JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1990, p. 2616–2620
0095-1137/90/122616-05$02.00/0

Vol. 28, No. 12

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

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Received 11 June 1990/Accepted 10 September 1990

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 hydrogen peroxide and luminol in the presence of an enhancer, and the chemiluminescence was recorded by exposure to X-ray film. Slot blot hybridization demonstrated that the HRP-conjugated trivalent probe specifically hybridized with the DNA isolated from ETEC strains. The trivalent probe also specifically identified bacterial colonies of ETEC that produced LTh, STIa, STIb, LTh-STIa, or LTh-STIb. Treatment of targets with sodium dodecyl sulfate and proteinase K remarkably reduced nonspecific hybridization to DNAs of non-ETEC strains. 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 enterotoxins, which cause fluid accumulation in the intestine. ETEC produces two types of enterotoxin, 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 suckling mouse test for ST, the tissue culture method with the Chinease 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 radioactivity labeled nucleic acid probes are widely used to detect genes or pathogens. Radioactive probes, however, have a limited shelf life, are cumbersome to handle, and require costly disposable equipment and specialized facilities. Therefore, these probes are limited in their clinical and diagnostic applications. Nonradioactive probes have been synthesized by enzymatic incorporation of biotinylated nucleotides into polynucleotides (7). Hybridization of biotinylated DNA probes to their targets can be detected by complexing them with streptavidin linked to enzymes such as alkaline phosphatase (8) and by applying appropriate enzyme-substrate reactions.

We have previously demonstrated (1, 5) that ETEC strains that produce LTh, STIa, and STIb can be identified with biotinylated enterotoxin probes. However, we encountered some technical difficulties in these hybridization experiments. For example, the hybridization signal was weak when 157 polynucleotide bases were used as a STIa probe, and some strains that produce only STIa were undetectable. In addition, several digestion steps with restriction enzymes and elution from agarose gels were necessary to isolate and purify the probe fragments. Moreover, a complex enzymatic reaction was required to incorporate biotinylated nucleotides into the probe fragment. Thus, much time was required to prepare the enterotoxin probes.

In the present study, we constructed "cassette-probe plasmid" pKAD008, 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 those of a ^{32}P-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 thr 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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elers with diarrhea (2, 5) at the Osaka International Airport Quarantine. The *Staphylococcus aureus* and *Salmonella choleraesuis* strains used for stool hybridization studies were clinical isolates. Plasmids 1032H-19, pTE5014, and 53402T-1 encoding LTh, STIa, and STIb genes, respectively, were described previously (5) and provided the sources of enterotoxin probes with which to construct the cassette-probe plasmid pKAD008. pSP65 (10) and Bluescript SK(−) (13) (Toyobo, Tokyo, Japan) were used as the cloning vectors.

Media, chemicals, and enzymes. Colonies were grown on PAB (antibiotic medium 3; Difco Laboratories, Detroit, Mich.) supplemented with thymidine at 10 μg/ml and 1.5% agar. Proteinase K (Merck Co., Darmstadt, Federal Republic of Germany); restriction endnucleases, the Klenow fragment of *E. coli* DNA polymerase, and T4 DNA ligase (Toyobo, Tokyo, Japan); RNase inhibitor of human placenta and RNA polymerase (Takara-shuzo, Tokyo, Japan); [α-^{32}P]UTP (Amersham, Buckinghamshire, United Kingdom); and DNase (Boehringer GmbH, Mannheim, Federal Republic of Germany) were purchased commercially.

Enterotoxin assays of wild-type *E. coli*. *E. coli* strains producing LTh were detected by the reversed passive latex agglutination test (6) by using the VET-RPLA kit (Denkaseiken, Tokyo, Japan). The activities of ST were assayed by the fluid accumulation test in suckling mice (3) and were subtyped as STIa or STIb by hybridization tests with subtype-specific ^{32}P-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 with 10 μl of 2 N NaOH. After incubation for 10 min at room temperature, 25 μl of ammonium acetate were added. A total of 125 μl of the mixture thus prepared was slot blotted onto nylon membrane filters (3 by 8 cm; Hybond-N$^+$; Amersham) with a suction apparatus (MilliBlot-System; Millipore Ltd., Bedford, Mass.). Nylon filters were treated with 1 M ammonium acetate for 10 min before blotting. The filter was air dried at room temperature and baked at 80°C for 2 h to immobilize the DNA.

