**Title:** Examination of Colonies and Stool Blots for Detection of Enteropathogens by DNA Hybridization with Eight DNA Probes.

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**Report Date:** 10 JAN 1973

**Number of Pages:** 4

**Abstract:** We compared three methods for detecting enteropathogens in 416 children with diarrhea: (i) examination of 10 lactose-fermenting and all non-lactose-fermenting Escherichia coli (colony blots); (ii) examination of 360 colonies (replicate blots); and (iii) determination of the total bacterial growth of stools (stool blots). All specimens were spotted onto Salmonella bactirbers and hybridized with specific radiolabeled DNA probes. All patients with enteropathogens were detected in 38 patients by examining 10 lactose non-fermenting colonies, 360 colonies, and hybridization blots. Continued on page 2.
Examination of Colonies and Stool Blots for Detection of Enteropathogens by DNA Hybridization with Eight DNA Probes

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Received 23 June 1988/Accepted 2 November 1988

We compared three methods for detecting enteropathogens in 416 children with diarrhea: (i) examination of 10 lactose-fermenting and all non-lactose-fermenting Escherichia coli (colony blots); (ii) examination of 300 colonies (replicate blots); and (iii) determination of the total bacterial growth of stools (stool blots). All specimens were spotted onto Whatman 541 filters and hybridized with specific radiolabeled DNA probes. Enterotoxigenic E. coli was detected in 38 patients by examining colony blots, in 52 patients by examining replicate blots, and in 45 patients by examining stool blots. Enteropathogenic E. coli adhesin factor was detected in 12 patients by colony blots, in 25 patients by replicate blots, and in 16 patients by stool blots. E. coli that hybridized with the enterohemorrhagic E. coli probe was detected in 2 patients by colony blots, in 11 patients by replicate blots, and in 10 patients by stool blots. Shiga-like toxin-producing E. coli was detected in patients by colony blots, in 12 patients by replicate blots, and in 0 patients by stool blots. Shiga-like toxin-producing E. coli was detected in patients by colony blots, in 12 patients by replicate blots, and in 0 patients by stool blots. Shigella spp. were identified by standard bacteriological methods in 82 patients, and enteroinvasive E. coli was identified by colony blots in 11 patients (total, 93), by replicate blots in 56 patients, and by stool blots in 35 patients. Of 82 culture-confirmed Shigella infections, 45 were identified by examining replicate blots with the 17-kilobase-pair probe and 36 were identified by examining with the lpa probe (P < 0.05). Examining replicate blots with specific probes identified more enterotoxigenic E. coli (P < 0.005), enteropathogenic E. coli adhesin factor-producing E. coli (P < 0.001), and Shiga-like toxin-producing E. coli (P < 0.005) infections than examining colony blots. More Shigella and enteroinvasive E. coli infections were identified by standard bacteriological methods and examining colony blots with a specific probe than by examining replicate and stool blots (P < 0.001).

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replicate colony transfer pads (FMC Bioproducts, Rockland, Maine), and incubated at 37°C for 16 h. Colonies were transferred from MacConkey agar to filters (no. 541; Whatman, Inc., Clifton, N.J.) by pressing the filters evenly over the colonies. Filters were subsequently placed on Whatman no. 3 paper saturated with 0.5 N NaOH-1.5 M NaCl, steamed for 3 min in an autoclave, immersed in 1 M Tris-2 M NaCl (pH 7.4) for 4 min, and air dried (10).

For replicate blots, 0.1 ml of 10-fold serial dilutions of 1 g of stool vortexed in 2 ml of sterile phosphate-buffered saline was spread on MacConkey agar with a sterile glass triangle probe were washed in 6X SSC, three times for 30 min each at 50°C, and air dried. Filters examined with the polyacrylamide gel electrophoresis (PAGE) method. Among 38 children with ETEC, examining replicate colony transfer pads (FMC Bioproducts, Rockland, Maine), and incubated at 37°C for 16 h. Colonies were transferred from MacConkey agar to filters (no. 541; Whatman, Inc., Clifton, N.J.) by pressing the filters evenly over the colonies. Filters were subsequently placed on Whatman no. 3 paper saturated with 0.5 N NaOH-1.5 M NaCl, steamed for 3 min in an autoclave, immersed in 1 M Tris-2 M NaCl (pH 7.4) for 4 min, and air dried (10).

