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Detection of Plasmid-Mediated β-Lactamases with DNA Probes

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β-Lactamase identification by colony hybridization with ^{32}P-labeled DNA probes for TEM-1, SHV-1, OXA-1, OXA-2, PSE-1, PSE-2, and PSE-4 was compared with isoelectric focusing in 122 clinical isolates making a variety of enzyme types. All strains producing a probe-type enzyme gave a positive hybridization reaction. Cross-hybridization was observed between TEM-1 and TEM-2 or TLE-1, between SHV-1 and SHV-2, between OXA-1 and OXA-4, between OXA-2 and OXA-3 (weak), between PSE-2 and OXA-6 or OXA-5 (weak), and among PSE-1, PSE-4, and CARB-3. With allowance for such cross-hybridization, only six strains gave false-positive reactions, and the procedure was 99% specific.

Biochemical studies have established that plasmids in gram-negative bacteria determine a variety of β-lactamase types. The enzymes can be differentiated by substrate profile, reactions with inhibitors, molecular weight, and particularly isoelectric point (pI) into at least 25 kinds (16, 19, 32). Some, such as TEM-1 and SHV-1, have broad-spectrum but relatively unspecialized activity. As their name implies, the OXA set hydrolyzes oxacillin and related β-lactams with particular efficiency, whereas CARB enzymes, also given PSE designations because they were first found in *Pseudomonas aeruginosa*, are notably effective in carbenicillin hydrolysis. Distinguishing between the enzymes is of value for epidemiological studies where the presence of a less common β-lactamase type may allow a particular strain or plasmid to be traced (20). The distribution of β-lactamase types in different organisms may also help elucidate where these enzymes originated and how they have spread in response to the use and elaboration of β-lactam antibiotics.

Studies relying on isoelectric focusing have demonstrated TEM-1 as the predominant β-lactamase in ampicillin-resistant *Escherichia coli* and *Salmonella* spp., whereas SHV-1 is preponderant in *Klebsiella* spp. (29). In *P. aeruginosa* carbenicillin or ticarcillin resistance due to β-lactamase production is the result mainly of PSE-1 or PSE-4 (24, 33).

If β-lactamase genes are as diverse as the enzymes they encode, it should be possible to develop DNA probes for β-lactamase identification that would allow more rapid studies with large numbers of strains. DNA fragments suitable as probes for TEM-1 (3, 12, 22), OXA-1 (12, 23), OXA-2 (2), and PSE-1 (12) have been described and have been studied for specificity by utilizing purified plasmid DNA. The purpose of this investigation was to apply such probes to clinical isolates to evaluate their sensitivity and specificity.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the reference β-lactamase-producing strains used as controls in hybridization and isoelectric focusing experiments.

A total of 102 unique clinical gram-negative isolates resistant to ampicillin, ticarcillin, or both were obtained from the Massachusetts General Hospital bacteriology laboratory during August and September 1986. Resistance was first defined by the disk diffusion method (1). Subsequently, all strains were demonstrated to grow on plates containing 25 μg of ampicillin per ml and to produce β-lactamase as determined by the nitrocefin test (21). Because no probe-type β-lactamases were detected among *P. aeruginosa* isolates from this source, 20 *P. aeruginosa* clinical isolates with known plasmid-mediated β-lactamases collected by A. Philippon in France before 1981 were also included in hybridization studies.

Analytical isoelectric focusing. Extracts were prepared by sonication from cultures grown exponentially in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and frozen at −20°C until use. Analytical isoelectric focusing on polyacrylamide gels was carried out as described by Matthew et al. (17). β-Lactamases isolated from control strains were used as pI standards. Current was applied at a constant power of 1 W with voltage limit of 320 V at 4°C for 17 h (LKB 2103 Power Supply; LKB Instruments, Inc., Rockville, Md.) and then for an additional hour at 400 V. Filter paper soaked in nitrocefin (500 μg/ml; BBL Microbiology Systems, Cockeysville, Md.) was overlaid on the gel, and photos were taken with Polaroid MP-4 camera loaded with type 55 film with a Tiffen 658 dark green filter.

DNA probes. DNA probes (Table 2) were prepared from pBR322 (31), pMON21 (2), pMON301 (12), and derivatives of plasmids described by Levesque et al. (12) as follows: pMON811, an *Ava*I deletion derivative of pMON810 making PSE-1; pMON234, an *Ava*I deletion derivative of pMON2301 encoding PSE-2; pMON709, a deletion derivative related to pMON701 producing PSE-4; and pMON38, an *Ava*I-*Cla*I deletion derivative of pMON31 containing the cloned SHV-1 gene (M. Boissinot, J. Mercier, M. Lafond, and R. C. Levesque, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, A-130, p. 22). Probes for TEM-1 and OXA-2 are intragenic (4, 31). An intragenic probe was also developed for the PSE-2 *bla* gene based on the recent determination of its DNA sequence (7).

