ANTIMICROBIAL RESISTANCE

Detection by polymerase chain reaction of genes encoding aminoglycoside-modifying enzymes in methicillin-resistant *Staphylococcus aureus* isolates of epidemic phage types

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Summary. The polymerase chain reaction (PCR) was used to identify the *aacA*–*aphD*, *aphA3* and *aadC* genes, encoding the aminoglycoside-modifying enzymes AAC(6′)–APH(2″), APH(3′)III and ANT(4′ 4″), respectively, and the methicillin resistance determinant *mecA*, in epidemic aminoglycoside and methicillin-resistant isolates of *Staphylococcus aureus*. In total, 37 isolates collected in the period 1980–1985 and 81 isolates from the period 1991–1992 were obtained from 10 different Belgian hospitals. Epidemic isolates from the earlier period were characterised by phage type C (6/47/54/75) of phage group III, whereas two other epidemic phage types of group III—types A (77) and B (47/54/75/77/84/85)—were commonest in isolates from the second period. The bifunctional AAC(6′)–APH(2″) was the enzyme encountered most frequently. The prevalence of APH(3′)III decreased significantly in the 1991–1992 period, while ANT(4′ 4″) was found solely in isolates from this period. Resistance mechanisms were more complex in isolates from the 1991–1992 period and the *mecA* gene was detected in all isolates. The PCR results corresponded well with those obtained in the radiochemical phosphocellulose paper binding assay. Isolates from the 1991–1992 period were shown to express significantly higher levels of acetyltransferase activity than isolates from the 1980s.

Introduction

Staphylococcus aureus is a versatile human pathogen that continues to be an important cause of nosocomial infection. Methicillin-resistant strains of *S. aureus* (MRSA) were recognised soon after the clinical introduction of methicillin.¹ In 1982, epidemic MRSA strains showing multiple antibiotic resistance and an enhanced capacity to cause widespread outbreaks of infections were described.² Such strains seem to have a special capacity to colonise patients and staff.^{3,4} Although enhanced pathogenicity has often been suggested for epidemic strains of MRSA, there is no firm evidence that these strains are more or less virulent than other strains.^{5,6} The fundamental cause of their pathogenicity remains elusive. Nevertheless, there seems to be a general consensus that infections caused

by this type of strain are often associated with a high degree of morbidity and mortality.^{6,7} Furthermore, the presence of additional multiple resistance to a wide range of antibiotics, including aminoglycosides, represents a considerable therapeutic challenge.^{6,8–11}

Endemic and epidemic outbreaks of nosocomial infections caused by MRSA have been reported at many centres, and the continuing increase in the incidence of these infections represents an important problem in many countries, including Belgium.^{9,11–15} All these findings justify surveillance programmes in which specific epidemiological markers are used to type the strains and to identify the major source of infection and the main routes of dissemination.

Aminoglycoside resistance mediated by aminoglycoside-modifying enzymes (AMEs) is often plasmid- or transposon-mediated and is, therefore, of considerable clinical importance. The present study describes the identification by polymerase chain reaction (PCR) of genes encoding AMEs in epidemic and laboratory isolates of MRSA in Belgium.

Materials and methods

Bacterial isolates

In total, 118 isolates of *S. aureus* were studied. The isolates were resistant to methicillin and one or more clinically used aminoglycosides. Resistance was determined routinely in the collaborating laboratories by disk diffusion methods.¹⁶ The isolates were from two different periods.

Period 1980–1985 ("1980 period"). Eight clinical isolates ("IPB isolates") of no known epidemic character were chosen from our laboratory collection for their relatively high level of resistance to aminoglycosides. Also examined were 29 epidemic MRSA isolates from five different hospitals. Eleven of these strains were obtained in 1981 from a hospital in Brussels (HB5); the remaining strains were isolated from septicaemic patients in four other hospitals during a Belgian surveillance study conducted in 1983–1985 by the Institute of Hygiene and Epidemiology, Brussels, Belgium.¹³

Period 1991–1992 ("1991 period"). Eighty-one unrelated epidemic MRSA isolates (obtained from various clinical specimens) were collected in 10 different hospitals as part of a second surveillance study conducted during this period by the Belgian Study Group of Hospital Infections (GDEPIH/GOSPIZ).

