

Detection of a Heat-Labile Enterotoxin Gene in Enterotoxigenic *Escherichia coli* by Densitometric Evaluation Using Highly Specific Enzyme-Linked Oligonucleotide Probes

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Two alkaline phosphatase-conjugated 24-mer oligonucleotide probes were developed to detect the heat-labile enterotoxin gene in enterotoxigenic *Escherichia coli*. Probes were antisense codon sequences, which are transcribed into mRNA, of the heat-labile enterotoxin gene of enterotoxigenic *Escherichia coli* of human origin. Using dot-blot hybridization, probes were tested with 100 clinical isolates and evaluated by a reflectance-type densitometer. Results agreed very well with those of an immunological test, the Biken test, and a ³²P-labelled recombinant DNA probe. The oligonucleotide probes did not react with nucleic acids prepared from other diarrhoeagenic bacterial pathogens. Thus, the alkaline phosphatase-conjugated oligonucleotide probes seem to be highly sensitive and specific for detection of heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli*. Moreover, the results indicate a potential usefulness for densitometric evaluation of DNA hybridization.

Enterotoxigenic *Escherichia coli* (ETEC) is an important causative agent of diarrhoeal diseases in humans and some domestic animals (1). ETEC is defined as *Escherichia coli* that produces heat-labile (LT) and/or heat-stable (ST) enterotoxins and is usually identified by either biological (2, 3) or immunological (4-7) assays, both based on enterotoxin detection. However, bioassays for ETEC are impractical for routine clinical laboratories since large numbers of animals or the culture of tissue cells is required. While immunoassays have been developed as more practical alternatives to bioassays for LT identification, they require highly specific standard antibody against enterotoxins. Moreover, culture conditions often affect LT production (unpublished data).

Radio-labelled, cloned DNA probes (restriction DNA fragments of enterotoxin genes) (8-14) or synthetic oligonucleotide probes (14-22) have been developed to identify ETEC isolates. Radio-labelled probes, however, have several disadvantages in routine identification (23): (a) probes have a short half-life of radioactivity, (b) there are

frequently difficulties in the handling of radioactive isotopes, and (c) there is usually a requirement for restricted laboratory areas in the use of these probes. Thus, these DNA probes remain unacceptable as routine methods in small clinical laboratories.

Alternatively, non-isotopical gene probes have several advantages. They are safe to handle and may be stored for long periods of time. Non-radioactively-labelled restriction DNA fragments have been previously developed for probes to detect enterotoxin genes of ETEC (20, 24-26); methods to link detection enzymes to oligonucleotides have also been devised (27, 28). Oligonucleotide probes directly linked to detection enzymes require fewer steps and a shorter hybridization time for assay than DNA-fragment probes and are more stable. Such advantages may make the method more acceptable in clinical laboratories as a routine technique for detection of ETEC. Several studies (28-32) have shown that non-radiolabelled oligonucleotides are useful for detection of ETEC. However, we often encountered difficulties in detecting LT genes in some LT-producing ETEC strains using commercially available kits. Moreover, information on nucleotide sequences of commercially available oligonucleotide probes for LT enterotoxin genes is lacking (27).

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Another problem encountered with hybridization methods is the objective evaluation of data. In this paper we describe two antisense-strand oligonucleotide probes, both of which were highly sensitive and specific for the LT gene when alkaline phosphatase (ALP) was covalently bound to the C-5 position of a modified uridine base. We also developed a densitometrical reading method to obtain objective results.

Materials and Methods

Strains. One hundred *Escherichia coli* strains, all isolated from cases of travellers' diarrhea, were obtained from the Osaka Airport Quarantine Station, Japan. Isolates were stored on Dorset egg medium at room temperature. All strains were tested for LT production by an immunological test (Biken test) as described previously (4, 5). *Escherichia coli* JM109 (33) was used for molecular cloning of the LT probe DNA. Strains used for preparation of whole DNA were obtained from the RIMD Culture Collection, Research Institute for Microbial Diseases, Osaka University, Japan.

