ANTIMICROBIAL RESISTANCE

An 8-year study of resistance to amikacin in gram-negative bacilli isolates from patients with nosocomial infection at one hospital in Argentina

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Summary. Administration of either gentamicin or amikacin induced an increase in the number of amikacin-resistant (AR) isolates of certain Enterobacteriaceae and *Acinetobacter* species in a hospital in Buenos Aires. A total of 127 AR isolates was selected to study the molecular mechanisms of resistance involved. The *aac(6′)-Ic* gene was found by dot-blot hybridisation in every *Serratia marcescens* isolate. A gene different from *aac(6′)-Ia*, *aac(6′)-Ib* and *aac(6′)-Ic* encoding the AAC(6′)-I activity was found in a 15.5-kb plasmid in *Acinetobacter* spp. Plasmids from 27 Enterobacteriaceae contained an *aac(6′)-Ib* gene and 26 of these carried sequences related to the Tn1331 transposon, whereas one *Escherichia coli* plasmid showed homology in another fragment of the Tn1331 transposase. Because plasmids bearing the *aac(6′)-Ib* gene were heterogeneous, dissemination of the *aac(6′)-Ib* gene may have been due to transposition of Tn1331 rather than the spread of an epidemic plasmid. The rate of AR isolates varied within each species in spite of the presence of Tn1331, and it is likely, therefore, that this transposon may not be the sole factor responsible for the observed variation. The *aph(3′)-VIa* gene (originally described in *Acinetobacter* spp.) was found with high frequency (80%) in this *Acinetobacter* population. Furthermore, this gene was found also in plasmids from 20% of other gram-negative organisms commonly involved in nosocomial infections in this hospital.

Introduction

Resistance to aminocyclitol aminoglycosides is an important clinical problem since these antibiotics are widely used in the treatment of serious infections.^{1,2} Amikacin has been the drug of choice for treating nosocomial infections refractory to other aminoglycosides.^{3,4} In recent years, resistance to amikacin due to production of 3′-aminoglycoside-phosphotransferases, 2′-adenyltransferases and aminoglycoside-6′-N-acetyltransferases has been reported.^{5–8} In nosocomial isolates, genes encoding resistance to amikacin most frequently belong to the *aac(6′)-I* group and can be encoded by plasmids or have a chromosomal locus, as is the case with *Serratia marcescens*.^{5,8} The frequent presence of genes encoding modifying enzymes in plasmids or transposons,^{9,10} and, consequently, their

capacity for dispersion, further increases the clinical problems posed by antibiotic resistance. It appears, from studies limited in each case to one institution, that the substitution of another aminoglycoside by amikacin may not necessarily be followed by a concomitant increase in resistance to the antibiotic. According to one study, predominant use of amikacin may result in a general reduction in the number of aminoglycoside-resistant isolates.³ However, other studies have described a correlation between increased amikacin use and the occurrence of enzyme-mediated resistance in gram-negative bacilli.⁴ In Argentina, as in many hospitals from other non-developed countries, it is very difficult to establish a relationship between clinical observations and the spread of antibiotic resistance because many variables are involved and not all can be controlled appropriately. This study was designed to analyse molecular changes that occurred in association with the emergence of resistance to amikacin over a period of 8 years in nine bacterial species isolated from patients hospitalised in the J. A. Fernández Hospital of Buenos Aires. The appearance

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of enzymes, plasmids, sequences related to Tn1331 and the gene responsible for amikacin resistance were investigated.