The isolates from the two periods were selected to resemble as closely as possible the strain distribution in the hospitals investigated. In total, isolates were obtained from 14 hospitals—one in Aalst (HA), five in Brussels (HB1, HB2, HB3, HB4 and HB5), one in Charleroi-Gilly (HC), one in Geel (HG), one in Ieper (HI), two in Leuven (HL1 and HL2), one in Montigny-le-Tilleul (HM), one in Tournai (HT) and one in Yvoir (HY) (tables I and II).

Isolates from the 1980 period were kept lyophilised; isolates from the 1991 period were sent frozen by the GDEPIH and kept at -80°C . When required, isolates were rehydrated or thawed and inoculated on to Mueller-Hinton Agar (MHA; Gibco).

S. aureus strains FK422 and FK170 and *S. epidermidis* strain FK109 were used as reference strains and were kindly supplied by F. H. Kayser (University of Zurich, Switzerland).

Determination of MICs

MICs of aminoglycosides were determined with a Cooke Dynatech MIC2000 Dispenser and Inoculator and an inoculum size of $1\ \mu\text{l}$. The isolates were first grown overnight at 35°C on MHA. A few colonies were then suspended in 5 ml of Mueller-Hinton broth (MHB; Gibco) and incubated for 3–5 h at 35°C . These cultures were adjusted to match a McFarland No. 1 Standard, then diluted 1 in 20 and inoculated into microdilution plates (Sarstedt) containing $100\ \mu\text{l}$ of MHB with doubling antibiotic dilutions to give a final inoculum of $c. 1.5 \times 10^5$ cfu/ml. These plates were then incubated at 35°C for 18 h. In every plate, one row

without antibiotics served as a control for growth. *S. aureus* strain ATCC25923 was included as a control for the MIC results. The plates were examined on a Cooke Dynatech viewing box and the MIC was recorded as the lowest concentration inhibiting visible growth. Interpretation of resistance was based on the criteria suggested by the French Society for Microbiology.¹⁷ MICs of the following antibiotics were determined: amikacin, gentamicin, netilmicin and tobramycin.

Enzymic assays for aminoglycoside-modifying enzymes (AMEs)

Cell-free enzyme extracts were first prepared by growing strains in 200 ml of MHB with aeration (200 rpm) at 37°C until the culture was in the logarithmic phase of growth. The bacterial cells were harvested by centrifugation and washed twice in cold Haas No. 1 buffer.¹⁸ The cells were lysed by the addition of $150\ \mu\text{g}$ of lysostaphin (Sigma) and incubation for 60 min at 37°C . In some experiments, cells were also disrupted with an MSE 150-W ultrasonic disintegrator (three 30-s bursts at 0°C and an amplitude of $15\ \mu\text{m}$). After centrifugation at $100\,000\ g$ for 1 h, the supernate was decanted carefully and stabilised with dithiothreitol at a final concentration of 10 mM. Portions ($500\ \mu\text{l}$) of these suspensions were stored at -80°C .

Aminoglycoside acetylating, nucleotidylating and phosphorylating assays were by the phosphocellulose paper binding assay¹⁸ with appropriate radiolabelled substrates: [^{14}C]acetyl-co-enzyme A ($0.86\ \text{mM}$; specific activity $9.7\ \text{Ci/mol}$); [^{14}C]ATP ($1.2\ \text{mM}$; specific activity $8.36\ \text{Ci/mol}$); and [^{32}P]ATP ($0.75\ \text{mM}$; specific activity $13.3\ \text{mCi/mol}$) (Amersham International). Assays were allowed to proceed for 30 min at 35°C . The amount of radioactivity associated with each aminoglycoside tested was then estimated in a Betaszzint BF5000/300 Liquid Scintillation Counter (Berthold, Wildbad, Germany). Controls without antibiotics and without enzyme preparations were included in each assay run.

In general, two or three aminoglycosides were used to detect the presence of AMEs: amikacin, gentamicin and kanamycin A for acetylating activity; gentamicin and kanamycin A for phosphorylating activity; and kanamycin A and tobramycin for nucleotidylating activity. For a more precise identification of the enzymes in particular strains, a set of 15 different aminoglycosides was used.

The following aminoglycosides were included in the assays: gentamicin, isepamicin, netilmicin, sisomicin, butirosin, lividomycin, neomycin, ribostamycin, amikacin, kanamycin A and B, apramycin, tobramycin, dibekacin and paromomycin.

