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Detection of *aac(6′)-I* Genes in Amikacin-Resistant *Acinetobacter* spp. by PCR

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The distribution of *aac(6′)-I* genes in 62 strains of *Acinetobacter* spp. resistant to amikacin, netilmicin, and tobramycin and susceptible to gentamicin, a phenotype compatible with synthesis of an AAC(6′)-I enzyme, was studied by PCR and by DNA hybridization. Both methods gave similar results. Among the 51 *Acinetobacter baumannii* strains, *aac(6′)-Ib* was found in 19 isolates and *aac(6′)-Ih* was found in the remaining strains. The *aac(6′)-Ig* gene was present in all 10 *A. haemolyticus* strains studied and was detected only in this species. A pair of degenerate oligonucleotides complementary to conserved regions of *aac(6′)-Ic*, *-Id*, *-If*, *-Ig*, and *-Ih* enabled detection of these genes and also of *aac(6′)-Ij*, recently recognized in *Acinetobacter* sp. strain 13.

Aminoglycosides, in particular amikacin, are widely used in the treatment of nosocomial infections due to gram-negative bacteria. Since the introduction of these antibiotics in clinical practice, bacterial resistance that most often results from enzymatic modification has been reported (17). In *Acinetobacter* spp., amikacin resistance is due to production of either 6′-*N*-acetyltransferase type I [AAC(6′)-I] or 3′-*O*-phosphotransferase type VI [APH(3′)-VI] (8, 9, 11). The AAC(6′) family of enzymes is of particular significance because it modifies aminoglycosides of therapeutic importance such as amikacin, gentamicin, netilmicin, sisomicin, and tobramycin. On the basis of enzymatic substrate profiles, two types of AAC(6′) have been distinguished: type I confers resistance to amikacin but not to gentamicin, whereas type II confers resistance to gentamicin but not to amikacin. A minimum of 11 genes, designated *aac(6′)-Ia* to *-Ij*, encode AAC(6′)-I enzymes (17), whereas only *aac(6′)-IIa* and *-IIb*, apparently confined to *Pseudomonas aeruginosa*, encode AAC(6′)-II enzymes (15, 17). Comparison of AAC(6′) protein sequences indicates that the proteins can be classified in three subfamilies (17). The first one consists of AAC(6′)-Ia from *Citrobacter diversus* (20) and AAC(6′)-Ii from *Enterococcus faecium* (3). The second includes AAC(6′)-Ib, -IIa, and -IIb (15, 17, 22) and AAC(6′)-Ie, the amino-terminal portion of the AAC(6′)-APH(2″) bifunctional enzyme of gram-positive cocci (5). The third and largest subfamily comprises AAC(6′)-Ic, -Id, -If, -Ig, -Ih, and -Ij (10, 11, 14, 18, 21). Interestingly, AAC(6′)-Ic, -Ig, and -Ij were found to be specific to *Serratia marcescens*, *Acinetobacter haemolyticus*, and *Acinetobacter* sp. 13, respectively (10, 11, 18, 19).

Study of the distribution of the corresponding structural genes by DNA hybridization requires specific probes. In addition, the genetic diversity in the third subfamily renders likely the existence of yet-undetected related genes, in particular in the genus *Acinetobacter*, which harbors species-specific *aac(6′)-I* genes. The use of degenerate primers in PCR allows detection of antibiotic resistance genes despite substantial nucleotide sequence diversity (1, 23). Recently, it was proposed to use PCR to detect *aac(6′)-Ia*, *-Ib*, *-If*, and *-IIa* genes in gram-negative bacteria (24). The investigators confirmed that *aac(6′)-Ib* is widely distributed, but they did not detect any genetic determinants in the majority of amikacin-resistant strains of *S. marcescens* and *Acinetobacter* spp., probably because they did not look for the presence of *aac(6′)-Ic*, *-Ig*, and *-Ih* genes. In this study, we compared PCR and dot blot hybridization as methods of detection of *aac(6′)-I* genes in *Acinetobacter* spp. In addition to primers specific for *aac(6′)-Ia*, *-Ib*, *-Id*, *-If*, *-Ig*, and *-Ih*, we developed a pair of degenerate oligodeoxynucleotides for the genes encoding a subfamily of related AAC(6′)-I enzymes which includes AAC(6′)-Ic, -Id, -If, -Ig, and -Ih.

