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Detection of Enterotoxigenic Escherichia coli in Stool Samples by Using Nonradioactively Labeled Oligonucleotide DNA Probes and PCR

CONSTANCE SCHULTSZ,1,2* GIEL J. POOL,1 RUUD VAN KETEL,1 BOB DE WEVER,1 PETER SPEELMAN,2 AND JACOB DANKERT1

Department of Medical Microbiology1 and Department of Infectious Diseases, Tropical Medicine and AIDS,2 Academic Medical Centre, Amsterdam, The Netherlands

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The detection of heat-labile enterotoxin LT-A and heat-stable enterotoxin ST la and ST lb genes from enterotoxigenic Escherichia coli (ETEC) by using oligonucleotide DNA probes and the PCR was evaluated in reconstruction experiments and by testing stool specimens from 29 healthy subjects and from 50 patients with diarrhea who had returned from the (sub)tropics. ETEC strains were detected in concentrations ranging from 10⁶ to 10⁸ CFU/g of feces when oligonucleotide probes were applied to colony blots from five randomly picked E. coli-like colonies from CLED (cystine lactose electrolyte deficient) agar plates inoculated with the feces. When these probes were applied to blots from whole stool cultures collected from the agar plates (sweep blot), the detection limit was 10⁶ CFU/g of feces. PCR of the sweep material could detect toxin genes when the concentration of ETEC strains was 10⁵ CFU/g of feces. Results obtained with stool specimens from 29 healthy control subjects were negative. Testing stool specimens from 50 patients confirmed the observation that the number of samples containing ETEC enterotoxin genes was higher when PCR of sweeps was used than when oligonucleotide DNA probe hybridization of either sweep blots or colony blots was used. Furthermore, PCR of sweeps is an easy and rapid method which does not require DNA extraction and purification from fecal specimens.

Enterotoxigenic Escherichia coli (ETEC) is a major cause of diarrhea in travelers to (sub)tropical areas. ETEC strains, which cause diarrhea by producing a heat-labile enterotoxin (LT), heat-stable enterotoxins (ST la or ST lb), or both, can be detected by testing isolates for enterotoxin production with bioassays or immunossays (1, 5). Alternatively, ETEC strains can be identified by detection of genes encoding enterotoxin production, utilizing DNA hybridization techniques or the PCR. In general, 5 to 10 E. coli-like colonies are randomly picked from an agar plate and tested by either method. Stool blot hybridization assays also have been applied for use in DNA hybridization techniques. In such assays, toxin genes are detected directly in bacteria grown from stool specimens on a membrane overlaying an agar medium by using polynucleotide (2) or oligonucleotide DNA probes (9).

Oligonucleotide probes are specific (9), and in contrast to polynucleotide probes, laborious techniques are not required for preparation of the probe. When results obtained with oligonucleotide probes and polynucleotide probes in hybridization of colony blots are compared, the results are similar, whereas results obtained with stool blots are not unequivocal (3, 14). With either probe, the detection rates of ETEC by using stool blots or colony blots are also not unequivocal. In one study in which volunteers were challenged with one ETEC strain, resulting in stool samples containing large numbers of ETEC, hybridization of stool blots showed a higher sensitivity than hybridization of colony blots (7). Opposite results were found in children with diarrhea with relatively low numbers of ETEC in their stools (14). Reasons for the discrepancy in the detection rate of ETEC between hybridization of colony blots and hybridization of stool blots may be the variation in the number of ETEC organisms present in the stool sample and in the number of colonies picked for hybridization.

We needed a method for detection of ETEC in stool samples from patients who returned from the (sub)tropics with diarrhea. Such patients often have mild diarrhea of variable duration (13), and the number of ETEC strains in their stools, even in the case of infection, may be relatively small.

The PCR is a very sensitive method for detection of specific genes. When applied directly to stool samples, the PCR may be inhibited by fecal substances. In a preliminary study, we were not able to obtain reproducible results in reconstruction experiments using the PCR performed as described by Frankel et al. (4). Victor et al. (18) described a screening method that used the PCR for detection of LT-producing ETEC in a scrape of mixed growth on a MacConkey agar plate. This method proved to be sensitive, specific, and easy to perform.