Chemicals, Solutions and Media. Restriction enzymes, modifying enzymes for DNA and a random primer labelling kit were all purchased from Toyobo, Japan. [α - 32 P]-dCTP was obtained from Amersham Japan, Japan. Hybridization and washing buffers (SSC, SSPE) and Luia-Bertani medium (LB) were prepared as described previously (33). LB medium was used for manipulation of *Escherichia coli*. Brain heart infusion agar was obtained from Difco Laboratories, USA. GAM medium (Nissui Seiyaku, Japan) was used for cultivation of anaerobic bacteria.

Molecular Cloning. Preparation and cloning of DNA, plasmid vectors, *Escherichia coli* strains used for transformation, labelling of DNA with 32 P by random primers, and other general genetic engineering techniques were performed as described (33).

Preparation of Nucleic Acids. Bacteria were cultured on appropriate agar media at 37 °C overnight. Colonies were harvested and suspended in 0.3 ml of 0.1 M NaHPO₄, pH 7.0 in a 1.5 ml Eppendorf tube. Thirty microliters of protease K solution (10 mg/ml in 50 mM Tris[hydroxymethyl]-aminomethane[Tris]-HCl, 1 mM CaCl₂, pH 8.0) and 0.6 ml of lysing solution (8 M urea, 0.25 % sodium dodecyl sulfate [SDS], 0.25 % sodium lauryl sarcosine, 50 mM ethylenediaminetetraacetic acid [EDTA], pH 7.6) were added to the cell suspension. After incubation at 60 °C for 60 min with gentle shaking, nucleic acids were extracted once with buffer-saturated phenol and several times with chloroform. Nucleic acids were precipitated with ethanol, dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at a concentration of 100 µg/ml, and stored at -20 °C.

Dot-Blotting of DNA. One microgram of *Escherichia coli* nucleic acid, dissolved in 100 µl of TE buffer, was denatured with 4 µl of 2N HCl at room temperature for 5

min, then neutralized with 4 µl of 2N NaOH. Denatured DNA was blotted onto a nylon membrane (Gene Screen Plus, New England Nuclear-DuPont, USA) using a dot blotter (Bethesda Research Laboratories, USA). In order to minimize errors of data between experiments, control DNA for LT gene (alkali-denatured pKY195, see below) was blotted on each sheet as a standard. The membrane was rinsed with 5 x SSC and then dried at room temperature to fix nucleic acids.

Preparation of Alkaline Phosphatase-Conjugated Oligonucleotide Probes. Linker-armed oligonucleotide probes with ligands were synthesized by the phosphoramidite method using a DNA synthesizer (Type 381A, Applied Biosystems, USA) and purified on a Mono-Q column (Pharmacia, Sweden). Purified linker-armed oligonucleotide was covalently cross-linked with alkaline phosphatase as described previously (27). Conjugates were then dissolved in conjugate buffer (30 mM Tris-HCl, 3 M NaCl, 1 mM MgCl₂, 0.1 mM ZnCl₂, 0.05 % NaN₃, pH 7.6) at 10 µg DNA/ml and stored at 4 °C in the dark until used. Enzyme activity was stable for at least six months.

Hybridization with ALP Conjugate. Five to ten sheets of membranes (9 cm x 9 cm) fixed with DNA were soaked in 5 x SSC for 5 min, then prehybridized in 15 ml of hybridization buffer (5 x SSC, 0.5 % bovine serum albumin, 0.5 % polyvinylpyrrolidone, 1 % SDS) at 50 °C for 15 min in plastic hybridization bags (Bethesda Research Laboratories). Fifty nanograms of the ALP conjugate were added to the membranes followed by incubation for 10 min at 50 °C. Membranes were washed twice in 1 x SSC (containing 1 % SDS) for 5 min at 50 °C with gentle shaking. This procedure was repeated using 1 x SSC (containing 1 % Triton X-100); 1 x SSC (containing 1 % Triton X-100) for 5 min at room temperature, and finally twice with 1 x SSC for 5 min at room temperature. Washed membranes were immersed in 7.5 ml of substrate buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.1 M MgCl₂, 0.1 mM ZnCl₂, 0.05 % NaN₃, pH 8.5) containing 33 µl of nitro-blue-tetrazolium (75 mg/ml in 70 % dimethylformamide) and 44 µl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in dimethylformamide) and incubated at 37 °C for 2-3 h.

