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

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Received 29 January 2001/Returned for modification 31 March 2001/Accepted 15 June 2001

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-phosphotransferase III [APH(3’)-III] (7), aminoglycoside-4’-O-phosphotransferase I [ANT(4’)-I] (14) and aminoglycoside-6’-N-acetyltransferase/2”-O-phosphotransferase [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 MS55. 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.
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 10^6 CFU/ml with buffered saline containing gelatin. The bacterial suspensions were delivered by an inoculator (Sakuma Seisaku, Tokyo, Japan) with an inoculum size of 10^4 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.

**Coagulase typing.** 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 were 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 3 min. 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 amplified from total DNA isolated from reference strains. Lanes: 1, maker DNA (αX174 HaeIII digest); 2, S. aureus MS353(pMS18); 3, S. aureus MS353(pMS91); 4, S. aureus MS353(pMS555); 5, S. aureus MS353 as a negative control.

**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 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 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.

**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). 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 of supernatant was aliquoted 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.
was present in combination with either the aac(6\')-aph(2\') and ant(4\')-I genes. The most frequent combination of genes was ant(4\')-I with aac(6\')-aph(2\') (48%). The aac(6\')-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 aac(6\')-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 (≥8 μg/ml), and most of them were also resistant to tobramycin (≥8 μg/ml). A total of 322 isolates with the ant(4\')-I gene were highly resistant to tobramycin (≥128 μg/ml); 34 isolates with the aac(6\')-III gene were highly resistant to lividomycin (≥1,024 μg/ml); on the other hand, the isolates with the ant(4\')-I gene but without the aac(6\')-III gene were only mildly resistant to lividomycin (8 to 128 μg/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-
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′)/H11032-I gene were of coagulase type II or III but not type I, IV, or VII. The isolates carrying 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 provisionally established as follows: streptomycin, 32 μg/ml; lividomycin, 256 μg/ml; and

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

<table>
<thead>
<tr>
<th>AME genes present</th>
<th>Total no. of strains</th>
<th>No. of strains with coagulase type of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>aac(6′)-aph(2′)</td>
<td>ant(4′)-I</td>
<td>aph(3′)-III</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
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<tr>
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<tr>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>381</td>
</tr>
</tbody>
</table>

* ND, not determined.
The frequencies of genes encoding AME was studied in 381 Japanese isolates. The \(\text{ant}(4')-I\), \(\text{aac}(6')-\text{aph}(2')\), and \(\text{aph}(3')-\text{III}\) genes were evident in 84.5, 61.7, and 8.9% of isolates, respectively. One of the reasons why the \(\text{ant}(4')-I\) 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 \(mecA\) gene (5, 31). In the present work, a relation 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.

**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 role in the USA. This gene was found only in isolates with the \(\text{aac}(6')-\text{aph}(2')\) gene and showed high resistance (\(\geq 512 \mu\text{g/ml}\)) to gentamicin (data not shown).

### TABLE 3. AME and aminoglycoside resistance

<table>
<thead>
<tr>
<th>AME gene present</th>
<th>Total no. of strains</th>
<th>% of isolates resistant to*</th>
</tr>
</thead>
<tbody>
<tr>
<td>aac(6')-aph(2')</td>
<td>381</td>
<td>61.7, 95.3, 8.9</td>
</tr>
<tr>
<td>ant(4')-I</td>
<td></td>
<td>14.2%</td>
</tr>
<tr>
<td>aph(3')-III</td>
<td></td>
<td>28.0%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Gm, gentamicin; Tob, tobramycin; Lvdm, lividomycin; Sm, streptomycin; Km, kanamycin; Abk, arbekacin; the cutoff MIC (in micrograms per milliliter) is given in parentheses.
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’)-II gene were encountered less frequently among isolates from Japan. The aac(6’)-aph(2’)-II 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 conjugal 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’)-II 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 Tn5854 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, showed excellent antibacterial activity against tobramycin-

ACKNOWLEDGMENTS

We thank the Working Group for collecting isolates of MRSA. We also thank Toshiko Hashizume and Mizuyo Kurazono for technical assistance.

This study was supported in part by the All Kitasato Project Study, Kitasato University, and the Working Group for MRSA cooperated by Meiji Seika Kaisha, Ltd., Tokyo, Japan.

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