Choice of synthetic oligonucleotide primers

The primer sequences were chosen with a PCR primer selection program¹⁹ within the nucleotide

sequences of the published regions of the three genes: *aacA-aphD*, *aphA3* and *aadC*, encoding the AAC(6')-APH(2''), APH(3')III and ANT(4', 4'') enzymes, respectively.²⁰⁻²² The primers for the *mecA* gene, encoding methicillin resistance, were chosen according to Murakami *et al.*²³ The genes were aligned with an "Align" program to enhance the specificity of the assays by selecting non-identical regions in these genes.

In the sequences of the sense and antisense primers, the numbers in brackets indicate the starting position of the sequence based on the numbering proposed in GenBank under the specific access number for each gene. The sequences chosen were as follows:

aacA-aphD: 5'(2022)-CCAAGAGCAATAAGG-GCATACC-3' and 5'(2369)-CACACTATCATAAC-CACTACCG-3' (yielding an amplicon of 222 bp);

aphA3: 5'(329)-CTGATCGAAAAATACCGCT-GC-3' and 5'(597)-TCATACTCTTCCGAGCAAA-GG-3'(269 bp);

aadC: 5'(605)-CTGCTAAATCGGTAGAAGC-3' and 5'(777)-CAGACCAATCAACATGGCACC-3'(174 bp);

mecA: 5'(1282)-AAAATCGATGGTAAAGGTT-GGC-3' and 5'(1814)-AGTTCTGCAGTACCGG-ATTTCG-3'(533 bp).

DNA isolation and polymerase chain reaction

DNA was isolated by a rapid one-step DNA isolation technique. Bacteria were grown overnight at 37°C on MHA containing a suitable aminoglycoside. A few (two or three) colonies were suspended in 50 µl of PCR mixture—50 mM KCl, 10 mM Tris-HCl, pH 9.0, 2.5 mM MgCl₂, gelatin 0.01 % v/v, Triton X-100, 0.1 % v/v, 300 µM of each dNTP (Promega) and 0.5 µM of each primer. The PCR mixture was then heated to 99°C for 10 min to lyse the bacterial cells. After cooling to 26°C, 1.5 U of *Taq* Polymerase (Promega) was added to each sample, followed by a drop of mineral oil to prevent evaporation. The samples were then subjected to 32 amplification cycles of 1 min at 94°C, 2 min at 60°C and 3 min at 72°C, followed by a 5-min extension period at 72°C. Thermal cycling was performed in a Programmable Heat Block (Hybaid, Teddington). After amplification, 10-µl portions were electrophoresed through a gel of Nusieve 3:1 agarose (FMC, Rockland, ME, USA) 4% w/v in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and the PCR products were visualised by staining with ethidium bromide 0.05 % w/v. In each run, a negative control to detect any contamination of the reagents and *Hae*III-digested ϕ X174RF DNA size markers (BRL-Gibco) were included. All isolates were tested at least twice before they were considered as positive.

Phage-typing

Bacteriophage typing was with the International Typing Set at the routine test dilution (RTD) and

RTD $\times$ 100. Phages used included lytic-group I (phages 29, 52, 52A, 79 and 80), group II (phages 3A, 3C, 55 and 71), group III (phages 6, 42E, 47, 53, 54, 75, 77, 83A, 84 and 85), group V (phages 94 and 96) and a non-allocated (miscellaneous) group (phages 81 and 95). *S. aureus* isolates were considered to have different phage types if there was either a difference in phage lytic group or if there was a difference in two or more lytic reactions for isolates belonging to the same group. The strain was considered as non-typable if there was no lysis at RTD or RTD $\times$ 100 for each of the phages tested.

An epidemic situation in a hospital was surmised when at least three isolates with the same phage type were found among 10 successive MRSA isolates.

Statistical evaluation

Statistical evaluation was with the χ^2 test, the Mann-Whitney U test, Student's *t* test and Spearman's rank correlation coefficient r_s when indicated. The level of significance was set at $p \leq 0.05$.

Results

Susceptibility data

The distribution of MICS of the different aminoglycosides for MRSA isolates from the two periods are shown in fig. 1. There was a significant shift to higher MICs of netilmicin ($0.02 > p > 0.01$), amikacin and tobramycin (both $p < 0.001$) for MRSA isolates in the 1991 period. There was a non-significant tendency ($0.2 > p > 0.1$) towards higher MICs of gentamicin.

