JOURNAL OF CLINICAL MICROBIOLOGY, July 1989, p. 1697–1699
0095-1137/89/071697-03$02.00/0

Vol. 27, No. 7

Identification of *Escherichia coli* That Produces Heat-Stable Enterotoxin ST_A by a Commercially Available Enzyme-Linked Immunoassay and Comparison of the Assay with Infant Mouse and DNA Probe Tests

SYLVIA M. SCOTLAND,* GERALDINE A. WILLSHAW, BENGÜ SAID, HENRY R. SMITH, AND BERNARD ROWE

Division of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, England

Received 28 December 1988/Accepted 11 April 1989

By using the infant mouse test and a commercially available competitive enzyme-linked immunosorbent assay (ELISA), 100 strains of *Escherichia coli* carrying ST_{A1} or ST_{A2} genes were shown to produce the heat-stable enterotoxin ST_A. An additional 100 strains were negative in both tests. The ELISA was easy to perform, and results were available within 24 h. Testing strains with an enzyme-linked DNA probe kit that incorporated both ST_{A1}- and ST_{A2}-specific oligonucleotides showed that the 100 strains positive in the mouse test and ELISA also hybridized with the mixed probe. Two strains carrying ST_{A1} genes but negative in the mouse test and ELISA also hybridized with the mixed probe.

Enterotoxigenic strains of *Escherichia coli* (ETEC) are an important cause of diarrheal disease in humans, especially in developing countries (13). ETEC infecting humans produces either a heat-labile enterotoxin, LT, or a heat-stable enterotoxin, ST_A, or both toxins. Many tests have been developed for detecting production of LT and, as LT is antigenic, these include enzyme-linked immunosorbent assays (ELISAs) and a modified Elek test (5). In contrast, ST_A is routinely detected by an infant mouse assay which requires the intragastric injection of at least two infant mice followed by dissection of the intestine (2). This assay is expensive and laborious and is not suitable for the testing of large numbers of strains. ST_A is poorly antigenic, and this has delayed the development of immunological assays. Antisera to ST_A have been prepared by using partially purified ST_A rendered antigenic by polymerization with glutaraldehyde (29) or by conjugation with bovine serum albumin or bovine immunoglobulin G (4, 6). ELISAs and radioimmunoassays for ST_A using these antisera have been described, but the reagents for these tests, which may require a supply of purified toxin in addition to antiserum, have not been readily available. In this paper, we describe a competitive ELISA for ST_A (COLI ST EIA) that is commercially available (Denka Seiken Co. Ltd., Tokyo, Japan). This uses a peroxidase-conjugated monoclonal antibody to ST_A together with ST_A prepared by peptide synthesis. Results of this test are compared with those obtained from the infant mouse test and from an alkaline phosphatase-conjugated oligonucleotide probe for ST_A genes.

Two hundred strains of *E. coli* were tested for production of ST_A by using the COLI ST EIA. One hundred strains, which were received from Y. Takeda in 1981, were used previously in a multilaboratory collaborative study to evaluate the Biken test for LT (27). An additional 100 strains were from the collection of the Division of Enteric Pathogens, Central Public Health Laboratory, London, England. The strains had been kept on Dorset egg slopes at room temperature. Production of ST and LT is plasmid encoded and may be an unstable characteristic in some strains; therefore, cultures were used that had been prepared recently from a single colony in order to ensure that, as far as possible, all bacteria within the culture were the same with respect to toxin production (22). By using the Y1 adrenal cell test (23) for LT production and the infant mouse test (2, 23) for ST_A production, the strains were characterized as 50 producing ST_A and LT (ST_A^+ LT^+), 50 producing ST_A only (ST_A^+ LT^-), 40 producing LT only (ST_A^- LT^+), and 60 nonenterotoxigenic strains (ST_A^- LT^-). The 100 strains from the Division of Enteric Pathogens were chosen to represent a wide range of serogroups. ST_A^+ LT^- or ST_A^+ LT^+ strains belonged to 32 different O serogroups. ST_A^- LT^+ strains belonged to six different O serogroups. Nonenterotoxigenic strains (ST_A^- LT^-) included strains belonging to enteropathogenic *E. coli* serogroups and Vero cytotoxin-producing strains.

For the COLI ST EIA, a strain was inoculated into 2 ml of Casamino Acids (Difco Laboratories, Detroit, Mich.)-yeast extract broth (3), adjusted to pH 8.0 as recommended in the instruction leaflet, and grown with shaking at 37°C for 18 h. The supernatant obtained after centrifugation of cells was used within 1 day for the test which was carried out according to the instructions of the manufacturer. This allows for the testing of each sample in only one well. Microdilution wells were supplied which had been precoated with synthesized ST_A. After a well was washed once with the supplied buffer, 200 μl of the supernatant to be tested was added; 10 μl of monoclonal antibody conjugated with peroxidase was added immediately, and the well contents were mixed for 1 min using a Micro Shaker (Dynatech Laboratories, Inc., Billingshurst, Sussex, United Kingdom). After 90 min at room temperature, the contents were removed, and the well was washed five times with buffer. Enzyme substrate solution (100 μl), prepared by adding a hydrogen peroxide solution to a vial containing *o*-phenylenediamine, was added. The well contents were mixed by micromixer for a few seconds, and the plate was left at room

* Corresponding author.

