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Comparison of Three DNA Hybridization Methods for Detection of the Aminoglycoside 2″-*O*-Adenylyltransferase Gene in Clinical Bacterial Isolates

THOMAS D. GOOTZ,[1]†* FRED C. TENOVER,[1,2,3] STEPHEN A. YOUNG,[2]‡ KATHERINE P. GORDON,[3] AND JAMES J. PLORDE[2,3]

Departments of Microbiology and Immunology[1] and Laboratory Medicine,[2] University of Washington, Seattle, Washington 98195, and Veterans Administration Medical Center, Seattle, Washington 98108[3]

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Two rapid DNA hybridization methods in which whole-cell lysates fixed to nitrocellulose were used were compared with Southern hybridization of purified plasmid or chromosomal DNA for the ability to identify the 2″-*O*-adenylyltransferase [ANT(2″)] gene in 42 enzymatically defined isolates of gram-negative bacilli. A DNA restriction fragment isolated from an ANT(2″) gene cloned into pBR322 and radiolabeled with ^{32}P was used as the probe in all three procedures. Under conditions of high stringency, agreement was obtained between the Southern hybridization method and detection of the ANT(2″) enzyme by the phosphocellulose paper binding assay or resistance phenotype in 39 of the 42 strains tested. By using these characterized strains, colony hybridization was shown to be unsatisfactory as a rapid technique for detecting the ANT(2″) gene, due to the high number of false-positive and -negative signals obtained. Compared with Southern hybridization, however, spot hybridization (SPH) proved highly reliable for detecting the ANT(2″) gene in both members of *Enterobacteriaceae* and *Pseudomonas aeruginosa* harboring R factors ranging in size from 23 to 150 kilobases. The relatively low copy number of the 150-kilobase plasmids decreased the sensitivity of SPH, necessitating a minimum cell density of 5×10^6 cells per spot. SPH proved to be a very useful method for rapidly screening large numbers of clinical isolates for this resistance determinant.

The most common mechanism of resistance to aminoglycoside antibiotics in bacteria is by enzymatic modification leading to restricted uptake of antibiotic into the bacterial cell (2, 7–10, 26). The tendency for the structural genes of the modifying enzymes to be located on plasmids and transposons has greatly increased the spread of these resistance determinants throughout the family *Enterobacteriaceae* and in many *Pseudomonas* species (3–5, 20, 25, 31). This has led to the isolation of organisms that frequently contain several different modifying enzymes, thus demonstrating complex aminoglycoside resistance phenotypes. Such patterns have complicated efforts at following the dissemination of individual modifying enzymes by antibiogram alone. The phosphocellulose paper binding assay (PPBA) has been used to identify specific aminoglycoside-modifying enzymes (13) but is time consuming and may fail to detect enzymes expressed in low levels in the bacterial cell (23, 29). We have recently used DNA hybridization techniques (29) for this purpose by cloning the structural gene for a 2″-*O*-adenylyltransferase [ANT(2″)] modifying enzyme that was responsible for an epidemic of aminoglycoside resistance at the Seattle Veterans Administration Medical Center. A 310-base-pair (bp) restriction fragment isolated from the interior of the cloned ANT(2″) gene was radiolabeled and used in Southern hybridization (SH) analysis to identify this resistance determinant on plasmids isolated from clinical strains (29). The resulting DNA probe proved to be specific for the ANT(2″) gene. It also was used to identify this determinant in some resistant strains in which the standard PPBA (13) failed to detect low levels of ANT(2″) modifying enzyme activity (29). Since SH identified the ANT(2″) gene on plasmids isolated from eight different genera of bacteria, we wished to determine whether DNA hybridization could be used as a rapid method for screening large numbers of clinical isolates for this resistance determinant. In this study, we compared hybridization results obtained by using colonies grown on nitrocellulose filters (colony hybridization [CH]) and overnight broth cultures spotted directly on filters (spot hybridization [SPH]) with those obtained with isolated plasmid and chromosomal DNA in SH. Good agreement was obtained between the rapid hybridization techniques, SH of isolated plasmid or chromosomal DNA, and identification of the ANT(2″) gene by enzymology or resistance phenotype analysis (17). The rapid DNA hybridization methods described constitute a convenient approach by which genes encoding specific antibiotic resistance determinants can be followed throughout large populations of clinical microorganisms.