In both periods, netilmicin had the lowest MIC values and was significantly more active against MRSA isolates than the other aminoglycosides ($0.02 > p > 0.01$ compared to amikacin in the 1991 period; $p < 0.001$ for all other comparisons). Netilmicin also showed the lowest prevalence of resistance—0% in the 1980 period and 1.2% in the 1991 period. Isolates with intermediate resistance to netilmicin comprised 10.3% in the 1980 period and 9.9% in the 1991 period. Resistance to amikacin was found in 6.9% of the isolates from the 1980 period and 6.2% from the 1991 period, with a significant increase in strains with intermediate resistance to amikacin (from 0% to 24.7%; $0.01 > p > 0.001$). For gentamicin, the level of resistance remained practically identical in the two periods—89.7% and 86.4%. For tobramycin, there was a significant increase in resistant strains in the 1991 period (from 62.1% to 95.1%; $p < 0.001$), accompanied by a significant decrease in strains with intermediate resistance (from 27.6% to 1.2%; $p < 0.001$).

Aminoglycoside-modifying enzymes (AMEs)

The results of assays for AMEs are presented in tables I and II. Acetyltransferase activity varied

Fig. 1. Distribution of in-vitro MICs of aminoglycosides for epidemic methicillin-resistant *S. aureus* isolates from the "1980 period" (▨) and the "1991 period" (■). AK, amikacin; GM, gentamicin; TM, tobramycin; NT, netilmicin.

Table 1. Aminoglycoside-modifying enzymes and phage types in aminoglycoside-resistant *mecA*-positive *S. aureus* isolates from Belgian hospitals (1980–1985)

Hospital*	Total number of strains	Aminoglycoside resistance profile (number of strains)	Number of strains expressing each enzyme†			Phage type‡ (number of strains)
			AAC(6')-APH(2'')	ANT(4', 4'')	APH(3')III	
HA	8	ak Gm nt TM (5)	5	0	5	C (5)
		ak Gm tm (1)	1	0	0	C (1)
		Other (2)	0	0	2	C (2)
HB4	8	Gm Tm (7)	7	0	7	C (4); C* (2); C ^{oo} (1)
		Ak Gm Nt Tm (1)	1	0	1	C (1)
HB5	11	Gm Tm (8)	8	0	8	C (4); C* (2); C ^{oo} (1); E (1)
		ak Gm Tm (2)	2	0	2	C* (1); E (1)
		Ak Gm Nt Tm (1)	1	0	1	C* (1)
HL2	1	Gm Tm (1)	1	0	1	A (1)
HM	1	Gm Tm (1)	1	0	1	A (1)
JPB	8	ak Gm Tm (4)	4	0	4	C (2); D (2)
		Ak Gm Tm (4)	4	0	0	C (2); D (1); E (1)

Ak, amikacin; Gm, gentamicin; Nt, netilmicin; Tm, tobramycin; ak, gm, nt and tm indicate intermediate resistance.

*See text.

†Determined by the phosphocellulose paper binding assay and PCR.

‡Determined at the routine test dilution (RTD) for phage types A, C, D and E, and at RTD × 100 for phage type C^{oo}.

markedly amongst the isolates from the two periods. Fig. 2 compares the enzymic modification profile of an isolate (Sta. 810416s) from the 1980 period—with low acetylating activity—with the profile of an isolate (Sta. 920098s) from the 1991 period with high acetylating activity. Fig. 3 compares the acetylating activity, with amikacin as the substrate, of isolates expressing the

bifunctional AAC(6')-APH(2'') enzyme. A significantly higher level of acetyltransferase activity was found in isolates from the 1991 period compared with isolates from the 1980 period ($p < 0.001$). A significant increase in phosphorylating activity was not found ($0.7 > p > 0.5$). There was no correlation between acetylating activity and the MIC of amikacin ($p > 0.9$

Table II. Aminoglycoside-modifying enzymes and phage types in aminoglycoside-resistant *mecA*-positive *S. aureus* isolates from Belgian hospitals (1991–1992)

