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Trends in the gentamicin and arbekacin susceptibility of methicillin-resistant *Staphylococcus aureus* and the genes encoding aminoglycoside-modifying enzymes

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Abstract It is generally accepted that methicillin-resistant *Staphylococcus aureus* (MRSA) is also resistant to aminoglycoside antibiotics. We investigated trends of gentamicin and arbekacin susceptibilities and the prevalence of the genes encoding aminoglycoside-modifying enzymes (AMEs) for a total of 218 strains of MRSA isolated from blood specimens obtained from 1978 through 2002 in one hospital. The minimum inhibitory concentrations of gentamicin at which 50% of the strains were inhibited (MIC_{50}) were ≥128 and 32 μg/ml for isolates obtained from 1978 to 1984 and from 1985 to 1989, respectively, and 0.5 μg/ml for isolates obtained from 1990 to 2002. The MIC_{90} of gentamicin was consistently ≥128 μg/ml. Investigation of the occurrence of AME revealed that the MIC_{50} of gentamicin was highly correlated with the presence of *aac(6′)/aph(2″)* encoding aminoglycoside acetyl/phosphotransferase. The MIC_{50} of arbekacin was 2 μg/ml for strains isolated in 1978–1984 and ≤0.5 μg/ml for strains isolated from 1985 to 2002. The MIC_{90} of arbekacin was 8 μg/ml for the strains isolated in 1978–1989 and 1 to 2 μg/ml for strains isolated in 1990–2002. Though it has been established that AAC(6′)/APH(2″) modifies arbekacin, the trend of arbekacin resistance was not necessarily consistent with the presence of this enzyme. However, the prevalence of both *aac(6′)/aph(2″)* and *aph(3′)-III* in the strains isolated from 1978 through 2002 was correlated with the MIC_{90} values of arbekacin. Thus, it is most likely that APH(3′)-III, in addition to AAC(6′)/APH(2″), is somehow involved in arbekacin resistance in *S. aureus*. Our results imply that gentamicin- and arbekacin-resistant MRSAs have consistently decreased for the past 25 years and that this finding is, most likely, attributable to the declining prevalence of genes encoding for AMEs.

Key words Methicillin-resistant *Staphilococcus aureus* (MRSA) · Arbekacin · Gentamicin · Drug resistance

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen causing pneumonia, infections in operations, and septicemia; it is refractory to antibiotic chemotherapy.[1] The frequency of MRSA isolation in Japan in the years 1998, 1999, and 2000 was reported to be 61.7%, 53.1%, and 56.2%, respectively.[2] A few antibiotics, including vancomycin, teicoplanin, arbekacin, and linezolid have been used for the treatment of MRSA infections in Japan. Vancomycin or teicoplanin may be used as the last bullet in the armamentarium for the treatment of multidrug-resistant MRSA, and these agents are generally effective.[2–5] However, more recently, the emergence of vancomycin- and teicoplanin-resistant, and -intermediate resistant MRSA has been reported.[6–10]

Although arbekacin has been the treatment of choice for MRSA in Japan since 1990, the occurrence of bacteria with a low degree of arbekacin resistance has been reported.[2,3,11,12] Most, if not all, aminoglycoside antibiotics act on the bacterial ribosome, inhibiting protein synthesis.[13–15] MRSA gains resistance to aminoglycoside antibiotics mainly by producing aminoglycoside-modifying enzymes (AMEs) such as phosphotransferases, acetyltransferases, and adenyltransferases, encoded by *aph*, *aac* and *aad,* respectively.[14,16] It was reported that gentamicin-resistant and arbekacin-resistant

MRSAs produce AAC(6′)/APH(2″) and AAC(6′)/APH(2″) plus additional enzyme(s), respectively.[12,17,18] Therefore, whether MRSA harbors gene(s) encoding these AMEs could be a key to their aminoglycoside resistance.

