Detecting the frequency of aminoglycoside modifying enzyme encoding genes among clinical isolates of methicillin-resistant *Staphylococcus aureus*

Zahra Shokravi¹, Laleh Mehrad¹, Ali Ramazani²

¹ Microbiology Department, Faculty of Basic Sciences, Islamic Azad University, Arak, Iran  
² Biotechnology Department, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran

**Introduction**

Methicillin-resistant staphylococci are the most common cause of antibiotic-resistant healthcare-associated infections in hospitals worldwide.¹ Methicillin-resistant staphylococci, particularly *Staphylococcus aureus* (MRSA), are now recognized as the main pathogens of nosocomial bacteremia associated with orthopedic equipment colonization and intravascular catheter as well as postoperative infections in general. Over the past few decades, MRSA strains have become not only resistant to beta-lactam antibiotics, but also acquired additional resistance to non-beta-lactam antibiotics, including aminoglycosides.²,³ Tobramycin and gentamicin are the most efficient aminoglycosides against MRSA which are commonly used in combination with other types of antibiotics, especially in the treatment of staphylococcal endocarditis.⁴ Enzymatic modification of aminoglycosides is the most frequently encountered mechanism of resistance to aminoglycosides in staphylococci, which is predominantly mediated by five groups: aminoglycoside-6'-N-acetyltransferase/2'-O-phosphoryltransferase ([AAC(6')/APH(2')] encoded by the aac(6')/aph(2') gene; aminoglycoside-3'-O-phosphoryltransferaseIII[APH(3')-III] encoded by aph (3')-IIIa gene; aminoglycoside-4'-O-phosphoryltransferase I [ANT(4')-I] encoded by ant

**Abstract**

**Introduction:** Methicillin-resistant *Staphylococcus aureus* (MRSA) plays an important role in causing many serious nosocomial infections. In this study, the antimicrobial susceptibility and the frequency of aminoglycoside modifying enzyme encoding genes among clinical isolates of methicillin-resistant *Staphylococcus aureus* was investigated from two university hospitals of Zanjan province of Iran.

**Methods:** In this study, the antimicrobial susceptibility of MRSA isolates to various antibiotics was investigated by the disk diffusion method. Multiplex PCR assays were used for the determination of aminoglycoside modifying enzyme (AME) genes and staphylococcal cassette chromosome mec (SCCmec) types in MRSA strains.

**Results:** All 58 MRSA isolates were sensitive to vancomycin. Resistance to penicillin G, oxacillin, gentamicin, erythromycin, clindamycin, kanamycin, and tobramycin was found in 96.4%, 98.3%, 51.7%, 53.4%, 55.2%, 62% and 58.6% of the isolates, respectively. The most prevalent AME genes were *aac(6')/aph(2')* (48.3 %) followed by *ant(4')-Ia* (24%). The *aph(3')-Ia* gene was the least frequent AME gene among MRSA isolates (19%). Of the 58 tested MRSA isolates, 5 (8.6%) were harboured SCCmec type I, 11 (19%) SCCmec type II, 20 (34.5%) SCCmec type III, 17 (29.3%) SCCmec type IVa, 1 (1.7%) SCCmec type IVb, 2 (3.4%) SCCmec type IVc, 11 (19%) SCCmec type IVd, and, 18 (31%) SCCmec type V. Nineteen isolates were not typeable.

**Conclusion:** In conclusion, the *aac (6')/aph (2')* was the most common aminoglycoside modifying enzyme gene and SCCmec type II and V were the most frequent types detected in hospital isolates, respectively.

*Corresponding author: Ali Ramazani, Email: ramazania@zums.ac.ir*
Clinically, the most important of these enzymes are ANT(4′)-Ia, AAC(6′)/APH(2″) and APH(3′)-IIIa, as well as the distribution of staphylococcal cassette mec (SCCmec) types, among meticillin-resistant Staphylococcus aureus isolates from two university hospitals in Zanjan, Iran.

Materials and methods

Chemicals and reagents

All experiments were performed with chemicals in analytical-reagent grade. PCR Master Mix (2X) was obtained from Fermentas (Waltham, USA). Primers were purchased from Generay Biotech (Shanghai, China). Ethanol, Tris, EDTA, phenol/chloroform and other chemicals were obtained from Merck (Darmstadt, Germany).