Preparation of targets for colony and stool hybridizations. Bacteria grown on a nitrocellulose filter (3 by 8 cm; type BA85; Schleicher and Schuell, Dassel, Federal Republic of Germany) that was sterilized by autoclaving before use were the targets for hybridization. For colony hybridization, 21 test strains were inoculated onto the filter. For stool hybridization, an aliquot of 10 μl of the 1 ml of suspension containing 500 mg of feces from volunteers and 10^9 to 10^3 of the test bacterial cells from the serial dilutions were spotted onto the filters. The filters were incubated on PAB agar plates at 37°C for 8 h for colony hybridization 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 mg 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 *Xba*I fragment containing LTh, STIa, and STIb 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 (10 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 *Xba*I and was transcribed in the presence of [α-^{32}P]UTP. The 1,268-base RNA transcript labeled with ^{32}P 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 by 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 ^{32}P-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 (Δ*Eco*RI) contained a 676-bp *Xba*I-*Eco*RI portion of the subunit A gene of LTh (14) and was derived from plasmid 1032H-19 by deletion of the *Eco*RI fragment. pKAD001 and pKAD002 contained some of the structural genes of STIa (14) and STIb (11), respectively. pKAD001 was constructed by cloning a 157-bp *Hin*fI fragment of pTE5014, and pKAD002 was constructed by cloning a 220-bp *Hpa*II fragment of plasmid 53402T-1. For the constructions of pKAD001 and pKAD002, the respective restriction fragments with an *Eco*RI linker at both termini were inserted into plasmid pSP65 at the *Eco*RI site. To generate pKAD007, the probe regions of LTh, STIa, and STIb from plasmids 1032H-19 (Δ*Eco*RI), pKAD001, and pKAD002, respectively, were inserted into pSP65 at the *Xba*I and *Eco*RI sites. To construct the cassette-probe plasmid pKAD008, a 1,258-bp *Xba*I-*Eco*RI fragment was excised from pKAD007 and was inserted into the Bluescript SK(−) plasmid at the corresponding sites, and then the *Eco*RV site of the vector plasmid was converted to an *Xba*I site by the ligation of an *Xba*I linker.

Sequence analysis of pKAD007 and pKAD008 demonstrated that one copy each of the LTh (676 bp) and STIb (236 bp) probe regions and two copies of the STIa (173 bp) probe region were contained in these plasmids. The LTh, STIa, and STIb probes were arranged in the order LTh-STIa-STIa-