Here we report on our investigation, using 218 MRSA strains obtained from blood specimens of patients with septicemia, carried out to determine the chronological frequency of the isolation of gentamicin- and arbekacin-resistant MRSAs over the past 25 years, and the prevalence of genes encoding AMEs.

Materials and methods

Bacterial strains

S. aureus strains were isolated by standard procedures such that blood specimens were streaked on mannitol-NaCl agar, and the colonies grown on the medium were subjected to the methicillin susceptibility test, or more recently, the oxacillin susceptibility test. Strains that were *mecA*-gene positive and for which the minimum inhibitory concentration (MIC) of methicillin was ≥16 μg/ml or that of oxacillin was ≥4 μg/ml were classified as MRSA. A total of 218 strains were collected at one 700-bed geriatric hospital in Tokyo from 1978 to 2002 and they were divided chronologically into five groups: 1978–1984, 1985–1989, 1990–1994, 1995–1999, and 2000–2002, which consisted of 45, 45, 49, 49, and 30 strains, respectively. Because the MRSA isolated in 1978 was the very first strain isolated in Japan, and because only 1 MRSA strain was isolated in 1978–1979, this was included in the isolates in 1978–1984. The strains were kept at –80°C in brain heart infusion broth (Nippon Becton Dickinson, Tokyo, Japan) supplemented with 40% glycerol.

Antibiotics

Arbekacin was kindly supplied by Meiji Seika (Tokyo, Japan). Gentamicin was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Determination of minimum inhibitory concentrations (MICs) of antibiotics

The MICs of antibiotics were determined by the agar-dilution method according to the Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards (CLSI/NCCLS) 2005 guidelines (19). Bacteria were grown overnight in Mueller–Hinton broth (Nippon Becton Dickinson) at 35°C and diluted to $A_{578nm} = 0.3$. Cells were further diluted 10-fold and a 5-μl fraction was inoculated on Mueller–Hinton agar (Nippon Becton Dickinson) plates impregnated with gentamicin or arbekacin, using a microplanter (Sakuma, Tokyo, Japan), and cell growth was scored after 18 h of incubation at 35°C. Strains with gentamicin MICs of 8 and ≥16 μg/ml were judged as gentamicin-intermediate-resistant and -resistant, respectively, according to the CLSI 2005 criteria. Strains with arbekacin MICs of ≥4 μg/ml were assessed as arbekacin-resistant, based on an earlier report.[20]

Polymerase chain reaction (PCR) amplification of the genes coded for AMEs and *mecA*

Multiplex PCR was carried out using a pair of primers described by Tsuchizaki et al.,[21] incorporating an earlier method.[22,23] We made the following modification. Briefly, DNA was extracted from the heat-killed cells at 100°C for 10 min with a phenol-chloroform (1:1) mixture, and centrifuged at 12 000 g for 1 min. The PCR mixture, in a total aliquot of 50 μl, consisted of 1 μl of the extracts, 1 U of Amplitaq Gold polymerase (Applied Biosystems, Foster City, CA, USA), 5 μl of 10×PCR buffer, 5 μl of 25 mM $MgCl_2$, 5 μl of 2 mM deoxynucleoside triphosphates, and 0.1 μl of 100 mM primers. The thermal cycle was set as 94°C for 30 s, 52°C for 1 min, and 72°C for 1 min and run for a total of 40 cycles after running at 95°C for 12 min in the beginning, and at 70°C for 10 min at the end; a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems Japan, Tokyo, Japan) was used. The products were analyzed by agarose gel electrophoresis.

Results

All the strains used in this study were subjected to the *mecA* test and it was confirmed that they had the *mecA* gene. Taken together with methicillin/oxacillin resistance, they were identified as MRSA.