MATERIALS AND METHODS

Bacterial strains. A total of 42 organisms were evaluated in this study. Five gram-negative bacilli previously shown to contain R factors encoding the ANT(2″) enzyme (29) were used as positive controls for hybridization studies. These included *Serratia marcescens* PS1, *Klebsiella pneumoniae* PS930, *Providencia stuartii* PS330, *Citrobacter freundii* PS319, and an *Escherichia coli* PS944 transconjugant of PS319 (29). Another positive *E. coli* PS942 transconjugant of PS330 was used in SH studies but was not evaluated by SPH or CH. Four strains, *E. coli* PS436, PS736, PS1649 (ANT 3″), and *S. marcescens* PS532 (AAC6′I) were previously shown (29) to lack the ANT(2″) determinant and served as negative controls for hybridization. Thirty-two additional clinical iso-

* Corresponding author.
† Present address: Pfizer Central Research, Groton, CT 06340.
‡ Present address: West Virginia University, Morgantown, WV 26505.

lates were obtained from the Clinical Microbiology Laboratory at the Seattle Veterans Administration Medical Center to serve as test strains for hybridization. These were chosen for analysis based upon their reaction with the ANT(2") probe in SH. The test strains included eight *Pseudomonas aeruginosa*, six *C. freundii*, five *E. coli*, four *S. marcescens*, three *Enterobacter cloacae*, three *K. pneumoniae*, two *Providencia* spp., and one *Morganella morganii*. Twenty-three of the clinical isolates were resistant to gentamicin, kanamycin, and tobramycin (19) by the disk diffusion method of Bauer et al. (1). All strains were screened for the presence of plasmids by the method of Portnoy et al. (22). The expression of the ANT(2") gene was studied by moving selected plasmids into *E. coli* K-12 by conjugation (21) or transformation (6). Clinical isolates were also tested by R. Hare (Schering Corp., Bloomfield, N.J.) for the presence of the ANT(2") determinant by using the resistance phenotype method of Miller et al. (17). The eight *Pseudomonas aeruginosa* isolates were also analyzed for adenylating activity by the PPBA as previously described (29).

Source of ANT(2") probe DNA. The recombinant plasmid pFCT3103, a derivative of pBR322 containing an intact ANT(2") structural gene, served as a source of probe DNA (29). A 310-bp *Ava*I restriction fragment from within the interior of the ANT(2") structural gene was prepared as previously described (29) and labeled with [^{32}P]dCTP (800 Ci/mmol) by nick translation (24), using a commercial kit (New England Nuclear Corp., Boston, Mass.).

Hybridization studies. SH with the radiolabeled 310-bp fragment as probe was chosen as the reference hybridization method for identifying the ANT(2") gene in all strains tested. Briefly, plasmid or chromosomal DNA was denatured after electrophoresis in 0.7% agarose gels and transferred to nitrocellulose by the method of Southern (27). Filters were prehybridized for 60 min as described by Wahl et al. (33). Hybridization was carried out at 37°C for 18 h with 2×10^5 cpm of ^{32}P-labeled probe DNA per filter under stringent conditions in 50% formamide (33). After posthybridization washing, filters were dried and exposed to Kodak X-Omat AR X-ray film (Eastman Kodak, Rochester, N.Y.) with an intensifying screen for 48 to 72 h at −70°C.

Genomic DNA was prepared by a sodium dodecyl sulfate-pronase lysis method (16) from all strains that were probe negative in the initial Southern plasmid hybridizations. Partial restriction digestions of chromosomal DNA were made by incubating approximately 7.5 μg of DNA overnight at 37°C in the presence of 7 U of *Bam*HI. The restricted DNA was electrophoresed in agarose, transferred to nitrocellulose filters, and hybridized with the ANT(2") probe as described above.

