Simplified and Accurate Nonradioactive Polynucleotide Gene Probe Assay for Identification of Enterotoxigenic Escherichia coli

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Received 4 May 1989/Accepted 25 September 1989

The present study describes a colony hybridization setup for identification of enterotoxigenic Escherichia coli obviating the need for advanced equipment and radioactive isotopes. With a modest laboratory arrangement, polynucleotide gene probes were produced in large quantities. The probes were labeled with digoxigenin and, after hybridization, detected with an antidigoxigenin alkaline phosphatase conjugate. With an established isotope-based oligonucleotide hybridization assay as reference, a blinded study on a large battery of enterotoxigenic and nonenterotoxigenic bacteria revealed a satisfactory sensitivity and specificity of the nonradioactive assay.

Enterotoxigenic Escherichia coli (ETEC) is among the most important microbial causes of childhood diarrhea in developing countries (1, 13). Thus, methods for accurate detection and classification of ETEC are essential for clinical and epidemiological studies of diarrhea. The three E. coli enterotoxins most commonly encountered in human disease are the two heat-stable enterotoxins STaII (STh) and STaI (STp) and the human heat-labile enterotoxin LTh. LTh shares important structural and functional features with cholera toxin (3, 25, 28), while Yersinia enterocolitica and Vibrio cholerae non-O1 have been found to produce enterotoxins similar to E. coli STa (26, 27). The plasmid-borne genes encoding STaII, STaI, and LTh have been cloned and sequenced (4, 15, 28), thereby enabling the production of polynucleotide (14, 22) as well as oligonucleotide (16, 23) diagnostic gene probes. Compared with standard bioassays for identifying ETEC, the efficiency of colony hybridization has been satisfactorily (14, 16, 22, 23). DNA hybridization assays are well suited for large-scale epidemiological studies (1, 2). However, laboratories in developing countries often lack advanced equipment such as ultracentrifuge and certain reagents, e.g., radionucleotides, that are traditionally used in the production and utilization of diagnostic gene probes.

Hybridization assays with nonradioactive ETEC probes have been described earlier (10, 12, 17–20). Tests with alkaline phosphatase-conjugated oligonucleotide ETEC probes show considerable variation with regard to sensitivity and specificity (12, 17–20). These tests are mostly supplied in kits that include expensive reagents such as proteinase K. One of the assays did not require proteinase K but was only applicable for the detection of STaII (17). Kirri et al. (10) have shown that biotinylated probes effectively identify LT-producing ETEC strains. However, only a limited number of STa-producing strains were examined, revealing a suboptimal sensitivity (85%) of the STaI probe. Moreover, treatment of the lysed colonies with proteinase K was essential to avoid nonspecific binding of streptavidin.

Colony hybridization assays yield some degree of background signals. As four of these studies (10, 17, 19, 20) were not performed blindly, the specificities of the assays therefore seem somewhat difficult to evaluate.

We developed a simplified and accurate colony hybridization setup for detecting and classifying ETEC. The technique obviates ultracentrifugation, radionucleotides, and proteinase K. To evaluate the sensitivity and specificity of the test against an established oligonucleotide hybridization assay (22, 23), we examined a large battery of ETEC, the other enterotoxigenic bacteria V. cholerae O1, V. cholerae non-O1, and Y. enterocolitica, and nonenterotoxigenic E. coli. To avoid registration bias, the results were read by two independent scientists using a coded procedure.

MATERIALS AND METHODS

Probe preparation. pDAS100, pDAS101, and pEWD299 were constructed for STaII, STaI, and LT probe isolation, respectively, as described previously (22). To eliminate the ultracentrifugation on a cesium chloride gradient that otherwise is applied in large-scale preparations of plasmid DNA (8, 11, 22), we used the method of Frei et al. (8) simplified by Anders Fjose and Karl Henning Kalland at the Laboratory of Biotechnology, University of Bergen. Thus, after the plasmid preparations were treated with RNase (8), the DNAs were purified directly by phenol-chloroform extraction (11) followed by isopropanol precipitation with a 0.4 volume of 5 M NH4Cl, and 2 volumes of 100% isopropanol added to 1 volume of the plasmid-containing water phase. The resulting pellets were thereafter dissolved in appropriate volumes of TE (10 mM Tris hydrochloride, 0.1 mM EDTA, pH 7.5). The plasmid DNA was ethanol precipitated (11) and redissolved in TE before the yield was measured in a DNA fluorometer (Hoechst TK0100; Hoefer Scientific Instruments, San Francisco, Calif.). The STaII insert was cleaved from pDAS100 by using BamHI and HindIII, while pDAS101 and pEWD299 were digested with restriction endonucleases as described previously (22). The resulting DNA fragments (probe templates) were separated on ethidiun bromide-stained 1.5% agarose gels, trapped in DEAE membrane strips (NA45; Schleicher & Schuell, Inc., Keene, N.H.) as suggested by the manufacturer, and eluted in a solution of 1 M NaCl and 50 mM arginine (pH 9.0) at 70°C for 1.5 h.