Color densities of deposits were estimated with a reflectance-type color difference meter (Chromameter Type CR-221, Minolta, Japan) at E*ab mode according to the manufacturer's directions. Color density was represented by an arbitrary value, NBS (National Bureau of Standards, USA) unit. Color density was adjusted by dividing each value by the ratio of density of control (pKY195) DNA in an individual experiment to the mean value of the controls of the all experiments in this study.

Preparation of a 32 P-Labelled Cloned DNA Probe for LT. The structural gene of porcine-origin LT in pEWD299 (34) has approximately 90 % homology to human-origin LT gene (34-37). This gene was used to detect the LT gene of human-origin ETEC. pEWD299 was completely digested with *Xba*I and then partially digested with *Hind*III. DNA fragments were separated by polyacrylamide gel electrophoresis (PAGE). A 1.3 kb *Xba*I-*Hind*III DNA fragment was cut off from the gel, electroeluted, and cloned into the *Xba*I-*Hind*III site of plasmid vector pHGS398 (38) (designated pFL48). pFL48 was further digested with *Sma*I, and a 0.9 kb in-

ternal fragment of the structural gene for LT was subcloned to the *Sma*I site of pUC18 (33) (designated pKY195). pKY195 was purified by CsCl₂ ultracentrifugation and then digested with *Sma*I. The 0.9 kb *Sma*I fragment was separated by PAGE and extracted from the gel. The extracted fragment was treated with phenol-chloroform and precipitated with ethanol. The probe DNA fragment was then labelled with ³²P using random primers.

Hybridization with ³²P-Labelled Cloned DNA Probe. A dot-blotted nylon membrane was prehybridized with hybridization buffer (5 x SSPE, 40 % formamide, 1 % SDS, 3 x Denhardt's solution, 100 µg/ml alkali-denatured sonicated salmon sperm DNA) for 2 h. The membrane was hybridized at 37 °C for 18 h with the same buffer containing 5 ng/ml of the DNA probe labelled with ³²P. The membrane was washed three times with 2 x SSPE (containing 1 % SDS) for 5 min at room temperature, then incubated at 60 °C for 1 h, rinsed in 2 x SSPE, dried at room temperature and finally, exposed to x-ray film at -70 °C by fluorography.

Synthesis of Alkaline Phosphatase-Conjugated Probes. There are several evolutionarily conserved nucleotide sequences among LT (37) and cholera toxin (CT) (39–41) gene families. We expected DNA sequences that code for relatively common amino acid sequences to be useful in the detection of LT genes in many strains isolated from a wide variety of sources, since heterogeneity may be unexpected, for the most part, in these regions compared to the rest of the gene. We also expected heterologous DNA sequences between LT and CT genes to be useful for differentiating CT and LT genes. Thus, we selected DNA sequences for probes that are highly heterologous in DNA sequences between LT and CT genes but code for relatively common amino acid sequences between both toxins. We chose three sense-strand sequences (the same nucleotide sequence as mRNA except for the thymine base) in such regions for synthesis of the LT oligonucleotide probes (Table 1, probes A-1, A-2 and B-1). Alternatively, we also prepared antisense-strand probes (A-3 and B-2), which may be transcribed into m-RNA, at the same position of A-2 and B1, respectively.

Table 1: Nucleotide sequence selected for LT probes. Probes A-1, A-2 and A-3 are for subunit A gene, and B-1 and B-2 are for subunit B gene of heat-labile enterotoxin of enterotoxigenic *Escherichia coli*. Probes A-3 and B-2 are antisense sequences of A-2 and B-1, respectively. X represents 5-position linker-armed deoxyuridine, which is covalently attached to ALP through 12 atoms spacer.