Inoculation of nitrocellulose filters with colonies or broth culture spots. Two rapid hybridization methods were compared by inoculating nitrocellulose filters to produce discrete colonies or by spotting suspensions of cells directly onto filters. The CH method of Grunstein and Hogness (12) was used as modified by Moseley et al. (18). Sterile nitrocellulose filters (82 mm; pore size, 0.45 μm; Schleicher & Schuell, Inc., Keene, N.H.) were placed on the surface of a brain heart infusion agar (BBL Microbiology Systems, Cockeysville, Md.) plate and inoculated from an 18-h plate culture of each organism by using a standard bacteriological loop. Care was taken to keep the inoculum confined to a circle not greater than 3 mm in diameter, permitting the inoculation of between 20 and 25 organisms per plate. Plates were then incubated overnight at 37°C, and the resulting colonies on the filter were lysed by the method of Moseley et al. (18).

An SPH method was also evaluated for use in screening large numbers of organisms for the ANT(2") gene. To determine the minimum number of cells per spot required to give a positive signal with the ANT(2") probe, eight strains previously assayed for ANT(2") modifying enzyme activity by the PPBA were tested after serial dilution in saline. Cells taken from overnight plate cultures were suspended in sterile saline to an optical density at 570 nm (Coleman Jr. II spectrophotometer) corresponding to a viable count of 10^9 CFU/ml as determined by direct plate counts on brain heart infusion agar. Twofold dilutions of this standardized suspension were made in saline, and 10 μl of each dilution was spotted on nitrocellulose, air dried, and lysed. SPH was also evaluated as a routine screening procedure with preparations from overnight cultures of strains in brain heart infusion broth.

Lysis of colonies and spot inoculations. Lysis of colonies and spot inoculations were performed as described by Moseley et al. (18) with sequential treatments of filters with 0.5 N NaOH–1 M Tris (pH 7) followed by a solution of 1 M Tris (pH 7)–1.5 M NaCl. In a modification of this procedure, filters were treated sequentially with pronase and chloroform before the 1 M Tris–1.5 M NaCl step. After treatment with the 1 M Tris solutions, filters were floated for 30 min in a petri dish containing 1 ml of a 10-mg/ml pronase solution (Sigma Chemical Co., St. Louis, Mo.) prepared in 1 M Tris. Filters were then air dried for 10 min and washed by submersing in a dish containing chloroform (J. T. Baker Chemical Co., Phillipsburg, N.J.) for 2 min with gentle agitation. They were then treated with 1 M Tris–1.5 M NaCl.

Nitrocellulose filters were prehybridized and then hybridized as described above, with 10^5 cpm of probe per filter. After overnight hybridization and washing, the dried filters were exposed to X-ray film at −70°C for 48 to 72 h.

Reproducibility studies. To test the batch-to-batch precision of the ANT(2") probe, the 10 control strains and 32 clinical isolates were inoculated onto five replicate sets of filters by the CH and SPH methods. Five separate lots of probe were prepared and hybridized to the filters on consecutive weeks. Probe results for each filter were judged by comparison to known positive and negative controls included on each filter.

RESULTS

Comparison of resistance phenotype analysis and enzymology with SH. All 42 strains included in the study were tested with the ANT(2") probe by SH. The six control strains previously shown to contain the gene (29) were positive by this method, and as expected, the four negative controls showed no homology with the probe. These results agree with those obtained previously, showing the radiolabeled *Ava*I fragment to be specific for the ANT(2") gene (29).

Among the 32 clinical strains tested, the ANT(2") probe hybridized with plasmids ranging in size from 23 to 150 kilobases (kb), with 62% of these being 150 kb. The 150-kb plasmids were found in a wide range of isolates, including strains of *E. coli*, *C. freundii*, and *Providencia stuartii*. Restriction digests of 150-kb plasmids isolated from three different genera showed a significant degree of heterogeneity (data not shown). The smallest plasmids encoding the ANT(2") gene were 23 and 53 kb from isolates of *Pseudomonas aeruginosa*. No evidence for a chromosomal location of the ANT(2") gene was observed in any of the strains tested. Three plasmid-containing isolates, *Providencia stuartii* PS330 (control) and *Pseudomonas aeruginosa* PS351 and PS435, failed to demonstrate adenylating activity by