Antimicrobial susceptibility

The MIC_{50} values of gentamicin in the strains isolated in 1978–1984, 1985–1989, 1990–1994, 1995–1999, and 2000–2002 appeared to be >128, 32, 0.5, 0.5, and 0.5 μg/ml (Fig. 1a), clearly indicating that the MIC_{50} of gentamicin-resistant MRSAs was decreasing. The values were steadily low in the years in 1990–2002. The MIC_{50} of gentamicin in the strains isolated from 1990 through 2002 was at least 512 times lower than that in the strains isolated in 1978–1984. The MIC_{90} values of gentamicin were consistently high, at ≥128 μg/ml. The frequency of occurrence of gentamicin-resistant strains appeared to be 77.8%, 62.2%, 46.9%, 40.8%, and 46.7% in 1978–1984, 1985–1989, 1990–1994, 1995–1999 and 2000–2002, respectively (Fig. 2). The chronological variation in the prevalence of gentamicin-resistant strains was roughly correlated with that of the gentamicin MIC_{50} (Fig. 1a and Fig. 2).

The MIC_{50} values of arbekacin in the strains isolated in 1978–1984, 1985–1989, 1990–1994, 1995–1999, and 2000–2002 were 2, ≤0.5, ≤0.5, ≤0.5, and ≤0.5 μg/ml, respectively (Fig. 1b). It is not very clear from these data whether the arbekacin susceptibility of the MRSA strains varied during the years surveyed. The MIC_{90} values of arbekacin in the

strains isolated in 1978–1984, 1985–1989, 1990–1994, 1995–1999, and 2000–2002 showed clear differences: the values were 8, 8, 1, 1, and 2 μg/ml, respectively (Fig. 1b).

Prevalence of the genes encoding AMEs

The above study showed a chronological decrease in gentamicin and arbekacin resistance in strains isolated in the past 25 years. As it is well established that aminoglycoside resistance is mainly attributable to the expression of AMEs, we analyzed the occurrence of the genes encoding AMEs in all 218 strains by PCR. We employed PCR primers designed to amplify plasmid-borne genes encoding AME known in *S. aureus* because *S. aureus* lacks the chromosomal gene coded for AME. To investigate the linkage of gentamicin resistance and the presence of *aac(6′)/aph(2″)* as reported earlier,[12] we tested for the presence of the *aac(6′)/aph(2″)* gene. The results showed that the chronological prevalence of the *aac(6′)/aph(2″)* gene was well correlated with the occurrence of gentamicin-resistant strains (Table 1) and with the MIC_{50} of gentamicin (Fig. 1a). Although the trend of the prevalence of the *aph(3′)-III* gene seemed to be correlated with arbekacin resistance, it is unlikely that this enzyme alone confers this resistance, because arbekacin lacks a modification site.

A combination of *aac(6′)/aph(2″)* plus *aph(3′)-III* is reported to be responsible for arbekacin resistance.[17] Therefore, we searched for cells that had both *aac(6′)/aph(2″)* and *aph(3′)-III*; we found that 53.3%, 35.6%, 2.0%, 4.1%, and 3.3% of the strains isolated in 1978–1984, 1985–1989, 1990–1994, 1995–1999, and 2000–2002, respectively, were *aac(6′)/aph(2″)* plus *aph(3′)-III*-positive (Fig. 3). Trends in the prevalence of both *aac(6′)/aph(2″)* and *aph(3′)-III* appeared to be consistent with the MIC_{90} of arbekacin (Figs. 1 and 3) and with the occurrence of resistant strains (Table 1).

The prevalence of *aac(6′)/aph(2″)* plus *aad(4′, 4″)* was 11.1%, 22.2%, 40.8%, 34.7%, and 40.0%, in 1978–1984, 1985–1989, 1990–1994, 1995–1999, and 2000–2002, respectively, showing that the prevalence of this combination of AMEs increased from 1978 to about 1994 and kept steady