A-1	5'-ACCGT CGT GCT GAC XCT AGA CCCAC-3'
A-2	5'-AACAGG GAA XAC AGA GACCGG TAT-3'
A-3	5'-ATA CCG GTC TCX GTA TTCC CTGTT-3'
B-1	5'-G GAC ACA TXA AGA ATC ACA TAT CT-3'
B-2	5'-AG ATA TGT GAT TCX TAA TGT GTCC-3'

Results

Immunological and Hybridization Tests with the Radioisotope-Labelled DNA Probe for *Escherichia coli* Isolates. To determine whether the test isolates not only produced LT but harbored the LT gene, immunological detection of LT (Biken test) (4, 5) and a dot-hybridization test with a ³²P-labelled DNA probe, respectively, were performed (Figure 1). Forty-six of the 100 test strains, isolated from patients with travellers' diarrhea, were positive in both dot-hybridization (Figure 1A) and Biken test (Figure 1B). The remaining 54 strains were negative in both tests.

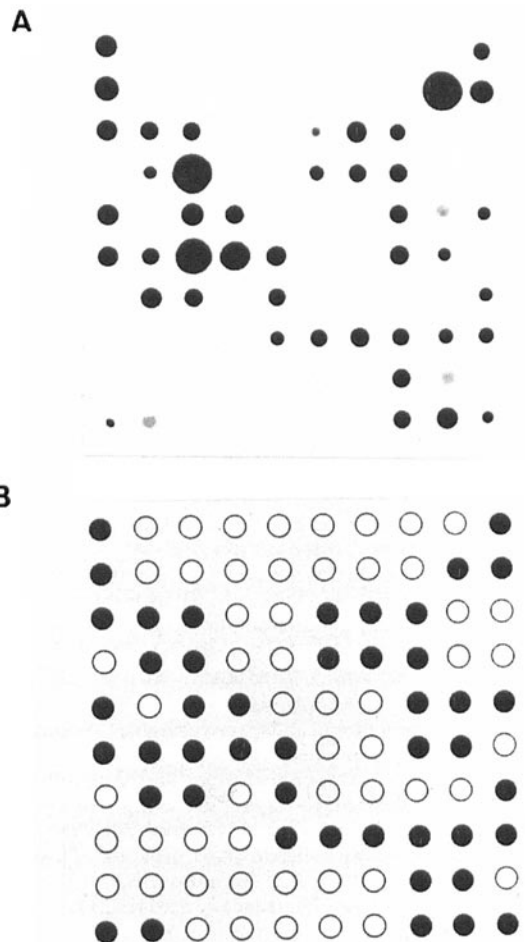


Figure 1: Results of dot-hybridization of *Escherichia coli* DNA with ³²P-labelled LT gene fragment (A) and results of the Biken test (B). LT-positive (●) and LT-negative (○) strains in Biken test (B) are present in the corresponding position in dot-hybridization (A).

