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Oligonucleotide Probes (TEM-1, OXA-1) versus Isoelectric Focusing in β-Lactamase Characterization of 114 Resistant Strains

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Oligonucleotide probes specific for detection of the TEM-1 and OXA-1 β-lactamase genes were compared with isoelectric focusing in 114 gram-negative β-lactamase-producing strains representing at least 16 species. Correlations of 96 and 100% with isoelectric points were found for the TEM-1 and OXA-1 probes, respectively.

More than 25 plasmid-mediated β-lactamases (EC 3.5.2.6) have been described among gram-negative bacteria (7, 11, 12). These enzymes are characterized by substrate and inhibition profiles, isoelectric focusing, and primary structure determination (1, 9). There are now some β-lactamases with very subtle differences in isoelectric point, such as TLE-1, TEM-2, and BRO-1; OXA-1 and OXA-4; and OXA-5, OXA-6, and OXA-7 (12, 13, 16). Identification by these criteria will become increasingly difficult and a source of error. DNA probes for characterization and as epidemiological tools for the detection of antibiotic resistance genes in bacteria are now widely used (18).

Several β-lactamase gene probes have now been developed (3, 7, 13, 14) which provide a complement and alternative to isoelectric focusing for β-lactamase identification. DNA fragment probes sometimes cross-hybridize with unrelated β-lactamase genes, whereas oligonucleotide probes are much more specific and can be used to discriminate between two genes differing by a single nucleotide, such as TEM-1 and TEM-2 (13).

Recently, Jouvenot et al. (5) described the correlation of molecular hybridization with a fragment probe versus isoelectric focusing to determine TEM type β-lactamase in gram-negative bacteria. We have previously described the synthesis and specificity of oligonucleotides for the detection of TEM and OXA β-lactamase genes (13, 14). In this paper we describe the use of these oligonucleotide probes in an epidemiological study and compare their concordance with isoelectric point determination in β-lactamase characterization.

A total of 114 β-lactamase-producing gram-negative bacteria, isolated from different geographical areas, belonging to at least 16 species, were used (Table 1). β-Lactamases were characterized by analytical isoelectric focusing on polyacrylamide gels as described earlier (9, 15) and by substrate and inhibition profiles and antisera. The TEM-1 β-lactamase was found in each of these genera (Table 1), confirming its wide dispersion. The TEM-2 β-lactamase was more frequent than TEM-1 in *Proteus* spp.; its predominance in this species has been reported previously (11). In *Klebsiella pneumoniae*, the predominant enzyme had a pI near 7.7, which most probably represents the SHV-1 enzyme (10). In *Pseudomonas aeruginosa*, most β-lactamases were carbenicillinases, mainly PSE-1. This β-lactamase is widespread in *P. aeruginosa* in France (4, 17).

The TEM-1 probe, 5′-CCCAACTGATCTTCA-3′, is complementary to a part of the TEM-1 coding strand in which the codon containing the central base (CAG, glutamine) differs from TEM-2 (AAG, lysine). The TEM-1 probe discriminates against strains carrying the TEM-2 gene as well as strains carrying the "standard" plasmids coding for OXA-1, -2, and -3, PSE-1, -2, -3, and -4, HMS-1, and SHV-1 (13). The OXA-1 probe, 5′-CCAAAGACGTGGATG-3′, is complementary to a unique sequence in the OXA-1 structural gene. It hybridized to none of the strains above except those coding for OXA-1 (14). The probes were synthesized by using phosphoramidite chemistry on a Systec Microsyn 1460 synthesizer, purified on 20% polyacrylamide–7 M urea gels (14), and terminally labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP at 37°C for 30 min.

The probes were used in colony hybridization experiments: bacteria were grown for 4 to 6 h on a nylon membrane (Biodyne A, Pall) and lysed with sodium dodecyl sulfate, and the DNA was then denatured and fixed as described previously (13). Prehybridization for 2 h at 55°C and hybridization for 20 h at 37°C were done in 6× NET (1× NET is 0.15 M NaCl, 0.015 M Tris hydrochloride, 0.001 M EDTA), 0.5% sodium dodecyl sulfate, and 250 μg of single-stranded DNA per ml. The filters were washed three times at 4°C, once at room temperature, and twice at 39°C (TEM-1) or 45°C (OXA-1). All washes were done in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) for 10 to 15 min. Hybridization was revealed by autoradiography.

The TEM-1 oligonucleotide hybridized with all the bacteria harboring a TEM-1 gene, either alone or in association with another β-lactamase (Table 2). This probe did not hybridize with any of the TEM-2 genes, showing the specificity of the oligonucleotide probe. The TEM-1 probe hybridized with the DNA of six strains that were not found, by isoelectric point determination, to specify a TEM-1 β-lactamase. One of these six, a *K. pneumoniae* strain, was confirmed to produce a TEM-1 β-lactamase on a subsequent determination. Five *P. aeruginosa* strains producing only a PSE-1 β-lactamase, as determined by isoelectric focusing, hybridized consistently with the TEM-1 oligonucleotide probe. Two reasons could explain this nonspecific reaction: first, the presence of a cryptic sequence of the TEM-1 gene in the PSE-1-producing strains, or second, that the oligonucleotide recognized a homologous sequence found by chance in these strains. To test which hypothesis was correct, we

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TABLE 1. Distribution of β-lactamases in 114 gram-negative bacteria by isoelectric point

