JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1989, p. 2245–2248
0095-1137/89/102245-04$02.00/0

Vol. 27, No. 10

Evaluation of a Rapid Method of Extracting DNA from Stool Samples for Use in Hybridization Assays

PEDRO COLL,[1,2] KATHY PHILLIPS,[3] AND FRED C. TENOVER[3,4]*

Hospital de la Santa Creu i Sant Pau[1] and Department de Genetica i Microbiologia, Universitat Autonoma de Barcelona,[2] Barcelona, Spain; Laboratory Service, Veterans Affairs Medical Center, Seattle, Washington 98108[3]; and Department of Laboratory Medicine, University of Washington, Seattle, Washington 98195[4]*

Received 20 March 1989/Accepted 26 June 1989

The ability of the Extractor system (Molecular Biosystems, Inc., San Diego, Calif.) to isolate nucleic acid (NA) from stool samples for use in hybridization assays was investigated. Crude NA was recovered from 45 of 50 stool samples by using this system. The amount of NA recovered varied considerably depending on the microbial flora present in the sample (mean ± standard deviation, 50.2 ± 46.7 μg; range, 2 to 228 μg) but did not correlate with the consistency of the sample. Samples containing primarily gram-positive organisms or yeast cells gave lower yields of NA (<10 μg) than those containing gram-negative bacilli. The five samples which did not yield NA were sterile when cultured aerobically on blood agar plates. Samples of the 45 stools yielding NA were inoculated into broth and grown overnight, and a 10-μl sample of broth was spotted onto nitrocellulose filters. The NA samples recovered from the Extractor column were applied to nylon membranes by using the Centri-dot system. The NA on the broth blots and the NA on the Centri-dot filters were hybridized with a 310-base-pair probe specific for the 2″-*O*-aminoglycoside adenylyltransferase [ANT(2″)] resistance gene. The Extractor-Centri-dot system demonstrated 61.9% sensitivity and 95.8% specificity in detecting the ANT(2″) gene in stool samples containing colonies demonstrating the ANT(2″) phenotype. The positive and negative predictive values of the NA blot were 92.8 and 74.2%, respectively.

The technique of nucleic acid (NA) hybridization for the detection and identification of pathogenic microorganisms has been integrated into the workflow of many clinical microbiology laboratories (7). This technique plays a prominent role in the effort to identify fastidious or unusual pathogens in clinical samples, including such organisms as *Mycobacterium avium* (2) and enterotoxigenic *Escherichia coli* (3).

The development of methods for the chemical synthesis of large quantities of oligonucleotides with very high sequence fidelity has enabled companies to manufacture a wide spectrum of highly specific reagents for detecting and identifying infectious microorganisms (7). However, specimens such as stool often have proved problematic for hybridization assays (9), and the need for improved methods of processing specimens has been recognized.

One novel approach to specimen preparation is to extract NA from the sample before hybridization. Seriwatana et al. (6) and Tompkins and Krajden (9) noted increased sensitivity of hybridization assays for enterotoxigenic *E. coli* and *Campylobacter jejuni*, respectively, when purified DNA was used as a substrate in place of colonies grown overnight on filter paper. Rapid NA extraction has been used in conjunction with the polymerase chain reaction to detect the presence of enterotoxigenic *E. coli* in stool samples (5) and has been incorporated into commercial kits for the detection of *C. jejuni* (V. Lauderdale and D. Roszak, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 394, 1988). While preliminary reports of these tests are encouraging, the studies to date have been limited in scope. Furthermore, the method of the sample preparation, the Extractor column method, has not been evaluated independently of the probe tests.

This study was undertaken to compare the sensitivity of a broth blot hybridization assay (4) in which a sample of stool was incubated overnight in broth, spotted onto nitrocellulose, lysed to release NA, and hybridized, with that of a hybridization method in which NA was isolated from a stool sample by the Extractor method and immobilized on nylon filters by using the Centri-dot system. A DNA probe specific for the 2″-*O*-aminoglycoside adenylyltransferase [ANT(2″)] resistance gene, a gene common among gram-negative bacilli, was used. Here, we report the results of these studies.

