

# Identification of Aminoglycoside-Modifying Enzymes by Susceptibility Testing: Epidemiology of Methicillin-Resistant *Staphylococcus aureus* in Japan

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**A multiple-primer PCR was used to identify genes encoding aminoglycoside-modifying enzymes in 381 clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA). The technique used three sets of primers delineating specific DNA fragments of the *aph(3')-III*, *ant(4')-I*, and *aac(6')-aph(2'')* genes, which influence the MICs of gentamicin, tobramycin, and lividomycin. Isolates with none of the three genes detected were susceptible to all three agents. Isolates with the *aph(3')-III* gene showed resistance to lividomycin (MIC > 1,024 µg/ml), and those with the *ant(4')-I* gene were resistant to tobramycin (MIC ≥ 8 µg/ml). Isolates with only the *aac(6')-aph(2'')* gene were resistant to gentamicin (MIC ≥ 8 µg/ml) and tobramycin in decreasing order; those with both the *ant(4')-I* and *aac(6')-aph(2'')* genes also were resistant to gentamicin and tobramycin, but in increasing order. Susceptibility testing, then, could detect specific genes. In 381 Japanese MRSA isolates, the *ant(4')-I*, *aac(6')-aph(2'')*, and *aph(3')-III* genes were prevalent in 84.5, 61.7, and 8.9%, respectively. Isolates with only the *ant(4')-I* gene had coagulase type II or III, but isolates with both the *ant(4')-I* and *aac(6')-aph(2'')* genes included all coagulase types. Most isolates with coagulase type IV or VII carried the *aac(6')-aph(2'')* gene. Of the MRSA isolates with *ant(4')-I* and/or *aac(6')-aph(2'')* genes, 97% were resistant to aminoglycosides in clinical use, but a new aminoglycoside, arbekacin, had excellent activity against these isolates.**

Enzymatic modification of aminoglycosides is a common mechanism of resistance to these antibiotics shown by clinical bacterial isolates. Among gram-positive cocci such as staphylococci, streptococci, and enterococci, five kinds of aminoglycoside-modifying enzymes (AME) occur: aminoglycoside-6-*O*-nucleotidyltransferase I [ANT(6)-I] (21), aminoglycoside-9-*O*-nucleotidyltransferase I [ANT(9)-I] (16), aminoglycoside-3'-*O*-phosphoryltransferase III [APH(3')-III] (7), aminoglycoside-4'-*O*-phosphoryltransferase I [ANT(4')-I] (14) and aminoglycoside-6'-*N*-acetyltransferase/2'-*O*-phosphoryltransferase [AAC(6')/APH(2'')] (6, 24). APH(3')-III, ANT(4')-I, and AAC(6')/APH(2'') are of particular significance because they modify aminoglycosides of therapeutic importance, including kanamycin, tobramycin, and gentamicin, respectively. These modifying enzymes can be plasmid or chromosome encoded and often are encoded on transposable elements (3).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infection (10), and these bacteria have acquired multiple resistance to a wide range of antibiotics including aminoglycosides (9, 10, 37). AME produced by MRSA isolates can be determined by identifying the corresponding genes. Susceptibility profiles to selected aminoglycosides previously have been used to detect specific aminoglycoside resistance mechanisms. However, characterizing strains

containing several AME genes solely on the basis of aminoglycoside resistance profiles can be difficult, since one resistance profile is often partially duplicated thereby masking the presence of an additional profile. DNA hybridization and PCR amplification are sensitive and specific methods for the detection of genes including those encoding AME (34, 36, 38). However, such special techniques and the necessary equipment are not practical for the routine clinical laboratory, unlike conventional susceptibility tests.