Materials and methods

Bacterial strains and culture

Escherichia coli HB101 *pro leu thi lacY hsr hsm recA*¹¹ and *E. coli* C2110 *polA gyrA*¹² were provided by BioSidus Laboratory (Buenos Aires), and were used in this study as recipients in transformation and conjugation experiments, respectively. One hundred and twenty-seven gram-negative clinical isolates, comprising 49 amikacin-resistant (AR) *S. marcescens* (49 of 110), two AR *E. coli* (71 of 6986), two AR *Salmonella typhimurium* (12 of 35), two AR *Citrobacter freundii* (67 of 611), 18 AR *Klebsiella* spp. (1520 of 4253), two AR *Enterobacter cloacae* (225 of 1522), two AR *Proteus* spp. (45 of 907) and 50 AR *Acinetobacter* spp. (107 of 250) were selected for the study. Numbers in parentheses represent the total number of AR isolates and the total number of isolates of each species from hospitalised patients during the period 1984–1991. Several of these species (*Klebsiella* spp., *Serratia* spp., *Ent. cloacae* and *Acinetobacter* spp.) are frequently involved in nosocomial infections whereas others (*E. coli*, *Salmonella* spp., *C. freundii* and *Proteus* spp.) are responsible for sporadic infections in this hospital. Isolates were identified by standard methods.¹³ All AR isolates selected for the study were resistant to five or more antibiotics, including amikacin, and were isolated from patients with nosocomial infection, i.e., patients who had been hospitalised for at least 2 days before a sample revealed a positive bacterial culture. The symptoms of infection began at the hospital and were not present prior to admission. These isolates were grown in L-broth (Sigma) supplemented with amikacin 20 mg/L.

Antimicrobial susceptibility tests

Antibiotic resistance was investigated by the disk diffusion method¹⁴ in Mueller-Hinton Agar (Oxoid),

as recommended by the NCCLS.¹⁵ Quality control of this method was performed with *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 strains. The amikacin minimal inhibitory concentration (MIC), for strains characterised as intermediate or resistant according to the disk diffusion method by the NCCLS criteria, was determined by the agar dilution test with a Steers' replicator device to dispense a final inoculum of 10⁷ cfu/L, according to NCCLS recommendations.¹⁵ Organisms with an MIC of amikacin > 16 mg/L were considered to be resistant. Aminoglycoside resistance profiles (AGRP) were determined by analysing susceptibility to tobramycin, dibekacin, amikacin, 5'-episisomicin, netilmicin, 2'-N-ethylnetilmicin, sisomicin, kanamycin, neomycin, paromomycin, ribostomycin, butirosin, gentamicin and isepamicin, as described previously.¹⁶

Plasmids

The plasmids utilised in this study are listed in table I.^{8,10,17,18} Plasmid pUC18 was obtained from a commercial source (Bethesda Research Laboratories, Gaithersburg, MD, USA).

Conjugation and transformation

Transfer of R plasmids from isolates of *S. marcescens*, *E. coli*, *S. typhimurium*, *C. freundii*, *K. pneumoniae*, *K. oxytoca*, *Ent. cloacae*, *Proteus* spp. and *Acinetobacter* spp. to *E. coli* C2110 was performed in L-broth at 37°C. Transconjugants were selected on L-agar (Sigma) containing the following antibiotics at the indicated concentrations: nalidixic acid 25 mg/L, and amikacin 20 mg/L or ampicillin 50 mg/L. Transformation of *E. coli* HB101 with plasmid DNA was performed by the method of Hanahan.¹⁹ All transformants were assayed for susceptibility to amikacin, tobramycin, streptomycin, gentamicin, netilmicin, ampicillin, chloramphenicol, cefotaxime and cefoxitin by the disk-diffusion method, as described above.

Isolation of plasmid DNA and restriction endonuclease analysis

Plasmid DNA was prepared by the method of Birnboim and Doly.²⁰ Restriction endonuclease cleavage of plasmid DNA was performed as recommended by the supplier (BRL). *Hind*III-digested λ -phage DNA and *Hae*III-digested phage $\phi \times 174$ DNA (Pharmacia, Uppsala, Sweden) were included as molecular mass standards. Electrophoresis of DNA fragments was performed in horizontal agarose 0.8% w/v gels.²¹ Gels were stained with ethidium bromide 0.5 mg/L and photographs were obtained with Polaroid type 667 film under UV exposure. Cloning was performed according to a standard procedure described elsewhere.²¹