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Probe labeling, hybridization, standardization of washing conditions, and immunological detection of digoxigenin-labeled probes. The oligonucleotide probes (StaI, 5'-GAA CTT TGT TGT AAT CCT GCC TGT GGA TGT-3'; StaII, 5'-GAA TTG TGT TGT AAT CCT GCT TGT ACC GGG TGC-3'; LT, 5'-A CTG TCC GGA GGT CTG ATG CCC AGA GGG CAT AAT-3') were produced and radiolabeled as previously described (23). Their hybridization and washing conditions were carefully calibrated to obtain optimal sensitivity and specificity. In short, using 3x SSC (1x SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate, the optimal temperatures of stringent washing for each probe were found to be 65, 65, and 56°C, respectively (23).

To optimize specificity, we individually calibrated the washing conditions of the StaII, StaI, and LT polynucleotide probes as described earlier (23), with one modification. Thus, after radiolabeling of the polynucleotide probes and hybridization (22), instead of adjusting the temperatures of stringent washing, the ionic strength was decreased stepwise from 5x SSC to 0.1x SSC with a constant temperature of 65°C. This approach was chosen because less well equipped laboratories often have only one water bath. For the StaII, StaI, and LT probes, the lowest monovalent ionic strengths giving satisfactory intensities of positive signals were achieved by using 3x SSC, 1x SSC, and 0.2x SSC, respectively, in the stringent washing solutions.

For the nonradioactive hybridization assay, the polynucleotide probes were labeled with digoxigenin in a random hexanucleotide-primed Klenow enzyme-mediated reaction (7), using a gene probe labeling and detection kit provided by Boehringer GmbH (catalog number 1093 657; Mannheim, Federal Republic of Germany) according to the enclosed instructions, with one modification. Scaling up reaction volumes by a factor of 2 to maximize the incorporation ratio of digoxigenin-dUTP, we added 600 ng of template DNA, thus anticipating 240 ng of digoxigenin-labeled probe. After the denatured target DNA immobilized on BA85/20 nitrocellulose membranes (NCMs) (Schleicher & Schuell) was submerged in a prehybridization solution containing 50% formamide at 42°C for 1.5 h, the labeled, denatured probes were added to a final concentration of 2.4 ng/ml. Hybridization was performed for 11 h at 42°C. After being washed, the digoxigenin-labeled probes were detected immunologically as described by the manufacturer, with one alteration. Even though the NCMs had been prehybridized in a solution containing the provided blocking reagent, it was found essential to perform a repeated blocking with the same substance before adding the antidigoxigenin alkaline phosphatase conjugate. Otherwise, a massive background was observed on the NCMs.

Examination of purified DNA. pDAS100, pDAS101, and pEWD299 were used as StaII, StaI, and LT target sequences, respectively. Decreasing concentrations of DNA, measured by DNA fluorometry, were examined with the radiolabeled oligonucleotides (23) and with the digoxigenin-labeled cloned probes along guidelines described previously (22). The optimized stringent washing conditions were used in both assays.

Bacterial strains. A battery of 147 human and 10 porcine *E. coli* isolates were examined in parallel by the nonradioactive colony hybridization assay (NCHA) and the oligonucleotide reference assay. The strains were selected from earlier performed studies on ETEC (1, 21, 22, 23).

For further evaluation of probe specificity, we included three *V. cholerae* O1 strains (courtesy of J. Lassen, National Institute of Public Health, Oslo), nine strains of *Y. enterocolitica*, of which eight were positive for StaI in the infant mouse assay (9), and two infant mouse-positive *V. cholerae* non-O1 strains. In addition, the nonotoxigenic *E. coli* G101, harboring pUC8, and HB101, harboring pBR313, the plasmid vectors of the cloned StaI and LT probes, respectively, were examined to exclude the possibility of probe contamination by vector DNA.