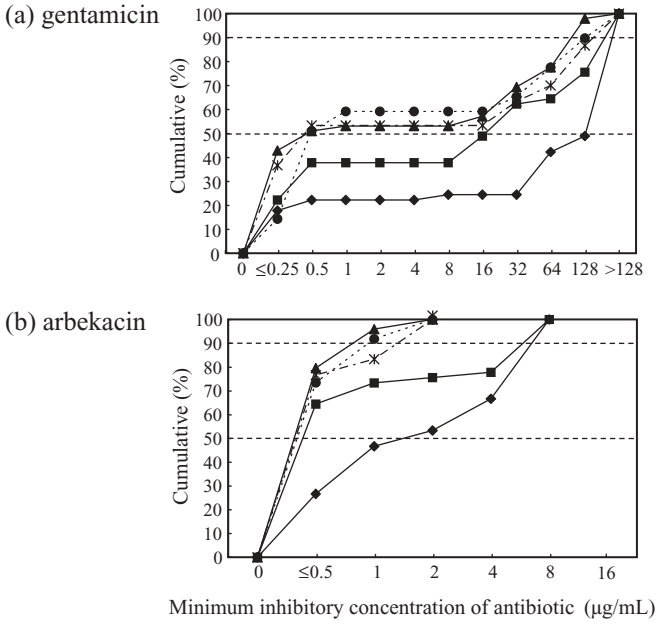

Fig. 1a,b. Cumulative minimum inhibitory concentrations (MICs) of aminoglycoside antibiotics (**a** gentamicin, **b** arbekacin). MICs of antibiotics were determined as described in the "Materials and methods" section. MIC_{50} and MIC_{90} denote that 50% and 90%, respectively, of the strains tested showed the indicated MIC value. *Diamonds*, 1978–1984; *squares*, 1985–1989; *triangles*, 1990–1994; *closed circles*, 1995–1999; *asterisks*, 2000–2002

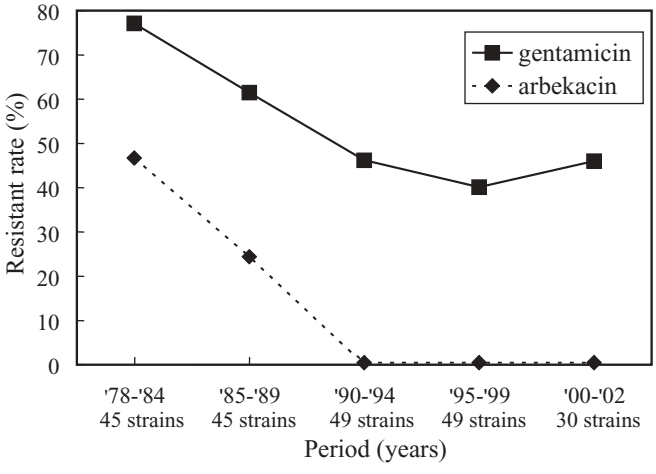

Fig. 2. Periodic isolation frequency of aminoglycoside-resistant strains, showing the percentage of aminoglycoside-resistant strains in the period indicated. Total numbers of strains tested are indicated on the *horizontal axis*. *Squares*, gentamicin; *diamonds*, arbekacin

Table 1. Linkage between the occurrence of different types of AME and gentamicin/arbekacin resistance

	Gentamicin				Arbekacin			
	MIC_{50} (μg/ml)	MIC_{90}	Range	Resistant strains (%)	MIC_{50} (μg/ml)	MIC_{90}	Range	Resistant strains (%)
aac(6′)/aph(2″) (-)[a] (*n* = 98)[b]	≤0.25	0.5	≤0.25–1	0	≤0.5	≤0.5	≤0.5–1	0
aac(6′)/aph(2″) (+) and *aph(3′)-III* (-)[a] (*n* = 75)[b]	64	128	16–>128	100	1	2	≤0.5–2	0
aac(6′)/aph(2″) (+) and *aph(3′)-III* (+)[a] (*n* = 45)[b]	>128	>128	8–>128	100	8	8	≤0.5–8	71.1