Probe DNA was prepared by using appropriate restriction endonucleases (New England Biolabs, Beverly, Mass.). DNA fragments were separated by electrophoresis on 6% polyacrylamide gels (14), electroeluted, precipitated with ethanol, extracted once with chloroform, and precipitated again with ethanol. DNA (0.5 to 1 μg) was labeled with [^{32}P]dATP and [^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.) by nick translation (28) employing reagents from Amersham. Labeled DNA was purified by utilizing Sephadex G50 columns (Pharmacia, Solna, Sweden) as described by Maniatis et al. (14). Probe specific activity was

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TABLE 1. Bacterial plasmids used for hybridization testing[a]

Plasmid	β-Lactamase	Isoelectric point	Reference
R1	TEM-1	5.4	9
RP4	TEM-2	5.6	9
pMG204b	TLE-1	5.55	18
R1010	SHV-1	7.6	9
pBP60-1	SHV-2	7.6	11
R997	HMS-1	5.2	9
pUZ8::Tn*1412*	LCR-1	5.85	9
pMK20::Tn*1414*	NPS-1	6.5	13
pDS075	OHIO-1	7.0	30
pMON401	ROB-1	8.1	12
RGN238	OXA-1	7.4	9
R46	OXA-2	7.7	9
RIP64	OXA-3	7.1	8
pMG203	OXA-4	7.5	9
pMG54	OXA-5	7.62	9
pUZ8-pMG39	OXA-6	7.68	9
pMG202	OXA-7	7.65	9
pMG217	PSE-1	5.7	9
pUZ8-R151	PSE-2	6.1	9
Rms149	PSE-3	6.9	9
pUZ8-DAL	PSE-4	5.3	9
pUZ8::Tn*1408*	CARB-3	5.75	9
pMK20::Tn*1413*	CARB-4	4.3	26
pUK657	SAR-1	4.9	27
Ω7711[b]	AER-1	5.9	6

[a] All plasmids were transferred to *E. coli* C600.
[b] Ω7711 is a cluster of resistance genes including that for AER-1 β-lactamase transferred en bloc from *Aeromonas hydrophila* to the *E. coli* chromosome.

controlled to be more than 1.0×10^8 cpm/μg (10^7 cpm/ml of hybridization buffer); probes were denaturated by boiling for 10 min before being added to hybridization buffer.

DNA hybridization. Colony hybridization was carried out as described by Grunstein and Wallis (5). Colonies were grown to a diameter of 1 to 2 mm on 0.45-μm-pore-size nitrocellulose filters (Schleicher & Schuell Co., Keene, N.H.) and lysed with 10% sodium dodecyl sulfate (SDS), 0.5 M NaOH–1.5 M NaCl, and 1.5 M NaCl–0.5 M Tris chloride (14). Filters were baked in an 80°C oven for 90 min and prehybridized in buffer containing 5× Denhardt solution (14)–0.5% SDS–5× SSPE (14)–200 μg of denaturated, sonicated salmon sperm DNA per ml for 60 min at 70°C. Hybridization was performed for 3.5 h at 70°C in the same buffer plus 10% dextran sulfate. A 10-ml sample of hybridization solution was used for 300 cm^2 of filter surface. After hybridization filters were washed for 10 min in 1× SSPE–0.1% SDS at room temperature, for 30 min in 0.1× SSPE–0.1% SDS at 65°C, for 10 min in 0.1× SSPE–0.1% SDS at room temperature, and finally for 10 min in 3 mM Tris base (pH 9.5) at room temperature. Filters were dried under a heat lamp and exposed to Kodak X-Omat AR film with intensifying screen for 10 min to 18 h at −70°C.

RESULTS

Hybridization with reference strains. Cross-hybridization was found between a few β-lactamase genes that produce biochemically similar enzymes (Table 2). Colony hybridization gave positive results between genes for TEM-1 and TEM-2 or TLE-1, between OXA-1 and OXA-4, and between PSE-1 and PSE-4 or CARB-3, confirming prior findings with purified DNA (12). The OXA-2 probe also reacted weakly with an OXA-3 control strain as previously reported (2), and an SHV-1 probe hybridized as well with closely related SHV-2 (11). Unexpectedly, a PSE-2 probe, one known from sequencing to lie within the structural gene, reacted with OXA-6 and weakly with OXA-5 DNA.