In the present study, we compared aminoglycoside resistance profiles to PCR data to determine whether susceptibility tests could reproducibly detect specific AME in MRSA. We then used the results to study the epidemiology of AME in Japanese MRSA isolates.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The reference strains used in this study were three transductants, pMS18, pMS91, and pMS555. Each of these was transduced by S2 phage in *S. aureus* MS353. The pMS18 transductant is known to carry two genes, *ant(6)-I* and *aph(3')-III*; the pMS91 transductant is known to carry three genes, *ant(6)-I*, *aph(3')-III*, and *aac(6')-aph(2'')*; and the pMS555 transductant carries one gene, *ant(4')-I* (27).

A total of 381 MRSA strains were collected from various medical settings in different parts of Japan. MRSA strains were identified by growth on plates containing culture medium supplemented with 6 µg of oxacillin (Sigma, St. Louis, Mo.) per ml and 4% NaCl.

**Antibiotics and chemicals.** Reference samples of various aminoglycosides and other antimicrobial agents of known potency were kindly supplied as powders by the manufacturers, as follows: kanamycin, streptomycin, and arbekacin were from Meiji Seika Kaisha, Tokyo, Japan; gentamicin was from Schering-Plough Japan,

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Osaka, Japan; and tobramycin was from Shionogi Pharmaceutical, Osaka, Japan. Lividomycin was obtained commercially (Sigma).

**Determination of MICs.** MICs were determined by the twofold agar dilution method in Sensitivity Disk Agar N (Nissui, Tokyo, Japan). The bacteria were grown overnight in Sensitivity Test broth (Nissui) at 35°C. The culture was diluted to a final concentration of 10<sup>6</sup> CFU/ml with buffered saline containing gelatin. The bacterial suspensions were delivered by an inoculator (Sakuma Seisaku, Tokyo, Japan) with an inoculum size of 10<sup>4</sup> CFU/spot on agar plates. Inoculated plates were incubated for 18 h at 35°C. The MIC was defined as the lowest concentration of the compound that prevented visible growth.

**DNA isolation.** Each strain was subcultured overnight at 35°C on brain heart infusion agar (Nissui). Bacteria grown on plates were suspended in 100 µl of lysing solution (20 mM Tris-HCl, 140 mM NaCl, 5 mM EDTA [pH 8.0]) containing 250 µg of lysostaphin (Sigma) and incubated at 37°C for 30 min. After the suspensions were cooled on ice, 200 µl of distilled water was added to each, and they were heated at 65°C for 5 min. Subsequently, phenol-chloroform extraction and ethanol precipitation were performed as described by Okamoto et al. (20). The pellet was dried briefly in a vacuum desiccator and dissolved in 100 µl of distilled water. A 10-µl volume of a 1:10 dilution of the total DNA solution was used for PCR.

**PCR experiments.** Heat-stable *Taq* polymerase, the four deoxynucleoside triphosphates, and PCR buffer were purchased from Takara Shuzo (Otsu, Japan). As primers for PCR (see below), 20-mer oligonucleotides were used; these were purchased from Takara Shuzo. Cell lysates as processed above (10 µl) were added to a PCR mixture containing each primer at 0.1 µM, 10 µL of a 10-fold concentrate of PCR buffer, deoxynucleoside triphosphates (each at 200 µM), and 2.5 U of *Taq* polymerase in a final volume of 90 µl of distilled water. To prevent evaporation, 2 drops of mineral oil (Sigma) was added to each mixture.

A thermal cycler (Perkin-Elmer Cetus, Emeryville, Calif.) was used for amplification of DNA. The cycling program included 30 cycles of a denaturing step at 94°C for 1 min, an annealing step at 57°C for 2 min, and an extension step at 72°C for 30 s. Then 5-µl volumes of the samples were taken for analysis by electrophoresis on 2% agarose gels (FMC BioProducts, Rockland, Maine) in Tris-borate-EDTA buffer. The PCR products were detected by ethidium bromide staining under UV illumination.

