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Comparative Study of Colony Hybridization with Synthetic Oligonucleotide Probes and Enzyme-Linked Immunosorbent Assay for Identification of Enterotoxigenic *Escherichia coli*

HALVOR SOMMERFELT,[1]* ANN-MARI SVENNERHOLM,[2] KARL HENNING KALLAND,[3] BJØRN-IVAR HAUKANES,[3] AND BJARNE BJORVATN[1]

Section for Medicine of Developing Countries, Medical Department B, University of Bergen, Haukeland Hospital, 5021 Bergen,[1] and Laboratory of Biotechnology, University of Bergen, 5001 Bergen,[3] Norway, and Department of Medical Microbiology, University of Gøteborg, 413 46 Gøteborg, Sweden[2]

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On the basis of the published nucleotide sequences of the genes that code for the heat-labile toxin LTh and the heat-stable toxins STaI and STaII of human enterotoxigenic *Escherichia coli*, a 34-mer and two 33-mer oligonucleotide probes were synthesized. To compare their relative efficacies in the detection and differentiation of enterotoxigenic *E. coli*, a colony hybridization technique using these probes and a GM1 ganglioside enzyme-linked immunosorbent assay using monoclonal anti-LT and anti-ST antibodies were used with 76 strains of *E. coli* with known enterotoxin profiles. For further evaluation of probe specificity, the enterotoxigenic bacteria *Vibrio cholerae* O1 and non-O1 and *Yersinia enterocolitica* were examined with the colony hybridization technique. The sensitivity of colony hybridization compared favorably with that of GM1 ganglioside enzyme-linked immunosorbent assay, and the two assays showed a high level of concordance in specific detection and differentiation of *E. coli* with various enterotoxin profiles (kappa = 0.906, $P < 0.00001$). The probes did not hybridize with DNAs from strains of *V. cholerae* O1 or non-O1 or *Y. enterocolitica*.

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrheal disease among humans and young animals (17). *E. coli* enterotoxins are classified into two main groups, heat-stable toxins (ST) and heat-labile toxins (LT). The ST include two classes, STa (STI) and STb (STII) (3). The STa class consists of two chemically similar toxins, the STaI (STIa or STp) and the STaII (STIb or STh). STaI is produced by both human and porcine isolates of ETEC, whereas STaII is produced by human isolates only (19, 32). The other class of ST, termed STb (STII), is only rarely produced by ETEC strains isolated from humans (7). Compared with ST, LT are homogeneous with regard to gene structure, DNA sequence, and biochemical properties (14, 32). Furthermore, LT share important structural and functional features with cholera toxin (4, 25, 33). Two LT of ETEC have been characterized genetically (5, 14, 32, 34) as well as immunologically (2, 29). LTh is found exclusively in ETEC of human origin, whereas LTp is found in isolates from animals only (32).

Methods for accurate detection and classification of ETEC are essential for studies on the etiology and epidemiology of diarrheal disease. Furthermore, such methods seem indispensable in the evaluation of future vaccination trials against ETEC-induced diarrhea. Numerous methods for ETEC detection have been described. Of these, GM1 ganglioside enzyme-linked immunosorbent assays (GM1-ELISAs) and detection of the plasmid-borne toxin genes using gene probes have proven most valid. We describe here a comparison of GM1-ELISA, using monoclonal antibodies, and colony hybridization, using three newly constructed oligonucleotide probes, for detecting ETEC and determining their enterotoxin profiles. Furthermore, we evaluate the specificity of the probes by using a panel of other enterotoxigenic bacteria.

* Corresponding author.

MATERIALS AND METHODS

Construction and synthesis of oligonucleotide probes. The required information on nucleotide sequences was obtained from the publications of So and McCarthy (22), Moseley et al. (19), and Yamamoto and Yokota (34). Each of the three DNA probes (STaI [5′ GAA CTT TGT TGT AAT CCT GCC TGT GCT GGA TGT 3′], STaII [5′ GAA TTG TGT TGT AAT CCT GCT TGT ACC GGG TGC 3′], and LT [5′ A CGT TCC GGA GGT CTT ATG CCC AGA GGG CAT AAT 3′]) was constructed to give 100% homology with a region of the corresponding gene and at the same time the highest possible number of nucleotide mismatches as compared with other sequenced genes. The oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems 380A) by using the phosphite triester coupling method (R. I. Letsinger, J. L. Finnan, G. A. Hearner, and N. B. Lunsford, Letter, J. Am. Chem. Soc., **97**:3278–3279, 1975) and subsequently purified by reverse-phase high-pressure liquid chromatography with a C18 column.