To detect ETEC in stool samples we evaluated stool samples by DNA hybridization with oligonucleotide probes as well as by PCR. For labeling of the oligonucleotide probes, we used ³²p as well as digoxigenin (DIG); the latter has been shown previously to be a sensitive, nonradioactive label (12, 16).

MATERIALS AND METHODS

Bacterial strains. ETEC strains E7476 (O166:H27; ST) and E5798 (O7:H18; LT) (kindly provided by B. Rowe, Central Public Health Laboratory, Colindale, United Kingdom) H10407 (O78; LT and ST) and H25/67 (O8:K200; LT) (provided by W. Jansen, National Institute for Public Health and Environmental Protection, Bilthoven; The Netherlands), and 4083 (O78; LT and ST) (provided by C. M. A. Rademaker,
University Hospital, Utrecht, The Netherlands) were used as control strains and in reconstruction experiments. The enterotoxin-negative control strain 14RS59 (E. coli K-12) was provided by B. Rowe. Previously, the presence of the various toxin genes in these strains had been determined by using bisulphate and/or polynucleotide probes.

**Oligonucleotide probes and PCR primers.** Oligonucleotide probes and PCR primers are listed in Table 1. Probe and primer sequences were based on the published sequences for the E. coli LT-A subunit (LT-A); LT-B subunit (LT-B), and ST la and ST lb genes of enterotoxigenic E. coli of human (LT-A, LT-B, ST lb) and porcine (ST la) origin (8, 15, 19). The oligonucleotide probes were shown to be sensitive and specific compared with bioassays (9, 10). The LT and ST lb PCR primers were chosen as described by Frankel et al. (4), with modification of primer 1 of ST lb. The ST la primer sequences were derived from the region of the ST la gene which corresponds to the region of the ST lb gene from which the ST lb primers were derived. The oligonucleotide probes also served as internal probes for the detection of PCR products. All oligonucleotides were purchased from Eurosequence BV, Groningen, The Netherlands, and were custom synthesized with an Applied Biosystems DNA synthesizer (model 380 B).

Oligonucleotides were labeled with either 32P or DIG for reconstruction experiments. For detection of ETEC in stool samples from patients, only DIG-labeled probes were used. Probes were labeled with 35P (Amersham Nederland BV, *s* Heraeus, Almere, The Netherlands). For labeling with DIG, oligonucleotide probes were 3′-end labeled by using T4 polynucleotide kinase (Boehringer Mannheim BV, Almere, The Netherlands). For labeling with DIG, oligonucleotide probes were 3′-end labeled by using terminal transferase provided in the DIG Oligonucleotide 3′-End Labeling Kit, as specified by the manufacturer (Boehringer Mannheim).

**Processing of samples.** (i) Reconstruction experiments. Stools were collected from a healthy 4-year-old Dutch child. ETEC strains were added to the stools in concentrations, measured spectrophotometrically by using MacFarland's opacity standard, ranging from 108 to 1011 CFU/g of feces. From the original sample and from each stool sample to which ETEC was added, a loopful was streaked on a CLED cystine lactose electrolyte deficient (CLED) agar plate and grown for 18 h at 37°C. Five E. coli-like colonies were picked. All of the remaining bacterial growth on the CLED agar plate was collected with a sterile cotton swab and designated sweep.

(ii) Samples from patients and control subjects. Stool samples were obtained from 30 consecutive patients who visited the outpatient Department of Tropical Medicine, Academisch Medisch Centrum, Amsterdam, The Netherlands, because of complaints of diarrhea, within 1 year after return from the tropics. Samples were submitted by the clinicians only when the following definition of diarrhea was met: four or more loose stools in 24 h, or any number of loose stools accompanied by at least one of the following symptoms and signs: nausea, vomiting, fever of >38°C, abdominal cramps, or tenesmus. Stool samples were collected in sterile containers, immediately transported to the Laboratory of Medical Microbiology, Academisch Medisch Centrum, and stored at 4°C. Samples were generally processed within 2 h after arrival at the laboratory and always within 24 h. Each stool specimen was tested for Salmonella, Shigella, Yersinia, and Campylobacter species by using standard identification methods. Stool samples were also examined for amebas, ova, and cysts by direct microscopic examination and after concentration (by the modified Ridley's method). Additionally, a CLED agar plate was inoculated, and after incubation for 18 h at 37°C, five individual E. coli-like colonies and a sweep were collected, suspended in glycerol-peptone, and stored at −70°C. Patients were asked prior to use of antimicrobial agents by their clinician. A total of 29 stool samples from 29 healthy Dutch subjects were obtained and processed as mentioned above to detect ETEC from a sweep by PCR.