MATERIALS AND METHODS

Stool specimens. A total of 50 stool samples submitted to the Seattle Veterans Affairs Medical Center Microbiology Laboratory were studied. All specimens in the study were obtained from different patients. A sample of stool was plated on Columbia agar plates containing 5% sheep blood (Prepared Media Laboratories, Tualatin, Oreg.) and on two MacConkey agar plates (Difco Laboratories, Detroit, Mich.), one containing 5 μg of tobramycin per ml and a second containing 3 μg of gentamicin per ml, to screen for gram-negative organisms harboring an ANT(2″) gene (8). Gentamicin was obtained from Schering Corp., Bloomfield, N.J., and tobramycin was obtained from Eli Lilly and Co., Indianapolis, Ind. Plates were examined for the presence of growth after 18 and 48 h of incubation. Antimicrobial susceptibility testing of gram-negative bacilli was performed by the disk diffusion method of Bauer et al. (1).

Extraction of DNA. Crude NA was prepared from stool samples by using Extractor columns (Molecular Biosystems, Inc., San Diego, Calif.) as described by the manufacturer. This method involves a mixed matrix column with both ion-exchange and molecular sizing properties and uses increasing salt concentrations to bind, purify, and elute NA. Thus, NA can be separated from proteinaceous and carbohydrate-containing material by passing the processed stool sample through the column matrix. Specifically, a sample of

* Corresponding author.

stool the size of a grain of rice (approximately 0.1 g of formed stool) or 100 μl of liquid stool was diluted in 1 ml of 100 mM phosphate buffer, pH 7.5. The samples were lysed by the addition of 100 μl of a solution containing 20 mg of proteinase K per ml (Amresco, Solon, Ohio) followed by the addition of 2 ml of lysis reagent which contained 8.0 M urea, 0.25% sodium dodecyl sulfate, 0.25% sodium lauroylsarcosine, and 50 mM EDTA, pH 8.0. All reagents were obtained from Sigma Chemical Co., St. Louis, Mo. The mixture was heated at 60°C for 30 min and centrifuged for 5 min at 750 × *g*. The supernatant was applied to the Extractor column and washed with 15 ml of 20 mM Tris–200 mM NaCl in 40% ethanol (pH 7.5). A second 5-ml wash containing 20 mM Tris–250 mM NaCl (pH 7.5) was then applied to the column. NA were eluted from the column with 2 ml of 20 mM Tris–500 mM NaCl (pH 7.5). The sample application, washing, and elution steps were performed with a Harvard Mini Infuser 150XL pump (C. R. Bard, Inc., North Reading, Mass.). The concentration of NA in the eluate was determined by A_{260} in a Gilford Systems spectrophotometer (CIBA Corning Diagnostics Corp., Oberlin, Ohio), and its relative purity was determined by the ratio of A_{260} to A_{280}.

After addition of the elution buffer to the column, nine 10-drop fractions were collected from the column and 10 μl of each fraction was subjected to electrophoresis through a 0.7% agarose gel to determine the elution profile of the NA. Serial dilutions of pKS007 DNA (see below) were spotted onto nitrocellulose filters to determine the minimum concentration of DNA required to produce a positive signal on X-ray film after hybridization with a ^{32}P-labeled probe.

ANT(2″) probe preparation. The recombinant plasmid pKS007, a derivative of pBR322 containing a 310-base-pair *Ava*I fragment from the open reading frame of the ANT(2″) structural gene (8), was the source of probe DNA. (pKS007 was obtained from K. Shaw, Schering Corp.) Plasmid DNA was purified by CsCl-ethidium bromide density centrifugation, and the 310-base-pair *Ava*I fragment was prepared as previously described (8). The probe was labeled with [^{32}P]dCTP (800 Ci/mmol) by random priming using a commercial kit (Bethesda Research Laboratories, Gaithersburg, Md.).