Table I. DNA hybridisation probes

Probe*	Intragenic DNA fragment
<i>aac</i> (6')-Ia; <i>aacA1</i>	405-bp <i>Sst</i> I fragment from pFCT1165 ¹⁷
<i>aac</i> (6')-Ib; <i>aacA4</i>	180-bp <i>Sst</i> I/ <i>Kpn</i> I fragment from pJHCMW1 ¹⁰
<i>aac</i> (6')-Ic	357-bp <i>Bam</i> HI/ <i>Eco</i> RV fragment from pSCH2014 ⁸
<i>aph</i> (3')-VIa; <i>aphA6</i>	370-bp <i>Eco</i> RI/ <i>Xho</i> I fragment from pSCH2010 ¹⁸
Intragenic fragment of Tn1331	3000-bp <i>Bam</i> HI/ <i>Bam</i> HI fragment from pJHCMW1 ¹⁰
Intragenic fragment of the Tn1331 transposase	2616-bp <i>Eco</i> RV/ <i>Eco</i> RV fragment from pJHCMW1 ¹⁰

*Please note that *aacA1*, *aacA4* and *aphA6* are alternative, old-style nomenclature.

Southern blot hybridisation and labelling of plasmid DNA

Restriction fragments were transferred to nylon membranes by the Southern blot method.²² Intragenic probes of *aac(6')-Ia*, *aac(6')-Ib*, *aac(6')-Ic* and *aph(3')-VIa* were prepared as described elsewhere.^{8,10,17,18} The intragenic probe of the *Tn1331* transposase was a 2616-bp *EcoRV* DNA fragment obtained from pJHCMW1.¹⁰ A second probe specific for *Tn1331* comprised a 3.0-kb *BamHI* DNA fragment obtained from pJHCMW1.¹⁰ All fragments were isolated by electro-elution from agarose gels. Probes were labelled by the random-primer technique with ³²P dCTP.²¹ All hybridisation experiments were performed under maximum stringent conditions as described previously.²¹ Dot-blot hybridisation experiments were performed by a standard procedure, as described elsewhere.²¹

Results

Incidence of amikacin resistance

Amikacin resistance increased steadily in several species throughout the study. From 1982 to the present time, amikacin has been used as the aminoglycoside of second choice, except in 1985 when it was selected as the aminoglycoside of first choice while gentamicin became the second choice. Resistance to gentamicin was a clinical problem during the study (1984–1991). The frequency of gentamicin-resistant isolates in different species was 60–70% in *Klebsiella* spp., 55–65% in *Ent. cloacae*, 90% in *S. marcescens*, 65% in *C. freundii* and 75–85% in *Acinetobacter* spp. These frequencies remained high throughout the study.

Table II summarises data on the incidence of amikacin resistance in Enterobacteriaceae and *Acinetobacter* spp. There was a 2–4-fold increase in the prevalence of amikacin resistance in isolates of *Ent. cloacae*, *Klebsiella* spp. and *Acinetobacter* spp., between 1984 and 1991 with a baseline of ≥ 10% in 1984. Among *E. coli*, *Proteus* spp. and *C. freundii* isolates, the average percentages of amikacin resis-

tance were 2, 6 and 12%, respectively, and remained relatively constant throughout the study (8 years). Resistance to amikacin in *S. typhimurium* appeared in a few isolates (< 1%) in 1990. Rates of amikacin resistance in *E. coli*, *Proteus* spp., *C. freundii* and *S. typhimurium* did not differ significantly from those described in other hospitals from this country (table II). Rates of amikacin resistance in *S. marcescens* were the highest when compared with those from other species in 1984 (42%) and reached 70% in 1986. It should be noted that the incidence of resistance to amikacin in *Klebsiella* spp. and *Acinetobacter* spp. increased steadily throughout the study, and was higher overall than in *S. marcescens*.

Genes involved in amikacin resistance

A total of 127 clinical isolates from patients with nosocomial infection was selected in this period for further investigation. These isolates were resistant to five or more common antibiotics, including amikacin. Although it was not considered a selection criterion, 90% of the isolates were also resistant to gentamicin. The genes encoding amikacin resistance found in this study are summarised in table III. The presence of enzymes belonging to the AAC(6')-I class is characterised by resistance to tobramycin, dibekacin, amikacin, 5'-episisomicin, netilmicin, 2'-ethylnetilmicin and sisomicin. At least six genes encoding this AGRP have been identified.⁶ We analysed the bacterial population with probes currently available for the *aac(6')-Ia*, *aac(6')-Ib* and *aac(6')-Ic* genes. The frequency of these genes varied among the different species investigated and the *aac(6')-Ia* gene was never found in 10 *Acinetobacter* isolates, 17 *K. pneumoniae*, two *E. coli* and 10 *S. marcescens* isolates. The *aac(6')-Ic* gene was detected by dot-blot hybridisation in every *S. marcescens* isolate and was the only amikacin resistance marker in 72% of these isolates. The *aac(6')-Ib* gene was found in five *S. marcescens*, one *E. coli*, one *Ent. cloacae*, 13 *K. pneumoniae*, one *K. oxytoca*, two *C. freundii*, two *Proteus* spp. and two *S. typhimurium* isolates. Excluding *Serratia* and *Acineto-*