**Preparation of membranes and hybridization.** Individual colonies and sweeps from reconstruction experiments and clinical specimens were grown on CLED agar medium. For the plates on which individual colonies had been inoculated, one colony was suspended in 1 ml of sterile phosphate-buffered saline (PBS; pH 7.4), and 5 μl of the suspension was spotted onto two nylon membranes (Hybond-N; Amersham Nederland BV), overlaying nutrient agar plates, and grown for 6 h at 37°C. The entire sweep was suspended in 4 ml of PBS, diluted 10-fold, and further processed as described for the colonies (sweep blot). Negative and positive control strains were applied equally to each membrane. Membranes were removed, and bacterial growth was lysed and denatured, essentially as described by Sambrook et al. (11). Membranes were baked at 80°C for 1 h to fix the DNA.

Hybridization with 32P-labeled oligonucleotide probes was done in hybridization buffer containing 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.5× sodium dodecyl sulfate (SDS), 5× Denhardt's solution, and 100 μg of denatured salmon sperm DNA per ml. For hybridization with DIG-labeled oligonucleotide probes, membranes were prehybridized at 55°C in hybridization solution containing 5× SSC, 0.1% laurylsarcosine, 0.02% SDS, and 1% blocking reagent (Boehringer Mannheim), treated with proteinase K (100 μg/ml) for 60 min, washed twice in 2× SSC-0.1% SDS, and prehybridized a second time. The LT-A and LT-B probes were added together as well as the ST la and ST lb probes.

Hybridization with both the 32P- and DIG-labeled probes was done at 55°C for 1 h. After hybridization, the membranes were washed under stringent conditions as described previously (17). Membranes were air dried and exposed to X-ray film for 24 h at −70°C, or when DIG-labeled probes were used, detection of hybrids was performed as described in the instructions of the DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

**PCR.** In reconstruction experiments, PCR was performed on the sweeps from the original sample and from each fecal sample containing increasing concentrations of ETEC. For all patient and healthy subject stool samples, PCR was performed on the sweeps.
The sweep from a CLED agar plate was suspended in 4 ml of PBS and then diluted 10-fold in PBS. Suspensions were heated at 95°C for 10 min to release and denature the DNA and placed on ice. No further treatment was done. For control strains, a single colony was suspended in 1 ml of PBS and treated the same as the sweep.

The amplification procedure was performed in a final volume of 100 ul. The reaction mixture contained 10 mM Tris-hydrochloride (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.001% gelatin, 200 μM each of the deoxyribonucleotides, 300 ng each of the oligonucleotides, and 2.5 U of Tag polymerase (Perkin-Elmer, Gouda, The Netherlands), to which 10 μl of the bacterial suspension was added. The mixtures were processed in a programmable DNA thermal cycler (Perkin-Elmer) and subjected to 35 cycles of amplification. One cycle consisted of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. A control, containing water instead of template DNA, was included in each experiment to exclude the possibility of reagent contamination. To prevent cross-contamination of reagents and samples, precautions were taken essentially as described by Kwok and Higuchi (6).

Electrophoresis was carried out on 10 μl of amplified sample in 2% agarose gels with a 123-bp molecular mass marker. DNA was blotted onto nylon membranes under alkaline conditions (0.4 N NaOH) and hybridized by using DIG-labeled oligonucleotide probes as described above.

Statistical analysis. Differences between groups were calculated by using the χ² test or Fisher's exact test when appropriate.

### TABLE 2. ETEC infections detected by hybridization of DIG-labeled oligonucleotide DNA probes and by PCR

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Oligonucleotide DNA probes</th>
<th>PCR</th>
<th>Other pathogens present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LT</td>
<td>ST</td>
<td>LT</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>6</td>
<td>-</td>
<td>-</td>
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<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* a Both assays were performed on bacterial growth collected from CLED agar plates (sweep).

* b As detected by direct examination or culture of stool specimen.

* c Sweeps were hybridized with pooled probes for detection of ST la and ST lb genes.