Bacterial isolates

In current research, nasal specimens were collected between November 2011 and February 2012 from health care workers (HCW) of two university-affiliated hospitals in Zanjan, Iran. Study participants were 260 HCW who were working in various departments of the hospitals. Nasal samples were collected from nostrils with collection swabs, and directly streaked on mannitol salt agar, then incubated at 37°C for 24 h in order to isolate the specimens. Initial identification was further done using conventional methods, including Gram staining, colony morphology, slide coagulation test and catalase test, and further confirmatory test was done by amplification of the species-specific primer for Sa442 and femA genes. Nucleotide sequences belonging to primers used in this investigation are shown in Table 1. For this purpose, staphylococcal DNA was extracted using the phenol/chloroform method and used as templates; DNA extractions were stored at −20°C until use. The presence of the mecA gene was investigated by PCR method using a set of previously described primers.

Antimicrobial susceptibility testing

The phenotypic resistance to various antibiotics in MRSA isolates was determined using the standard disk diffusion method.
method according to Clinical and Laboratory Standards Institute guidelines.10

**Multiplex PCR**

All MRSA isolates were investigated by multiplex PCR strategy to determine staphylococcal cassette chromosome mec types and subtypes (SCCmec), according to previously described methods.11 In addition, three sets of primers specific for ant(4′)-Ia, aac(6′)/aph(2′′) and aph(3′)-IIIa were chosen from published sequences.12,13 All MRSA isolates were analyzed by polymerase chain reaction (PCR) with a PCR master mix (Fermentas, K01071) containing 500 ng genomic DNA, 20 pM primer mix (10 pM for each primer), and distilled water to a final volume of 25 µL. Negative and positive controls were also included in PCR assays. The reaction was carried out in a thermocycler (iCycler, BIO-RAD, USA) according to the following program: 94˚C for 5 min; 35 cycles of 94˚C for 1 minute, 55˚C for 45 s, 72˚C for 40 s; and a final extension at 72˚C for 7 min. After amplification, PCR products were subjected to electrophoresis in 1.5% agarose. The gels were then stained with DNA robust staining dye (SyberGreen) and photographed under UV light.

**Results**

Among the 260 nasal swabs obtained from HCW, 104 of the isolates were identified as *S. aureus* through biochemical procedures. All the 104 isolates were Sa442 and femA positive (implying they were *S. aureus*), and the meca gene (encoding PBP2a, a penicillin-binding protein that mediate meticillin resistance) was found in 58 of the 104 staphylococcal isolates based on PCR analysis (Fig. 1).7 These high rates of MRSA isolates are comparable with other reports that show similar rates.14 All 58 MRSA isolates were sensitive to vancomycin; conversely, phenotypic resistance patterns of the strains to aminoglycosides were as follows: 96.4% to penicillin G, 98.3% to oxacillin, 53.4% to erythromycin, 55.2% to clindamycin, 51.7% to gentamicin, 58.6% to tobramycin, and 62% to kanamycin.

The rates of prevalence of AME gene were as follows: *aac(6′)/aph(2′′)*, 48.3%; *ant(4′)-Ia*, 24%; and the *aph(3′)-Ia* gene was the least frequent AME gene among MRSA isolates (19%).

Thirty-four (57%) isolates carried one of these genes either alone or in combination with other genes (Fig. 2). Two resistance genes of *aac(6′)/aph(2′′) and aph(3′′)-III* were simultaneously presented in 9 (15.5%) of the isolates. *Ant (4′)-Ia* and *aac(6′)/aph(2′′) genes* coexisted in 7 (12%) of the MRSA isolates. The triple combination of *ant (4′)-I, aac(6′)-aph(2′′) and aph(3′′)-III* was determined in 3.4% of the isolates, and among these MRSA strains 37 (64%) isolates had at least one of these genes.

