

Detection of tetracycline resistance genes, aminoglycoside modifying enzymes, and coagulase gene typing of clinical isolates of *Staphylococcus aureus* in the Southwest of Iran

Seyed Sajjad Khoramrooz ^{1*}, Samin Alipoor Dolatabad ², Fatemeh Mostafapour Dolatabad ³, Masoud Marashifard ⁴, Mehdi Mirzaii ⁵, Hossein Dabiri ⁶, Azam Haddadi ², Seyed Mohammadreza Rabani ⁷, Hamid Reza Ghaffarian Shirazi ¹, Davood Darban-Sarokhalil ⁸

¹ Cellular and Molecular Research Center, Yasuj University of Medical Sciences, Yasuj, Iran

² Department of Biology, Faculty of Science, Karaj Branch, Islamic Azad University, Karaj, Iran

³ Department of Basic Sciences, Islamic Azad University, Yasooj Branch, Yasooj, Iran

⁴ Student Research Committee, Yasuj University of Medical Sciences, Yasuj, Iran

⁵ School of Medicine, Shahrood University of Medical Sciences, Shahrood, Iran

⁶ Department of Medical Microbiology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁷ Beheshti Teaching Hospital, Yasuj University of Medical Sciences, Yasuj, Iran

⁸ Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Objective(s): The aim of the present study was to determine the aminoglycoside modifying enzymes (AMEs) encoded genes, tetracycline resistance genes, and the *coa* based typing of *Staphylococcus aureus* isolates in the Southwest of Iran.

Materials and Methods: Antimicrobial susceptibility of isolates was carried out by agar disk diffusion methods. Two sets of multiplex PCR mixture were used for detection of AME genes and *tet* genes. All of the isolates were typed with the coagulase gene typing method. Of the 121 isolates, 29.75% and 47.93% were resistant to at least one aminoglycosides and tetracyclines, respectively.

Results: The *aac(6')-Ie-aph(2'')* was the most frequent gene (97.22%), and *aph(3')-IIIa* and *ant(4')-Ia* genes were detected in 61.11% and 11.11% of aminoglycoside resistant isolates, respectively. The *tetK* and *tetM* genes were detected in 82.75% and 56.9% of tetracycline resistant isolates, respectively. Overall 31.4% of isolates were MRSA. Totally 17 distinct *coa* gene RFLP patterns, numbered C1 to C17, were observed. The C5 was the most frequent *coa* type with 31 isolates.

Conclusion: The *aac(6')-Ie-aph(2'')* and *aph(3')-IIIa* genes were the most important genes contributing to aminoglycosides resistance, while resistance to tetracyclines was mediated by *tetK* and *tetM* genes. Interestingly all *S. aureus* with C5 as the most prevalent *coa*-type were resistant to at least one of the aminoglycoside antibiotics and tetracycline simultaneously. Moreover, 30 out of 31 isolates with this *coa* type were MRSA, indicating the importance of the C5 *coa*-type in MRSA strains and also in isolates that were resistant to aminoglycosides and tetracycline.

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Introduction

Staphylococcus aureus is one of the most important causes of nosocomial infections around the world and can cause a variety of diseases such as skin and soft tissue infections, pneumonia, bloodstream infection, osteomyelitis, endocarditis, and also toxin mediated diseases (1). *S. aureus* has shown resistance to different classes of antibiotics, which complicates the treatment of infections (2, 3). Aminoglycosides by inhibiting the bacterial protein synthesis show bactericidal activity. This group of antibiotics especially gentamycin and tobramycin in combina-

tion with beta-lactam or glycopeptides antibiotics have synergical effects on treatment of *S. aureus* infection, particularly endocarditis (4). Resistance to aminoglycosides occurs mainly by drug inactivation via bacterial aminoglycoside modifying enzymes (AMEs) that are encoded by the genes located on plasmids or transposons (4, 5). AMEs are classified into four groups according to the modification imposed on aminoglycoside antibiotics: acetyltransferases (AACs), phosphotransferases (APHs), nucleotidyltransferases (ANTs), and adenylyltransferases (AADs). The most important enzymes which confer resistance to amino-

*Corresponding author: Seyed Sajjad Khoramrooz. Cellular and Molecular Research Center, Yasuj University of Medical Sciences, Yasuj, Iran. Next to Imam Sajad Hospital, Shahid Ghorbanali Jalil Blvd, Yasuj, Iran. Tel/Fax: +98-743-323- 5153; email: Khoramrooz@gmail.com

glycosides among staphylococci isolates are AAC(6')=APH(2''), APH(3')-III, and ANT(4')-I, which are encoded by *aac(6')-Ie=aph(2'')*, *aph(3')-IIIa*, and *ant(4')-Ia* genes, respectively (6, 7).

