Trivalent Heat-Labile- and Heat-Stable-Enterotoxin Probe Conjugated with Horseradish Peroxidase for Detection of Enterotoxigenic Escherichia coli by Hybridization

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A 1,268-bp polynucleotide probe for heat-labile and heat-stable enterotoxins (LTh, STIa, STIb) was conjugated with horseradish peroxidase (HRP). The HRP-conjugated trivalent probe was applied to the detection of enterotoxigenic Escherichia coli (ETEC) by colony and stool hybridizations. The binding of the probe to its targets was assayed by the addition of HRP substrates to samples with and without ETEC. The hybridization signal was weak when LTh, STIa, STIb, or LTh-STIa, or STIa-STIb. Treatment of targets with sodium dodecyl sulfate and proteinase K reduced the nonspecific hybridization signal to DNA isolated from ETEC. Furthermore, this probe was able to detect stool specimens seeded with 10^2 original ETEC cells per 5 mg of feces. These results suggest that the HRP-conjugated trivalent probe is a candidate for use in the clinical laboratory to detect ETEC.

Enterotoxigenic Escherichia coli (ETEC) causes diarrhea in humans and animals. ETEC infection in infants and young children is one of the most common causes of diarrhea in tropical developing countries. Two virulence factors are required to cause diarrhea by ETEC infection. One is fimbrial adhesins (colonization factor antigens), which promote colonization of the small intestinal tract by ETEC. The second virulence factor is enterotoxin, which cause fluid accumulation in the intestine. ETEC produces two types of enterotoxins, heat-labile enterotoxin (LT), heat-stable enterotoxin (ST), or both. The most frequent enterotoxins found in human ETEC are LT type h (LTh) and ST types Ia (STIa) and Ib (STIb).

Laboratory tests for enterotoxin production in ETEC include animal models, such as the ligated rabbit intestinal loop method for LT or the sucking mouse test for ST, the tissue culture method with the Chinese hamster ovary (CHO) cell line, the immunological method with anti-enterotoxin antibodies, or the DNA-DNA hybridization method with radioactively labeled enterotoxin gene probes. Because of their high specificity and sensitivity, hybridization reactions with radioactive labeled nucleic acid probes are widely used to detect genes or pathogens. However, when selecting an appropriate enzyme-substrate reaction, one must consider several factors, including the enzyme-substrate reaction, trivalent horseradish peroxidase (HRP) used for hybridization experiments. We have previously demonstrated (1, 5) that ETEC strains that produce LTh, STIa, and STIb can be identified by biotinylated enterotoxin probes. However, we encountered some technical difficulties in these hybridization experiments. For example, the hybridization signal was weak when LTh-STIa, or STIa-STIb, or LTh-STIb. Treatment of targets with sodium dodecyl sulfate and proteinase K remarkably reduced the nonspecific hybridization signal to DNA isolated from ETEC. Furthermore, this probe was able to detect stool specimens seeded with 10^2 original ETEC cells per 5 mg of feces. These results suggest that the HRP-conjugated trivalent probe is a candidate for use in the clinical laboratory to detect ETEC.

In the present study, we constructed "cassette-probe plasmid" pKA008, which enabled rapid and simple preparation of LTh-STIa-STIa-STIb gene probes for the detection of ETEC strains. The trivalent gene probe conjugated with horseradish peroxidase (HRP) was used for hybridization reactions. We found that the HRP-conjugated probe detected ETEC in colonies and stool specimens seeded with ETEC with a specificity and sensitivity similar to that of the 32P-labeled trivalent probe. The potential usefulness of the HRP-conjugated trivalent probe for diagnostic purposes is described.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli C600 (hsd thy leu thi lacY tonA supE44) was used for the construction and propagation of recombinant plasmids. Wild-type E. coli strains used for hybridization experiments were isolated from trav-

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E. coli. E. coli strains producing LTH were detected by the reversed passive latex agglutination test (6) by using the VET-RPLA kit (Dennkaseiken, Tokyo, Japan). The activities of ST were assayed by the fluid accumulation test in suckling mice (3) and were subtyped as ST1a or ST1b by hybridization tests with subtype-specific 32P-labeled probes, as reported previously (5).