In the present study, 5 (8.6%) samples harbored SCCmec type I, 11 (19%) SCCmec type II, 20 (34.5%) SCCmec type III, 17 (29.3%) SCCmec type IVa, 1 (1.7%) SCCmec type IVb, 2 (3.4%) SCCmec type IVc, 11 (19%) SCCmec type IVd, and 18 (31%) SCCmec type V (Table 2). Nineteen isolates were not typeable.

Contrary to previous reports, 25 (43%) strains showed multiband as follows: 1 (1.7%) isolate for SCCmec types I and II, 2 (3.4%) isolates for SCCmec types II and III, 3 (5.2%) isolates for SCCmec types II and V, 2 (3.4%) isolates for SCCmec types III and V, 3 (5.2%) isolates for SCCmec types IVa and V, 2 (3.4%) isolates for SCCmec types IVd and V, 1 (1.7%) isolate for SCCmec types II, III and V, 1 (1.7%) isolate for SCCmec types I, III and IVa, 1 (1.7%) isolate for SCCmec types II, III and IVa, 1 (1.7%) isolate for SCCmec types II, III and V, 2 (1.7%) isolate for SCCmec types II and IVa, 1 (1.7%) isolate for SCCmec types II, III and IVa, 1 (1.7%) isolate for SCCmec types II, III and IVa, 1 (1.7%) isolate for SCCmec types II and IVa, 1 (1.7%) isolate for SCCmec types II, III and IVa, 1 (1.7%) isolate for SCCmec types II and IVa, and 1 (1.7%) isolate for SCCmec types III, IVa and V.

The nucleotide sequences of some of the staphylococcal isolates reported in this study have been deposited in the GenBank database under accession numbers of JX426257, JX426258, JX142145, JX142143, JX142141, JX142146, JX142144, JX142142, JX437190, JX426259, and JX437191.
**Table 2. Distribution of staphylococcal cassette chromosome mec types and subtypes in MRSA isolates and resistance profile**

| SCCmec types | No. (%) of isolates | VAN | PEN | OX | CLI | ERY | G | T | K |
|--------------|---------------------|-----|-----|----|-----|-----|---|---|---|
| Type I       | 5 (8.6)             | 0   | 5 (100) | 5 (100) | 2 (40) | 0 | 3 (60) | 5 (100) | 4 (80) |
| Type II      | 11 (19)             | 0   | 11 (100) | 11 (100) | 9 (75) | 5 (19) | 6 (54.5) | 7 (63.6) | 4 (36.4) |
| Type III     | 20 (34.5)           | 0   | 20 (100) | 19 (95) | 12 (60) | 20 (34.5) | 10 (50) | 18 (90) | 14 (70) |
| Type IVa     | 17 (29.3)           | 0   | 15 (86.7) | 17 (100) | 13 (76.5) | 17 (29.3) | 7 (41.2) | 10 (86.7) | 10 (58.8) |
| Type IVb     | 1 (1.7)             | 0   | 1 (100) | 1 (100) | 0 | 0 | 0 | 0 | 0 |
| Type IVc     | 2 (3.4)             | 0   | 2 (100) | 2 (100) | 0 | 0 | 0 | 0 | 0 |
| Type IVd     | 11 (19)             | 0   | 11 (100) | 11 (100) | 6 (54) | 4 (19) | 6 (54.5) | 6 (54.5) | 8 (72.7) |
| Type V       | 18 (31)             | 0   | 18 (100) | 18 (100) | 7 (38.9) | 18 (31) | 7 (39) | 13 (72.2) | 11 (61.1) |
| Total        | 58                  | 0   | 56 (96.4) | 57 (98.3) | 32 (55.2) | 31 (53.4) | 30 (51.7) | 34 (58.6) | 36 (62) |

VAN, vancomycin; PEN, penicillin G; CLI, clindamycin; OX, Oxacillin; ERY, erythromycin; G, gentamicin; T, tobramycin; K, kanamycin.

**Discussion**

Antibiotic resistance is a major problem in the world. A wide distribution of the mecA gene and AMEs genes in *S. aureus* has been demonstrated in previous studies. The current investigation also demonstrated the prevalence of the SCCmec types and subtypes along with the most clinically important aminoglycoside resistance genes in MRSA isolates. The mecA gene was detected in 58 of the 104 staphylococcal specimens. Among the mecA-positive isolates there was one *Staphylococcus* isolate which was not phenotypically sensitive to oxacillin, revealing again that the exhibition of different phenotypical and genotypical profiles is possible.