Hospital*	Total number of strains	Aminoglycoside resistance profile† (number of strains)	Number of strains expressing each enzyme‡			Phage type§ (number of strains)
			AAC(6')-APH(2'')	ANT(4', 4'')	APH(3'')III	
HG	8	ak Tm (8)	0	8	0	C ^{oo} (8)
HB1	7	Ak Gm Nt Tm (7)	7	7	0	A (7)
HB2	9	Ak Gm nt Tm (4)	4	4	0	B (3); G (1)
		Gm Tm (5)	5	0	0	A (3); C (1); H (1)
HB3	9	ak Gm nt Tm (4)	4	4	0	A (4)
		Gm nt Tm (3)	3	0	0	A (3)
		Ak Gm nt Tm (1)	1	0	0	E (1)
		Gm Nt tm (1)	1	1	0	D ^{oo} (1)
HB4	10	ak Gm nt Tm (5)	5	5	5	A (2); B (2); D (1)
		Gm tm (1)	1	1	1	B (1)
		ak Gm nt Tm (3)	3	0	0	B (2); C ^{oo} (1)
		Ak Gm nt Tm (1)	1	0	1	D (1)
HC	9	ak Gm nt Tm (9)	9	9	6	A (7); B (2)
HI	8	Ak Gm nt Tm (8)	8	8	3	A ^{oo} (4); NT (3); B (1)
HL1	4	ak Gm nt Tm (4)	4	0	0	B (2); B ⁺ (1); E ⁺ (1)
HY	10	ak Gm nt Tm (7)	7	7	0	A (5); B (1); F (1)
		Gm nt Tm (3)	3	0	0	A (2); B (1)
HT	7	ak Gm nt Tm (7)	7	7	0	B (7)

*See text.

†See table I.

‡Determined by the phosphocellulose paper binding assay and PCR.

§Determined at the routine test dilution (RTD) for phage types A–H, and at RTD × 100 for phage types A^{oo}–D^{oo}; NT, not typable. **Fig. 2.** Enzymic modification profiles of two staphylococcal isolates—(a) 810416s and (b) 920098s—synthesising the bifunctional AAC(6')-APH(2'') enzyme. AK, amikacin; AP, apramycin; BT, butirosin; DB, dibekacin; GM, gentamicin; KM A and B, kanamycin A and B; LV, lividomycin; NM, neomycin; NT, netilmicin; PM, paromomycin; RM, ribostamycin; SS, sisomicin; TM, tobramycin.

for the 1980 period and $0.3 > p > 0.2$ for the 1991 period).

PCR experiments

Specificity. The specificity of the different sets of primers was tested with DNA extracts of the reference

Fig. 3. Comparison of the N-acetyltransferase activities—expressed as pmol/μg dry weight with amikacin as the substrate—of isolates expressing the AAC(6')-APH(2'') enzyme from the 1980 period (■) and the 1991 period (●).

strains FK422, FK170 and FK109 containing, respectively, the *aacA-aphD*, *aphA3* and *aadC* genes. Primers for the *mecA* gene were tested with DNA from strain Sta.810442s from our laboratory stock. The reference strains generated a band of amplified DNA with the corresponding specific sets of primers (fig. 4, lanes 1–4).

Detection of AMEs in clinical isolates. The PCR technique was applied to the clinical isolates to detect the presence of genes encoding AMEs. The results of these experiments are shown in tables I and II. Fig. 4 illustrates the results of PCR experiments with two clinical isolates. Isolate Sta.810442s from the 1980 period contains the *aacA-aphD* and the *aphA3* genes (fig. 4, lane 5), whereas isolate Sta.920399s contains the *aacA-aphD* and the *aadC* genes (fig. 4, lane 6). Isolates reported as methicillin-resistant by the participating laboratories each generated a specific DNA band following amplification with the *mecA* primers, and the detection of AME-encoding genes by PCR

Fig. 4. Ethidium bromide-stained agarose gel of amplified DNA fragments from reference strains and clinical MRSA isolates. Lane 1, *S. aureus* FK422 (*aacA-aphD*); 2, *S. aureus* FK170 (*aphA3*); 3, *S. epidermidis* FK109 (*aadC*); 4, MRSA Sta.810442s (*mecA*); 5, MRSA Sta.810422s (*mecA*, *aacA-aphD*, *aphA3*); 6, MRSA Sta.920399s (*mecA*, *aacA-aphD*, *aadC*); 7, negative control; 8, *Hae*III-digested ϕ X174 size markers. AMEs indicated in parenthesis were those whose presence was indicated following amplification with the corresponding primer sets. Isolates in lanes 5 and 6 were amplified with the four sets of primers simultaneously.

