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

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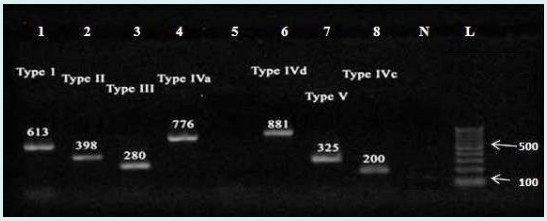
Abstract



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* (SCC*mec*) types in MRSA strains.

Results: All 58 MRSA isolates were sensitive to vancomycin. Resistance to penicillin G, oxacilin, 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 SCC*mec* type I, 11 (19%) SCC*mec* type II, 20 (34.5%) SCC*mec* type III, 17 (29.3%) SCC*mec* type IVa, 1 (1.7%) SCC*mec* type IVb, 2 (3.4%) SCC*mec* type IVc, 11 (19%) SCC*mec* type IVd, and, 18 (31%) SCC*mec* type V. Nineteen isolates were not typeable.

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

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 bacterima 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.[3]

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*-phosphoryltransferase III [APH(3′)-III] encoded by *aph (3′)-IIIa* gene; aminoglycoside-4′-*O*-phosphoryltransferase I [ANT(4′)-I] encoded by *ant*

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(4′)-Ia gene; aminoglycoside-9-*O*-nucleotidyltransferase I [ANT(9)-I] encoded by the *ant (6)-I*, and aminoglycoside-6-*O*-nucleotidyltransferase I [ANT(6)-I] encoded by the *ant (6)-I*) gene.[4]

Clinically, the most important of these enzymes are ANT (4′)-I, AAC (6′)/APH(2″) and APH (3′)-III because these enzymes modify aminoglycosides of therapeutic significance, including tobramycin, gentamicin and kanamycin, respectively.[4,5] Many of these modifying enzymes are carried on the transposons or on small plasmids and can be integrated into the staphylococcal chromosomal cassette *mec* (*SCCmec*) of *Staphylococcus aureus* and coagulase negative staphylococci isolates.[3,4] In this study, we investigated the distribution of the most clinically significant aminoglycoside resistance genes *ant (4′)-Ia*, *aac(6′)/ aph(2″)* and *aph(3′)-IIIa*, as well as the distribution of staphylococcal cassette chromosome *mec* (SCC*mec*) 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.[6,7] 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.[8] The presence of the *mecA* gene was investigated by PCR method using a set of previously described primers.[9]

Antimicrobial susceptibility testing

The phenotypic resistance to various antibiotics in MRSA isolates was determined using the standard disk diffusion

Table 1. Oligonucleotide sequences of primers used in PCR amplification of different genes investigated in this study

Target genes		Oligonucleotide sequences	Size of target region (bp)	Reference
FemA	Forward Reverse	5′- TTATCTCGCTTGTTATGTG -3′ 5′- TTACTGCTGTACCTGTTATG -3′	686	7
Sa442	Forward Reverse	5′- AATCTTTGTCGGTACACGATATTCTTCACG-3′ 5′- CGTAATGAGATTTCAGTAGATAATACAACA -3′	107	7
mecA	Forward Reverse	5′-CTGGAACTTGTTGAGCAGAG-3′ 5′- TGGCTATCGTG TCACAATCG -3′	310	8
aac(6′)/aph(2″)	Forward Reverse	5′-CAGAGCCTTGGGAAGATGAAG -3′ 5′- CCTCGTGTAATTCATGTTCTGGC -3′	348	12
ant(4′)-Ia	Forward Reverse	5′-AATCGGTAGAAGCCCAA-3′ 5′-GCACCTGCCATTGCTA-3′	135	12
aph(3′)-IIIa	Forward Reverse	5′- GGCTAAAATGAGAATATCACCGG -3′ 5′- CTTTAAAAAATCATACAGCTCGCG -3′	523	12
Type I	Forward Reverse	5′- GCTTTAAAGAGTGTCGTTACAGG -3′ 3′-GTTCTCTCATAGTATGACGTCC-5 ′	613	11
Type II	Forward Reverse	5′--CGTTGAAGATGATGAAGCG-3′ 3′-CGAAATCAATGGTTAATGGACC-5′	398	11
Type III	Forward Reverse	5′-CCATATTGTGTACGATGCG-3′ 3′-CCTTAGTTGTCGTAACAGATCG-5′	280	11
Type IVa	Forward Reverse	5′-GCCTTATTCGAAGAAACCG-3′ 3′-CTACTCTTCTGAAAAGCGTCG-5′	776	11
Type IVb	Forward Reverse	5′-TCTGGAATTACTTCAGCTGC-3′ 3′-AAACAATATTGCTCTCCCTC-5′	493	11
Type IVc	Forward Reverse	5′-ACAATATTTGTATTATCGGAGAGC-3′ 3′-TTGGTATGAGGTATTGCTGG-5′	200	11
Type IVd	Forward Reverse	5′-CTCAAAATACGGACCCCAATACA-3′ 3′-TGCTCCAGTAATTGCTAAAG-5	881	11
Type V	Forward Reverse	5′-GAACATTGTTACTTAAATGAGCG-3′ 3′-TGAAAGTTGTACCCTTGACACC-5	325	11

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 (SCC*mec*), 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 oxacilin, 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 SCC*mec* type I, 11 (19%) SCC*mec* type II, 20 (34.5%) SCC*mec* type III, 17 (29.3%) SCC*mec* type IVa, 1 (1.7%) SCC*mec* type IVb, 2 (3.4%) SCC*mec* type IVc, 11 (19%) SCC*mec* type IVd, and 18 (31%) SCC*mec* typeV (Table 2). Nineteen