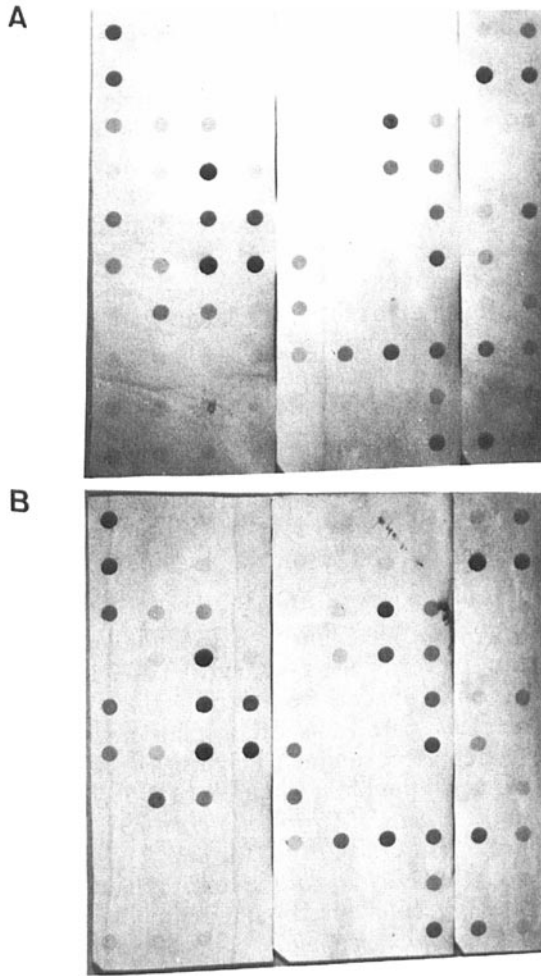


Figure 2: Dot-hybridization test of *Escherichia coli* DNA with alkaline phosphatase-conjugated oligonucleotide probe. LT-A probe (A-3) (A) and LT-B probe (B-2) (B) were used.

Dot-Hybridization with Alkaline Phosphatase-Conjugated Probes. We tested the five aforementioned probes for dot-blot hybridization of ETEC nucleic acids. Six ETEC strains confirmed to be

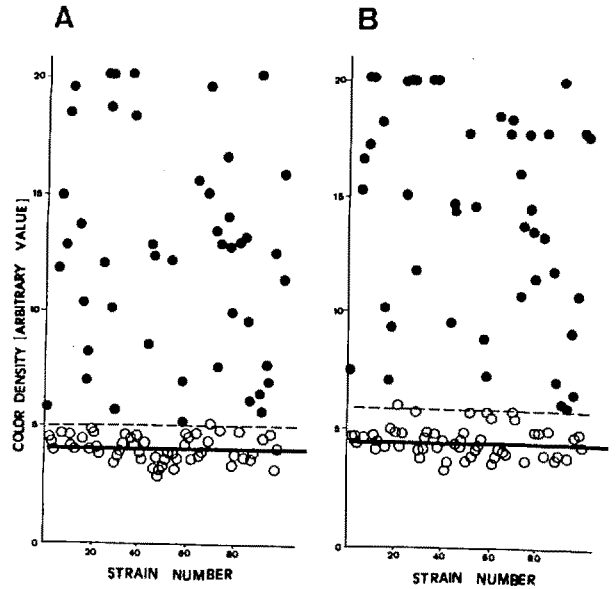


Figure 3: Densitometric measurement of dot-hybridization with alkaline phosphatase-conjugated oligonucleotide. Color densities of dots in Figure 2 were measured with a chromometer. Closed and open circles represent positive and negative strains, respectively, in hybridization with ³²P-DNA and the Biken test. Solid lines represent mean color densities of negative strains. Dotted lines represent cut-off values (mean + 2 SD). The arbitrary values are NBS units as described in Materials and Methods.

LT producers by ELISA were tested for the probes. Four strains were positive with all of the probes, but two strains were negative with any probes when nucleic acids were denatured with alkali. However, when the nucleic acids were acid-denatured, all strains were positive when A-3 and B-2 antisense probes were used. Thus, in the studies to follow, we used probes A-3 and B-2. As A-3 and B-2 are antisense sequence probes, hybridization between the DNA probes and m-RNA is also expected as DNA-DNA hybridization. Thus, acid denaturation of dotted

Table 2: Comparison of ALP-conjugated probes and a ³²P-labelled DNA probe for detection of enterotoxigenic *Escherichia coli* by the dot hybridization test. Identical results were obtained by an immunological test (Biken test).