[a] (+) and (–) denote that the respective gene was detectable and undetectable, respectively
[b] (*n*), Number of strains classified as having the respective AME

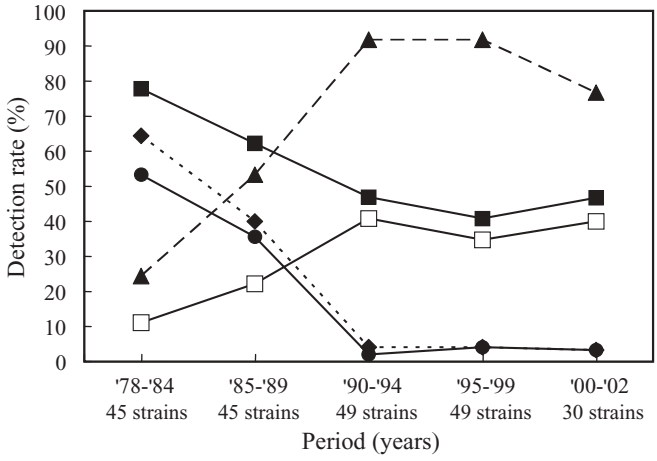

Fig. 3. Periodic detection frequency of the genes encoding aminoglycoside-modifying enzyme, showing the percentages of strains that had a positive result for the gene encoding aminoglycoside-modifying enzyme in the period indicated. Total numbers of strains tested are indicated on the *horizontal axis*. *Closed squares*, *aac(6′)/aph(2″)*; *diamonds*, *aph(3′)-III*; *triangles*, *aad(4′, 4″)*; *closed circles*, *aac(6′)/aph(2″)* plus *aph(3′)-III*; *open squares*, *aac(6′)/aph(2″)* plus *aad(4′, 4″)*

until 2002 (Fig. 3). Only one strain having both *aph(3′)-III* and *aad(4′, 4″)* was found in 1986 and only one strain having all *aac(6′)/aph(2″)*, *aph(3′)-III*, and *aad(4′, 4″)* genes was found in 1984. Therefore, it is difficult to judge the connection between the presence of the gene and aminolycoside resistance. Throughout 1978–2002, nine strains had none of these AMEs.

The prevalence of *aad(4′, 4″)*, which is known to be linked with kanamycin, tobramicin, and amikacin resistance, was 24.4%, 53.3%, 91.8%, 91.8%, and 76.7%, in the MRSA isolated in 1978–1984, 1985–1989, 1990–1994, 1995–1999, and 2000–2002, respectively (Fig. 3). The prevalence of this gene increased from 1978–1984 and the trend continued until 1995–1999 and then decreased a little. Thus, the presence of *aad(4′, 4″)* alone seems unrelated with either gentamicin or arbekacin resistance. The prevalence of the *aph(3′)-III* gene, which encodes the enzyme linked to kanamycin resistance, was 64.4%, 40.0%, 4.1%, 4.1%, and 3.3% for the years 1978–1984, 1985–1989, 1990–1994, 1995–1999, and 2000–2002, respectively (Fig. 3).

Discussion

We reported, in this study, on arbekacin- and gentamicin-resistant MRSA isolated in a single geriatric hospital in Tokyo and we also reported the prevalence of genes coding for AMEs. The reason that the survey was limited to one hospital is that a long-term survey in a single hospital reflects trends in the prevalence of resistant strains, rather than showing the mixtures of strains from many hospitals, as reported earlier.[24]

AMEs were reported as factors that confer aminoglycoside resistance to *S. aureus*.[14,25] In the present study, trends of gentamicin and arbekacin resistance in MRSA strains collected from 1978 through 2002 showed a chronological decrease in resistant MRSA strains. Our survey of the prevalence of the genes encoding for AMEs, including *aac(6′)/aph(2″)*, *aad(4′, 4″)*, and *aph(3′)-III*, showed that the prevalence of *aac(6′)/aph(2″)* alone, *aac(6′)/aph(2″)* plus *aph(3′)-III*, and *aph(3′)-III* alone decreased chronologically and this decrease was nearly parallel with the chronological decrease in the MIC_{50} values of gentamicin and the MIC_{90} values of arbekacin (Figs. 1 and 3). The prevalence of genes encoding for AMEs, including *aad(4′, 4″)* alone and *aac(6′)/aph(2″)* plus *aad(4′, 4″)* did not correspond with the MIC_{50} values of gentamicin or the MIC_{90} values of arbekacin. Therefore, these genes were excluded from further consideration.