Hybridization procedures using the Centri-dot system. The Centri-dot system (Molecular Biosystems, Inc.) consists of a six-well plastic chamber that fits inside a 50-ml conical centrifuge tube. The inner unit contains a removable nylon membrane. Six samples can be processed in each Centri-dot, which concentrates the NA in a small spot by low-speed centrifugation. The nylon membranes were prewet with distilled water, and the NA in the eluate were denatured by heating at 95°C for 10 min. After denaturation, the samples were applied to the nylon membrane contained in the Centri-dot unit and centrifuged at 750 × *g* for 20 min at 4°C. The membrane was removed from the Centri-dot, and the NA was fixed by placing the membrane on a Chromato-Vue midrange transilluminator (Ultra-Violet Products, Inc., San Gabriel, Calif.) for 1 min while it was still wet. The filters were then hybridized with the ANT(2″) probe as described by Wahl et al. (10). Briefly, filters were prehybridized for 1 h at 42°C in a buffer containing 50% formamide, 1% glycine, 50 mM phosphate buffer (pH 6.5), 100 μg of sonicated calf thymus DNA, 5× Denhardt solution (1× Denhardt solution equals 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.02% Ficoll), 5× SSPE (1× SSPE contains 0.18 M NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1.1 mM disodium EDTA, and 5.5 mM NaOH, pH 7.7), and 0.5% sodium dodecyl sulfate. This solution was discarded and replaced with a hybridization buffer containing 50% formamide, 1% glycine, 20 mM phosphate buffer (pH 6.5), 1× Denhardt solution, 100 μg of calf thymus DNA, 5× SSPE, 10% dextran sulfate, and 0.5% sodium dodecyl sulfate containing 10^6 cpm of ^{32}P-labeled probe. Hybridization was carried out at 42°C overnight. Filters were washed at room temperature three times in 2× SSPE–0.1% sodium dodecyl sulfate and twice at 50°C in 0.1× SSPE–0.1% SDS. After washing, filters were dried and exposed to Kodak X-Omat AR X-ray film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens for 2 to 24 h at −70°C.

Broth blots. A portion of each stool sample was placed in 3 ml of Trypticase soy yeast extract broth (BBL Microbiology Systems, Cockeysville, Md.) and incubated without shaking at 37°C overnight. After incubation, a 10-μl sample of each broth was spotted onto a nitrocellulose filter and lysed and fixed as described by Gootz et al. (4). Each broth was also subcultured onto two MacConkey agar plates, one containing 3 μg of gentamicin per ml and a second containing 5 μg of tobramycin per ml, to screen for bacteria containing the ANT(2″) gene, as described above. All hybridization tests were performed in duplicate.

RESULTS

Recovery of NA from stool samples by using the Extractor column. A total of 50 stool samples were processed with Extractor columns, with a new column used for each sample. Five of the samples did not yield detectable amounts of NA; all of these failed to produce colonies on blood agar plates when incubated under aerobic conditions. The total amount of NA obtained from the remaining 45 samples ranged from 2 to 228 μg (Fig. 1), with a mean concentration of 50.2 ± 46.7 μg (mean ± standard deviation). The consistency of the stool sample (liquid versus formed) did not correlate with the yield of NA. Samples extracted in triplicate gave similar quantities of NA. Those samples yielding less than 10 μg of NA showed a predominant growth of gram-positive organisms or yeast cells on the blood agar plate. Attempts to obtain NA from pure cultures of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, or *Candida albicans* by the stool extraction procedure also produced low yields of NA (data not shown). Of the 45 NA samples recovered, 42 produced absorbance ratios between 1.5 and 2.1. Three samples gave ratios of >2.4. These samples were not noticeably different from the other stools in terms of their consistency or color (presence or absence of visible blood). All three grew a mixture of gram-negative and gram-positive flora on the blood agar plate. Agarose gel electrophoresis of the column eluate demonstrated that the first 20 drops collected after the elution procedure was initiated contained no detectable DNA (data not shown). The majority of DNA was contained in drops 20 to 60 (approximately 1.3 ml). The use of the Harvard infusion pumps greatly facilitated the washing and collection procedures and enabled one person to run multiple columns simultaneously.

