal samples, on the other hand, gave doubtful results in 4 of 26 samples investigated (Table 1). One sample gave questionably positive reactions by both assay systems, while the other three samples were questionably positive only by ELISA, but negative by the suckling mouse assay. *E. coli* strains isolated from the fecal sample giving doubtful results by both ELISA and the suckling mouse assay produced ST_a by both methods. On the other hand, *E. coli* strains isolated from the three samples which were negative by the suckling mouse assay, but were questionably positive by ELISA, were always negative by both tests. One possible explanation of the discrepancy is that the bacteria causing the questionable positive reactions by ELISA, but not by the suckling mouse assay, were outnumbered by ST_a-negative *E. coli* or other species of bacteria in the same samples, and thus were not detectable in the less-sensitive suckling mouse assay. If this is the case, the small number of bacteria causing these weak reactions by ELISA are of no diagnostic significance, since the observed clinical symptom is probably caused by other dominant pathogens or toxic substance(s) than ST_a in the same samples. Another possibility is that some other substance(s) than ST_a produced in the three samples is responsible for the weak reactions by ELISA, which is unlikely since no other fecal samples, primary cultures, or isolated *E.*

coli strains cultivated in the same medium gave such reactions. In addition, cocultivation of ST_a-positive *E. coli* strains with nonenterotoxigenic *E. coli* strains and other bacterial strains may cause loss of the ability to produce ST_a by the ST_a-positive *E. coli* strains (13).

ST_a-positive *E. coli* strains have been shown to produce ST_a in the early logarithmic growth phase (7, 15). This suggests that the entire test procedure, including cultivation in liquid medium, can be performed within 1 working day (8 h). Ten different primary cultures already tested for ST_a production were cultivated in CAYE medium for 3 h instead of 18 h. After centrifugation the supernatants were assayed for ST_a by the suckling mouse assay and ELISA. None of the 10 different supernatants showed any sign of ST_a activity by the two methods. Perhaps this failure to detect ST_a in supernatants from primary cultures after 3 h of incubation is due to the cultivation conditions used in this study. In studies in which ST_a has been reported to be present in the early logarithmic growth phase (7, 15), *E. coli* isolates were grown under controlled conditions (temperature, aeration, agitation, and pH) in 10-liter fermentors. In the present study, a loop with a mixture of bacterial strains was inoculated in tubes containing 10 ml of broth, resulting in a much longer lag phase compared with controlled growth in a fermentor, and thus a delayed occurrence of ST_a in the medium. Apparently, the most convenient and reliable (with regard to ST_a production in primary cultures) test procedure involves overnight incubation (18 h) in liquid medium and an enterotoxin assay the following day.

Three ELISAs for the detection of *E. coli* ST_a have been reported recently (8, 10, 19) with the same high sensitivity and specificity. The competitive ELISAs (10, 19) differ from our method in the solid-phase antigen used, among other things. We used native, unmodified toxin as the solid-phase antigen, while the other ELISAs have ST_a conjugated to BSA (19) or human serum albumin (10). Interestingly, our experience is that unmodified ST_a binds to plastic and retains its ability to bind antibodies, but the antibodies bind more easily to ST_a conjugated to BSA. On the other hand, it was not possible to compete with soluble, native ST_a (either purified or from culture supernatant) when ST_a conjugated to BSA was used as the solid-phase antigen, and at the same time retain the sensitivity of the assay. This discrepancy might be explained by the coupling procedure used to conjugate ST_a to BSA. In our attempts to use the ST_a-BSA conjugate as the solid-phase antigen, ST_a was coupled to BSA exactly as described previously for coupling to human thyroglobulin, with *N*-succinimidyl 3-(2-pyridyl-dithio)propionate (SPDP) (14), while in the other ELISAs ST_a was conjugated to BSA with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloric acid (19) or to human serum albumin with glutaraldehyde (10). The method of conjugating ST_a to protein carrier when used as a solid-phase antigen in ELISA therefore may be ascribed a certain value.

Certain antibodies raised against ST_a-human thyroglobulin conjugates formed with SPDP may be directed to the cross-linking reagent or determinants formed by the reaction of SPDP with ST_a and bind with a higher affinity to SPDP-coupled ST_a-BSA conjugates than to free ST_a in solution.

Independent of which form of ST_a is most accurate when used as a solid-phase antigen in a competitive ELISA, results of this study demonstrate the usefulness of such an assay, using native, unmodified toxin as the solid-phase antigen and polyclonal antibodies against a human ST_a, for detection of ST_a-producing *E. coli* strains of porcine origin.

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