### RESULTS

#### Reconstruction experiments

To compare detection levels of the colony blot method with those of the sweep method, stools were spiked with either strain H25/67 (for detection of LT genes), H10407 (for detection of ST la genes), or 4083 (for detection of ST lb genes). When testing five colony blots from individual colonies, ETEC strains were detected in stool samples which were spiked with 10⁶ to 10⁷ CFU/g of feces. For samples containing 10⁸ bacteria per g, in all cases at least one colony blot was positive, whereas for some samples containing 10⁹ bacteria per g, all five colony blots were negative. The detection levels were identical for 32P- and DIG-labeled probes (Fig. 1). When testing sweep blots, ETEC strains could be detected in stool samples which were spiked with 10⁶ CFU/g of feces by using 32P-labeled LT, ST la, and ST lb probes and in stool samples spiked with 10⁷ to 10⁸ CFU/g of feces by using DIG-labeled probes. Treatment of membranes with proteinase K in conjunction with washing and prehybridization was included in the hybridization procedure of the DIG-labeled probes to reduce background staining of the sweep blots. After hybridization of sweep blots, all stains of DIG-labeled oligonucleotides, generally stronger signals were obtained with the pooled LT probes than with the pooled ST probes.

Sweeps prepared in reconstruction experiments for hybridization with oligonucleotide probes were also subjected to PCR analysis. PCR of sweep material proved to be a highly sensitive method. LT-A or ST la genes could be detected specifically with the corresponding primer sets in stool samples spiked with only 10⁶ CFU/g of feces (Fig. 1). The ST lb primer set performed marginally less sensitively than the other two primer sets. Specificity of the PCR bands was proven by hybridization after alkaline blotting with the corresponding oligonucleotide probe.

#### Patient samples and healthy control samples

One stool sample obtained from 50 patients was tested. Samples were screened by hybridization of sweep blots with DIG-labeled oligonucleotide probes and PCR analysis of sweeps. Three (6%) samples hybridized with the LT probes, two (4%) samples hybridized with the pooled ST probes. From six (12%) samples, the sweeps were positive by PCR for LT-A, and from seven (14%) samples, the sweeps were positive by PCR for ST la and/or ST lb (Table 2). All samples which were positive in the hybridization assay were also positive by PCR. All PCR-
positive samples were submitted to repeated PCR on the sweeps stored at -70°C. All samples were again PCR positive. A total of 29 sweeps from stool samples from healthy Dutch individuals were subjected to PCR analysis and were negative for all three primer sets. For each sample in which ETEC could be detected by hybridization of sweep blots and/or PCR of the sweep, five blots from randomly picked colonies were hybridized. For each sample in which the sweep blot hybridized with one or more of the probes, at least one of the colony blots also hybridized with the corresponding probes. However, none of the colony blots obtained from samples for which the sweep blot was negative but the PCR was positive hybridized with any of the probes.

For stool samples from 8 (16%) patients, other enteropathogens were isolated by culture. Two samples harbored Campylobacter jejuni, two samples harbored Campylobacter sp., and one sample each harbored Vibrio cholerae, Shigella flexneri, Campylobacter coli, and Campylobacter fetus. In one case, both C. coli and ETEC were detected. In this case, four of the five separate colonies hybridized with the LT probes, and three of five colonies hybridized with the ST probes. All of these colonies were characterized biochemically as E. coli. Parasitological examination showed six (12%) samples containing Giardia lamblia cysts, six (12%) samples containing Blastocystis hominis, and one sample each containing Entamoeba histolytica cysts and cyclosporin. In two samples, both G. lamblia cysts and ETEC were detected. In the other cases in which a pathogen could be identified, no ETEC were detected. However, when a decreasing number of ETEC bacteria are present in the stool, the chances that no ETEC bacteria are present among the five randomly picked colonies become higher. The sensitivity of the sweep blot may be less prone to chance.

The DIG-labeled oligonucleotide probes appeared to be equally efficient as radioactively labeled oligonucleotide probes when applied to colony blots and almost as efficient when applied to sweep blots. The difference in detection levels of ETEC in sweep blots between 32P- and DIG-labeled oligonucleotide probes may be explained by background staining of bacterial proteins present on the membrane when DIG-labeled probes are used. A weak signal, due to the presence of relatively low numbers of ETEC, can be detected with 32P-labeled probes but may be indistinguishable from background staining when using the DIG-labeled probes. We obtained less background staining with the DIG-labeled probes after treatment of membranes with proteinase K.