β-Lactamase distribution in clinical isolates. As determined by isoelectric focusing, the predominant β-lactamase in the 102 ticarcillin-resistant clinical isolates from the Massachusetts General Hospital was TEM-1 (59 strains), with OXA-2 identified in 9 isolates, OXA-1 identified in 5 isolates, SHV-1 identified in 4 isolates, and TEM-2 identified in 1 isolate. The distribution of enzyme types in different species is shown in Table 3. Other β-lactamases seen but not characterized further included enzymes at pI 5.2 in three *Citrobacter* strains, at pI 6.9 in *Citrobacter* strains, *P. aeruginosa*, and *Enterobacter* spp., at pI 7.0, 7.65, and 7.65 to 7.68 in three strains of *E. coli*, and at pI 7.1 in four strains of *Serratia marcescens*. Eleven isolates produced TEM-1 and a second β-lactamase.

No probe-type β-lactamases were produced by the 17 ticarcillin-resistant *P. aeruginosa* isolates. Accordingly the sample was supplemented with 20 *P. aeruginosa* strains from France that produced plasmid-mediated β-lactamases. Eight of these strains made PSE-1, four made TEM-1, two made PSE-4, two made CARB-3, two made OXA-1, one made OXA-3, and one made PSE-2.

Hybridization results with clinical isolates. The 102 clinical isolates and the 20 strains from France were tested by colony hybridization with ^{32}P-labeled probes representing the seven β-lactamase types. Cross-hybridizations known from sample experiments (Table 2) were not considered to be false-positives.

Every strain producing a probe-type β-lactamase was identified. Thus, the sensitivity of the technique was 100%. Six strains gave false-positive reactions, i.e., a positive hybridization reaction for an enzyme not demonstrated to be present by isoelectric focusing. The overall accuracy of

TABLE 2. Probes used for hybridization and reactions with other β-lactamase genes

β-Lactamase	Plasmid	Fragment	Cross-hybridization[a]
TEM-1	pBR322	0.42-kb *Bgl*I-*Hin*cII[b]	TEM-2, TLE-1
SHV-1	pMON38	0.78-kb *Pvu*II-*Pvu*II[c]	SHV-2
OXA-1	pMON301	0.31-kb *Bgl*II-*Bgl*II	OXA-4
OXA-2	pMON21	0.51-kb *Hin*cII-*Hin*cII[b]	(OXA-3)
PSE-1	pMON811	1.3-kb *Bam*HI-*Bgl*II	PSE-4, CARB-3
PSE-2	pMON234	0.46-kb *Rsa*I-*Xba*I[b]	OXA-6, (OXA-5)
	pMON234	1.2-kb *Ava*I-*Ava*I	OXA-6, (OXA-5)
PSE-4	pMON709	0.18-kb *Bgl*II-*Xba*I[d]	PSE-1, CARB-3

[a] β-Lactamases in parentheses hybridized weakly.
[b] Fragments known to lie within β-lactamase structural gene.
[c] Probe perfected by J. Mercier.
[d] Probe perfected by M. Boissinot.

TABLE 3. Distribution of β-lactamase types by isoelectric focusing and DNA hybridization

Species[a] (n)	No. positive in isoelectric focusing/no. positive in hybridization with β-lactamase type:				
	TEM-1	SHV-1	OXA-1	OXA-2	PSE-1
Escherichia coli (47)	40/40	1/2[b]	5/5	2/3[c]	0/1[c]
Pseudomonas aeruginosa (17)				0/1[d]	
Citrobacter spp. (10)	4/4				
Klebsiella pneumoniae (9)	6/6	3/3		4/4	
Serratia marcescens (8)	4/4			2/2	
Enterobacter spp. (7)	3/3			1/1	
Proteus mirabilis (2)	2/2[e]				
Pseudomonas putida (2)	1/1				
Pseudomonas aeruginosa[f] (20)	4/4		2/2		12/14[g]

[a] Some strains produced only chromosomal β-lactamase, whereas a few produced more than one plasmid-mediated enzyme (see text for details).
[b] Strains with false-positive hybridization produced β-lactamase with pI 7.0.
[c] Strains with false-positive hybridization produced TEM-1 β-lactamase.
[d] Strains with false-positive hybridization produced only chromosomal β-lactamase.
[e] One strain produced TEM-2 β-lactamase.
[f] The collection from France including one PSE-2 and one OXA-3 producer as well.
[g] Included eight PSE-1, two PSE-4, and two CARB-3 producers as determined by isoelectric focusing. False-positive reactors made OXA-1 β-lactamase.

colony hybridization compared with isoelectric focusing was 99.2% (Table 4).