In the present study, maximum resistance of MRSA isolates was observed towards oxacillin and penicillin G followed by kanamycin, tobramycin, clindamycin, erythromycin, and gentamicin. Nearly 76% of our isolates were resistant to at least four or more antimicrobial agents tested; namely, oxacillin, penicillin G, erythromycin, clindamycin, and three of which were aminoglycosides.

Aminoglycosides have been used in the treatment of staphylococcal infections, but emergence of the resistant strains, mostly caused by AMEs, reduce their effectiveness. The aac(6′)/aph(2″) gene, conferring gentamicin resistance, was determined in 48% of our methicillin-resistant isolates followed by the ant(4)-Ia and aph(3′)-IIIa genes, respectively. These results are in accordance with previous studies which reported that prevalence of aac(6′)/aph(2″) gene is higher than that of two other AME genes, ant(4)-Ia and aph(3′)-IIIa, in MRSA isolates. Our results showed a correlation between the presence of aac(6′)/aph(2″) gene and gentamicin resistance. Of 30 isolates which were susceptible to gentamicin resistance determined by the disk diffusion method, only two strains were negative for the presence of aac(6′)/aph(2″) gene.

The prevalence rate of aac(6′)/aph(2″) gene in this study (48%) was significantly lower than the rate reported from two hospitals in the capital city, Tehran, (i.e., 83%) and higher than a report from Turkey (i.e., 28%). These conflicting findings can be caused by changes in antibiotic policies.

The second most frequent AME detected in this research was ant (4′)-Ia, which conferred resistance to kanamycin and was in agreement with the report from Tehran, Iran. The majority of the isolates carrying this gene (83%) were also resistant to kanamycin. We detected the resistance gene aph (3′)-IIIa in 11 (19%) of our isolates; The 82% of these isolates harbored aac(6′)/aph(2″) in combination. Sixty-seven percent of isolates negative for AME genes were susceptible to all aminoglycosides tested. In the present study, 62% of MRSA isolates carry one SCCmec types I, II or III, which are traditionally associated with HA-MRSA. According to the previous studies which have reported that isolates containing HA-MRSA harbor more resistance genes than other SCCmec types, our data showed that isolates with SCCmec III were more resistant to non-beta-lactam antibiotics such as ciprofloxacin, clindamycin and erythromycin, but the differences were not statistically significant for all antibiotics (Table 1). The SCCmec type V and IVa were the most prevalent types identified among community-associated MRSA (CA-MRSA), which accounted for 31% and 29.3% of the isolates. The data shows the tendency of SCCmec types V and IVa strains, the smallest structural types, to transmit in healthcare facilities as mentioned previously.

**Conclusion**

In summary, in this study a high level of resistance of MRSA to multiple classes of antibiotics and upward trend in emergence of CA-MRSA strains in hospitals was observed. Introduction of CA-MRSA strains in hospitals and other health care settings has become a threat to public health. In order to prevent transmission of these CA-MRSA strains, accurate and rapid monitoring techniques should be administered in both clinical and community settings.

**Acknowledgements**

We thank Research Deputy of Zanjan University of Medical Sciences for the financial support of this project.
Research Highlights

What is current knowledge?
✓ Aminoglycosides still play an important role in antistaphylococcal therapies.
✓ $\text{Aac}(6')\text{-I-aph}(2')$, ant(4')-Ia and $\text{aph}(3')$-IIIa genes are the most clinically prevalent aminoglycoside-modifying enzymes (AME) among \textit{Staphylococcus aureus} isolates.

What is new here?
✓ The $\text{aac}(6')\text{-I-aph}(2')$ was the most common aminoglycoside modifying enzyme gene among Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) isolates.
✓ SCCmec type II and V were the most frequent types detected in hospital isolates, respectively.
✓ The study results showed that isolates with SCCmec III were more resistant to non beta-lactam antibiotics.

Ethical issues
This study was approved by the Ethical Committee of Zanjan University of Medical Sciences.

Competing interests
Authors declare no conflict of interests.

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