**Design of primers for PCR.** Three sets of primers were designed to detect the three different genes encoding AME in a single test. All primer sequences were chosen from a site within the nucleotide sequence of the AME gene region known to be specific for an enzyme. The primers for detection of the *aph(3')-III* gene were based on the nucleotide sequences reported by Gray and Fitch (7). The 5' primer was CGATGTGGATTGCGAAAACCT; the 3' primer was CAC CGAAATAACTAGAACCC. Primers for detection of the *aac(6')-aph(2'')* gene were based on the nucleotide sequences reported by Ferretti et al. (6) and Rouch et al. (24). The 5' primer was CATTATACAGAGCCTTGGGA; the 3' primer was AGGTTCTCGTTATTCCCGTA. Primers for detection of the *ant(4')-I* gene were based on the nucleotide sequences reported by Matsumura et al. (14). The 5' primer was ATGGCTCTTGGTTCGTCAG; the 3' primer was TAAG CACACGTTCTGGCTG. The primers did not interact with one another or with genes encoding other AME.

**Coagulase typing.** Coagulase types were discerned by inactivation of coagulase activity type-specific antisera (33). The specific antisera and normal rabbit plasma for coagulase typing were purchased from Denka Seiken (Tokyo, Japan). Clinical isolates were grown overnight in 5 ml of brain heart infusion agar at 35°C. After a 30-min centrifugation at 1,600 × *g* 0.1 ml of supernatant was aliquotted into each of nine tubes. Type-specific antiserum (0.1 ml) or control serum was added to each tube, and the mixtures were incubated at 37°C for 1 h. Finally, 0.2 ml of normal rabbit plasma was added to all tubes. Coagulase types were determined by inhibition of clotting after incubation at 37°C for 1 to 48 h.

## RESULTS

**Primer specificity.** An agarose gel separation of DNA fragments amplified from total DNA isolated from reference strains is shown in Fig. 1. The primers for the *aph(3')-III* gene yielded a fragment of 175 bp. This DNA fragment was amplified from total DNA isolated from *S. aureus* MS353(pMS18) and *S. aureus* MS353(pMS91). The primers for the *aac(6')-aph(2'')* gene yielded a fragment of 279 bp. This DNA fragment was amplified only from total DNA isolated from MS353(pMS91). The primers for the *ant(4')-I* gene yielded a fragment of 367 bp. This DNA fragment was amplified only

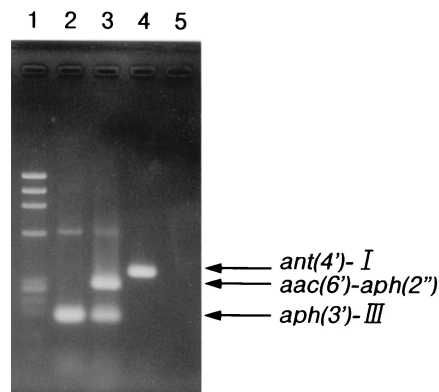


FIG. 1. Agarose gel electrophoresis of amplified DNA fragments from reference strains. Lanes: 1, maker DNA ( $\phi$ X174 *Hae*III digest); 2, *S. aureus* MS353(pMS18); 3, *S. aureus* MS353(pMS91); 4, *S. aureus* MS353(pMS555); 5, *S. aureus* MS353 as a negative control.

from total DNA isolated from *S. aureus* MS353(pMS555). Then different primers for the three genes were mixed and used to test the specificity of these primers with mixed DNA isolated from MS353(pMS18), MS353(pMS91), and MS353(pMS555). As expected, three different sizes of amplified DNA, of 175, 279, and 367 bp, were detected. These results indicated that the PCR products following amplification and the aminoglycoside resistance profiles were in correct agreement. Therefore, the specificity of the primers selected for this study was confirmed, as well as the specificity and sensitivity of the method for detection of these three genes encoding AME.