TEM-1 β-lactamase was detected by hybridization in all 63 strains (59 from the Massachusetts General Hospital and 4 from France). One *Proteus mirabilis* producing TEM-2 gave a positive reaction as well (Table 3). Four SHV-1 β-lactamase producers were positive by hybridization, but one *E. coli* strain producing a β-lactamase of pI 7.0 was false-positive with the SHV-1 probe. All seven OXA-1 producers were correctly identified, as were nine strains making OXA-2. However, the OXA-2 probe gave false-positive reactions with an *E. coli* strain producing TEM-1 by isoelectric focusing and with a *P. aeruginosa* making only a chromosomal-type β-lactamase. The PSE-1 probe detected all eight PSE-1 β-lactamase producers as well as two *P. aeruginosa* strains making PSE-4 and two strains making CARB-3. Three strains gave false-positive results with the PSE-1 probe: an *E. coli* strain making TEM-1 and two *P. aeruginosa* strains making OXA-1 β-lactamase. The 1.2-kilobase (kb) PSE-2 probe used initially gave false-positive reactions with three strains producing OXA-2 and two making TEM-1. The intragenic 0.46-kb PSE-2 probe gave no false-positive reactions and correctly identified a PSE-2-producing *P. aeruginosa* isolate. The PSE-4 probe reacted with the same 12 strains correctly identified by the PSE-1 probe but gave no false-positive reactions.

DISCUSSION

Although several groups have developed DNA probes for β-lactamase detection, the reliability of this approach for clinical isolates has previously been evaluated only for a TEM-1 probe. Jouvenot et al. (10) analyzed 328 ampicillin- or ticarcillin-resistant bacterial isolates and found that 166 made TEM-1 or TEM-2 whereas 174 reacted with probe DNA. False-negative results were seen with 8 strains, and 16 isolates of various types were probe positive but did not make a TEM β-lactamase, raising the possibility that these strains might contain a silent, unexpressed TEM β-lactamase gene. Such strains were not seen in our collection with a TEM-1 probe, perhaps because the sample was smaller or because we used an intragenic 0.42-kb TEM-1 probe unlike the 1-kb probe employed by Jouvenot et al., which contains approximately 200 bases external to the β-lactamase gene.

With the probes evaluated here, all strains making a probe-type β-lactamase by isoelectric focusing were positive, there were no false-negative reactions (100% sensitivity), and the frequency of false-positive reactions was low so that the overall specificity was 99%. Only 6 of 122 strains gave hybridization reactions inconsistent with the β-lactamase identified by biochemical testing. Further studies will be needed to establish whether these false-positive reactions, which involved SHV-1, OXA-2, and PSE-1 probes, are the result of errors inherent to the methodology or are due to the more interesting possibility of silent β-lactamase genes.

The utility of a probe containing only coding sequence for a gene is illustrated by PSE-2, for which our initial 1.2-kb probe gave false-positive reactions that disappeared when an intragenic 0.46-kb probe was employed. Sequence analysis has shown that the larger probe contains 101 base pairs external to the PSE-2 gene that are 100% identical to the sequence proximal to the OXA-2 *bla* gene (7). Three of five strains falsely reacting with the 1.2-kb PSE-2 probe made

TABLE 4. Accuracy of colony hybridization compared with isoelectric focusing in determining β-lactamase type

Test results		No. of isolates with indicated test results					
Hybridization	Isoelectric focusing	TEM-1	SHV-1	OXA-1	OXA-2	PSE-1, PSE-4, and CARB-3	PSE-2
+	+	64[a]	4	7	9	12	1
+	−	0	1	0	2	3	0
−	+	0	0	0	0	0	0
−	−	58	117	115	111	107	121

[a] Includes one TEM-2 producer.

OXA-2. The finding that the smaller PSE-2 probe hybridizes with OXA-6 and weakly with OXA-5 implies a closer relationship between these genes than might have been anticipated from their names. However, PSE-2 is unique among the β-lactamases efficiently hydrolyzing carbenicillin in also readily attacking oxacillin, cloxacillin, and methicillin (15, 25) and has considerable amino acid sequence similarity with the OXA-2 enzyme (7). Since the 0.46-kb PSE-2 probe did not hybridize with the OXA-2 β-lactamase gene, an even closer relationship between the nucleotide sequence of PSE-2 and that of OXA-5 and OXA-6 can be predicted from the hybridization result. As the sequence of more plasmid-mediated β-lactamase genes is established, more such relationships are likely to become evident, and more specific probes can be devised along the lines of oligonucleotide probes that can discriminate between such closely related genes as those for TEM-1 and TEM-2 (22).

At present isoelectric focusing is the "gold standard" for plasmid-mediated β-lactamase identification. Its drawbacks are the limited number of isolates that can be tested per day and the increasing number of enzymes with barely distinguishable isoelectric points that require further tests to differentiate (19). Colony hybridization is more demanding technically and utilizes ^{32}P-labeled nucleotides with short half-lives but is efficient when large numbers of strains need to be analyzed. Use of a nonradioactive probe would facilitate routine application, but attempts to utilize biotin labeling in place of ^{32}P gave unacceptably high false-positive and false-negative results, in agreement with the experience of others (34). If the limited cross-hybridization reactions inherent with the currently available probes are taken into account, the frequency of false-positive reactions is low; improved probes can be anticipated as more β-lactamase genes are sequenced.

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