³² P-labelled DNA probe	No. of strains			
	ALP-conjugated LT-A Probe (A-1)		ALP-conjugated LT-B Probe (B-2)	
	Positive	Negative	Positive	Negative
Positive	46	0	46	0
Negative	1	53	1	53

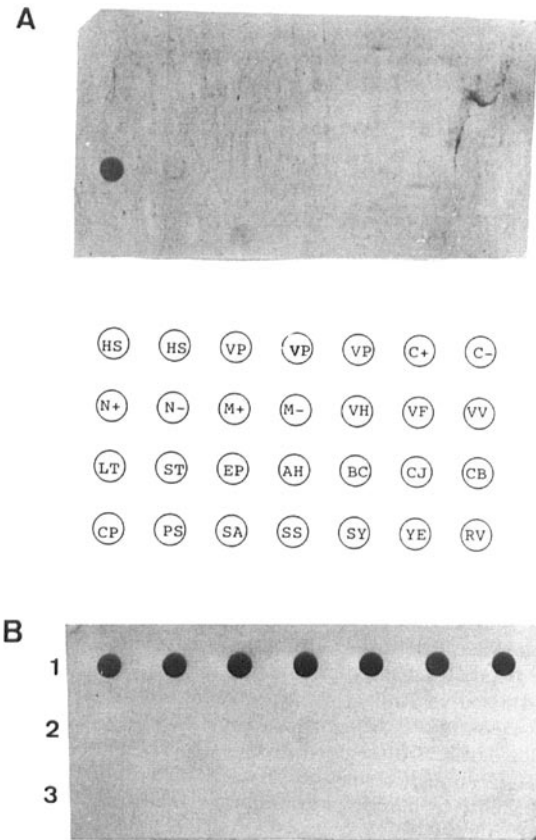


Figure 4: Cross-hybridization test with enteropathogenic bacteria other than ETEC (A) and cholera toxin-producing and non-producing *Vibrio cholerae* (B). Probe was a mixture of LT_A (A-3) and LT_B (B-2). Nucleic acids were extracted from human stool (HS), *Vibrio parahaemolyticus* (VP), cholera toxin (CT)-positive *Vibrio cholerae* (C+), cholera toxin-negative *Vibrio cholerae* non-O1 (C-), thermostable direct hemolysin (TDH)-like hemolysin-positive *Vibrio cholerae* non-O1 (N+), TDH-like hemolysin-negative *Vibrio cholerae* non-O1 (N-), TDH-like hemolysin-positive *Vibrio mimicus* (M+), TDH-like hemolysin-negative *Vibrio mimicus* (M-), *Vibrio hollisae* (VH), *Vibrio fluvialis* (VF), *Vibrio vulnificus* (VV), LT-positive ETEC (LT), ST-positive ETEC (ST), enteropathogenic *Escherichia coli* (EP), *Aeromonas hydrophila* (AH), *Bacillus cereus* (BC), *Campylobacter jejuni* (CJ), *Clostridium botulinum* (CB), *Clostridium perfringens* (CP), *Plesiomonas shigelloides* (PS), *Staphylococcus aureus* (SA), *Shigella sonnei* (SS), *Salmonella typhi* (SY), *Yersinia enterocolitica* (YE) and Rotavirus (RV). B: Row 1, LT-positive *Escherichia coli* strains; row 2, CT-negative *Vibrio cholerae* non-O1 strains; row 3, CT-positive *Vibrio cholerae* O1 strain. CT production was detected by ELISA as described previously (6).

nucleic acids on nylon membranes, which may not destroy m-RNA, was employed instead of alkali denaturation of nucleic acids. Results of dot-blot

hybridization with probes A-3 and B-2 are shown in Figure 2.

Figure 3 shows the distribution of the color densities of the deposits measured by the chromometer. Cut-off values were set at the mean value + 2 SD of the color density obtained with ³²P-labelled DNA probe negative strains. Those values were 5.0 and 6.0 for the A-3 and B-2 probes, respectively. *Escherichia coli* isolates with values lower than the cut-off values were all negative for the LT gene using the radioisotope-labelled DNA probe and the Biken test. Results are summarized in Table 2. The LT-A probe had a sensitivity of 100 %, a specificity of 98.1 %, a positive predictive value of 97.8 % and a negative value of 100 %. The LT-B probe had a sensitivity of 100 %, a specificity of 98.1 %, a positive predictive value of 97.8 % and a negative value of 100 %.