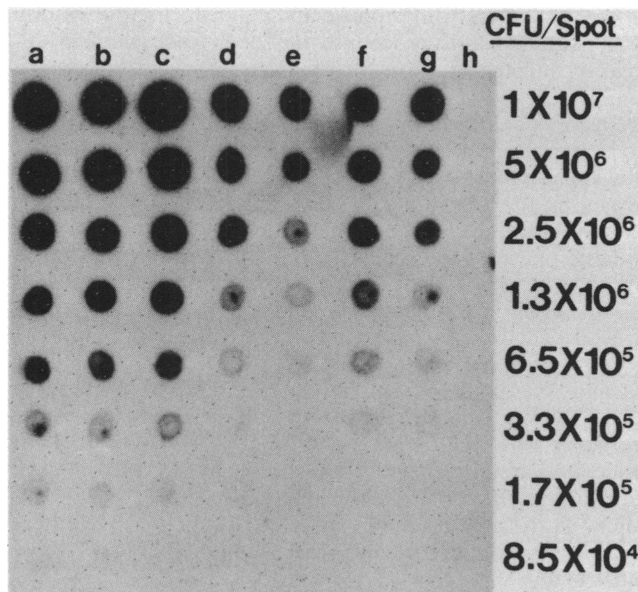

FIG. 1. Exposed film of spot dilution scheme of strains diluted in saline and hybridized with the ANT(2") probe. Sensitivity of probe is decreased for strains containing a 150-kb plasmid that hybridizes. Lane a, *E. coli*(pFCT3103), 5.9 kb; lane b, *S. marcescens*(pLST1000), 68 kb; lane c, *K. pneumoniae* PS930, 79 kb; lane d, *C. freundii* PS319, 150 kb; lane e, *E. coli* PS944 transconjugant of strain PS319, 150 kb; lane f, *Providencia stuartii* PS330, 150 kb; lane g, *E. coli* transconjugant of PS330, 150 kb; lane h, aminoglycoside-susceptible *S. marcescens* PS532.

either the PPBA or the resistance phenotype method but hybridized with the ANT(2") probe in SH. An *E. coli* transconjugant of *Providencia stuartii* PS330 (PS942) and an *E. coli* transformant of *Pseudomonas aeruginosa* PS435 demonstrated expression of the ANT(2") gene by the PPBA and resistance phenotype methods, respectively. In the remaining 39 isolates, there was complete concordance between presence of the ANT(2") enzyme and demonstration of homology with at least one plasmid with the probe in SH (21 positives, 18 negatives). These 39 concordant strains were used in subsequent studies to compare the reproducibility of the rapid hybridization procedures after standardization of the SPH and CH procedures.

Relationship of target replicon size and sensitivity of the ANT(2") probe. The sensitivity of the ANT(2") probe in SPH was determined by hybridizing saline dilutions of strains containing the ANT(2") gene on plasmids ranging in size from 5.9 to 150 kb. *E. coli* PS1170 containing the multicopy 5.9-kb recombinant plasmid, pFCT3103, showed detectable hybridization with as little as 3.3×10^5 cells per spot (Fig. 1, lane a). In contrast, strains containing the ANT(2") gene on a 150-kb plasmid required nearly 10-fold more cells per spot to obtain a positive signal of similar intensity (Fig. 1, lanes d through g). From these data, the minimum inoculum of cells required to obtain a positive signal in SPH was determined to be 5×10^6 cells per spot. In all subsequent studies with the SPH procedure, a 10-μl inoculum taken from an overnight culture was used, thus delivering approximately 5×10^6 to 1×10^7 cells, an amount compatible with this degree of ANT(2") probe sensitivity.

Evaluation of signal patterns produced by CH and SPH. In Fig. 2, the signal patterns obtained for a group of clinical strains tested by the CH and SPH methods are compared. In situ lysis and hybridization of colonies often resulted in uneven or weak signal patterns with CH, likely the result of flaking and loss of colony material during posthybridization washing (Fig. 2A). Loose colony material frequently washed to extraneous areas of the filter, causing a false-positive signal. This problem was most frequently encountered with mucoid strains of *Klebsiella* sp. and organisms such as *Citrobacter* sp. that produced inordinately large colonies. Treatment of colony filters with pronase and chloroform had no effect on fixing the DNA to the nitrocellulose or improving the reproducibility of CH. More uniform and reproducible signal patterns were obtained with SPH when the appropriate cell density was maintained (Fig. 2B). Despite the application of fewer cells, clearer signal patterns were obtained with SPH, and no cross-contamination of inocula was observed.