TABLE 1. Results of a competitive ELISA (COLI ST EIA) for ST_A

A_{490}	No. of strains of type[a]:			
	ST^+ LT^+	ST^+ LT^-	ST^- LT^+	ST^- LT^-
0–0.099	43	48		
0.1–0.199	7	2		
0.2–0.299				2
>0.4			40	58

[a] ST production was determined in the infant mouse assay. LT production was determined in the Y1 cell test. A total of 31 ST^- LT^+ and 51 ST^- LT^- strains gave an absorbance value greater than 0.9.

temperature for 30 min, with light excluded. H_2SO_4 (1.5 N, 100 μl) was added to stop the reaction. The plates were read by eye and spectrophotometrically at 490 nm by using a Dynatech MR600 microplate reader. The manufacturers indicate that the positive controls should give an A_{490} of <0.2 (as this is a competitive assay) and the negative controls should give an A_{490} of approximately 1.0. In five tests, the positive control gave A_{490}s of less than 0.02 and were virtually colorless. In five tests, the negative control gave A_{490}s between 0.82 and 1.14 (mean, 0.95). All 100 strains which were positive in the infant mouse test gave A_{490}s of <0.2 (Table 1). All 100 strains which were negative in the infant mouse test gave A_{490}s of >0.2, although the values ranged from 0.255 to 1.464 (Table 1). The test was easy to read by eye, and in only two tests with values of 0.255 and 0.281 were the estimations of color development difficult to assess by eye. The only additional equipment needed was a shaker capable of taking tubes and a centrifuge, although a plate mixer might be useful. Filtration of the supernatants was not required.

Two types of ST_A have been identified, ST_{A1} (or STp or STIa) and ST_{A2} (or STh or STIb), and probes for these genes have been developed (10, 16). Strains of *E. coli* isolated from human infections usually produce ST_{A2}, although strains producing ST_{A1} alone or with ST_{A2} have also been described (14, 16). The 100 ST_A-producing strains in our study were examined to ensure that they included both ST_{A1} and ST_{A2} producers to verify that the COLI ST EIA could detect both toxins. Strains were grown overnight at 37°C and spotted on nylon membranes (Hybond-N; Amersham International, Amersham, United Kingdom) placed on MacConkey agar plates. Controls of ST_{A1}^+, ST_{A2}^+, and ST^- strains were included on each membrane. Plates were incubated at 37°C for 5 h and then prepared for hybridization by the method of Maniatis et al. (15). The presence of ST_{A1} sequences specifically was detected with a cloned DNA probe consisting of a ^{35}S-labeled 157-base-pair *Hin*fI-generated fragment of plasmid pRIT10036 (16). Hybridization and washing were under stringent conditions (17), and probe-positive colonies were detected by autoradiography. To detect the presence of ST_{A2} sequences specifically, the alkaline phosphatase-conjugated ST_{A2} oligonucleotide kit, SNAP hybridization system (NEP-010; Du Pont (UK) Ltd. NEN Products Div., Stevenage, United Kingdom), was used according to the instructions of the manufacturer. A total of 75 strains carried ST_{A2} gene sequences, and 24 carried ST_{A1} gene sequences; 1 strain hybridized with both individual probes. It was concluded that the COLI ST EIA successfully detected both ST_{A1} and ST_{A2}.

The NEP-010 nucleotide probe for ST_A (11) is 26 bases long and constitutes part of the ST_{A2} gene sequence but differs in two bases from the analogous region of the ST_{A1} sequence. Previous reports have shown that this probe is satisfactory in colony hybridization tests for the detection of strains with the ST_{A2} gene but does not identify all strains carrying the ST_{A1} gene (18, 25). A mixed probe incorporating both ST_{A1} and ST_{A2} enzyme-linked oligonucleotides was provided by Du Pont, NEN Research Products, Boston, Mass., at our request and was used to test the 200 strains. The procedures were those in the protocol supplied with the Du Pont kit NEP-010 except that hybridization and the high temperature washes were at 50°C. The mixed ST_A probe test was rapid and easy to perform. All 100 strains producing toxin in the infant mouse test and ELISA hybridized with the mixed probe. However, two strains that were negative in both the mouse test and the ELISA also hybridized with the probe. These two strains belonged to serotype O17.H45, produced LT, and carried ST_{A1} gene sequences. Strains giving similar results have been obtained in other surveys (1, 26). The ST genes in these strains may be incomplete, although they possess sequences in common with the probe. Alternatively, although the toxin genes may be complete in these strains, other genetic information needed for their full expression may be lacking. We have shown in an earlier study that not all wild-type strains into which the same ST_A-encoding plasmid had been inserted produced detectable amounts of the toxin by the infant mouse assay (21).

ETEC producing only ST have been recognized in several studies as an important cause of diarrhea (7, 9, 24, 28). Strains of *E. coli* producing only ST_A and belonging to serotypes such as O27.H20, O78.H12, and O159.H20 have also caused a number of outbreaks of diarrhea (8, 12, 19, 20). Many laboratories detect LT-producing strains, and thus they indirectly detect those ETEC producing LT together with ST_A. Owing to the lack of a simple test for ST_A, strains producing only this toxin have been identified in fewer laboratories. Both the ELISA and mixed oligonucleotide probe test are suitable alternatives to the infant mouse test, although it must be recognized that the probe test may detect a small proportion of strains that do not produce toxin.

This work was supported by a grant from the Diarrhoeal Diseases Control Programme of the World Health Organization.

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