The duplexes formed by oligonucleotide probes and their target DNAs dissociate at a given temperature, which can be estimated as described by Lathe (13). Thus, at a monovalent cation concentration of 0.6 M, the dissociation temperatures for the STaI, STaII, and LT probes were calculated to be 71.4, 72.7, and 75.2°C, respectively.

Bacterial strains. The study comparing colony hybridization with GM1-ELISA included 71 human and 5 porcine isolates of *E. coli*, all with toxin profiles established by reference techniques. ST production was determined with the suckling mouse assay (9). LT production was assessed by the Chinese hamster ovary cell assay (11) and GM1-ELISA using anti-LT antibodies (27). Of the human strains, 48 were kindly provided by Y. Takeda, University of Tokyo, 8 were from I. Adlerberth, Department of Clinical Immunol-

ogy, University of Gøteborg, and 15 were isolated during an epidemiological study in Dacca, Bangladesh (10). Of the 71 human *E. coli* strains, 25 produced ST only (ST^+ LT^-), 15 produced LT only (ST^- LT^+), 9 produced both ST and LT (ST^+ ST^+), and 22 produced neither ST nor LT (ST^- LT^-). The five porcine strains were originally provided by J. A. Morris, Weybridge, Great Britain. These strains all produced ST, including three strains that also produced LTp, as shown by LTp-specific monoclonal antibodies (29).

For further evaluating the specificity of the LT probe, we included three *Vibrio cholerae* O1 strains: NCTC 8021 (Ogawa, classical), NCTC 3661 (Inaba, El Tor) (courtesy of J. Lassen, National Institute of Public Health, Oslo, Norway), and 569 B (Inaba, classical). Because *Yersinia enterocolitica* and *V. cholerae* non-O1 strains have been found to produce ST with structural and functional similarities to *E. coli* STa (30, 31), 12 strains of *Y. enterocolitica*, of which 9 were positive in the infant mouse assay, and 2 infant mouse assay-positive *V. cholerae* non-O1 strains, all isolated from humans with diarrhea, were included in the evaluation of the ST probes.

Cultivation of bacteria and preparation of nitrocellulose membranes. Initially, all of the strains were grown at 37°C overnight on agar plates: *E. coli* and *Y. enterocolitica* on MacConkey agar and *V. cholerae* O1 and non-O1 strains on blood agar. Throughout the study, after coding, three typical colonies from each of the *E. coli* strains were examined in parallel by GM1-ELISA and colony hybridization. Thus, from each bacterial sample, three colonies were inoculated into three separate GM1-coated, bovine serum albumin-blocked wells of microtiter plates containing 100 μl of CAYE medium with 45 μg of lincomycin per ml and 2.5 mg of glucose per ml for culture at 37°C overnight on a rotating platform (24). To ensure comparable results between the GM1-ELISA and the hybridization assay, remaining material from these three colonies was inoculated in triplicate onto three nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.) overlying fresh MacConkey agar, one membrane for each of the above-described gene probes. Up to 60 colonies (representing 20 strains) were inoculated onto each of the membranes, which were then incubated at 37°C for 5 h. The membranes were then removed from the agar, the bacterial cells were lysed, and the DNA was denatured and fixed to the membranes essentially as described by Moseley et al. (20).

To evaluate the specificities of the probes, three colonies from each of the three *V. cholerae* O1 and the two non-O1 strains were inoculated on nitrocellulose membranes as described above, along with the 76 strains of *E. coli*. Similarly, the 12 strains of *Y. enterocolitica* were incubated at 25°C for 18.5 h before lysis and DNA denaturation.