Colony hybridization. To avoid discrepancies caused by spontaneous loss of toxin genes (21, 23), we cultivated the same three clones of each bacterial strain on six parallel NCMs. Three membranes were used in the oligonucleotide StaII, StaI, and LT reference assay (23), and three were used in the corresponding NCHA. In repeated pretrials of the NCHA with low-stringency (22) as well as high-stringency washing conditions, a subset of the described bacterial strains, including G101 and HB101, were probed on nylon membranes (NY 13 N; Schleicher & Schuell) as well as on NCMs. When examining the main battery of test strains for evaluation of the NCHA, we included seven nonenterotoxigenic strains of *E. coli* as negative controls. Furthermore, on each NCM, four control strains were included, two colonies of H10407+ with toxin genes for StaII, StaI, and LT (23), a nonpathogenic strain of *E. coli*, as well as G101 and HB101. The results were recorded by the main investigator 2 and 4 h after initiation of the color reaction. After 20 h of development, the results were registered by the main investigator as well as by a scientist not otherwise involved in the study. Registration was performed before breaking the reference code. The previously defined criteria (22) for interpretation of colony hybridization assay results were applied, with modifications. Thus, a strain from which all the three examined clones showed inadequate growth would be excluded from the study. Furthermore, signals were only to be classified as positive or negative. A standardized form for registration of bacterial growth and hybridization results simplifies such interpretation (Fig. 1).

Rehybridization with 32P-labeled oligonucleotide probes. A subset of the study strains were first examined for StaII, StaI, and LT by the NCHA. Stripping the digoxigenin-labeled probes off their target was performed by rinsing the membranes in 0.1% sodium dodecyl sulfate at 95°C twice for 5 min each time. The target sequences were reexamined with the corresponding oligonucleotide toxin gene probes labeled with 32P.

RESULTS

Simplified production of cloned probes. The yields of pDAS100, pDAS101, and pEWD299 were 880, 2,600, and 1,100 μg, respectively, 1.5, 2.4, and 2.9 times greater than with the previously described standard procedure (22). The high yield could not be explained by increased contamination of chromosomal DNA (Fig. 2).

Detection limits of purified target DNA. In the examination of cloned toxin gene sequences, the oligonucleotides detected 98 pg of StaII, 24 pg of StaI, and 49 pg of LT plasmid DNA. After 2 h of color development, the corresponding figures for the nonradioactive hybridization assay were 391, 1,562, and 781 pg; after 4 h they were 195, 781, and 391 pg; and after 20 h they were 98, 195, and 195 pg, respectively.

Identification of ETEC by colony hybridization with digoxigenin-labeled polynucleotide probes. In the pretrials of the NCHA, nylon membranes yielded considerably higher background levels than did NCMs. The latter were therefore used in the study. Furthermore, low-stringency washing condi-
EcoRI and borderline trifugation with digested tive, suggesting on DNA lactose-fermenting colonies, not E. coli. Growth is for two NCMB, and LT probes, respectively. The signals obtained after color development (see Fig. 3A) or after autoradiography (see Fig. 3B and C) are registered under Hybrid. res. + is exemplified by Fig. 3, StAI, interception of column 5 and row B. ++ is exemplified by Fig. 3, StAI, interception of column 6 and row E.

FIG. 1. Standardized form for registration of bacterial growth and hybridization results simplifies the examination of fecal specimens for ETEC. The figure shows the first half of the form filled in after examination of 24 specimens (three colonies from each) using a set of three NCMB. Plate Growth is for recording an abbreviated description of colony appearance on MacConkey agar plates after overnight incubation of stool samples. E, Pure growth of E. coli suspect colonies; MCE, mixed culture dominated by E. coli suspect colonies; LF, lactose-fermenting colonies, not E. coli suspect. Col indicates column and Row indicates row; their interception defines points of inoculation on the NCMB. NCMa growth, NCMB growth, and NCMc growth are for recording the growth of colonies to be examined with the StAI (StTh), StAI (StP), and LT probes, respectively. The signals obtained after color development (see Fig. 3A) or after autoradiography (see Fig. 3B and C) are registered under Hybrid. res. + is exemplified by Fig. 3, StAI, interception of column 5 and row B. ++ is exemplified by Fig. 3, StAI, interception of column 6 and row E.