**PCR identification of genes encoding AME in clinical isolates.** The genes encoding AME were subjected to PCR amplification and to agarose gel electrophoresis. The frequencies of the genes encoding AME detected by PCR are shown in Fig. 2 for the 381 isolates. PCR products were amplified from 375 of the 381 isolates but not from the remaining 6 isolates

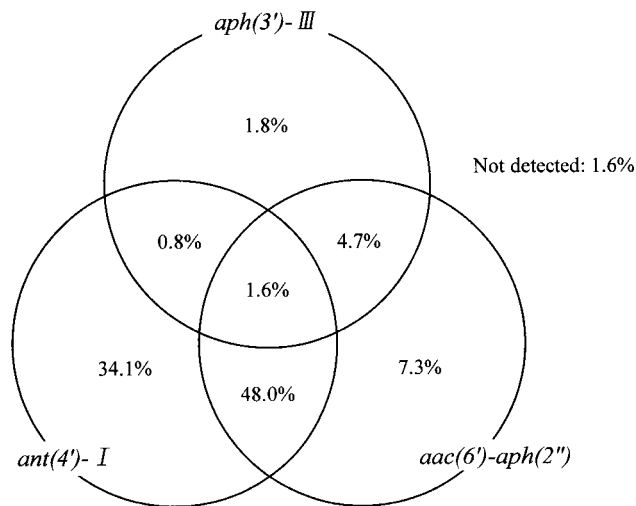


FIG. 2. Distribution of genes encoding AME as determined by PCR detection in 381 isolates of MRSA.

TABLE 1. PCR detection of genes encoding AME and MIC distributions in clinical MRSA isolates

Detection by PCR			Total no. of strains	antibiotic	No. of strains for which the MIC ( $\mu\text{g/ml}$ ) is:												
<i>aac(6')-aph(2'')</i>	<i>ant(4')-I</i>	<i>aph(3')-III</i>			0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024
+	+	+	6	Gentamicin Tobramycin Lividomycin								1	2	3			
												2	1	3			6
+	+	-	183	Gentamicin Tobramycin Lividomycin				4	3	2	45	86	16	14	8	5	
								7	92	77	6	1	7	28	92	44	12
+	-	+	18	Gentamicin Tobramycin Lividomycin						1	2	3	5	4	2		1
										1	2	4	5	5		1	18
+	-	-	28	Gentamicin Tobramycin Lividomycin					5	6	1	6	5	2	2		1
								5	22	1			5	1			
-	+	+	3	Gentamicin Tobramycin Lividomycin		3								3			
																	3
-	+	-	130	Gentamicin Tobramycin Lividomycin	6	104	20							7	54	63	6
										4	74	49	3				
-	-	+	7	Gentamicin Tobramycin Lividomycin		6	1										
						6	1										7
-	-	-	6	Gentamicin Tobramycin Lividomycin		5	1										
						5	1										
								3	3								

(1.6%). The *ant(4')-I* gene was encountered most frequently (84.5%), and 59.6% of isolates carried this gene in combination with one or both of the others. The *aph(3')-III* and *aac(6')-aph(2'')* genes were present in 8.9 and 61.7% of isolates, respectively. The most frequent combination of genes was *ant(4')-I* with *aac(6')-aph(2'')* (48%). The *aph(3')-III* gene was present in combination with either the *aac(6')-aph(2'')* gene or the *ant(4')-I* gene in 4.7 and 0.8% of isolates respectively. The triple combination of *aph(3')-III*, *ant(4')-I*, and *aac(6')-aph(2'')* was present in 1.6% of isolates.

**Correlation of aminoglycoside susceptibilities and the presence of AME genes.** The MICs of three aminoglycosides, gentamicin, tobramycin, and lividomycin, for the 381 isolates characterized above by PCR are shown in Table 1. All 235 isolates with the *aac(6')-aph(2'')* gene were resistant to gentamicin ( $\geq 8 \mu\text{g/ml}$ ), and most of them were also resistant to tobramycin ( $\geq 8 \mu\text{g/ml}$ ). A total of 322 isolates with the *ant(4')-I* gene were highly resistant to tobramycin ( $\geq 128 \mu\text{g/ml}$ ); 34 isolates with the *aph(3')-III* gene were highly resistant to lividomycin ( $\geq 1,024 \mu\text{g/ml}$ ); and 6 isolates with none of the three genes were susceptible to gentamicin ( $\leq 1 \mu\text{g/ml}$ ), tobramycin ( $\leq 1 \mu\text{g/ml}$ ), and lividomycin ( $\leq 8 \mu\text{g/ml}$ ).