Specificity of Alkaline Phosphate-Conjugated Probes. To verify the specificity of A-3 and B-2 probes, nucleic acids from various bacterial diarrhoeagenic pathogens other than *Escherichia coli* were also examined (Figure 4). These included *Vibrio parahaemolyticus*, *Vibrio hollisae*, *Vibrio mimicus*, *Vibrio cholerae* O1, *Vibrio cholerae* non-O1, *Vibrio fluvialis*, *Vibrio vulnificus*, *Escherichia coli*, enterotoxigenic (ST+) *Escherichia coli*, enteropathogenic *Escherichia coli*, *Campylobacter jejuni*, *Shigella sonnei*, *Salmonella typhi*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Bacillus cereus*, *Clostridium perfringens* and *Clostridium botulinum*. No cross-hybridization was observed with these particular bacteria (Figure 4A). This indicates that the LT-A (A-3) and LT-B (B-2) probes were highly specific, hybridizing only with nucleic acid from LT-producing ETEC. LT has about 80 % homology in amino acid sequences with cholera toxin (39–41). We tested nucleic acids from six cholera toxin producing *Vibrio cholerae* O1 strains to determine if they cross-hybridized with the A-3 and B-2 probes. Figure 4B shows that neither the LT-A nor the LT-B probe cross-hybridized with the *Vibrio cholerae* strains examined.

Discussion

In a comparison of three methods for detection of LT-producing ETEC, the Biken test, a ³²P-labelled cloned DNA probe (Figure 1) and ALP-labelled synthetic oligonucleotide probes (Figure

2), the last one gave good sensitivity and specificity (Figures 2 and 3) and showed no cross-reactivity with the various other diarrhoeagenic bacteria tested (Figure 4A). Furthermore, the probes were not reactive even with cholera toxin producing *Vibrio cholerae* strains, indicating that the two probes were highly specific for LT (Figure 4B). These specificities may be because the A-3 and B-2 probes were designed to have seven mismatches in 24 base pairs with the cholera toxin gene (39–41). Seven nucleotide mismatches decrease more than 30 °C of melting temperature. This must have caused selective hybridization at high stringent temperature. Such high specificities of the ALP-labelled synthetic oligonucleotide probes, which have the two selected sequences, suggest that they may be useful for detection of LT-producing ETEC.

Some workers (10, 42, 43), however, claim that enzyme-linked oligoprobes are not as sensitive as radioisotope-labelled oligonucleotide probes. Specificities of the enzyme-linked oligoprobes examined here were strongly affected by the sequences used; such effects were not as apparent with the ³²P-labelled oligonucleotide (data not shown). This might cause interference of large enzyme molecules on the combined oligonucleotide probes. Thus, DNA sequence selection and the linked position of nucleotide to enzymes may be important for sensitivity and specificity of the probes.

Non-isotopic detection of genes with poly- or oligonucleotide probes was developed as an easier and safer alternative. The use of biotin-labelled restriction DNA fragments might solve this problem, but the cost for fragment preparation and enzymatic or chemical labelling is expensive (44). Alternatively, the oligonucleotide probe can be synthesized automatically, prepared in large quantities (45), and any part of the sequence may be chosen for the probe. Thus, the non-radioactively labelled oligonucleotide probe is superior to other currently available methods in many respects. Detection of ETEC by this type of probe would be a most practical tool for clinical diagnostic laboratories.

Several methods have been developed to determine DNA hybridization quantitatively by immobilizing DNA on discs of nitrocellulose membrane (46), on latex particles (47) or in microtiter wells (48). In these methods, optical densities of soluble products in test tubes or microtiter wells were measured after enzymatic reaction. However, they require segmented hybridization in tubes or

wells. Instead, dot-spotted membranes can be hybridized with probes in mass and washed easily, and a separate enzymatic reaction is unnecessary. We demonstrated that determination by a color difference meter of color deposits of dot-hybridization yielded more reliable and objective results. The color difference meter used in this study was accurate, portable and relatively inexpensive. This may lead to automated evaluation of genetic markers in DNA hybridization tests on membrane.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (No. 03304030) and a Grant-in-Aid for Developmental Scientific Research from the Ministry of Education, Science and Culture of Japan.

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