After GM1-ELISA assessment of enterotoxin production, an independent scientist selected 11 of the coded *E. coli* strains for standardization of washing conditions in the hybridization assay. Of these strains, four were ST^+ LT^-, two were ST^- LT^+, and one, a porcine strain, was ST^+ LT^+; the remaining four strains were toxin negative. Each strain was prepared in triplicate for hybridization as described above.

GM1-ELISA for detection of enterotoxins. Detection of LT and ST from the same cultures was performed as described elsewhere (24, 26, 28).

DNA colony hybridization. The gene probes were radioactively 5′ end labeled with ^{32}P by using T4 polynucleotide kinase as described by Maniatis et al. (15). The specific activity was approximately 10^9 cpm/μg of probe, as determined by the trichloracetic acid precipitation method (15). Hybridization was performed as described by Maniatis et al. (15), with the following modifications. In the prehybridization and hybridization solutions, formamide was replaced by H_2O. Washing was performed at room temperature in 0.45 M sodium chloride plus 0.045 M sodium citrate (3× SSC), giving a monovalent cation concentration of 0.6 M, and subsequent stringent washing was in 3× SSC plus 0.1% sodium dodecyl sulfate at the temperatures defined below. The concentrations of radioactive probe varied from 2.4×10^5 to 1.4×10^6 cpm/ml of hybridization fluid, depending on the probe and the time after labeling.

TABLE 1. Parallel examination of 76 *E. coli* strains by GM1-ELISA and oligonucleotide probes[a]

Phenotype determined by GM1-ELISA	No. of strains determined by hybridization with oligonucleotide probes to be:			
	ST^+ LT^-	ST^- LT^+	ST^+ LT^+	ST^- LT^-
ST^+ LT^-	20	0	2	1
ST^- LT^+	0	14	1	0
ST^+ LT^+	0	1	6	0
ST^- LT^-	0	0	0	31

[a] Original designations showed that 27 of the strains were ST^+ LT^-, 15 were ST^- LT^+, 12 were ST^+ LT^+, and 22 were ST^- LT^-. In the hybridization assay, ST included STaI, STaII, or both. kappa = 0.906, $P < 0.00001$.

Standardization of hybridization and washing conditions for the three oligonucleotide probes. The DNAs of the 11 selected strains immobilized on the nitrocellulose membranes were used to assess the optimal temperatures of stringent washing for each probe. Starting at 54°C, washing was performed at stepwise-increasing temperatures. For the STaI, STaII, and LT probes, the temperatures giving minimal background and no false-negative readings were 65, 65, and 56°C, respectively.

Interpreting the test results of GM1-ELISA and colony hybridization. The cutoff values for the GM1-ELISA were determined as described elsewhere (27, 28). Before GM1-ELISA and genetic probing were performed, growth in the culture medium and on the nitrocellulose membranes, respectively, was registered. The following criteria for interpretation were defined in advance. A strain would be considered toxin negative if two or three colonies showed adequate growth and the test results for these were negative. If at least one of the colonies gave a positive test result, the strain would be considered toxin positive, regardless of growth pattern. If two of the colonies did not show adequate growth and the third gave a negative test result, both assays would be repeated. The results of the GM1-ELISA and the hybridization assay were recorded before breaking of the reference code.

Statistical procedure. The kappa statistic (21) was calculated to quantify the concordance between the results of GM1-ELISA and colony hybridization.

RESULTS

Parallel detection of toxin production by GM1-ELISA and toxin genes by colony hybridization. Parallel analyses of the 76 *E. coli* strains (71 human and 5 porcine) showed a high level of concordance between the two methods with regard to identification and enterotoxin profiles (Table 1). GM1-ELISA and colony hybridization gave discordant results in the analyses of five strains. Two strains originally reported

TABLE 2. Comparison of GM1-ELISA and oligonucleotide probes for detection of toxins and toxin genes, respectively, of ETEC

Assay	% Proficiency		
	Sensitivity	Specificity	Accuracy for positive prediction
ST GM1-ELISA	100 (28/28)	97 (34/35)	97 (28/29)
ST[a] hybridization	100 (28/28)	97 (34/35)	97 (28/29)
LT GM1-ELISA	91 (19/21)	100 (42/42)	100 (19/19)
LT hybridization	100 (21/21)	100 (42/42)	100 (21/21)