The gentamicin resistance in MRSA isolates was associated only with the *aac(6')-aph(2'')* gene, and the cutoff MIC of gentamicin between susceptible and resistant isolates was 8  $\mu\text{g/ml}$ . The lividomycin resistance in MRSA isolates was associated with the *aph(3')-III* and *ant(4')-I* genes. All isolates with the *aph(3')-III* gene were highly resistant to lividomycin

( $\geq 1,024 \mu\text{g/ml}$ ); on the other hand, the isolates with the *ant(4')-I* gene but without the *aph(3')-III* gene were only mildly resistant to lividomycin (8 to 128  $\mu\text{g/ml}$ ). The tobramycin resistance in MRSA isolates was subjected to the genes carrying *ant(4')-I* and *aac(6')-aph(2'')*. However, from the determination of the MIC of tobramycin, it was difficult to identify these genes in tobramycin-resistant isolates.

**Relationship between the MICs of gentamicin and tobramycin in MRSA isolates with the *aac(6')-aph(2'')* gene.** As mentioned above, although all 235 isolates with the *aac(6')-aph(2'')* gene were resistant to gentamicin, it has not been clarified in susceptibility tests using a kind of aminoglycoside whether they also contained the *ant(4')-I* gene. However, determining whether the *aac(6')-aph(2'')* gene was combined with the *ant(4')-I* gene required a comparison of the MICs of gentamicin and tobramycin (Fig. 3). For most isolates (45 of 46) with the *aac(6')-aph(2'')* gene and without the *ant(4')-I* gene, the MIC of gentamicin was higher than that of tobramycin. All 189 isolates with both the *ant(4')-I* and *aac(6')-aph(2'')* genes were resistant to tobramycin and gentamicin, but for these bacteria the MIC of tobramycin was either higher than or equivalent to that of gentamicin. Using the above results, susceptibility tests for lividomycin, tobramycin, and gentamicin could reproducibly detect specific AME in MRSA.

**Coagulase typing and AME.** The coagulase types of 350 of the 381 tested strains were successfully determined using specific antisera against eight different types of coagulase in *S. aureus*. Type II predominated (83.7% of 381 isolates). In con-