Bacterial strains. A total of 74 unrelated clinical isolates of *Acinetobacter* were collected in Laïko Hospital in Athens, Greece, and in Hôpital Saint Michel in Paris, France. The strains consisted of 51 *Acinetobacter baumannii* isolates, 10 *A. haemolyticus* isolates, and 1 *Acinetobacter* sp. 13 isolate which were resistant to amikacin, netilmicin, and tobramycin and susceptible to gentamicin, a phenotype compatible with the synthesis of an AAC(6′) enzyme, and of 12 aminoglycoside-susceptible *A. baumannii* isolates. *A. baumannii* strains were identified by the ability to grow at 44°C and inability to hydrolyze gelatin. By contrast, *A. haemolyticus* strains and *Acinetobacter* sp. 13 hydrolyzed gelatin and did not grow at 44°C. The two latter species were differentiated by carbon source assimilation.

Design of oligodeoxynucleotide primers. Gene-specific primers were complementary to *aac(6′)-I* sequences (Table 1). Sequence alignments of the AAC(6′) proteins showed little homology among the three subfamilies but revealed the presence of regions of high homology with invariant amino acids in the third subfamily (10, 17). Two conserved motifs were selected to design the degenerate primers XL and XR (Fig. 1). Deoxyinosine, a nucleotide that can pair with any DNA base (2, 4, 7), was incorporated at hypervariable positions.

PCR procedure. Amplification was performed in 50-μl reaction mixtures consisting of 1× *Taq* DNA polymerase buffer [16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1.7 mg of bovine serum albumin per ml], 100 μM deoxynucleoside triphosphates, and

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TABLE 1. Oligodeoxyribonucleotides used for amplification

Primer	Sequence[a]	Location[b]	Strand	Size (bp) of product
AL	5′-**ATG**AATTATCAAATTGTG-3′	Beginning of *aac(6′)-Ia*	Coding	558
AR	5′-**TTA**CTCTTTGATTAAACT-3′	End of *aac(6′)-Ia*	Complementary	
BL	5′-TATGAGTGGCTAAATCGAT-3′	*aac(6′)-Ib* (115)	Coding	395
BR	5′-CCCGCTTTCTCGTAGCA-3′	*aac(6′)-Ib* (509)	Complementary	
DL	5′-**ATG**ATCGAAGCGTGTCACT-3′	Beginning of *aac(6′)-Id*	Coding	450
DR	5′-**TCA**TTCTGGCGCAAGCAT-3′	End of *aac(6′)-Id*	Complementary	
FL	5′-**ATG**GATGAAGCTTCTTTGA-3′	Beginning of *aac(6′)-If*	Coding	435
FR	5′-**TTA**ACCAAGCTATTCTTTT-3′	End of *aac(6′)-If*	Complementary	
GL	5′-**ATG**AATATTAAACCTGCAT-3′	Beginning of *aac(6′)-Ig*	Coding	438
GR	5′-**TTA**ATCTATTTTTTTACT-3′	End of *aac(6′)-Ig*	Complementary	
HL	5′-TCTGAATCACAATTATCA-3′	*aac(6′)-Ih* (19)	Coding	400
HR	5′-CACCACACGTTCAGTTTC-3′	*aac(6′)-Ih* (418)	Complementary	

[a] Start and stop codons are indicated in boldface.
[b] Positions of the 5′ ends of the internal oligonucleotides are indicated (position numbers are in parentheses) relative to the putative start codon of the corresponding *aac(6′)-I* gene.

approximately 25 ng of DNA prepared by boiling as described previously (6). Primer concentrations were 0.4 μM for degenerate primers and 0.14 μM for specific primers. PCR was performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Norwalk, Conn.). For amplification with specific primers, the samples were heated at 92°C for 3 min and then maintained at 55°C and 1 U of *Taq* DNA polymerase (Amersham International plc, Buckinghamshire, England) was added. Thirty-five cycles were then performed with an extension step at 72°C for 1 min, denaturation at 92°C for 30 s, and annealing at 55°C for 1 min. A final DNA extension step was performed for 5 min at 72°C. The PCR procedure with degenerate primers was identical to that with specific primers, except that the annealing temperature was 37°C. Ten-microliter samples were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