When the sweeps prepared in the reconstruction experiments were subjected to PCR analysis, 105 CFU/g of feces could be detected. Victor et al. (18) described a similar, easy, and rapid method for detection of LT-A genes in stool samples by PCR. However, the number of bacteria in the feces which could be detected was not reported.

Testing 50 clinical samples from patients with diarrhea confirmed the results of the reconstruction experiments. Three sweep blots were positive after hybridization with DIG-labeled oligonucleotide probes. In five additional samples, ETEC was detected by using PCR. None of five colony blots from randomly picked E. coli-like colonies from the latter samples hybridized with any of the oligonucleotide probes. In contrast, for five colonies obtained from the three samples which were positive after sweep blot hybridization, at least one colony blot was positive in all cases.

By using radioactively labeled oligonucleotide probes for detection of LT, ST Ia, and ST Ib genes, Sommerfelt et al. (14) found 100% correlation between hybridization of stool blots and hybridization of 12 colony blots, when the ratio of ETEC/E. coli organisms was >2:12. When the ratio of ETEC/E. coli organisms was ≤2:12, only 1 of 5 (20%) stool blots was positive. These data suggest that hybridization of stool blots or sweep blots can detect only large numbers of ETEC and may therefore be suitable only for detection of ETEC in the acute phase of diarrhea. PCR seems to be a suitable method for detection of large as well as small numbers of ETEC in sweeps and can distinguish between stools containing ETEC LT, ST Ia, and ST Ib genes. Furthermore, DNA extraction is not required, and the PCR is not inhibited by fecal substances.

The clinical relevance of our finding that five patients had low numbers of ETEC strains in their stool samples, as detected by PCR, is not known. In five of eight patients with ETEC in their stool samples, no other established enteropathogen was found. ETEC organisms could therefore have been the cause of diarrhea in these patients. However, a large number of patients used antimicrobial agents prior to stool sampling, and this may have influenced the results of the stool cultures. Although with our method mostly viable bacteria carrying the enterotoxin genes are detected, dead ETEC bacteria, resulting from the use of antimicrobial agents, may be present in the stool, originating from the first inoculation area, and may be detected by PCR. Further studies are warranted to establish the role of ETEC strains in the etiology of long-lasting diarrhea in travelers returning from the (sub)tropics and the effect of antimicrobial therapy.

**DISCUSSION**

Simple and rapid tests are required for detection of ETEC in routine laboratories. The most commonly used method for detection of ETEC is hybridization of colony blots with DNA probes specific for enterotoxin genes. Detection of ETEC in stool specimens by PCR has been described by several authors (4, 10, 18). To assess differences between hybridization techniques and PCR in the detection levels of ETEC in stool specimens, we performed reconstruction experiments, adding increasing concentrations of ETEC to stool samples from a healthy child. Results of these experiments indicate that after hybridization of sweep blots, amounts of ETEC in stool specimens (≥106 CFU/g) similar to those after hybridization of five colony blots from randomly picked E. coli-like colonies can be detected. However, when a decreasing number of ETEC bacteria are present in the stool, the chances that no ETEC bacteria are present among the five randomly picked colonies become higher. The sensitivity of the sweep blot may be less prone to chance.

**TABLE 3. Results of PCR for detection of ETEC, stool culture, and parasitological examination and relation to use of antimicrobial therapy prior to sampling**

<table>
<thead>
<tr>
<th>Results of stool examination</th>
<th>No. of patients (n = 50)</th>
<th>No. (%) of patients who used antimicrobial agents prior to sampling</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETEC PCR positive</td>
<td>12 (57)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>ETEC PCR negative</td>
<td>38 (86)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Routine culture positive</td>
<td>8 (13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Routine culture negative</td>
<td>42 (87)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parasites found</td>
<td>14 (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No parasites found</td>
<td>26 (52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total positive‡</td>
<td>27 (55)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total negative</td>
<td>23 (45)</td>
<td></td>
<td></td>
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</tbody>
</table>

‡ Multiple pathogens were found in three patients.
The application of PCR to bacterial growth on a selective agar plate, instead of to a fecal sample, provides a rapid, sensitive, and specific means for detection of ETEC in clinical samples.

ACKNOWLEDGMENTS

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