Comparison of SH, SPH, and CH procedures. In 30 of the 39 concordant strains (6 *Pseudomonas aeruginosa*, 33 *Enterobacteriaceae*), there was complete agreement between the three hybridization procedures. The nine strains demonstrating discrepant results are listed in Table 1. Since each organism shown was tested five consecutive times by both the spot and colony hybridization procedures, a total of 45 hybridizations were available for evaluation by each method. Forty-three of the 45 results obtained by SPH and 15 of 45 results obtained by CH were in agreement with those obtained by SH. Relatively few discrepancies were noted among the *Enterobacteriaceae*. SPH correctly identified the resistance determinant in all strains from this group, for a sensitivity and specificity of 100%. Three of the study strains of *Enterobacteriaceae*, however, demonstrated variable results by CH (Table 1). *C. freundii* PS674 and *E. coli* PS425 gave positive results only four of five and three of five times, respectively. Both bacterial strains produced large colonies (4 to 5 mm) on the nitrocellulose filter. *K. pneumoniae* PS42, a very mucoid clinical isolate, was positive only two of five times by CH.

The identification of the ANT(2") determinant in the remaining six *Pseudomonas aeruginosa* strains proved to be more difficult. None was positive for the determinant by enzymology or resistance phenotype. Four strains (PS139, PS339, PS163, and PS531) that were positive by CH in at least three of five tests were negative by both SH and SPH (Table 1). Two additional isolates (PS38 and PS335) were positive five of five times by CH, positive one of five

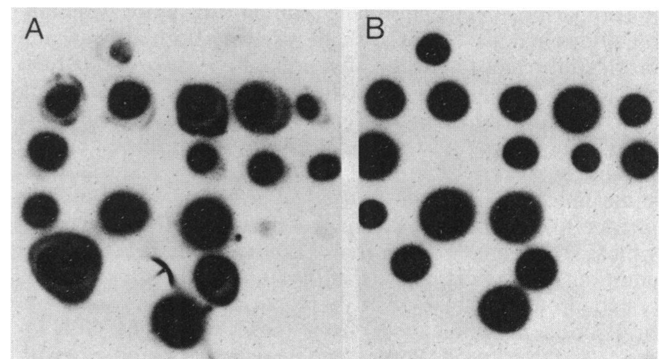

FIG. 2. Comparison of films prepared from strains analyzed by CH (A) and broth SPH (B). Nitrocellulose filters were treated through lysis and hybridization procedures in an identical fashion. Films were exposed with hybridized filters for 72 h at −70°C.

TABLE 1. Discrepancies observed between CH and SPH methods on 39 study strains

Organism	Strain	SH result[a]	No. of positive results by[b]:		Size (kb) of plasmid(s) positive by SH[c]
			SPH	CH	
Citrobacter freundii	PS674	+	5	4	135,68
Escherichia coli	PS425	+	5	3	146
Klebsiella pneumoniae	PS42	+	5	2	38
Pseudomonas aeruginosa	PS139	−	0	3	None
	PS339	−	0	5	None
	PS163	−	0	3	None
	PS531	−	0	3	None
	PS38	−	1[d]	5	None
	PS335	−	1[d]	5	None

[a] +, Positive result; −, negative result.
[b] Five tests were performed for each organism by each method.
[c] Plasmid showing homology with the ANT(2″) probe by SH.
[d] Equivocal positive result observed in one of five tests.

times by SPH, and negative by SH. The frequent false-positive results seen in *Pseudomonas aeruginosa* with the CH procedure could not be resolved by treating the filters with pronase and chloroform after colony lysis.

DISCUSSION

The current study has proposed the use of DNA hybridization for identifying and following the dissemination of specific aminoglycoside resistance genes in clinical isolates of bacteria. Although it has been relatively simple to document the increased occurrence of antibiotic resistance among clinical strains, it has been more difficult to identify and follow the specific resistance genes responsible. Cloning of the ANT(2″) structural gene onto pBR322 permitted the isolation of a unique restriction fragment from within the ANT(2″) gene that served as a specific DNA probe for this resistance determinant.