FIG. 2. Restriction enzyme-generated StAI (lanes 1 and 2), StAI (lanes 3 and 4), and LT (lanes 5 and 6) gene probe fragments (arrows) of pDAS100, pDAS101, and pEWD299, respectively, separated on ethidium bromide-stained 1.5% agarose gel. pDAS100 was digested with BamHI and HindIII, pDAS101 was digested with EcoRI and BamHI, and pEWD299 was digested with HindIII and EcoRI. DNA fragments of cesium chloride-purified plasmids (standard plasmid isolation procedure) were run in lanes 1, 3, and 5, while the corresponding fragments of plasmids isolated without ultracentrifugation (simplified procedure) were run in lanes 2, 4, and 6.
growth on the NCMs, classified as toxin gene negative in the oligonucleotide assay, was by the main investigator categorized as LT⁻, STaII⁻, and questionable, but probably negative, for STaI. On the other hand, the second observer considered it STaII⁻ STaI⁺ LT⁻, although only weakly positive for STaI. This was the only strain for which the two observers were hesitant to make a conclusive designation and the only strain for which they disagreed in the classifi-

![FIG. 3. Results of hybridization with digoxigenin-labeled polynucleotide (A) and 32P-labeled oligonucleotide (B) STaII, STaI, and LT gene probes. The vertical sequence of inoculation starts from the top of the second left column. Twenty-four test strains (three colonies from each) were examined along with five control colonies on the far left column (H10407+ harboring STaII, STaI, and LT genes; a nonenterotoxigenic strain of *E. coli*; HB101; G101; and H10407+). The autoradiograms in panel B were obtained after hybridization of NCMs inoculated with the same bacterial clones as those examined in the digoxigenin-labeled polynucleotide assay (A). Stripping the probes in 0.1% sodium dodecyl sulfate at 95°C from the membranes shown in panel A made the target sequences accessible to the corresponding oligonucleotide toxin gene probes (autoradiograms in panel C). The color precipitates on bacterial DNA hybridizing with the LT digoxigenin-labeled gene probe show an inconsistent smearing (A). Such smearing was also observed after rehybridization with the radiolabeled LT probe, indicating that this artifact was unrelated to the method of probe labeling or probe detection. In all three assays, 7 test strains were identified as STaII⁺ STaI⁺ LT⁻, 12 as STaI⁻ LT⁺, 1 as STaII⁻ STaI⁻ LT⁺, and 4 as STaII⁺ STaI⁻ LT⁻.

TABLE 1. Proficiency indices for detection of ETEC toxin genes in colony hybridization with digoxigenin-labeled polynucleotide gene probes

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Sensitivity after the following h of color reaction:</th>
<th>Specificity after the following h of color reaction:</th>
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<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>STaII</td>
<td>98.2 (54/55)</td>
<td>100 (55/55)</td>
</tr>
<tr>
<td>STaI</td>
<td>100 (25/25)</td>
<td>100 (25/25)</td>
</tr>
<tr>
<td>LT</td>
<td>100 (85/85)</td>
<td>100 (85/85)</td>
</tr>
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* After 20 h of color development, the signals were read by two observers, the second not otherwise involved in the study. When nothing is indicated, the two observers agreed in the interpretation of the hybridization results.

* According to the independent observer, the specificity was 99.2% (130 of 131).
Accordingly, compared to the oligonucleotide hybridization assay, the sensitivities and specificities of the NCHA were satisfactory (Table 1).

**Specificity of digoxigenin-labeled probes in differentiating ETEC from other toxigenic bacteria and from bacteria harboring their plasmid vectors.** The three *V. cholerae* O1, the two *V. cholerae* non-O1, the same *Y. enterocolitica*, and G101 and HB101 were all negative when tested in the hybridization assays, regardless of the probe used.

**Rehybridization with 32P-labeled oligonucleotide probes.** The results of the NCHA were reproduced after probe stripping and rehybridization with radiolabeled oligonucleotide probes (Fig. 3).

**DISCUSSION**

Major logistic obstacles such as lack of an ultracentrifuge and of radionuclides combined with uncertainties regarding the validity of hybridization assays (5, 6) have discouraged researchers from exploiting the full potential of this technology. The present study describes an ETEC hybridization assay eliminating the need for expensive equipment and radioactive isotopes. Using an established isotope-based oligonucleotide hybridization assay as reference, a blinded study on a large battery of enterotoxigenic and nonenterotoxigenic bacteria revealed satisfactory sensitivities and specificities of the NCHA. Registration of the hybridization results could be performed 2, 4, or 20 h after initiation of the color reaction without significantly affecting the sensitivity or specificity. This makes the test more rapid than its isotope-based precursor, without losing the flexibility of overnight color development. A false-negative reading was made after 2 h, while an apparently false-positive result was obtained after 20 h of development. However, as the false-positive signal was observed on the only strain from which all three colonies had shown weak growth on the NCMs, the same three clones were reexamined by the oligonucleotide assay, the NCHA, the Gm4 enzyme-linked immunosorbent assay (24), and the infant mouse assay (9). In both the repeated hybridization assays, all three clones, now showing normal growth on the NCMs, were classified as STaI-STaII LT+. Likewise, the immunological as well as the biological assay classified all three clones as STa LT+. Thus, in this strain, the finding of an apparently false-positive STaI reading of the NCHA seems to reflect a false-negative result of the reference oligonucleotide assay caused by weak growth, rather than by a suboptimal specificity of the NCHA. The growth of its three colonies on the NCMs was recorded as adequate, although weak. Accordingly, the strain was not excluded from the study. This emphasizes the importance of recording the colony growth and strictly applying the interpretation criteria defined above.