Preparation of targets for slot blot hybridization. Plasmid DNA for slot blot hybridization was isolated by the alkaline sodium dodecyl sulfate (SDS) lysis method (4) and was used as the target. Plasmid DNA was extracted from 1.5-ml cultures of test strains grown overnight in PAB. Isolated DNA was suspended in 90 μl of buffer containing Tris hydrochloride (pH 8.0) and 0.1 mM EDTA and was mixed of buffer containing Tris hydrochloride (pH 7.5) and 1 M NaOH before neutralization or overnight for stool hybridization. They were maintained on filter papers saturated with 10% SDS for 3 min, 0.5 N NaOH for 10 min, and 1 M Tris hydrochloride (pH 7.5) for 5 min. This denaturation (with 0.5 N NaOH) and neutralization (with 1 M Tris hydrochloride) procedure was repeated three times, after which the filters were placed on filter paper saturated with a solution containing 0.5 M Tris hydrochloride (pH 7.5) and 1.5 M NaCl for 5 min. The filter was immersed in 2 ml of proteinase K per ml at 37°C for 15 min. This proteinase K solution contained 0.05 M Tris hydrochloride (pH 7.5) and 0.5% Triton X-100. The filter was washed with 2× SSC solution (0.3 M NaCl plus 0.03 M trisodium citrate) for 1 min, air dried, and baked at 80°C for 2 h.

Preparation of DNA and RNA probes. A 1,268-bp XbaI fragment containing LTh, ST1a, and ST1b probe regions of pKAD008 (Fig. 1) was used as a DNA probe after conjugation with HRP according to the instructions of the manufacturer (ECL gene detection system; Amersham). The heat-denatured DNA in 10 μl of distilled water (30 μg of DNA per ml) was mixed with a DNA-labeling reagent mixture consisting of 10 μl of an HRP-p-benzoquinone-polyethyleneimine complex and 10 μl of glutaraldehyde solution, as described by Renz and Kurz (12). This mixture was incubated at 37°C for 10 min. A RNA transcript was prepared from pKAD007 in vitro (Fig. 1) by using SP6 RNA polymerase (10). pKAD007 DNA was linearized with XbaI and was transcribed in the presence of [α-32P]UTP. The 1,268-base RNA transcript labeled with 32P was used as the RNA probe.

Detection of ETEC by hybridization reactions. The ECL gene detection system (Amersham) was used for the detection of ETEC strains with the HRP-conjugated DNA probe. The filter with the target DNA was incubated with 5 ml of hybridization buffer. After incubation for 15 min at 42°C, it was further incubated at 42°C overnight with 100 ng of the HRP-conjugated probe. The filter was washed twice for 20 min at 42°C with 42 ml of washing solution consisting of 0.5× SSC, 0.4% SDS, and 6 M urea and was rinsed twice in 48 ml of 2× SSC at room temperature for 5 min. The following procedures were performed in a dark room. A total of 2.5 ml of a mixture containing equal volumes of detection reagents 1 and 2 was added to the filter and incubated for 1 min at room temperature. To detect chemiluminescence, the filter was wrapped with Saran Wrap and exposed to X-ray film (Hyperfilm-ECL; Amersham), for 10 to 30 min at room temperature. The detection of ETEC strains with the 32P-labeled RNA probe was performed by the method reported by Melton et al. (10).