Three hybridization methods were evaluated for use in detecting the ANT(2″) gene. In the reference method, isolated plasmid or total cellular DNA that was hybridized with the probe by the SH technique was used. Although this method has been shown to correlate well with the enzyme assay for detecting ANT(2″) activity (29), the time required for preparing, electrophoresing, and blotting DNA generally precludes its use in large-scale studies. Both colony and broth culture spot hybridization procedures have the distinct advantage of allowing more strains to be analyzed in a shorter period of time. Although CH has been applied to a broad range of assays (18, 28, 30), we found SPH to be more reliable in work with clinical isolates. Colony material from some mucoid strains of *Enterobacteriaceae* was lost from nitrocellulose filters during posthybridization washing, which sometimes resulted in false-negative reactions. In addition, two-thirds of the *Pseudomonas aeruginosa* strains tested produced false-positive reactions by CH, since hybridization of whole cells from these strains by SPH or genomic DNA by SH showed no evidence of homology with the ANT(2″) probe. It could be assumed that the false-positive CH reactions observed with *Pseudomonas aeruginosa* was due to nonspecific binding of the probe to colony material on the filter. This problem was not resolved by treating the filters with pronase and chloroform after colony lysis. Although this procedure has been successful in decreasing nonspecific binding of probe DNA to laboratory strains of *E. coli* (28), it may be less useful for CH of clinical strains of bacteria. In this sense, SPH appeared to provide a more appropriate cell inoculum for clearer, more reproducible signal patterns. A gene dosage effect was observed in measuring the sensitivity of the probe against whole cells spotted on nitrocellulose. The location of the ANT(2″) gene on large plasmids that are usually maintained in the cell in low copy number (11) necessitated the use of a standardized cell inoculum for obtaining the reproducible signal patterns of SPH. This phenomenon is relevant for identifying antibiotic resistance genes, since in our study, 62% of the plasmids that showed homology with the probe in SH were 150 kb in size. Other investigators have encountered similar problems with the reproducibility of DNA hybridization procedures resulting from variations in plasmid copy number with laboratory-constructed bacterial strains (14, 28, 30). Solutions to these problems have often involved amplification of plasmid copy number with chloramphenicol (28) or construction of a probe with a high specific activity (32). Large-molecular-weight plasmids in clinical strains, however, are not likely to be susceptible to chloramphenicol amplification. Nevertheless, under the conditions defined in this study, the sensitivity of SPH was equivalent to that of SH and produced only 1% false-positive blots.

Initial evaluation of the ANT(2″) probe showed that it was specific for the ANT(2″) gene, since it did not cross-react with genes encoding the ANT(3″) or ANT(4′) enzymes, the 3′ (I and II) phosphotransferases, or the 3′ (I and III)- and 6′-I-acetyltransferases (29). The current study supports this conclusion, since in the *Enterobacteriaceae* tested, the ANT(2″) resistance phenotype or enzyme activity was detected in all strains containing plasmids that showed homology with the probe and was not detected in probe-negative strains. Our previous work has indicated that the ANT(2″) probe is clearly more sensitive than the PPBA for detecting this enzyme in some clinical isolates (29). This was also observed in the present study for strains of *Pseudomonas aeruginosa*. None of the eight *Pseudomonas* strains tested demonstrated ANT(2″) activity by either the PPBA or the resistance phenotype method. However, two of these strains contained plasmids (23 and 53 kb) that hybridized with the probe in SH. Resistance phenotype analysis identified the ANT(2″) determinant in an *E. coli* transformant that contained the 23-kb plasmid but did not detect it in a transformant containing the 53-kb plasmid. Genes encoding other aminoglycoside-modifying enzymes have been shown to be expressed at very low levels in *Pseudomonas aeruginosa*, preventing their detection by standard assay methods (23). In this regard, rapid DNA hybridization methods would have extensive value for detecting genes whose proteins are expressed at levels below the sensitivity of existing biological assays (15).

In summary, with an appropriate inoculum, the SPH procedure is sufficiently simple, sensitive, and specific to warrant its use for rapidly screening large numbers of clinical isolates for the presence of the ANT(2″) structural gene. We are in the process of developing several DNA probes for various aminoglycoside-modifying enzyme genes for use in analyzing resistant strains by the SPH method. It is anticipated that a gene bank containing probes for the more common aminoglycoside modifying enzymes would have great utility in gaining greater insight into the origin and dissemination of these resistance determinants.

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