DNA probes. Plasmid DNA was isolated from E. coli K-12 containing pEWD299 (LT), pCDV419 (EHEC), pNN37-19 (ST), pNN110-18 (SLT II), pRM17 (EIEC), pW22 (IpA), and pMAR22 (EAF) as described by So et al. (22) and digested with appropriate restriction endonucleases (Bethesda Research Laboratories, Gaithersburg, Md.) under conditions specified by the manufacturer. A synthetic 23-mer oligonucleotide probe to detect genes coding for STA was constructed with a DNA synthesizer (Applied Biosystems, Foster City, Calif.) as previously described (4). The LT probe consisted of a 850-base HindIII digestion fragment of pEWD299 (12), the EHEC probe was a 3.4-kb HindIII fragment of pCDV419 (9), the EIEC probe was a 17-kb EcoRI digestion fragment of pRM17 (20), and the IpA probe was a 1.7-kb base EcoRI digestion fragment of pW22 (1). The SLT I probe was a BamHI 1.142-base fragment of pNN37-19, and the SLT II DNA probe was a SmaI-PstI 842-base fragment of pNN110-18 (15). The EAF probe was a BamHI-SaII I-kb digestion fragment of pMAR22 (14). The appropriate DNA fragments were separated by polyacrylamide gel electrophoresis, electroeluted from the gel, and labeled in vitro with [α-32P]deoxyribonucleotide triphosphate (Dupont, NEN Research Products, Boston, Mass.) by nick translation (11). The ST oligonucleotide probe was 5' end labeled with [γ-32P]deoxyribonucleotide triphosphate (11).

DNA hybridization. Filters examined with the polynucleotide-cloned probes were incubated in the following hybridization solution: 50% formamide, 5X SSC (1X SSC is 0.15 M NaCl and 0.015 sodium citrate), 0.1% sodium dodecyl sulfate, 1 mM EDTA, and Denhardt solution (0.02% Ficoll [Pharmac Fine Chemicals, Piscataway, N.J.], molecular weight, 400,000), 0.02% polyvinylpyrrolidone [molecular weight, 360,000], 0.2% bovine serum albumin). The filters were then transferred to fresh hybridization solution containing 106 cpm of heat-denatured DNA probe and 75 μg of sheared heat-denatured calf thymus DNA per ml and incubated at 37°C overnight. Hybridization with the γ-32P-labeled ST oligonucleotide probe was performed at 50°C overnight in 6X SSC-15% bovine serum albumin-1% polyvinylpyrrolidone-1 mM EDTA with 107 cpm of oligomer probe per ml.

The filters examined with the DNA polynucleotide probes were washed twice in 5X SSC-0.1% sodium dodecyl sulfate for 45 min at 65°C, rinsed in 2X SSC at 22°C., and air dried.餐具. Filters examined with the γ-32P-labeled ST oligonucleotide probe were washed in 6X SSC, three times for 30 min each at 50°C, and air dried. Filters were exposed to X-Omat-R X-ray film (Eastman Kodak Co., Rochester, N.Y.) with a single Cronex Lightning-Plus intensification screen (E.I. du Pont de Nemours & Co., Inc., Wilmington, Del.) for 24 h at -70°C. Films were developed according to the instructions of the manufacturer. E. coli strains that hybridized with the SLT I, SLT II, or EHEC probe were tested for VT production in the Vero cell assay (7). E. coli strains that hybridized with the E. coli adhesin factor (EAF) probe were tested for mannose-resistant adherence to HeLa cells as described by Scalsletsky et al. (17). E. coli strains that hybridized with the 17-kb or IpA Shigella-EIEC probe were tested by the Seryl test (18), and E. coli strains that hybridized with the LT and ST probes were tested for enterotoxin production (3, 16). No attempt was made to recover isolates that hybridized with DNA probes in replicate blots.

Statistical analysis was performed with the McNemar test for matched-pair analysis.

**RESULTS**

Comparison of colony, replicate, and stool blots with specific DNA probes. (1) ETEC. Among 416 children with diarrhea, 7 were infected with E. coli colonies that hybridized with both the LT and ST probes, 24 were infected with E. coli colonies that hybridized with only the LT probe, and 7 were infected with E. coli colonies that hybridized with only the ST probe. All of the E. coli colonies that hybridized with the enterotoxin gene probes produced enterotoxin(s), as determined by Y-1 adrenal and suckling mouse assays. Colonies that hybridized with the enterotoxin gene probes were identified in 38 children by testing 10 E. coli colonies (colony blots), in 52 children by testing 30 colonies (replicate blots) and in 45 children by testing stool blots (Table 1). ETEC was identified in 61 (15%) of 416 children by a method. Among 38 children with ETEC, examining replicate blots with the enterotoxin gene probes had a sensitivity of 92%, a specificity of 96%, a PPV of 67%, and a NPV of 99%. Examining stool blots with the enterotoxin gene probes had a sensitivity of 82%, a specificity of 96%, a PPV of 69%, and a NPV of 98%. The mean percentage of 300 colonies that hybridized with both the LT and ST probes was 93% (95% confidence limits, 88% to 98%), and the percentage that hybridized with the ST probe was 57% (95% confidence limits, 28 to 86%).