Table III. The distribution of phage patterns corresponding to MRSA phage types A–H in the 1980 and 1991 periods

Phage type	Sensitivity to phages	Number of strains	
		1980 period	1991 period
A	77	2	37
B	47/54/75/77/84/85	0	22
B ⁻	47/54/75/85	0	1
C	6/47/54/75	22	10
C ⁺	6/47/54/75/77/84 or 85	7	0
D	85	3	3
E	77/84/85	3	1
E ⁺	77/84/85/95	0	1
F	77/83A/85	0	1
G	47/75/77	0	1
H	79/6/42E/47/85/81/95	0	1
NT	Non-typable	0	3

was in agreement with results obtained by the phosphocellulose paper binding assay.

The *aacA-aphD* gene was encountered most frequently in the 1991 period, but the *aphA3* gene was more prevalent in the 1980 period. The *aadC* gene was found only in isolates from the 1991 period, whereas the *aphA3* gene was significantly more prevalent in the 1980 period compared with the 1991 period ($p < 0.001$). In both periods, the *aacA-aphD* gene was found mostly in combination with other genes. Of the isolates of the 1980 period, 90% harboured this bifunctional gene in combination with the *aphA3* gene, whereas this combination was found only in 1.2% of the 1991 isolates ($p < 0.001$). The most frequent combination of genes amongst isolates from the 1991 period was the *aacA-aphD* + *aadC* combination (47% of isolates). The triple combination of *aacA-aphD* + *aadC* + *aphA3* was not found in isolates from the 1980 period, but was present in 19% of isolates from the 1991 period ($0.05 > p > 0.02$) and was found

in isolates from three hospitals (HB4, HC and HI). In isolates from HG, only the *aadC* gene was detected. The presence of genes, or combinations of genes, encoding AMEs for which amikacin is a substrate was indicated in isolates considered as susceptible (29%) or of intermediate resistance (50%) in MIC tests; however, all isolates harbouring the *aacA-aphD* gene were clearly resistant to gentamicin in MIC tests.

Phage-typing

Phage types encountered amongst the MRSA isolates are listed in table III. The majority of the phage types belonged to phage group III, although two isolates with phage types E⁺ and H also showed significant reactions with phages belonging to other groups. Phage types C and C⁺ predominated in the 1980 period, whereas most strains of the 1991 period belonged to types A and B. Less than 2.5% (3 of 118) of the isolates were non-typable.

The phage-type distribution amongst the isolates of the different hospitals is shown in tables I and II. There was no particular correlation between the phage types and the presence of genes for AMEs. In the 1980 period, only two isolates belonged to phage type A—which spread epidemically in the 1991 period—while phage type C was epidemic in three hospitals (HA, HB4 and HB5). All eight isolates from HG, harbouring the ANT(4', 4'') enzyme, belonged to the rather peculiar phage type C^{oo}.

Discussion

MRSA play an important role in nosocomial infection. Epidemiological monitoring of MRSA is an essential step in the development of control programmes and a wide variety of epidemiological

markers is used, such as biotype, phage type, plasmid profile and antibiotype. The present paper reports the distribution and evolution of phage types and genetic determinants of AMEs in epidemic MRSA isolates from various Belgian hospitals. Furthermore, the utility of the PCR technique to detect and identify genes encoding AMEs was validated.

Phage typing of *S. aureus* has been standardised internationally and is regarded generally as a useful epidemiological tool, although its discriminating capacity has been questioned in some cases.^{9, 24, 25} In the present study, most isolates from the 1980 period belonged to phage group III, and particularly to phage type 6/47/54/75. Phage types A and B of phage group III, with sensitivity to phages 77 and 47/54/75/77/84/85, respectively, were most prevalent among isolates from the 1991 period. It should be noted that phage types A and B represent subclones that are closely related genetically. No particular linkage between phage types and AME genes was found, although the fact that all isolates that harboured only the *aadC* gene belonged to phage type C^{oo} may be worthy of further investigation.

Increased antibiotic resistance in MRSA has been reported worldwide and may compromise the therapeutic effectiveness of antimicrobial agents.^{6, 8–11, 25, 26} The simultaneous presence of genetic determinants coding for resistance to different antibiotics leads to an important narrowing of the therapeutic index. Aminoglycosides have been used in the treatment of staphylococcal infections, but the development of resistance, mostly caused by AMEs, will seriously limit their effectiveness. The bifunctional AAC(6′)–APH(2″) enzyme, which is restricted to gram-positive bacteria, inactivates a broad range of clinically useful aminoglycosides because it catalyses both acetyltransferase and phosphotransferase reactions. Various techniques, such as MIC ratios, the radiochemical phosphocellulose paper binding assay, and some molecular diagnostic methods, are used to identify AMEs or their corresponding genes.