[a] STaI, STaII, or both.

to produce ST and LT were positive in the ST GM1-ELISA and negative in the LT GM1-ELISA but positive in the ST and LT hybridization assays. One strain (Takeda 136), originally designated to produce only LT, was negative in the LT hybridization assay and the LT GM1-ELISA and negative in the ST hybridization assay but positive in the ST GM1-ELISA. Another of the originally ST^- LT^+ strains produced ST, as well as LT, in the GM1-ELISA, whereas only the LT probe showed positive hybridization. Finally, one of the strains, also reported to produce only LT, was positive in the LT GM1-ELISA and negative in the ST GM1-ELISA, whereas the hybridization assay was positive with the LT and STaI probes.

Either the GM1-ELISA or the colony hybridization assay was in agreement with the originally reported toxin profile for 63 of the 76 strains tested.

Of the 27 strains reported to produce only ST, 7 were negative in the ST GM1-ELISA, as well as in the colony hybridization assay, with either of the two ST probes. Similarly, 4 of the 12 strains reported to be ST^+ LT^+ showed a concurrent lack of toxin production and apparent absence of toxin genes in the colonies tested. Three strains, of which two were porcine isolates, were positive only for LT, and one was negative with regard to ST and LT in the GM1-ELISA, as well as in the hybridization assay. Of the 15 originally ST^- LT^+ strains, 2 (247250-3 and Takeda 136) were negative in both LT assays. Altogether, nine of the originally toxigenic strains were found to be toxin negative in both assays, whereas three originally ST^+ LT^+ strains were positive only for LT in the two parallel tests. However, when these 12 discordant strains were reanalyzed, along with concordant controls, by the reference techniques, the results of the GM1-ELISA and the hybridization assay were confirmed. For strain Takeda 136, the repeated reference assays confirmed the hybridization results. All of these 13 discordant strains were excluded when the test proficiencies of the GM1-ELISA and the hybridization assay were calculated and compared (Table 2).

In the ST hybridization, 21 strains were positive for STaII, 7 were positive for STaI, and only 1 (H10407) was positive for both types of STa. The three ST-producing porcine strains were all positive in the hybridization assay with the STaI probe and negative with the STaII probe.

Specificity of oligonucleotide probes. The 3 *V. cholerae* O1, the 2 *V. cholerae* non-O1, and the 12 *Y. enterocolitica* strains were negative when tested in the hybridization assay, regardless of the probe used. The three *E. coli* strains of porcine origin reported to produce LTp all hybridized with the LT gene probe. An attempt to differentiate between LTh- and LTp-producing strains by increasing the stringency of washing proved unsuccessful.

DISCUSSION

Parallel examination of 76 reference strains of *E. coli* with oligonucleotide probes and GM1-ELISA showed a correspondence in the ability to identify and differentiate toxigenicity (kappa = 0.906 [a kappa value of >0.75 indicates excellent correspondence]; $P < 0.00001$ for H_0 = kappa = 0 versus H_1 = kappa > 0) (21). However, on several occasions, these results were not in accordance with the original designations of the strains. Thus, 13 of the 54 strains reported to be toxigenic were negative in both the GM1-ELISA and the hybridization assay for one or both of the relevant toxins and toxin genes. However, these results were confirmed upon reexamination with the reference techniques, and the discordant strains were omitted when the proficiencies of the two assays were evaluated. Previous studies have shown that, during subcultivation, ETEC may loose genes that code for important virulence factors (1, 6, 12). Three strains that, upon subsequent subculture, yielded toxin-positive, as well as toxin-negative, clones were selected for a study of the mechanisms underlying concurrent loss of hybridization signals and toxin production. Examination of their plasmids and plasmid *PstI* endonuclease digests by Southern transfer hybridization revealed no loss of toxin gene-carrying plasmids. Instead, deletions of small DNA fragments harboring the relevant toxin genes were demonstrated (manuscript in preparation). Deletions of DNA fragments, as well as loss of entire plasmids carrying the toxin genes, have been described previously (6). Accordingly, gene loss seems to be a likely explanation for the observed discrepancy.