Provided access to a nucleotide synthesizer, oligonucleotides are convenient to prepare in large quantities. They are therefore well suited for use in clinical laboratories. However, DNA synthesizers are quite expensive, require elaborate service systems, and are accordingly located in well-equipped laboratories, seldom in developing countries. Furthermore, nonradioactive oligonucleotide colony hybridization assays for identification of ETEC are expensive and their accuracy is variable (12, 17–20). On the other hand, with a modest laboratory setup, a simplified method of preparing plasmid DNA generates specific polynucleotide probes suitable for economical nonradioactive labeling and detection. The method also has the advantage of high yield because loss of plasmid DNA on a cesium chloride gradient is eliminated. Thus, based on the amount of probe template obtained from a fraction of the vector plasmid produced for the present study, the calculated number of samples that may be examined for ETEC genes after a single large-scale plasmid preparation. Our low estimate is that 35,000 samples (105,000 colonies) may be examined for STaII, 110,000 (330,000 colonies) for STaI, and 80,000 (240,000 colonies) for LTH. Major advantages of the nonradioactive gene probes include safe handling and improved economy since unused probes may be stored for at least 1 year at −20°C. In contrast, 32P-labeled probes pose serious health hazards if used under unhygienic conditions and are generally useless after 6 weeks of storage.

After autoradiography, radiolabeled gene probes may easily be stripped from their target DNAs and the membranes reused for hybridization with other gene probes. Such rehybridization is economical when examining DNA for different nucleotide sequences. On the other hand, the described NCHA does not permit rehybridization with and detection of a second digoxigenin-labeled probe. However, in a small-scale rehybridization experiment, after the probes were stripped off their target, 32P-labeled oligonucleotides effectively identified the corresponding DNA sequences. Thus, if colonies are initially screened with the digoxigenin-labeled probes, the membranes may be sent to a reference laboratory for further examination with radiolabeled probes.

When producing cloned probes, omitting ultracentrifugation on cesium chloride gradients may theoretically produce probe templates contaminated by *E. coli* chromosomal DNA. After low-stringency washing, a nonenterotoxigenic strain harboring the LT probe vector sequences yielded variable hybridization signals. When washing was performed with calibrated increased stringency, the strain was consistently negative. This illustrates that optimizing washing conditions, found to be essential for oligonucleotide probes (23), may also be crucial when using polynucleotides, at least when the described simplified method of probe preparation is used.

The described method of probe production and hybridization provides an accurate tool for identification of ETEC to independent institutions in developing countries. This opportunity will hopefully strengthen clinical and epidemiological studies of diarrheal disease.

**ACKNOWLEDGMENTS**

We thank Steve L. Moseley, Department of Microbiology, School of Medicine, University of Washington, Seattle, for supplying the gene probes in recombinant plasmids and Ann-Mari Svennerholm, Department of Medical Microbiology, University of Göteborg, Göteborg, Sweden, for confirmatory phenotypic toxin assays. We also thank Yoshifumi Takeda, University of Tokyo, Tokyo, Japan, and Ann-Mari Svennerholm for supplying relevant bacterial strains. The constructive advice of Karl Henning Kalland, Anders Fjose, and Bjørn-Ivar Hauknes, Laboratory of Biotechnology, and Bjarne Bjorvatn, Institute of International Health, University of Bergen, as well as that of Ramesh Kumar, Department of Microbiology, All India Institute of Medical Sciences, is gratefully appreciated. We also thank Ranjana Srivastava and Savita Saini, Department of Pediatrics, All India Institute of Medical Sciences, for excellent technical assistance and enthusiastic cooperation.

The financial support of the Norwegian Research Council for Science and the Humanities and the Norwegian Ministry of Development Cooperation is gratefully appreciated.

**LITERATURE CITED**