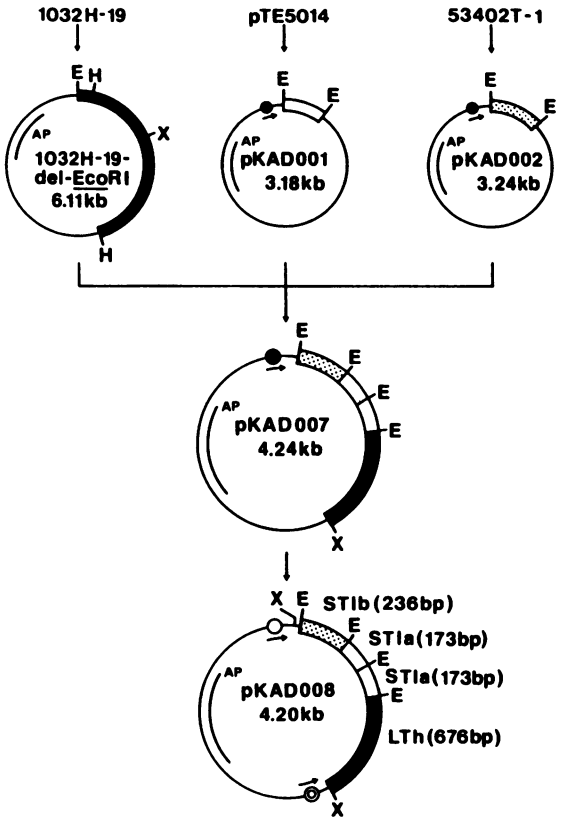

FIG. 1. Construction and structure of cassette-probe plasmid pKAD008. The vector plasmids are pBR322 for 10322H-19 (Δ*Eco*RI); pSP65 for pKAD001, pKAD002, and pKAD007; and Bluescript SK(−) for pKAD008. ●, Promoter of phage SP6 in pKAD001, pKAD002, and pKAD007; ○ or ◎, promoter of phage T7 or T3 in pKAD008, respectively. The arrows indicate the direction of transcription by these promoters. AP, Ampicillin resistance; X, *Xba*I; E, *Eco*RI; H, *Hin*dIII. Plasmids 1032H-19, pTE5014, and 53402T-1 have been described previously (5).

STIb on pKAD007 and pKAD008. According to the report by Short et al. (13), 10 bases exist between the *Eco*RV and *Eco*RI sites of Bluescript SK(−). We therefore predicted the size of the *Xba*I fragment of pKAD008 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 *Xba*I fragment of pKAD008 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 ^{32}P-labeled transcript from pKAD007 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 prove prepared from pKAD008 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 prove 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

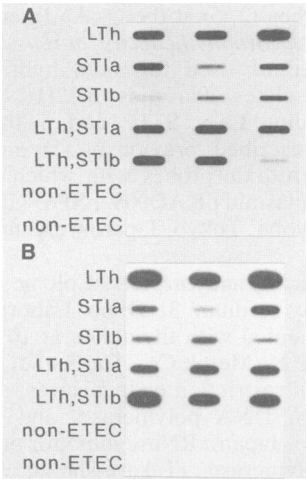

FIG. 2. Slot blot hybridization of ETEC strains with HRP-conjugated trivalent prove. Slot blot hybridizations with HRP-conjugated LTh-STIa-STIa-STIb DNA probe prepared from pKAD008 (A) and ^{32}P-labeled LTh-STIa-STIa-STIb RNA probe prepared from pKAD007 (B) were performed. Plasmid DNA from three strains producing LTh, STIa, STIb, LTh and STIa, and LTh and STIb and six non-ETEC strains were used as targets.

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 prove detects, by single-step colony hybridization, ETEC strains that produce LTh, STIa, STIb, LTh-STIa, or LTh-STIb.

Detection of ETEC in stool specimens. The HRP-conjugated trivalent probe was applied for stool hybridization. Feces (5 mg) was mixed with 10-fold serial dilutions of bacteria containing 10^7 to 10^1 cells of ETEC strains producing LTh, STIa, or STIb. These mixtures were incubated overnight and used for the hybridization studies as described in Materials and Methods. The trivalent probe detected the stool specimens when 10^2 or more original cells were contained in 5 mg of feces (Fig. 4A). The detection limit of the trivalent probe was 10^1 original bacterial cells if ETEC cells without feces were used as the targets (Fig. 4B).

A similar stool hybridization was performed with ETEC mixed with salmonellae or staphylococci (Fig. 5). The triva-

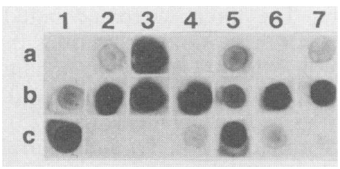

FIG. 3. Colony hybridization of ETEC strains with HRP-conjugated trivalent probe. Three strains producing LTh (1c, 3b, 5b), STIa (2a, 4c, 5a), STIb (1b, 6c, 7a), LTh and STIa (2b, 4b, 6b), and LTh and STIb (3a, 5c, 7b) and six non-ETEC strains (1a, 2c, 3c, 4a, 6a, 7c) were randomly inoculated. After incubation at 37°C for 8 h, they were used as targets for hybridization with the HRP-conjugated LTh-STIa-STIa-STIb DNA probe.