Table II. Percentages of amikacin-resistant isolates of Enterobacteriaceae and *Acinetobacter* spp. at the J. A. Fernández Hospital, and others, according to a local survey*

Species	Percentage of isolates that were AR in							
	1984	1985	1986	1987	1988	1989	1990	1991
<i>E. coli</i>	0 (3)	0 (2)	1 (1)	1 (2)	1 (1)	2 (1)	1 (1)	1 (6)
<i>Klebsiella</i> spp.	19 (9)	15 (9)	28 (11)	33 (6)	40 (25)	45 (25)	50 (24)	62 (12)
<i>Proteus</i> spp.	5 (6)	4 (3)	6 (2)	8 (4)	5 (2)	6 (13)	7 (5)	7 (3)
<i>Ent. cloacae</i>	10 (9)	15 (22)	17 (5)	18 (21)	21 (20)	23 (11)	20 (24)	21 (NA)
<i>C. freundii</i>	10 (8)	12 (6)	10 (14)	10 (10)	10 (14)	12 (0)	13 (21)	12 (NA)
<i>Salmonella</i> spp.	0 (2)	0 (6)	0 (0)	0 (0)	0 (1)	0 (0)	1 (15)	1 (NA)
<i>Serratia</i> spp.	42 (17)	31 (17)	70 (12)	41 (36)	28 (33)	0 (22)	40 (23)	0 (NA)
<i>Acinetobacter</i> spp.	19 (NA)	22 (NA)	25 (NA)	38 (NA)	49 (55)	56 (20)	63 (51)	75 (NA)

NA, not available.

*Numbers in parentheses represent data available from a local survey performed by the Comisión de Antibióticos, Asociación Argentina de Microbiología. The survey only included specimens retrieved from nosocomial infections.³³

Table III. Amikacin resistance genes found in 127 isolates

Species	Number of isolates	Frequency of appearance of each cloned gene			
		<i>aac(6')-Ib</i>	<i>aac(6')-Ic</i>	<i>aac(6')-I*</i>	<i>aph(3')-VIa</i>
<i>E. coli</i>	2	1	0	0	1
<i>K. pneumoniae</i>	17	13	0	0	4
<i>K. oxytoca</i>	1	1	0	0	0
<i>Proteus</i> spp.	2	2	0	0	0
<i>Ent. cloacae</i>	2	1	0	0	1
<i>C. freundii</i>	2	2	0	0	0
<i>S. typhimurium</i>	2	2	0	0	0
<i>S. marcescens</i>	49	5	49	0	9
<i>Acinetobacter</i> spp.	50	0	0	10	40

*This report.

bacter spp., the AAC(6')-I AGRP was found in combination with AGRPs associated with the AAC(3)-V enzymes in 40% of the isolates, with ANT(2'') in 20%, and with both AAC(3)-V and ANT(2'') in 40%. Most *Serratia* isolates (75%) had the triple combination of AAC(3)-V + AAC(6')-I + APH(3')-I AGRPs. The combination of these AGRPs causes the phenotype of resistance to gentamicin, tobramycin, netilmicin and amikacin, which were all aminoglycosides marketed in Argentina at the time of the study.