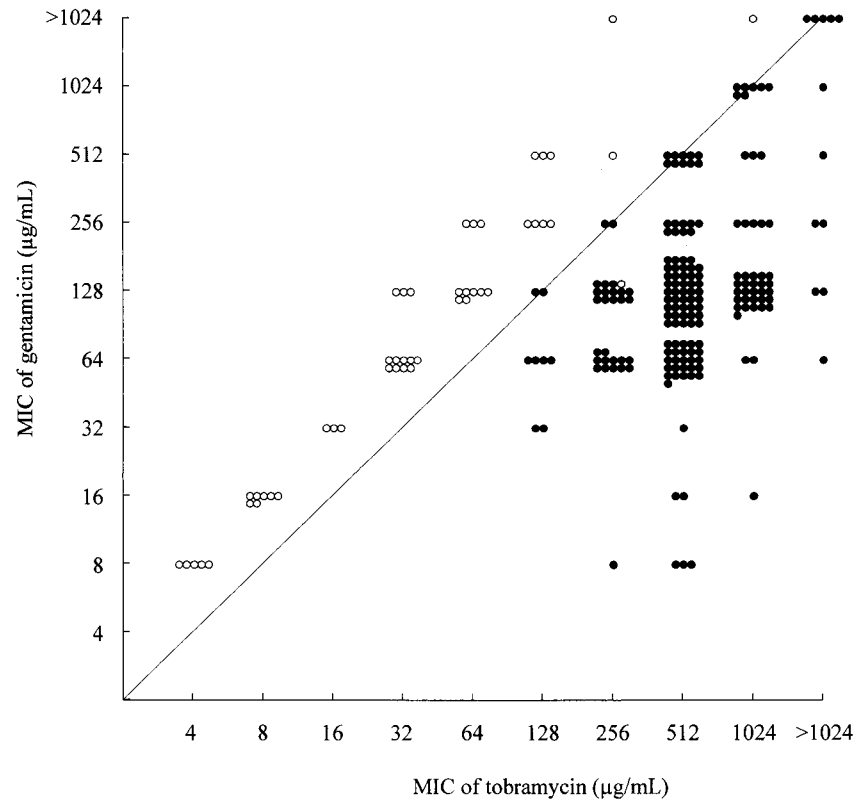


FIG. 3. Correlation between the MICs of gentamicin and tobramycin for 235 MRSA isolates with the *aac(6')-aph(2'')* gene. Each circle indicates one of strains. The solid circles indicate the isolates with both the *aac(6')-aph(2'')* and *ant(4')-I* genes, and the open circles indicate the isolates with the *aac(6')-aph(2'')* gene but not the *ant(4')-I* gene.

trast, the isolates with coagulase type III, IV, VII, or I were infrequent (3.7, 2.4, 1.3, and 0.8%, respectively). The coagulase types of 31 isolates were indistinguishable.

The relationship between coagulase type and genes encoding AME in MRSA isolates was examined (Table 2). Isolates with only the *ant(4')-I* gene were of coagulase type II or III but not type I, IV, or VII. The isolates carrying the both *ant(4')-I* and *aac(6')-aph(2'')* included all coagulase types. The results showed that isolates with the *aac(6')-aph(2'')* gene were more frequent among isolates with coagulase type I, IV, or VII than among those with type II or III.

**Drug resistance and AME.** The relationship between genes encoding AME and aminoglycoside resistance in MRSA isolates was examined (Table 3). The interpretive categories of gentamicin, tobramycin, and kanamycin resistance were recommended by National Committee for Clinical Laboratory Standards (NCCLS), and their cutoff MIC were 8, 8, and 32 µg/ml, respectively (18). For other aminoglycosides, such as streptomycin, lividomycin, and arbekacin, the interpretive categories were not listed in the NCCLS publication. Therefore, their interpretive categories were provisionally established as follows: streptomycin, 32 µg/ml; lividomycin, 256 µg/ml; and

TABLE 2. Relationship between AME Genes and coagulase type in MRSA isolates

AME genes present			Total no. of strains	No. of strains with coagulase type of:					ND <sup>a</sup>
<i>aac(6')-aph(2'')</i>	<i>ant(4')-I</i>	<i>aph(3')-III</i>		I	II	III	IV	VII	
+	+	+	6		2		1		3
+	+	-	183	1	147	7	1	4	23
+	-	+	18		13		3		2
+	-	-	28	2	21	1	3	1	
-	+	+	3		3				
-	+	-	130		123	5			2
-	-	+	7		5		1		1
-	-	-	6		5	1			
Total			381	3	319	14	9	5	31

<sup>a</sup> ND, not determined.



TABLE 3. AME and aminoglycoside resistance

AME gene present			Total no. of strains	% of isolates resistant to <sup>a</sup> :					
<i>aac(6')-aph(2'')</i>	<i>ant(4')-I</i>	<i>aph(3')-III</i>		Gm (8)	Tob (8)	Lvdm (256)	Sm (32)	Km (32)	Abk (8)
+	+	+	6	100	100	100	100	100	0
+	+	-	183	100	100	0	8.2	100	11.5
+	-	+	18	100	100	100	100	100	5.6
+	-	-	28	100	82.1	0	0	100	7.1
-	+	+	3	0	100	100	100	100	0
-	+	-	130	0	100	0	3.8	100	0
-	-	+	7	0	0	100	100	100	0
-	-	-	6	0	0	0	0	0	0
Total			381	61.7	95.3	8.9	14.2	98.4	6.3