RESULTS

Construction of the cassette-probe plasmid pKAD008. The construction and structure of cassette-probe plasmid pKAD008 are shown in Fig. 1. Plasmid 1032H-19 (ΔecORI) contained a 676-bp XbaI-EcoRI portion of the subunit A gene of LTh (14) and was derived from plasmid 1032H-19 by deletion of the EcoRI fragment. pKAD001 and pKAD002 contained some of the structural genes of ST1a (14) and ST1b (11), respectively. pKAD001 was constructed by cloning a 157-bp Hindll fragment of pTE5014 and pKAD002 was constructed by cloning a 220-bp HpalII fragment of plasmid 53402T-1. For the constructions of pKAD001 and pKAD002, the respective restriction fragments with an EcoRI linker at both termini were inserted into plasmid pSP65 at the EcoRI site. To generate pKAD007, the probe regions of LTh, ST1a, and ST1b from plasmids 1032H-19 (ΔecORI), pKAD001, and pKAD002, respectively, were inserted into pSP65 at the XbaI and EcoRI sites to construct the cassette-probe plasmid pKAD008, a 1,258-bp XbaI-EcoRI fragment was excised from pKAD007 and was inserted into the Bluescript SK(+) plasmid at the corresponding sites, and then the EcoRV site of the vector plasmid was converted to an XbaI site by the ligation of an XbaI linker.

Sequence analysis of pKAD007 and pKAD008 demonstrated that one copy each of the LTh (676 bp) and ST1b (236 bp) probe regions and two copies of the ST1a (173 bp) probe region were contained in these plasmids. The LTh, ST1a, and ST1b probes were arranged in the order LTh-ST1a-ST1b.
STIb on pKAD007 and pKADO08. According to the report by Short et al. (13), 10 bases exist between the EcoRV and EcoRI sites of Bluescript SK(-). We therefore predicted the size of the XbaI fragment of pKADO08 containing the trivalent probe region to be 1,268 bp.

Specificity of the HRP-conjugated LTh-STIa-STIa-STIb trivalent probe. A 1,268-bp XbaI fragment of pKADO08 was conjugated with HRP as described in Materials and Methods, and the specificity of this trivalent LTh-STIa-STIa-STIb probe was examined by slot blot hybridization. A ^32P-labeled transcript from pKADO07 was used as the control probe. All E. coli strains that produced LTh, STIa, and STIb, but not the non-ETEC strains, were detected by the RNA and DNA probes (Fig. 2). These results indicate that the trivalent probe prepared from pKADO08 was specific for the detection of E. coli strains that produce three types of enterotoxins, LTh, STIa, or STIb.

Detection of ETEC by colony hybridization by using HRP-conjugated trivalent probe. Results of colony hybridization with the HRP-conjugated trivalent probe are shown in Fig. 3. All strains that produced enterotoxins were clearly detected when the test bacteria were incubated for 8 h. A similar result was obtained (data not shown) by using targets prepared after overnight incubation of the bacteria. The hybridization signal was rather weak in some strains, especially those that produced only STIa or STIb or those that were incubated for 4 h (data not shown). SDS treatment of bacteria grown on nitrocellulose filters immobilized the target DNA onto the filter, and incubation of target DNA with proteinase K markedly reduced nonspecific hybridization. These two procedures were helpful in obtaining clear-cut results in colony hybridizations.

These results indicate that the HRP-conjugated trivalent probe detects, by single-step colony hybridization, ETEC strains that produce LTh, STIa, STIb, LTh and STIa, and LTh and STIb and six non-ETEC strains were used as targets.

FIG. 1. Construction and structure of cassette-probe plasmid pKADO08. The vector plasmids are pBR322 for 1032H-19 (ΔEcoRI); pSP65 for pKADO01, pKADO02, and pKADO07; and Bluescript SK(-) for pKADO08. ♦ Promoter of phage SP6 in pKADO01, pKADO02, and pKADO07; ○ or ♦, promoter of phage T7 or T3 in pKADO08, respectively. The arrows indicate the direction of transcription by these promoters. AP, Ampicillin resistance; X, XbaI; E, EcoRI; H, HindIII. Plasmids 1032H-19, pTE5014, and 53402T-1 have been described previously (5).