The results presented in this article are in accord with an independent study done by Japanese authors.[24] Because the clinical use of arbekacin is limited to Japan and a few other Asian countries, no worldwide survey information is available. On the other hand, in regard to the frequency of isolation of MRSA having AMEs in Turkey, European countries, and Korea, it was reported that *aac(6′)/aph(2″)* was the most frequent, followed by *aad(4′, 4″)* and *aph(3′)-III*.[26,27,28]

Which of the genes *aac(6′)/aph(2″)* alone, *aph(3′)* alone, or the combination of *aac(6′)/aph(3″)* plus *aph(3′)-III* are responsible for resistance to either gentamicin or arbekacin is not clear from the present data. Earlier studies have reported that AAC(6′)/APH(2″) modifies both gentamicin and arbekacin.[18,29] Our study confirmed an earlier report that trends in gentamicin and arbekacin resistance were well correlated with the prevalence of this gene. However, it is known that arbekacin is a poor substrate of AAC(6′)/APH(2″).[18] Thus, it is most likely that the chronological decrease that we found in gentamicin-resistant MRSA is attributable to the low prevalence of the *aac(6′)/aph(2″)* gene. However, arbekacin resistance is not so simple, because arbekacin-resistant MRSA was not isolated in the hospital in our study from 1990 through 2002, although arbekacin has been used for the treatment of MRSA infections in Japan since 1990, including at this hospital. About half of the MRSA strains isolated after 1990 had the *aac(6′)/aph(2″)* gene. Therefore, some other factor may be involved in arbekacin resistance. An earlier study suggested that the presence of *aac(6′)/aph(2″)* plus *aph(3′)-III* may be responsible for arbekacin resistance.[17] We confirmed in the present study that increasing susceptibility to arbekacin was well correlated with the trend of decreased prevalence of both the *aac(6′)/aph(2″)* and *aph(3′)-III* genes in MRSA. However, a problem is that APH(3′)-III alone does not modify arbekacin, due to a lack of the modification site. A plausible interpretation of this observation would be that the presence of *aph(3′)-III* in addition to *aac(6′)/aph(2″)* may induce additional unidentified factor(s) involved in resistance to arbekacin.

There were some exceptions to our general findings: (i) of the 45 *aac(6′)/aph(2″)* plus *aph(3′)-III* positive strains, 13 strains (28.9%) were arbekacin-susceptible (Table 1). This observation suggests that some of the genes were not expressed or were inactive. (ii) Of the arbekacin-susceptible MRSAs, 7.0% (13/ (173+13)) were *aac(6′)/aph(2″)* plus

aph(3′)-III-positive, which is inconsistent with the above conclusion. However, all the arbekacin-resistant MRSA strains, without exception, had the *aac(6′)/aph(2″)* plus *aph(3′)-III* genes (Table 1).

The hospital records showed that, since 1975, the use of gentamicin was to be limited. However, it took several years for this limited use to permeate the hospital.

In summary, our survey revealed that gentamicin and arbekacin resistance was well correlated with the presence of the *aac(6′)/aph(2″)* and *aac(6′)/aph(2″)* plus *aph(3′)-III* genes, respectively. Further surveys of arbekacin resistance and AMEs may be needed, because half of the recently isolated MRSA strains had the gene *aac(6′)/aph(2″)*, which is capable of modifying arbekacin.

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