<sup>a</sup> Gm, gentamicin; Tob, tobramycin; Lvdm, lividomycin; Sm, streptomycin; Km, kanamycin; Abk, arbekacin; the cutoff MIC (in micrograms per milliliter) is given in parentheses.

arbekacin, 8 µg/ml. The MRSA isolates with at least one of three genes were resistant to kanamycin. Of 381 isolates, 54 (14.2%) were resistant to streptomycin. All 34 isolates with the *aph(3')-III* gene were resistant to streptomycin, whereas only 5.8% of the isolates without this gene were resistant to streptomycin. Twenty-four isolates (6.3%) were resistant to arbekacin, and they were found only in isolates with the *aac(6')-aph(2'')* gene and showed high resistance ( $\geq 512$  µg/ml) to gentamicin (data not shown).

## DISCUSSION

Beginning in the late 1970s and continuing for the last 20 years, MRSA have been isolated in connection with outbreaks of nosocomial infection in many countries around the world (2, 13). In 1982 and 1983, MRSA began to increase in prevalence throughout Japan (10). MRSA typically are resistant to various antimicrobial agents such as penicillins, cephalosporins, macrolides, aminoglycosides, tetracyclines, and fluoroquinolones (9). Because of this multidrug resistance and tendency to spread in hospital populations, MRSA have a special clinical significance, requiring epidemiologic monitoring as a measure for control of nosocomial infection. Conventional methods commonly used in the clinical laboratory for typing of *S. aureus*, including phage typing (22), coagulase typing (33), and antibiotyping (19), often prove useless as epidemiologic tools since of most MRSA isolated in Japan have nonsensitivity to phage production of type II coagulase and resistance to many kinds of antibiotics (9, 10). In contrast to conventional methods of MRSA typing, genetic analyses such as pulsed field gel electrophoresis (23), the DNA hybridization techniques (15, 28), and the PCR technique (35) are sensitive and versatile tools. Vanhoof et al. (36) have reported that defining the genetic determinants of AME by PCR was useful for epidemiologic surveillance of MRSA. In the present work, a relationship was found between the PCR detection of genes encoding AME and aminoglycoside resistance patterns in clinically isolated MRSA; the distribution of AME, coagulase types, and antibiotic susceptibility patterns was studied in MRSA isolated from 30 hospitals widely distributed throughout Japan.

The PCR technique used three sets of primers delineating specific DNA fragments, *aph(3')-III*, *ant(4')-I*, and *aac(6')-aph(2'')*, defined as detecting and identifying AME genes in reference strains. PCR was performed in 381 clinical isolates to

identify AME genes. The 34 isolates with the *aph(3')-III* gene showed high resistance to lividomycin ( $\geq 1,024$  µg/ml), which has been reported to be a specific substrate of the enzyme APH(3')-III (26). Therefore, APH(3')-III production was determined by testing the susceptibility of strains to lividomycin. In this study, ANT(4')-I-producing strains showed low resistance to lividomycin (8 to 128 µg/ml). Although it has not been reported that lividomycin was inactivated by ANT(4')-I, this result suggested that it was inactivated only weakly by this enzyme. All isolates carrying the *aac(6')-aph(2'')* gene were resistant to gentamicin ( $\geq 8$  µg/ml); therefore, it was possible to detect AAC(6')/APH(2'') production by susceptibility testing with gentamicin. However, since most aminoglycosides are substrates of this enzyme, the additional production of ANT(4')-I is difficult to identify on the basis of antibiotic resistance patterns. Interestingly, the PCR results were almost always related to the MICs of gentamicin and tobramycin. In most isolates (45 of 46) with the *aac(6')-aph(2'')* gene but without the *ant(4')-I* gene, the MIC of gentamicin was higher than that of tobramycin; in the isolates with both the *aac(6')-aph(2'')* and *ant(4')-I* genes, the MIC of tobramycin was higher than or similar to that of gentamicin (Fig. 3). Ubukata et al. (30) have reported that AAC(6')/APH(2'') inactivates gentamicin more effectively than it inactivates tobramycin, and our results are compatible with and explained by this observation. Only 1 of the 235 isolates with the *aac(6')-aph(2'')* gene was exceptional in that the relationship between the PCR result and susceptibility testing did not show the same tendency. The reason for the discrepancy between PCR and MIC in this strain is not clear; it is possible that a mutation of the *aac(6')-aph(2'')* gene is incriminated in the change of substrate specificity. We should almost always be able to determine AME production in clinical isolates of MRSA by testing their susceptibility to lividomycin, gentamicin, and tobramycin, because the agreement between this method and the PCR method was 99.7% (380 of 381). Therefore, we recommend the use of this method in clinical laboratories in the epidemiologic study of MRSA.