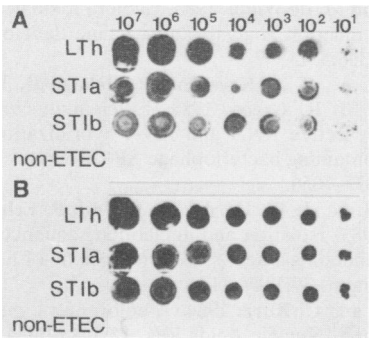

FIG. 4. Application of HRP-conjugated trivalent prove for stool hybridization. A total of 10^7 to 10^1 cells of ETEC and non-ETEC strains were incubated at 37°C overnight and used as targets for hybridization with the HRP-conjugated LTh-STIa-STIa-STIb probe. Hybridization were done with (A) and without (B) feces from a healthy volunteer.

lent probe hybridized only with stool specimens seeded with ETEC; *Salmonella choleraesuis* and *Staphylococcus aureus* did not interfere with the hybridization.

DISCUSSION

In our previous studies (1, 5) we showed that the sensitivities of biotinylated STIa probes in the detection of ETEC strains are lower than those of radioisotopic STIa probes. Two of 12 strains that produced only STIa were not detected by colony hybridization with the nonradioisotopic STIa probe (1, 5). In the present study, we included these two strains in colony hybridization tests, using LTh-STIa-STIa-STIb probes prepared from the cassette-probe plasmid. We found a more pronounced hybridization signal, and thus, with the HRP-conjugated trivalent probe, all strains producing STIa only were correctly identified.

The stronger hybridization signal might be required for the correct diagnosis of ETEC by hybridization, especially when nonradioisotopic probes are used. The strength of the hybridization signal is known to correlate with the size of the probes, because more biotin or HRP is incorporated in a 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.

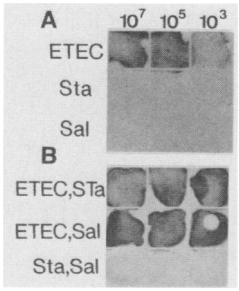

FIG. 5. Stool hybridization of ETEC mixed with a staphylococcus or a salmonella strain with HRP-conjugated trivalent probe. *E. coli*-producing LTh (ETEC), *Staphylococcus aureus* (Sta), and *Salmonella choleraesuis* (Sal) strains mixed with feces from a healthy volunteer were incubated at 37°C for overnight and used as targets for hybridization with the HRP-conjugated LTh-STIa-STIa-STIb DNA probe. In these experiments, the targets were not treated with SDS or proteinase K. (A) A total of 10^7 to 10^3 cells of ETEC, *Staphylococcus aureus*, and *Salmonella choleraesuis* strains were inoculated. (B) Mixtures of 10^7 to 10^3 cells of ETEC, *Staphylococcus aureus*, and *Salmonella choleraesuis* were inoculated.

Cassette-probe plasmid pKAD008 has several advantages, as follows. First, only two steps, digestion with *Xba*I and elution from an agarose gel, are needed to isolate a 1,268-bp fragment carrying the LTh-STIa-STIa-STIb gene probe. 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 prove 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 *Xba*I-*Eco*RI LTh probe region in the trivalent probe has 78.2% base sequence homology with the subunit A gene of *Vibrio cholerae* enterotoxin (CT) (9, 15). *V. cholerae* strains of serovar Inaba or Ogawa were used for colony and stool hybridizations with the HRP-conjugated trivalent probe. All the strains tested were detected under the same conditions as those used to detect ETEC strains (data not shown). Thus, five types of ETEC strains producing LTh, STIa, STIb, LTh-STIa, or LTh-STIb and a *V. cholerae* strain producing CT can be detected by a single hybridization experiment by using the trivalent prove.

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.

ACKNOWLEDGMENTS

We thank Makoto Ohashi and Yasuo Kudoh (Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan) for helpful discussions. The excellent technical assistance of Kazuo Iida (Kibun Co. Ltd., Tokyo, Japan) is acknowledged. We are grateful to Koomi Kanai and Dumrong Chiewsilp (National Institute of Health, Bangkok, Thailand) and Yasukiyo Nakase (Kitasato University, Tokyo, Japan) for encouraging this study.

A part of this study was financially supported by the Ohyama Health Foundation.

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