Hybridization of *Acinetobacter* DNA with *aac(6′)-I* probes. *aac(6′)-I* fragments with the predicted size were obtained by PCR with the primers described in Table 1 and DNA of recombinant plasmids pFCT1165 [*aac(6′)-Ia*] (20), pAZ505 [*aac(6′)-Ib*] (22), pSCH2013 [*aac(6′)-Ic*] (18), pUO492 [*aac(6′)-If*] (21), pAT475 [*aac(6′)-Ig*] (11), and pAT479 [*aac(6′)-Ih*] (10) as a template. PCR products were separated by agarose gel electrophoresis, purified with the Qiagen kit (Qiagen, Inc., Chatsworth, Calif.), and radiolabeled with a nick translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and [α-^{32}P]dCTP (Radiochemical Centre, Amersham, Buckinghamshire, England). Total DNA was pre-

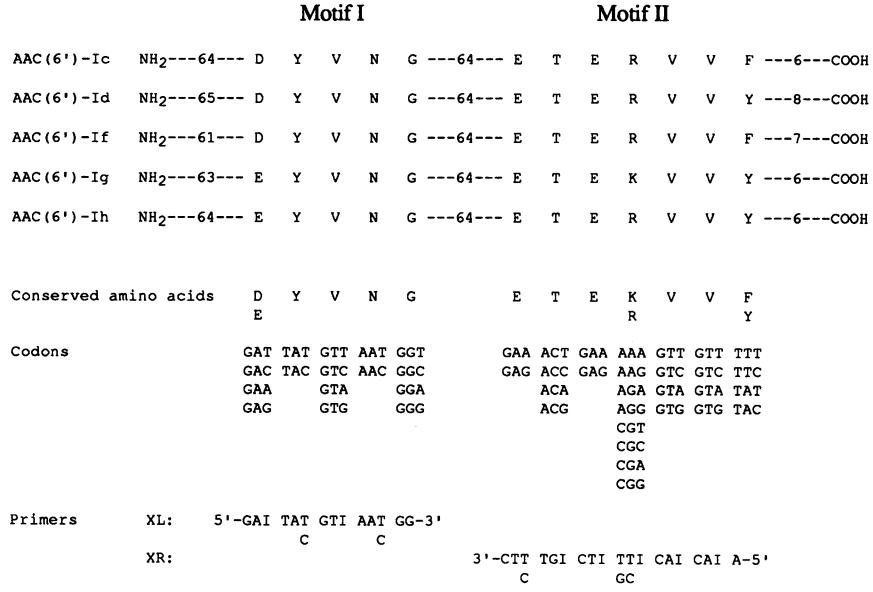

FIG. 1. Degenerate primers used to detect *aac(6′)-I* genes containing motifs I and II. Amino acid sequences are in the single-letter code. The numbers of amino acids between the NH_2 terminus and motif I, between motifs I and II, and between motif II and the COOH terminus are indicated. The nucleotide sequences of oligodeoxynucleotides XL and XR are indicated below the DNA sequence. I, deoxyinosine.

TABLE 2. Detection of *aac(6')-I* genes in *Acinetobacter* spp. by PCR and hybridization

Species (no. of strains)	*aac(6')-I* probe yielding hybridization (no. of strains)[a]	Specific primers giving a PCR product (no. of strains)[b]	No. of strains giving a PCR product with primers XL-XR
Resistant strains[c]			
A. baumannii (51)	*aac(6')-Ib* (19)	BL-BR (19)	0
	aac(6')-Ih (32)	HL-HR (32)	32
A. haemolyticus (10)	*aac(6')-Ig* (10)	GL-GR (10)	10
Acinetobacter sp. 13 (1)	None	None	1
Susceptible strains			
A. baumannii (12)	None	None	0

[a] The probes used were specific for the *aac(6')-Ia*, *-Ib*, *-If*, *-Ig*, and *-Ih* genes.
[b] The specific primers were AL-AR for *aac(6')-Ia*, BL-BR for *aac(6')-Ib*, DL-DR for *aac(6')-Id*, FL-FR for *aac(6')-If*, GL-GR for *aac(6')-Ig*, and HL-HR for *aac(6')-Ih*. No amplification products were obtained with primers AL-AR, DL-DR, and FL-FR from the 74 *Acinetobacter* strains studied.
[c] Strains with an aminoglycoside resistance phenotype compatible with the production of an AAC(6')-I enzyme.

pared as described previously (12). For dot blot and Southern hybridization, DNA was immobilized on Nytran membranes (Schleicher & Schuell, Dassel, Germany) as described previously (13). Prehybridization and hybridization were carried out for 5 and 15 h, respectively, at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate) containing 0.5% sodium dodecyl sulfate and 0.05% nonfat dry milk (13).