The frequencies of genes encoding AME was studied in 381 Japanese isolates. The *ant(4')-I*, *aac(6')-aph(2'')*, and *aph(3')-III* genes were evident in 84.5, 61.7, and 8.9% of isolates, respectively. One of the reasons why the *ant(4')-I* gene is the most frequent is that it adjoins the *mecA* gene (5, 31). In contrast, isolates with coagulase type I, IV, or VII did not carry

the *ant(4')-I* gene as frequently as did those with coagulase type II (Table 2). These results suggested that *mec* DNA regions differed between coagulase types and were compatible with other observations (M. Kurazono and T. Ida, unpublished data). AAC(6')/APH(2'') has been the enzyme most frequently found among MRSA isolated in Europe (25, 36). In contrast, gentamicin-resistant MRSA carrying the *aac(6')-aph(2'')* gene were encountered less frequently among isolates from Japan. The *aac(6')-aph(2'')* gene is encoded by transposon Tn4001 or Tn4001-like elements (11, 12, 24), and those have been detected in large plasmids in *S. aureus* (1, 29). The gentamicin resistance plasmids in *S. aureus* vary in conjugational transfer and have been isolated from different geographic areas (17). The reasons for the prevalence of the AAC(6')/APH(2'') enzyme in Japan may be more closely related to the spread of isolates with coagulase type II than to gentamicin resistance plasmids being conjugative or nonconjugative. The isolates carrying the *aph(3')-III* gene were not frequent among isolates from Japan. In 27 of 34 isolates with the *aph(3')-III* gene, this gene was combined with the *aac(6')-aph(2'')* gene and/or the *ant(4')-I* gene. Since AAC(6')/APH(2'') and ANT(4')-I are capable of inactivation for kanamycin, the *aph(3')-III* gene does not appear to be necessary for these isolates. However, all isolates with the *aph(3')-III* gene showed resistance to streptomycin, which is inactivated only by ANT(6)-I. The *aph(3')-III* and *ant(6)-I* genes are carried on transposon Tn3854 on the staphylococcal plasmid and chromosome (32). For these isolates, it may be more important to produce ANT(6)-I rather than APH(3')-III.

Most isolates in this study produced ANT(4')-I and/or AAC(6')/APH(2'') and were resistant to aminoglycosides used in clinical therapy. However, arbekacin, a derivative of dibekacin, showed excellent antibacterial activity against tobramycin- and gentamicin-resistant MRSA (Table 3) (8, 9), because arbekacin is modified very little by ANT(4')-I and/or AAC(6')/APH(2'') (30). In Japan, arbekacin has been approved for clinical use in MRSA infection since 1990, and no increase in the prevalence of arbekacin-resistant MRSA has been reported (4); nonetheless, a nosocomial infection caused by arbekacin-resistant MRSA has been reported at one hospital (9). Therefore, clinical laboratories should monitor the spread of arbekacin-resistant MRSA and the genes encoding AME. The susceptibility-based technique we describe and recommend here should facilitate the detection and characterization of AME.

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