FIG. 2. Slot blot hybridization of ETEC strains with HRP-conjugated trivalent probe. Slot blot hybridizations with HRP-conjugated LTh-STIa-STIa-STIb DNA probe prepared from pKADO08 (A) and ^32P-labeled LTh-STIa-STIa-STIb RNA probe prepared from pKADO08 (B) were performed. Plasmid DNA from three strains producing LTh, STIa, STIb, LTh and STIa, and LTh and STIb and six non-ETEC strains were used as targets.

FIG. 3. Colony hybridization of ETEC strains with HRP-conjugated trivalent probe. Three strains producing LTh (1c, 3b, 5b), STIa (2a, 4c, 5a), STIb (1b, 6c, 7a), LTh and STIa (2b, 4b, 6b), and LTh and STIb (3a, 5c, 7b) and six non-ETEC strains (1a, 2c, 3c, 4a, 6a, 7c) were randomly inoculated. After incubation at 37°C for 8 h, they were used as targets for hybridization with the HRP-conjugated LTh-STIa-STIa-STIb DNA probe.
FIG. 4. Application of HRP-conjugated trivalent probe for stool hybridization. A total of 10^2 to 10^4 cells of ETEC and non-ETEC strains were incubated at 37°C overnight and used as targets for hybridization with the HRP-conjugated LTh-STIa-STIb probe. Hybridization were done with (A) and without (B) feces from a healthy volunteer.

FIG. 5. Stool hybridization of ETEC mixed with a staphylococcus or a salmonella strain with HRP-conjugated trivalent probe. E. coli-producing LTh (ETEC), Staphylococcus aureus (Sta), and Salmonella choleraesuis (Sal) strains mixed with feces from a healthy volunteer were incubated at 37°C for overnight and used as targets for hybridization with the HRP-conjugated LTh-STIa-STIb DNA probe. In these experiments, the targets were not treated with SDS or proteinase K. (A) A total of 10^2 to 10^3 cells of ETEC, Staphylococcus aureus, and Salmonella choleraesuis strains were inoculated. (B) Mixtures of 10^2 to 10^3 cells of ETEC, Staphylococcus aureus, and Salmonella choleraesuis were inoculated.

longer probe than in a shorter one. The STIa probe region used in the present study is contained in the 1,268 bp of the trivalent probe, which is longer than the 157-bp probe used in our previous study (1, 5). This size difference between the biotin-labeled probe and the HRP-conjugated probe seemed to assist with improved diagnosis of STIa-producing ETEC in the present study, although comparison of the sensitivity between the two types of probes is necessary at the end of the experiment.

Cassette-probe plasmid pKAD008 has several advantages, as follows. First, only two steps, digestion with XbaI and elution from an agarose gel, are needed to isolate a 1,268-bp fragment carrying the LTh-STIa-STIb probe gene. Second, ligation of the STIa probe region (173 bp) with the LTh-STIb probe region enhances the specific activity of STIa by the incorporation of HRP throughout the trivalent probe region (1,268 bp). Third, two copies of the STIa probe region are cloned into the pKAD008 plasmid, resulting in dual STIa probe signals in the trivalent probe molecule. Compared with a monovalent, 173-bp STIa probe or another trivalent probe containing only one copy of the STIa probe region (data not shown), the trivalent probe prepared from pKAD008 generated a stronger chemiluminescence signal with strains that produced only STIa. Finally, the XbaI-EcoRI LTh probe region in the trivalent probe has 78.2% base sequence homology with the subunit A gene of Vibrio cholerae enterotoxin (CT).

The results obtained in the present study suggest that the HRP-conjugated trivalent probe prepared from cassette-probe plasmid pKAD008 has potential diagnostic application in the clinical laboratory. Study of practical applications of the HRP-conjugated trivalent probe is in progress in our laboratory, where we are evaluating stool specimens from patients suffering from diarrhea.

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LITERATURE CITED