Ten *Acinetobacter* isolates expressing the AAC(6')-I AGRP failed to hybridise with any of the three probes described above (table III). Therefore, a 2700-bp *Hind*III DNA fragment was cloned in pUC18 from a 15.5-kb *acinetobacter* plasmid. This fragment was able to encode the AAC(6')-I activity in *E. coli* HB101. Dot-blot hybridisation with this fragment as probe against 71 Enterobacteriaceae and 10 *Acinetobacter* isolates expressing the AAC(6')-I AGRP showed that only the *Acinetobacter* isolates hybridised with the probe. The fact that 71 Enterobacteriaceae, plus 20 other *Acinetobacter* isolates not expressing the AAC(6')-I activity, did not hybridise with the 2700-bp fragment demonstrated that the newly developed probe was specific enough for the purposes of this study. In *Acinetobacter* spp. the AAC(6')-I pattern occurred in combination with the AAC(3)-I AGRP.

The APH(3')-VI AGRP is associated with resistance to kanamycin, neomycin, paromomycin, ribostamycin, butirosin, gentamicin B, amikacin and isepamicin. It is known that there are at least two genes that encode this AGRP.⁶ The *aph(3')-VIa* gene was described by Lambert *et al.*²³ in *Acinetobacter* spp. and, in this study, a probe developed by others from pSCH2010 was used.⁶ This probe hybridised with 40 *Acinetobacter* spp. (80% of the *Acinetobacter* isolates), one *E. coli*, one *Ent. cloacae*, four *K. pneumoniae* and nine *S. marcescens* isolates (20% of Enterobacteriaceae isolates). These percentages are considerably higher than those described by other authors, who have reported that the APH(3')-VI AGRP was present in 35.5% of *Acinetobacter* spp., 0.2% of *Serratia* spp. and 0.8% of gram-negative bacteria excluding *Serratia* spp., *Acinetobacter* spp.

and *Pseudomonas* spp.^{6,18} One *K. pneumoniae* isolate had both the *aac(6')-Ib* and the *aph(3')-VIa* genes encoding resistance to amikacin. The APH(3')-VI AGRP appeared in combination with AAC(3)-I (20% of the isolates), AAC(3)-V (40%) or ANT(2'') (15%) AGRPs and determined the gentamicin-tobramycin-netilmicin-amikacin-isepamicin phenotype. No isolates with an AGRP consistent with the ANT(4')-I or the ANT(4'')-I resistance mechanisms¹⁸ were found in the bacterial population investigated.

Characterisation of amikacin resistance plasmids

The occurrence of plasmids conferring resistance to amikacin in 42 AR isolates was investigated by conjugation with *E. coli* C2110 or transformation of *E. coli* HB101, or both. These isolates included all Enterobacteriaceae that had hybridised with the *aac(6')-Ib*, or *aph(3')-VIa* genes. Ten *Acinetobacter* isolates were also tested but did not transfer any plasmid to *E. coli* C2110; transformation of *E. coli* with *acinetobacter* plasmids is, however, a rare event.^{23,24} The *aac(6')-Ib* gene was transferred, by transformation, from one *E. coli*, two *Proteus* spp., two *C. freundii*, one *Ent. cloacae*, five *S. marcescens*, 13 *K. pneumoniae*, one *K. oxytoca* and two *S. typhimurium* isolates to *E. coli* HB101. Amikacin resistance was associated with the presence of plasmid DNA between 10 and 20 kb in size. Five of these plasmids were mobilised in conjugation experiments with *E. coli* C2110. The plasmids recovered from transconjugants or transformants conferred resistance not only to amikacin, but also to tobramycin, streptomycin and ampicillin, and yielded 27 different restriction endonuclease fragment patterns after *Bam*HI digestion. Digestion of all 27 plasmids with *Bam*HI generated a 3000-bp fragment that hybridised with the *aac(6')-Ib* gene probe.