(2) EAFEC. E. coli strains that hybridized with the EAF probe and adhered to HeLa cells in a localized adherent pattern were isolated from 12 children with diarrhea. EAFEC was identified by colony blots in 12 children, by replicate blots in 25 children, and by stool blots in 16 children (Table 1). EAFEC infections were identified in 27 (6%) of 416 children with diarrhea by any method. The mean percentage of 300 colonies that hybridized with the EAF probe was 71% (95% confidence limits, 54 to 88%). Among

**TABLE 1. Identification of ETEC and EAFEC infections in 416 children with diarrhea by three methods**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Colony blot</th>
<th>Replicate blot</th>
<th>Stool blot</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETEC</td>
<td>38</td>
<td>52</td>
<td>45</td>
</tr>
<tr>
<td>EAFEC</td>
<td>12</td>
<td>25</td>
<td>16</td>
</tr>
</tbody>
</table>

The mean percentage of 300 colonies that hybridized with the EAF probe was 71% (95% confidence limits, 54 to 88%).
12 children with EAFEC, replicate blots examined with the EAFEC probe had a sensitivity of 83%, a specificity of 96%, a PPV of 40%, and a NPV of 99%. Examining stool blots with the EAFEC probe had a sensitivity of 83%, a specificity of 98%, a PPV of 62%, and a NPV of 99%.

(iii) E. coli that hybridized with the EHEC probe. E. coli that hybridized with the EHEC probe was identified by colony blots in 2 children, by replicate blots in 11 children, and by stool blots in 0 children. Infections with E. coli that hybridized with the EHEC probe were identified in 13 (45%) of 416 children with diarrhea by any method. Replicate blots from three children hybridized with the EHEC probe and SLT probes (SLT I and II, one child: SLT I only, one child; and SLT II only, one child). Eleven E. coli colonies that hybridized with the EHEC probe did not hybridize with the SLT probes or product VT. The mean percentage of 300 colonies that hybridized with the EHEC probe was 13% (95% confidence limits, 2 to 27%).

(iv) SLT-producing E. coli. None of the colony blots, 12 (2%) of the replicate blots, and none of the stool blots from children with diarrhea hybridized with the SLT probes. Five replicate blots hybridized with the SLT I probe, six hybridized with the SLT II probe, and one hybridized with both SLT probes. Of 300 colonies, a mean of 3% (95% confidence limits, 1 to 5%) hybridized with the SLT probes.

(v) Shigella spp. and EIEC. Shigella spp. were isolated from 82 children with diarrhea by standard bacteriological methods. Of these 82 culture-confirmed Shigella infections, 45 were identified by examining replicate blots and 28 were identified by examining stool blots with the 17-kb EcoRI probe (P < 0.01) (Table 2). From the same cases of shigellosis, 36 replicate blots and 24 stool blots hybridized with the Ipa probe (P < 0.05). More Shigella infections were identified by examining replicate blots with the 17-kb probe than with the Ipa probe (45 versus 36; P < 0.05); however, the difference between examining stool blots with the 17-kb probe versus examining blots with the Ipa probe (28 versus 24 infections identified) was not statistically significant. Among 82 patients with Shigella infection, examining replicate blots with the 17-kb and Ipa probes had sensitivities of 55 and 34%, specificities of 98 and 100%, PPVs of 83 and 82%, and NPVs of 91 and 89%. Examining stool blots from the same patients with the 17-kb and Ipa probes had sensitivities of 34 and 30%, specificities of 100 and 100%, PPVs of 100 and 96%, and NPVs of 86 and 87%.

EIEC was isolated from 11 children with diarrhea by testing E. coli for hybridization with the 17-kb probe and testing probe-positive isolates in the Sereny test. Nine children were infected with Lac⁺ and two with Lac⁻ EIEC.
identify Shigella spp. and EHEC in clinical specimens from patients with diarrhea.

The percentage of 300 colonies that hybridized with DNA probes for genes coding for pathogenic determinants was low in some children with diarrhea. This method may not always identify bacteria that are etiologically important. LT ETEC identified in 33% of 300 colonies have not been associated with diarrhea disease in children less than 5 years old (P. Echeverria, D. N. Taylor, U. Lekonmoen, M. Bhaibulaya, N. R. Blacklow, K. Tamhna, and R. Sakazaki, J. Infect. Dis., in press).

The 11 E. coli colonies that hybridized with the EHEC probe did not hybridize with the SLT1 and SLT2 probes or produce VT. Seriwatana et al. (19) previously described E. coli isolates that hybridized with the EHEC probe that did not produce VT. Although the EHEC probe hybridized with most EHEC isolated in Canada, Germany, the United States, and Chile, this probe hybridizes with E. coli that do not produce VT. It is possible that the E. coli isolates that hybridized with the EHEC probe previously contained phase-encoded genes for SLT.

DNA probes have been extremely useful in identifying genes coding for enterovirulent determinants in large numbers of E. coli. Examining 300 colonies in replicate blots increased the identification of diarrheagenic E. coli. How useful this will be in defining the relative importance of bacterial pathogens in patients with diarrheal disease remains to be determined.

LITERATURE CITED