Species or genus	No. of strains					
	TEM-1	TEM-2	TEM-1 + TEM-2	TEM-1 + others	OXA-1	Others
Acinetobacter calcoaceticus	3					
Citrobacter freundii	3		2			
Enterobacter cloacae	1		1	1		
Escherichia coli	4				6	6[a]
Klebsiella pneumoniae	2		1	6		10[b]
Levinea malonatica				1		2
Proteus spp.	2	7	3			
Providencia stuartii	2	2				
Pseudomonas aeruginosa	2	1				26[c]
Salmonella spp.	3	1		1	6	
Serratia marcescens	4	1				
Shigella spp.	2				3	

[a] K-12 strains harboring reference OXA-2, OXA-3, and PSE β-lactamases.
[b] Mainly SHV-1 β-lactamase.
[c] Mostly PSE-1 β-lactamase, some CARB-2 and -3; also strain PAO38 with PSE-2 and OXA-2, -3, -4, and -6 and CARB-4.

labeled a 656-base-pair (bp) *Hin*fI-*Taq*I fragment of pBR322 completely within the TEM structural gene (13). This probe hybridized with all the TEM-1- and TEM-2-producing strains but did not hybridize with the five *P. aeruginosa* strains reacting with the TEM-1 oligonucleotide.

The OXA-1 oligonucleotide probe hybridized with all the OXA-1-producing strains (Table 2). It also hybridized with a *Salmonella typhimurium* strain producing both TEM-1 and OXA-1. This oligonucleotide probe also hybridized with four strains, three *P. aeruginosa* and one *Escherichia coli*, producing the OXA-4 β-lactamase. The OXA-1 and OXA-4 β-lactamases are believed to have a molecular relationship like that of TEM-1 and TEM-2, as suggested by similarities of substrate and inhibition profiles (16). The hybridization of the OXA-1 oligonucleotide probe to the DNA of OXA-4-producing strains substantiates this claim. A fragment probe within the OXA-1 gene also hybridized with OXA-4 strains (7).

In conclusion, we have demonstrated the possibility of using oligonucleotide probes for the characterization of β-lactamase genes. Five strains of 114 which were not TEM-1 producers hybridized with the TEM-1 probe. This cross-hybridization is probably due to sequences homologous to the TEM-1 probe found in the same PSE-1-bearing strain, since these strains were isolated from the same hospital unit in Paris in 1977 to 1978 and had the same serotype. An easy way to circumvent this problem will be to increase the size of the oligonucleotide from 15 to 17 or 20 nucleotides. It is far more improbable to encounter by chance a perfect match of 17 to 20 nucleotides than 15 in the bacterial genome, yet one retains the ability to discriminate against a central 1-bp mismatch. The OXA-1 oligonucleotide hybridized with the OXA-1 and OXA-4 genes only. This result is not surprising, since these two genes are nearly identical (16). The results were thus 96% concordant for the TEM-1 probe and 100% for the OXA-1 probe. A high level of concordance for a TEM-1 fragment probe versus isoelectric focusing was also found by another group (5).

Isoelectric focusing has been extremely useful in the characterization of β-lactamases produced by gram-negative bacteria (9–11). Nevertheless, this technology does not permit rapid analysis of large numbers of clinical isolates and moreover does not yield any information on the relatedness of the enzymes studied. The latter is particularly important, since the number of different enzymes reported is steadily increasing and unrelated β-lactamases, such as SHV-1 and OXA-2, have the same isoelectric point, while closely related enzymes, such as TEM-1 and TEM-2, have different points. For these reasons, identification of β-lactamases by isoelectric point determination will become increasingly difficult and a source of error.

The DNA probe methodology, as discussed previously (13), is much faster than conventional isoelectric focusing. With appropriate probes, it is possible to discriminate between different β-lactamases having the same isoelectric point or structurally related β-lactamase genes having different points (7, 13, 14). In our present study, this is best exemplified by a strain of *Levinea malonatica* and a strain of *K. pneumoniae* having β-lactamases focusing at pI 5.4 (same as TEM-1) but which did not hybridize with our TEM-1 oligonucleotide probe. These two strains did not have the same substrate profile as TEM-1 and did not cross-react with TEM-1 antiserum, confirming our hybridization result. However, DNA probes are not problem free. Indeed, most of the new β-lactamases described recently have nearly identical structures but very different substrate specificities (6; S. Goussard, W. Sougakoff, G. Gerbaud, and P. Courvalin, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents

TABLE 2. Correlation between hybridization and isoelectric point in β-lactamase characterization of 114 strains

Determination method	No. of strains					
	TEM-1	TEM-2	TEM-1 + TEM-2	TEM-1 + others	OXA-1	Others
Total[a]	28	11	7	9	15	44
Hybridization probe TEM-1	28	0	7	9	0	6
Hybridization probe OXA-1	0	0	0	1	15	4[b]

[a] Total deduced by isoelectric focusing.
[b] Strains producing OXA-4.

Chemother., abstr. no. 517, 1987). Fragment probes can detect structurally related β-lactamases, but oligonucleotides might fail to detect a β-lactamase gene if a 1-bp change occurs in the region homologous to the oligonucleotide. For example, the TEM-3 (CTX-1) enzyme is identical to TEM-2 at the position at which they differ from TEM-1 (and for which our probes are made), but TEM-3 differs from TEM-2 at two positions (Goussard et al., 27th ICAAC), and a pair of probes for one of these will be necessary to differentiate TEM-2 from TEM-3. Finally, a drawback with DNA probe technology in resistance gene detection in the clinical laboratory is the use of radioactivity. However, we recently used a biotin-labeled fragment probe for the detection of TEM β-lactamase genes (8). It is also possible to use non-isotopically labeled oligonucleotides (2). Such developments should encourage the use of DNA probes in β-lactamase gene characterization.

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