Plasmid DNA samples from one *E. coli*, four *K. pneumoniae*, one *Ent. cloacae* and nine *S. marcescens* isolates that carried the *aph(3')-VIa* gene were all conjugative and all transferred resistance to amikacin and ampicillin. Twelve different plasmids, with sizes from 20 to 60 kb, were found in these 15 strains. One of these plasmids (35 kb) was found in four different

Figure. Analysis of *Tn1331* homology with pDCEC67 and pDCECL2; **a** and **b**, *Bam*HI, *Eco*RV/*Sst*I and *Bgl*II digestion and hybridisation of plasmid DNA. (**a**) Lane 1, pDCEC67 unrestricted; 2, pDCEC67 digested with *Bgl*II; 3, pDCEC67 digested with *Eco*RV and *Sst*I; 4, pDCEC67 digested with *Bam*HI; 5, molecular mass marker (λ -phage digested with *Hind*III); 6, pDCSP1 digested with *Bgl*II; 7, pDCSP1 digested with *Eco*RV and *Sst*I; 8, pDCSP1 digested with *Bam*HI; 9, pDCECL2 unrestricted; 10, pDCECL2 digested with *Bgl*II; 11, pDCECL2 digested with *Eco*RV and *Sst*I; 12, pDCECL2 digested with *Bam*HI. (**b**) Radio-autograph of Southern blot hybridisation of the gel in **a** with the 3-kb *Bam*HI pJHCMW1 fragment as probe. **c** and **d**, *Eco*RV digestion and hybridisation of plasmid DNA. (**c**) lane 1, molecular mass marker (λ -phage digested with *Hind*III); 2, molecular mass marker (phage $\phi \times 174$ digested with *Hae*III); 3, pJHCMW1 digested with *Eco*RV; 4, pDCECL2 unrestricted; 5, pDCECL2 digested with *Eco*RV; 6, pDCEC67 unrestricted; 7, pDCEC67 digested with *Eco*RV. (**d**) Radio-autograph of Southern blot hybridisation of gel **c** with the 2.616-kb *Eco*RV pJHCMW1 fragment as probe for the *Tn1331* transposase.

isolates. These four plasmids carried the *aph*(3')-*Vla* gene in a 4.7-kb *Bam*HI DNA fragment. One plasmid, from an *E. coli* isolate, also encoded resistance to cefotaxime and ceftiofite. The *aac*(6')-*Ib* and *aph*(3')-*Vla* genes, present in a single strain of *K. pneumoniae*, were located to two different plasmids.

Presence of DNA sequences related to *Tn1331*

Dot-blot analysis of the 127 amikacin-resistant isolates with an intragenic probe of the *Tn1331* transposase revealed that only those bearing the *aac*(6')-*Ib* gene hybridised with the probe. Plasmid DNA from the 27 transformants carrying the *aac*(6')-*Ib* gene was digested with *Bam*HI, *Bgl*II and *Eco*RV/

*Sst*I and hybridised with a 3-kb *Bam*HI DNA fragment specific for *Tn1331*. The results revealed that 26 of the plasmids exhibited a hybridisation pattern indicative of transposon *Tn1331* (figure). Plasmid pDCECL2 from one *Ent. cloacae* isolate, for example, exhibited a hybridisation pattern similar to that of pDCSP1²⁵ carrying *Tn1331* (figure). Plasmid DNA from all 27 isolates digested with *Eco*RV and hybridised against the intragenic probe of the *Tn1331* transposase revealed that 26 plasmids hybridised in the 2.6-kb *Eco*RV DNA fragment. Plasmid DNA from *E. coli* isolate 67 (EC67) hybridised in a 2.0-kb *Eco*RV DNA fragment. Plasmid DNA from EC67 (pDCEC67) exhibited a different *Tn1331* hybridisation pattern, although the plasmid shared certain homology in

different fragments (figure). In spite of these differences, pDCEC67 had a 3.0-kb *Bam*HI DNA fragment similar to that exhibited by Tn1331. To analyse sequences adjacent to the *aac(6')-Ib* gene, the 3.0-kb *Bam*HI fragment of PDCEC67 was cloned into pUC18. Restriction endonuclease analysis with *Bam*HI/*Ava*I, *Bam*HI/*Xho*I and *Bam*HI/*Kpn*I demonstrated that this recombinant plasmid had similar, if not identical, *aac(6')-Ib* sequences adjacent to Tn1331 (data not shown). Finally, although conditions for hybridisation were the same in both cases, undigested plasmid pDCECL2 hybridised with the 3.0-kb Tn1331 probe (figure, top panel, lane 9) but failed to hybridise with the 2616-bp probe (figure, bottom panel, lane 4), probably due to the low efficiency of undigested plasmid transference.²¹ However, digested pDCECL2 DNA hybridised with both probes, which permitted us to reach the conclusions presented here.