Screening stool samples for the ANT(2″) resistance gene. Aminoglycoside-resistant gram-negative bacilli were isolated on selective media from 20 of the stool samples. Organisms from 17 of the samples grew after direct inoculation on selective media, while 3 produced colonies only after incubation in broth. Organisms from 19 of the isolates grew on both gentamicin and tobramycin containing media, a profile consistent with carriage of the ANT(2″) gene. The remaining stool sample produced a small number of colonies

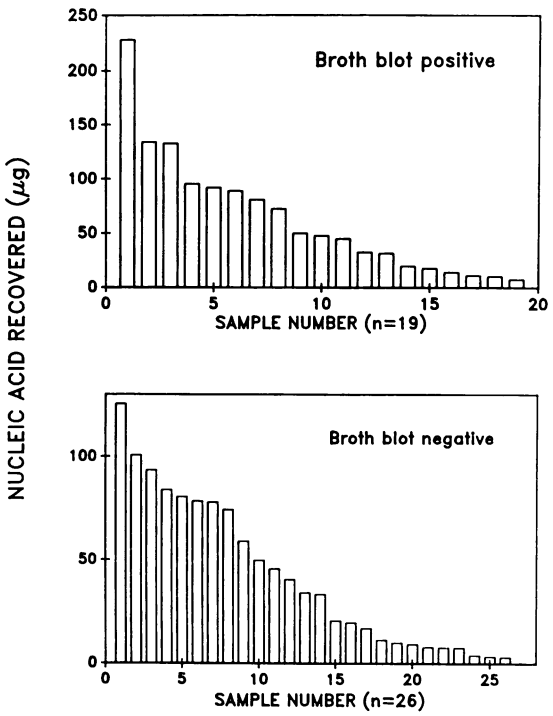

FIG. 1. Recovery of NA from stool samples that were positive (top) or negative (bottom) by broth blot hybridization with the ANT(2″) probe. Each bar represents the results of a single stool sample extraction.

on gentamicin agar only. Of these 20 stool samples, 18 were positive by the broth hybridization test. Serial dilutions of pKS007 DNA spotted onto nitrocellulose filters and hybridized with the ANT(2″) probe demonstrated that the minimal concentration of target required to produce a positive signal was 100 pg of DNA (data not shown). Two stool samples were negative by hybridization, but on selective media, they produced colonies containing gram-negative bacilli which produced disk diffusion patterns consistent with carriage of an ANT(2″) gene (resistance to gentamicin, kanamycin, and tobramycin). An additional stool sample did not produce aminoglycoside-resistant colonies but was positive by broth hybridization. Thus, a total of 21 of the 45 stool samples examined were considered to harbor ANT(2″)-containing organisms, as demonstrated by either a positive broth blot (n = 19) or growth on selective agar with antimicrobial susceptibility profiles consistent with the ANT(2″) gene (n = 2). The amount of NA recovered from the 19 broth blot-positive samples as well as the remaining 26 samples is depicted in Fig. 1.

Hybridization of the NA samples obtained from Extractor columns. Among the 45 NA samples obtained from stools using the Extractor column and blotted using the Centri-dot system, 14 hybridized with the probe. Of these 14 samples, 13 were among the 21 previously considered to harbor ANT(2″)-containing organisms (Table 1). The hybridization-negative samples were not those that yielded low quantities of DNA. On the contrary, the average amount of DNA obtained from these samples was 74.6 μg. The two samples that were negative by the broth blot hybridization test but produced gram-negative bacilli with the ANT(2″) phenotype were positive by the NA blot test. These samples had yielded 7.6 and 20.5 μg of DNA. One additional sample was positive twice in the NA blot but negative by broth blot and

TABLE 1. Ability of dot blot hybridization (NA blot) to detect the ANT(2″) resistance gene in NA samples isolated from stool[a]

Dot blot hybridization result	No. of stool samples with the following ANT(2″) test result[b]:	
	+	−
+	13	1
−	8	23

[a] Probe was the 310-base-pair *Ava*I fragment from pKS007.
[b] ANT(2″)-positive stools were defined as those which were positive with the ANT(2″) probe by broth blot hybridization (n = 19) or which yielded bacterial isolates on selective media with the ANT(2″) phenotype (see text).

did not produce colonies on either selective agar. If this sample is considered a false-positive, the sensitivity and specificity of the NA blot hybridization procedure compared with broth blots were 61.9 and 95.8%, respectively, while the positive and negative predictive values were 92.8 and 74.2%, respectively.