Detection of *aac(6')-I* genes in *Acinetobacter* clinical isolates. We have detected the *aac(6')-Ib* and *aac(6')-Ih* genes by PCR with specific primers and by dot blot hybridization with the corresponding probes in 19 and 32 strains of *A. baumannii*, respectively, and *aac(6')-Ig* also by both techniques in the 10 strains of *A. haemolyticus* (Table 2). These genes were never found together in the same strain. Interestingly, PCR products were obtained with the XL-XR primer pair and DNA from 43 *Acinetobacter* strains, including the 32 *A. baumannii* strains which contained *aac(6')-Ih* and the 10 *A. haemolyticus* strains. The remaining strain belonged to *Acinetobacter* sp. 13, in which the *aac(6')-Ij* gene had not yet been characterized (10). In order to test the specificity of the XL-XR pair, the amplification products were separated by agarose gel electrophoresis, transferred to nylon filters, and hybridized with the *aac(6')-Ig* and *-Ih* probes (Fig. 2 and data not shown). The results obtained were similar to those of dot blot hybridization (data not shown), confirming that the PCR products corresponded to the expected internal fragments of *aac(6')-I* genes. The XL-XR primer pair also allowed detection of *aac(6')-If* from pUO492 but not of *aac(6')-Ic* from pSCH2013. Annealing of XR with its target in *aac(6')-Ic* would require pairing of three deoxyinosines with three guanines. Inosine and guanine are structurally similar and form unstable pairs (2). It is thus not surprising that the XL-XR pair failed to prime amplification of the *aac(6')-Ic* gene. The ability of the XL-XR primer pair to detect *aac(6')-Id* could not be investigated because of the lack of the DNA target.

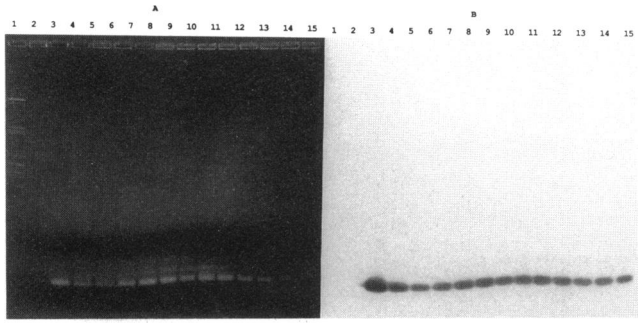

FIG. 2. Analysis of PCR-amplified DNA by agarose gel electrophoresis (A) and by hybridization (B). PCR products obtained with primer pair XL-XR were separated by electrophoresis in a 0.8% agarose gel (A), transferred to a Nytran filter, and hybridized to the in vitro ^{32}P-labeled *aac(6')-Ih* probe (B). Lanes: 1, bacteriophage λ DNA digested with *Pst*I as an internal size standard; 2, amplified DNA from *Escherichia coli* JM83 (pAT479); 3 to 15, amplified DNA from 13 strains of *A. baumannii* harboring the *aac(6')-Ih* gene. The expected 226-bp fragment hybridized to the probe.

The *aac(6')-I* resistance determinants of 61 strains of *Acinetobacter* with an AAC(6')-I phenotype were all detected by the set of specific primers used and by dot blot hybridization with specific probes, whereas the *aac(6')-Ij* gene of *Acinetobacter* sp. 13 was detected only by the degenerate primers. As expected, no *aac(6')-I* gene was found in the aminoglycoside-susceptible strains.

The *aac(6')-Ia*, *-Id*, and *-If* genes, considered rare in members of the family *Enterobacteriaceae* (16), were not found in the *Acinetobacter* isolates by PCR or DNA hybridization. Our study indicates that in *A. baumannii*, the *aac(6')-Ih* gene was more common than *aac(6')-Ib* and that oligonucleotides GL-GR are of particular interest since the *aac(6')-Ig* gene they amplify is specific for *A. haemolyticus* (11). The degenerate primers enabled detection of the *aac(6')-I* genes of the third subfamily, except for *aac(6')-Ic* from *S. marcescens*. In conclusion, PCR and hybridization are adequate to study the distribution of *aac(6')-I* genes and degenerate primers may be used to detect the genes encoding the subfamily of related AAC(6')-I enzymes that includes AAC(6')-Ic, -Id, -If, -Ig, -Ih, and -Ij.

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