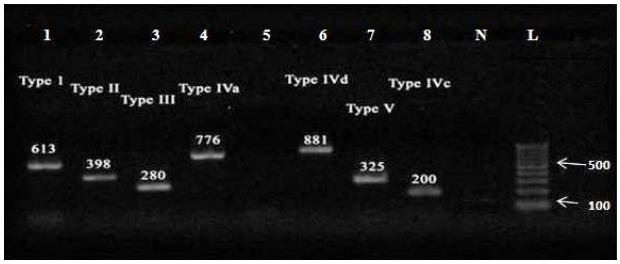

Fig. 1. Multiplex PCR for SCC*mec* typing of isolates. Multiplex PCR. Lane 1, SCCmec type I; lane 2, type II; lane 3, type III; lane 4, type IVa; lane 5, Not typeable; lane 6, type IVd; lane 7, type V; lane 8, type IVc; N: Negative control; L: 100 bp DNA Ladder.

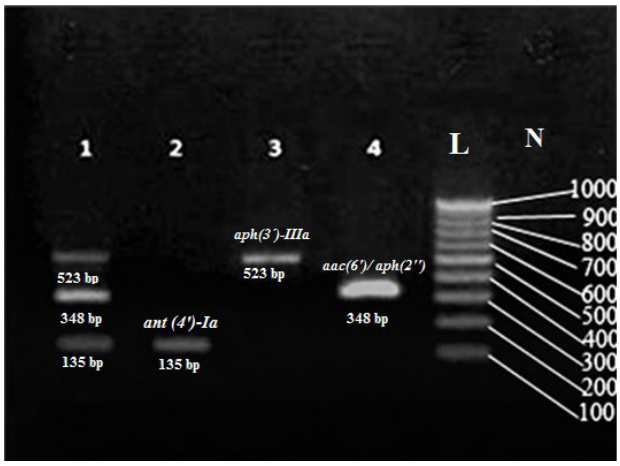

Fig. 2. Multiplex PCR for AME genes determined in agarose gel electrophoresis. Lanes 1 to 4, 4 representative isolates that possess one or more of the three genes studied. Lane1, triple combination of *ant (4′)-I*, *aac(6′)-aph(2′′)* and *aph(3′)-III* (see text). L: 100 bp DNA Ladder; N: Negative control; *ant (4′)-Ia* gene; 135 bp band, *aac(6′)/ aph(2″)*gene; 348 bp band, *aph(3′)-IIIa* gene; 523 bp band.

isolates were not typeable.

Contrary to previous reports, 25 (43%) strains showed multiband as follows: 1 (1.7%) isolate for SCC*mec* types I and II, 2 (3.4%) isolates for SCC*mec* types II and III, 3 (5.2%) isolates for SCC*mec* types II and V, 2 (3.4%) isolates for SCC*mec* types III and V, 3 (5.2%) isolates for SCC*mec* types IVa and V, 2 (3.4%) isolates for SCC*mec* types IVd and V, 1 (1.7%) isolate for SCC*mec* types II ,III and V, 1 (1.7%) isolate for SCC*mec* types I, III and IVa, 1 (1.7%) isolate for SCC*mec* types I, III, V and IVa, 1 (1.7%) isolate for SCC*mec* types II, III, V and IVa, 1 (1.7%) isolate for SCC*mec* types I, III and V, 2 (3.4%) isolates for SCC*mec* types III and IVa, 1 (1.7%) isolate for SCC*mec* types III and IVd, 1 (1.7%) isolate for SCC*mec* types IVa and IVd, 1 (1.7%) isolate for SCC*mec* types II and IVc, 1 (1.7%) isolate for SCC*mec* types I, IVa and V, and 1 (1.7%) isolate for SCC*mec* 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, JX 142145, JX 142143, JX 142141, JX142146, JX142144, JX142142, JX437190, JX426259, and JX437191.

Table 2. Distribution of staphylococcal cassette chromosome *mec* types and subtypes in MRSA isolates and resistance profile

SCC*mec* types	No. (%) of isolates	Antibiotics							
		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.[14-17] 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 SCC*mec* 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.[12,14]

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,[18] (i.e., 83%) and higher than a report from Turkey[14] (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.[18] The majority of the isolates carrying this gene (83%) were also resistant to kanamycin.[14]

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 percents of isolates negative for AME genes were susceptible to all aminoglycosides tested. In the present study, 62% of MRSA isolates carry one SCC*mec* 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 SCC*mec* types, our data showed that isolates with SCC*mec* 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).[15,19] The SCC*mec* 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 SCC*mec* types V and IVa strains, the smallest structural types, to transmit in healthcare facilities as mentioned previously.[20,21]

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

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Research Highlights

What is current knowledge?

√ Aminoglycosides still play an important role in antistaphylococcal therapies.

√ *Aac(6′)-Ie/aph(2″), ant(4′)-Ia* and *aph(3′)-IIIa* genes are the most clinically prevalent aminoglycoside-modifying enzymes (AME) among *Staphylococcus aureus* isolates.

What is new here?

√ The *aac (6′)/aph(2″)* was the most common aminoglycoside modifying enzyme gene among Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates.

√ SCC*mec* type II and V were the most frequent types detected in hospital isolates, respectively.

√ The study results showed that isolates with SCC*mec* 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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