DISCUSSION

There were three questions that we sought to answer during this study. Are the Extractor and Centri-dot systems suitable for use in a clinical laboratory, how effective is the Extractor column method in purifying NA from stool for hybridization assays, and how frequently is the ANT(2″) gene present but not expressed in clinical isolates of bacteria?

We found the extraction of NA from raw stool by the Extractor column procedure rapid and straightforward, requiring approximately 2 h for every three samples if Harvard infusion pumps were used (one pump for each column). Without the pumps, the procedure was tedious and multiple columns could not be run simultaneously.

The total amount of NA recovered from stool specimens on average was approximately 50 μg/0.1-g or 100-μl sample, with a range from 2 to 228 μg. Recovery appeared to depend on the microbial flora present in the stool rather than the consistency of the stool. The procedure produced consistent results, however, with samples extracted in triplicate giving similar yields of NA. The lowest yields of NA came from samples with a predominance of gram-positive organisms or yeast. Although NA can be recovered from staphylococci and streptococci by using different pretreatment steps, the lysis procedure for preparing stool samples was not adequate for this purpose. Both DNA and RNA can be recovered with the Extractor column; however, the stool lysis procedure used in this study favored the recovery of DNA. Investigators wishing to use this procedure for probes other than those provided by the manufacturer may need to alter the extraction procedure to maximize recovery of RNA. Such procedures are not provided with the kit and, in our experience, have been difficult to formulate.

There was close agreement between the presence of growth on antibiotic-containing media and the results of the broth hybridization procedure. However, we are unable to explain why the two samples that demonstrated growth on selective media and were positive by the NA blot method were negative twice by broth blot. The concentration of DNA in these samples should have been adequate to produce a positive hybridization signal. It is possible that the ANT(2″) gene, while present in high-enough copy numbers to be detected directly in the Extractor-processed sample, was present in strains that either did not grow well in broth

or did not lyse well with the spot blot procedure. Such problems have been noted in earlier hybridization studies, particularly with mucoid strains of *Klebsiella pneumoniae* (4), which proved much more difficult to lyse in situ than other members of the family *Enterobacteriaceae* with similar patterns of antimicrobial resistance.

We had hypothesized that the Extractor method would provide greater sensitivity than the broth blot method, since Seriwatana et al. (6) and Tompkins and Krajden (9) noted increased sensitivity of hybridization reactions carried out with purified DNA versus lysates of whole colonies. However, our results suggest that some type of amplification of either target or signal will be required before direct susceptibility testing of complex clinical samples will be feasible. This is consistent with the observations of Olive, who used Extractor columns in conjunction with polymerase chain reaction and oligonucleotide probes to detect enterotoxigenic *E. coli* in stool samples (5). Although stool is probably the most difficult sample from which to extract NA, it is likely that similar interfering substances, such as complex polysaccharides and proteins that may inhibit hybridization reactions, will be encountered in sputum and blood as well.

Our data, although admittedly from a small sample, suggest that organisms containing the ANT(2″) resistance gene are carried by a large percentage (42%) of patients seeking care at the Seattle Veterans Affairs Medical Center. The majority of the stool samples studied herein were obtained from inpatients who were admitted to the hospital for reasons other than diarrhea. This high rate of ANT(2″) carriage was surprising, since <25% of gram-negative enteric organisms isolated in the clinical microbiology laboratory show resistance to gentamicin or tobramycin (F. C. Tenover, unpublished observations). On the other hand, in a recent study of mechanisms of aminoglycoside resistance among gram-negative bacilli in the Seattle Veterans Affairs Medical Center, 83.1% of aminoglycoside-resistant isolates recovered from patients were noted by DNA hybridization to carry the ANT(2″) gene (R. K. Flamm, F. C. Tenover, K. L. Phillips, and J. J. Plorde, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, A81, p. 14). Thus, the gene, while known to be present in this population, was not expected in such high numbers of patients. The results presented here suggest not only that the ANT(2″) gene is present in a relatively large number of patients but also that it is virtually always expressed in these organisms. Thus, direct probe analysis for this gene with clinical samples, such as urine and blood, containing gram-negative bacilli may be of value for directing antimicrobial chemotherapy early in a patient's illness.