Discussion

A previous report has shown that the APH(3')-VI AGRP is associated primarily with *Acinetobacter* spp. (35.4%) and was rarely observed in the other gram-negative bacteria.⁶ At least two genes have been identified that encode this AGRP, *aph(3')-VIa* and *aph(3')-VIIb*.^{6,9} Amikacin resistance in strains of *Acinetobacter* spp. isolated from nosocomial infections was related to the presence of the *aph(3')-VIa* gene.²⁴ This gene has also been shown to be carried by Tn1528 in a *Providencia stuartii* isolate.²⁶ The *aph(3')-VIIb* gene was cloned from the *K. pneumoniae* plasmid pRPG101²⁷ but DNA hybridisation studies were not performed to show the relationship between this gene and the *aph(3')-VIa* from *Acinetobacter* spp.⁶ Surprisingly, in this study with Enterobacteriaceae, *aph(3')-VIa*, a gene confined so far to *Acinetobacter* spp., was also found in plasmids of *S. marcescens*, *K. pneumoniae*, *Ent. cloacae* and *E. coli* (20%). In the remaining 80%, as expected, the AAC(3)-V AGRP was responsible for resistance to amikacin. Two genes were found in the Enterobacteriaceae population encoding this AGRP. These were the *aac(6')-Ib* and the *aac(6')-Ic* genes.^{8,28} The former was described previously in plasmid DNA from *K. pneumoniae*, *E. coli* and *S. marcescens*.^{5,28-30} This gene was also found in plasmid DNA from *Proteus* spp., *S. typhimurium*, *Ent. cloacae*, *K. oxytoca* and *C. freundii*. The latter gene was found in 72% of *S. marcescens* isolates as the sole mechanism of resistance to amikacin. The only gene consistently found in both *Acinetobacter* spp. and Enterobacteriaceae was *aph(3')-VIa*. Furthermore, one *K. pneumoniae* isolate harboured two R-plasmids, one carrying the *aph(3')-VIa* and the other the *aac(6')-Ib* gene. The species that harboured the highest number of gene types conferring amikacin resistance was *S. marcescens*, which carried *aac(6')-Ib*, *aac(6')-Ic* and *aph(3')-VIa* genes.

DNA from 20% of those *Acinetobacter* isolates expressing the AAC(6')-I AGRP did not hybridise with *aac(6')-Ia*, *aac(6')-Ib* or *aac(6')-Ic* probes. Preliminary cloning studies from this laboratory revealed that this activity was located in a 15.5-kb plasmid from an *Acinetobacter* sp. A probe developed from this plasmid hybridised with those *Acinetobacter* isolates, whereas none of the Enterobacteriaceae isolates hybridised with this probe. This may represent the finding of a new gene, or another amongst other genes that have been reported recently, after this study was completed.^{6,31,32} Further studies are required to identify and characterise this gene. Although resistance to gentamicin was not used as a criterion for selecting AR strains, AAC(3)-V, ANT(2'')-I and AAC(3)-I AGRPs were found in 90% of the isolates, in combination with other mechanisms. The gentamicin-tobramycin-netilmicin-amikacin (the sole aminoglycosides marketed in Argentina during the study) phenotype was common in this survey because of the high incidence (55%) of isolates with the combination of AAC(3)-V with APH(3')-VI or AAC(6')-I AGRPs, or both. These combinations were found more frequently in our survey than in a survey reported by others.⁶ It should be noted that in our bacterial population the combination of AAC(3)-V with APH(3')-VI or AAC(6')-I AGRPs, or both, was not located on broad host range plasmids because the *aac(6')-Ib* and the *aph(3')-VIa* genes were always found in plasmids that did not transfer the gentamicin resistance.