Inconsistency between the results of the hybridization assay and the GM1-ELISA occurred with five strains. Two of the originally ST^+ LT^+ strains were found by GM1-ELISA to produce only ST, whereas the hybridization assay indicated the presence of both ST and LT genes. The results of the LT GM1-ELISA were borderline for both strains. Accordingly, the findings were interpreted as falsely negative. One strain (Takeda 136), reported to produce only LT, was negative in the hybridization assay with all three probes and negative in the LT GM1-ELISA but positive in the ST GM1-ELISA. Another strain originally designated to produce only LT, although positive only for the LT probe, was shown to produce LT, as well as ST, in the GM1-ELISA. The results of the ST GM1-ELISA were borderline and were therefore interpreted as falsely positive. Finally, one of the strains reported to produce only LT was positive only for LT in the GM1-ELISA while positive for LT and STaI in the hybridization assay. This finding can be explained by the presence of an unexpressed STaI gene. Isolates of *E. coli* that are positive in hybridization experiments using oligonucleotide probes but do not produce detectable enterotoxin have been described previously (8).

Compared with other oligonucleotides used for detecting ETEC (8, 12; R. D. Smith, C. M. Trepod, and E. Tu, Fed. Proc. **44**:527, 1985), our relatively long ST probes (33-mer) and LT probe (34-mer) minimize chances of nonspecific hybridization. According to recent literature (19, 22) and computer-based information (*EMBL Nucleotide Sequence Data Library; release 6.0*), the STaI and STaII probes have seven mismatches as compared with the gene sequences that code for STaII and STaI, respectively. Similarly, the LT probe has nine mismatches as compared with the corresponding nucleotide sequence of the closely related, so-called *ctxA* gene of *V. cholerae* (16, 34) and eight mismatches as compared with the reported nucleotide sequence

of the so-called *eltA* gene of LTp (23, 34). It was therefore surprising that this probe was unable to distinguish LTh-producing from LTp-producing strains, even at high stringency. However, a newly published nucleotide sequence of LTp (32) differs from that previously described by Spicer and Noble (23) in the relevant region. According to the newly published sequence (32), the LT probe of the present study has no mismatches as compared with the LTp gene.

Strain H10407 was the only strain that gave a positive result with both ST probes. Other workers have found this strain to possess genes that code for STaI as well as STaII (12, 35). The DNA prepared from the three porcine strains that were positive in the ST hybridization assay all hybridized with the STaI probe only. Keeping in mind that porcine strains have not been reported to contain genes coding for STaII (32), the findings of this study suggest that the two ST probes described hybridize specifically with their relevant genes.

None of the three *V. cholerae* O1 strains, the two *V. cholerae* non-O1 strains, or the nine toxigenic strains of *Y. enterocolitica* were positive in any of the hybridization assays. This shows that the probes of the present study differentiate ETEC genes from the related toxin genes of these bacteria.

The advantages of GM1-ELISA over the hybridization assay is that no advanced equipment is required for qualitative tests (24). Furthermore, for analysis of limited numbers of bacteria, it is quicker. With LTp-specific monoclonal antibodies, strains producing LTp can be differentiated from those producing LTh (29), a capability of practical epidemiological importance for examination of environmental sources of diarrheal pathogens. However, in contrast to the hybridization assay, the available GM1-ELISA cannot differentiate between STaI and STaII, considered to be important epidemiological markers (18). In epidemiological studies, in which it is convenient to analyze large numbers of isolates simultaneously, the hybridization technique has obvious advantages. Hybridization assays have been successful in detecting and differentiating ETEC in stool spotted onto nitrocellulose membranes (8, 18, 20). Furthermore, each nitrocellulose membrane is capable of harboring DNA representing up to 60 samples. The membranes may be stored for several months at room temperature (12) and sent by ordinary mail to the relevant laboratory. Three parallel membranes, as used in the present study, make it possible to perform hybridization with three probes simultaneously. Combining these advantages with the developing nonradioactive labeling techniques should provide the simple and accurate tool necessary for large-scale epidemiological studies.

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