The remaining issue concerns the one sample that was NA blot positive but negative for the ANT(2″) gene by all other parameters. Although this sample was scored as a false-positive, it is conceivable that it contained organisms harboring an ANT(2″) gene that is not expressed or perhaps incomplete. Thus, the specificity of the NA blot technique may be 100%. Further work using DNA sequence-specific amplification methods will be required to resolve this discrepancy.

In conclusion, we found the Extractor and Centri-dot systems to be a convenient and efficient means of recovering NA from stool samples. The NA recovered can be used directly in hybridization studies, although the sensitivity of these assays for certain genes, such as the ANT(2″) resistance gene, may be too low to be clinically useful without amplification of the target sequences or amplification of the resulting signal. Our studies suggest that the ANT(2″) resistance gene, in particular, is not present in high-enough concentrations to be detected by direct DNA hybridization analysis. However, we feel that further studies on direct susceptibility testing of clinical samples using DNA probes are warranted.

ACKNOWLEDGMENTS

We are grateful to Larry Carlson for his technical expertise and to Sam Morishima of Molecular Biosystems, Inc., for the generous gift of the Centri-dot systems and for helpful discussions.

This work was supported by the Medical Research Service of the Veterans Administration and a grant from the Microscan Division of the Baxter Healthcare Corporation.

LITERATURE CITED

1. **Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. **45**:493–496.
2. **Drake, T. A., J. A. Hindler, O. G. W. Berlin, and D. A. Bruckner.** 1987. Rapid identification of *Mycobacterium avium* complex in culture using DNA probes. J. Clin. Microbiol. **25**:1442–1445.
3. **Echeverria, P., J. Seriwatana, O. Sethabutr, and D. N. Taylor.** 1985. DNA hybridization in the diagnosis of bacterial diarrhea. Clin. Lab. Med. **5**:447–462.
4. **Gootz, T. D., F. C. Tenover, S. A. Young, K. P. Gordon, and J. J. Plorde.** 1985. Comparison of three DNA hybridization methods for detecting the aminoglycoside 2″-*O*-adenyltransferase gene in clinical isolates of bacteria. Antimicrob. Agents Chemother. **28**:69–73.
5. **Olive, D. M.** 1989. Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. J. Clin. Microbiol. **27**:261–265.
6. **Seriwatana, J., P. Echeverria, D. N. Taylor, T. Sakuldaipeara, S. Changschawalit, and O. Chivoratanond.** 1987. Identification of enterotoxigenic *Escherichia coli* with synthetic alkaline phosphatase-conjugated oligonucleotide DNA probes. J. Clin. Microbiol. **25**:1438–1441.
7. **Tenover, F. C.** 1988. Diagnostic deoxyribonucleic acid probes for infectious diseases. Clin. Microbiol. Rev. **1**:82–101.
8. **Tenover, F. C., T. D. Gootz, K. P. Gordon, L. S. Tompkins, S. A. Young, and J. J. Plorde.** 1984. Development of a DNA probe for the structural gene of the 2″-*O*-adenyltransferase aminoglycoside-modifying enzyme. J. Infect. Dis. **150**:678–687.
9. **Tompkins, L. S., and M. Krajden.** 1986. Approaches to the detection of enteric pathogens, including *Campylobacter*, using nucleic acid hybridization. Diag. Microbiol. Infect. Dis. **4**: 71S–78S.
10. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization using dextran sulfate. Proc. Natl. Acad. Sci. USA **76**:3683–3687.