The frequency of nosocomial infections due to AR gram-negative bacilli was documented in the USA in 1985 and the rates ranged from 0.8 to 3.5%.²⁻⁴ The rates found in Argentina³³ are remarkably higher than those found in the USA and Europe.^{2,6,24,34} Because, surprisingly, high rates of aminoglycoside resistance are found in isolates from most hospitals in Argentina, extreme care is routinely taken when assessing antimicrobial susceptibility. All isolates in this study were investigated by both disk diffusion and agar dilution tests and quality control was performed as recommended by NCCLS.¹⁵ The fact that not only in our hospital, but also in other hospitals in Argentina,³³ similar high rates of AR gram-negative bacilli have been found, lends credibility to our unusual findings and led us to design the present study. Thirty-one percent of all *S. marcescens* and 15% of all *Acinetobacter* and *Klebsiella* isolates from patients suffering from nosocomial infections in our hospital were resistant to amikacin in 1985. These resistance rates were not uniform and a steady increase in amikacin resistance was observed following modifications in aminoglycoside usage, although there is no proof that a cause-effect phenomenon has taken place. When aminoglycoside resistance mechanisms were investigated, it was seen that an unusually high number of isolates carried the combination of AAC(3)-V or AAC(3)-I with AAC(6')-I or APH(3')-VI AGRPs, which conferred resistance to both amikacin and gen-

tamicin. The species that carried the *aph(3')-VIa* gene with a frequency higher than that reported previously by others,⁶ were the ones usually involved in nosocomial infections and exhibited high rates of amikacin resistance. Ecological factors such as poor environmental control, which favour spreading of R-plasmids, may have also contributed to the maintenance of amikacin resistance. It is interesting to note that AAC(6')-I group enzymes are capable of inactivating amikacin but only cause resistance, according to the standards set by NCCLS, when the enzymic activity is expressed at high levels. Therefore, it is important to decide, according to the design of the study, the precise cut-off to determine which specimens would be considered. Because only isolates with an MIC > 16 mg/L were included in this study, the high percentage of those expressing the APH(3')-VI AGRP is not an artifact, due to missing specimens bearing AAC(6')-I, but a true estimate of the incidence of APH(3')-VI AGRP.

Previous reports have shown that outbreaks due to amikacin-resistant Enterobacteriaceae usually involve single plasmid species carrying the *aac(6')-I* genes.^{5,29} Hopkins *et al.* described four plasmids from 21 Enterobacteriaceae isolates.⁵ For another aminoglycoside gene, the same epidemic plasmid was found worldwide.³⁵ Enterobacteriaceae described in this report harboured at least 42 plasmid species mediating the *aac(6')-Ib* or the *aph(3')-VIa* genes; 48% of these plasmids were conjugative, which indicates conjugation as a likely mechanism mediating the spread of amikacin resistance.

Different *aac(6')-I* genes may be found in transposons of the Tn3 family.^{5,9,10} More recent data have suggested that Tn21 contains a specific region, the integron, into which many different resistance genes may be inserted.^{6,36} In the light of this information, dissemination of these transposons was investigated in

all AR isolates. Hybridisation strategies were used for this purpose, including an intragenic probe of the Tn1331 transposase that has a nucleotide sequence identical to that of Tn3 and Tn21.¹⁰ In this study we identified Tn1331-related sequences solely in plasmids harbouring the *aac(6')-Ib* gene. This gene was found in all 27 plasmids in a 3.0-kb *Bam*HI fragment. Cloning and restriction analysis of this *Bam*HI fragment with four endonucleases revealed that sequences adjacent to the *aac(6')-Ib* gene were identical in pDCEC67, a plasmid that displayed other genetic dissimilarities at the level of the Tn1331 transposase gene. Conversely, in a study that described four plasmids carrying Tn3-related transposons from 21 nosocomial outbreak isolates, Hopkins *et al.* showed that sequences adjacent to *aac(6')-I* alleles were different.⁵ Because all plasmids were different by restriction endonuclease analysis, several possible sources for Tn1331 are suggested: (i) the dissemination of Tn1331 may have been due to transposition mechanisms which occurred after intra- or inter-species transference; (ii) after transfer of a plasmid carrying Tn1331, a process of plasmid recombination may have been responsible for the appearance of Tn1331 in different plasmids; (iii) although unlikely, all isolates carrying different plasmids may have originated from different infection sources; or (iv) a combination of these mechanisms. Because the rate of AR isolates varied within each species in spite of the presence of Tn1331, it is concluded that this transposon may not be the sole factor responsible for the observed variation.

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