The PCR technique has been shown previously to be a reliable tool for the identification of AME genes in gram-negative bacteria^{27, 28} and was used in the present study to detect and identify genes encoding AMEs in epidemic MRSA isolates. For this purpose, three sets of primers delineating specific DNA fragments of the *aacA-aphD*, *aphA3* and *aadC* genes were defined. These sets of primers were assessed with reference strains encoding various AMEs. Amplification fragments specific for the AME genes were detected clearly from staphylococcal reference strains. No specific DNA fragments were detected when these primers were used on reference strains harbouring genes other than those mentioned above (data not shown). Furthermore, complete agreement was found between the results of the radiochemical phosphocellulose paper binding assay¹⁸ and the PCR technique.

The radiochemical technique is considered to be a reliable method for the detection of AMEs, but is a

costly and time-consuming technique which uses radiolabelled products. Furthermore, it may fail to detect low levels of intracellular enzymes, especially ANT(2″),²⁹ and results are not always easy to interpret when enzyme combinations of the same subclass are present. In the present study, isolates, especially from the 1980 period, with very low enzymic activity were found. Such borderline activity may be neglected or misinterpreted, and the more clear-cut results obtained with the PCR technique were much easier to interpret. However, the radiochemical technique does allow expression of enzymic activity to be followed. Significantly higher acetyltransferase activity was found in isolates from the 1991 period that produced AAC(6′)–APH(2″) compared with those from the 1980 period. The increased enzymic activity was paralleled by a significant shift towards higher MICs of amikacin and tobramycin, although there was no direct correlation between enzymic activity and MIC values. Moreover, combinations of AMEs were detected in MRSA isolates which would be regarded as amikacin-susceptible by internationally accepted sensitivity standards, indicating that MICs are not always indicative of the presence of particular AMEs. Interestingly all isolates expressing only the bifunctional AAC(6′)–APH(2″) enzyme were neomycin-susceptible (data not shown).

AAC(6′)–APH(2″) was the enzyme encountered most frequently among the isolates, as has been reported in other centres.^{30–36} The APH(3′)III enzyme decreased significantly in importance between the 1980 and 1991 periods. The ANT(4′, 4″) enzyme, which was not detected in the isolates of the 1980 period, was present in 75 % of the 1991 isolates. The occurrence of this enzyme resulted in more complex resistance mechanisms, since 18.5 % of the 1991 isolates possessed genes for the three distinct AMEs. The ANT(4, 4″) enzyme appears to have been relatively rare in European isolates at the beginning of the 1980s.^{30, 32, 35} Madsen *et al.*³⁵ observed no change in the distribution of AMEs in Danish staphylococcal isolates during 1979–1987, while an increasing prevalence of the ANT(4, 4″) enzyme was noted in a European collaborative study.^{31, 32} The European Study Group on Antibiotic Resistance (ESGAR)³² detected the ANT(4′, 4″) enzyme in 11.2 % of staphylococci isolated consecutively from blood and urine during 1984–1985, increasing to 46.1 % in the 1987–1988 survey.³¹ An important variation in the prevalence of AMEs in isolates from Central and Northern Europe compared with Southern Europe was reported in both studies. Interestingly, important differences in the distribution of bacterial resistance determinants have also been reported in more restricted geographic areas.^{33, 37} These changes in prevalence of AMEs can be caused by changes in antibiotic policies or can be explained by the introduction and consequent inter-hospital spread of resistant strains. Finally, it cannot be excluded that these resistance genes originated from an environmental source. Indeed, saprophytic staphy-

lococci have frequently been reported to carry resistance determinants, including those for AMEs,³⁰⁻³⁴ and can, therefore, function as a reservoir for the genetic determinants of these enzymes. Conjugational transfer of resistance determinants between *S. aureus* and *S. epidermidis*, leading to rapid dissemination of these determinants in the hospital environment, has been demonstrated.^{38,39}

In conclusion, the PCR technique was shown to be a reliable technique for studying the presence and evolution of antibiotic resistance markers in epidemic MRSA. The technique has the potential to be of great help for early detection of resistance genes in an epidemiological setting.

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