Tetracyclines are broad-spectrum antibiotics used in the treatment and prevention of bacterial infections and can be used for treatment of *S. aureus* caused infections such as skin and soft tissue infections (SSTIs) (8). Two main mechanisms of resistance against tetracyclines have been identified in *S. aureus*: active efflux, which is mediated by plasmid encoded *tetK* and *tetL* genes and ribosomal protection that is encoded by chromosomal or transposonal *tetM* or *tetO* genes (9). It has been shown that *S. aureus* isolates harboring the *tetK* gene are resistant to tetracycline but not minocycline while the *tetM* gene confers resistance to both of them (10).

Knowledge about the local epidemiology of *S. aureus* in a hospital setting is very important for prediction of trends in antibiotic-resistance patterns, detection of outbreaks and tracking the spread of infection, epidemiological surveillance, and hospital infection control (1, 11). Therefore various molecular techniques are available for identification and compression of different *S. aureus* strains. Of these techniques pulsed-field gel electrophoresis [PFGE], multilocus enzyme electrophoresis [MLEE], randomly amplified polymorphic DNA (RAPD) assay and a repetitive element sequence-based PCR (rep-PCR) and coagulase gene typing as well as sequence-based (multilocus sequence typing [MLST] and *spa* typing) techniques (12, 13) have been used. The coagulase gene (*coa*) typing is another technique in which the organism is typed based on the polymorphic region of the *coa* gene. Although the discriminatory power of the *coa* typing is lower than PFGE, MLEE, and *spa* typing methods (14), it is easier, faster, accurate, and enough reproducible for typing of *S. aureus* isolated from different sources (15). All of the *S. aureus* isolates produce the coagulase enzyme that is encoded by the *coa* gene. The *coa* typing is based on the heterogeneity of 81 bp tandem repeats that are located in the 3' coding region of coagulase gene. Amplification of that region using the PCR method results in DNA bands with diverse size and numbers in each strain. Furthermore, digestion of the amplified fragment with *AluI* and *HaeIII* restriction enzymes generates more DNA bands in different isolates which increases the discriminatory power of *coa* typing method (16, 17).

Considering the high frequency of *S. aureus* infections and contributed antimicrobial resistance in the region, there are a few studies addressing the AMEs genes and tetracycline resistance genes in accordance with the *coa* typing of *S. aureus* in Iranian hospitals. The aim of the present study was to determine the AMEs encoded genes, tetracycline resistance genes, and *coa* typing of *S. aureus* strains isolated from two major teaching hospitals in the southwest of Iran.

Materials and Methods

Bacterial isolates

A total of 121 *S. aureus* isolates were collected from hospitalized patients who were referred to teaching hospitals affiliated to Ahvaz Jundishapur University of Medical Sciences from May 2012 to January 2013. Identification of isolates at species level was done by conventional biochemical tests such as manitol fermentation, catalase, tube coagulase, and the DNase test (18). Confirmation of *S. aureus* isolates was performed using amplification of the *nucA* gene (19).

Antimicrobial susceptibility testing

Antimicrobial susceptibility of *S. aureus* isolates against 8 antibiotics was carried out by agar disk diffusion methods according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (20). The antibiotic panel included: aminoglycosides group (amikacin, gentamicin, tobramycin, and kanamycin), tetracyclines group (tetracycline, minocycline, and doxycycline), in addition to cefoxitin and erythromycin. All antibiotics were purchased from MAST, UK. *S. aureus* ATCC 25923 was used as the control strain.

Detection of AME encoded, *tet*, *mecA* and *nucA* genes

Total DNA genomic was extracted by boiling methods according to known procedure with some minor modification (21). The oligonucleotides primers and their expected size of PCR products for AME genes *aac(6')-Ie-aph(2'')*-I, *aph(3')-IIIa* and *ant(4')-Ia*, *tet* genes (*tetK*, *tetL*, *tetM* and *tetO*), *mecA* and *nucA* genes are presented in Table 1. Two sets of Multiplex-PCR mixture for detection of the AME genes and the *tet* genes were prepared separately. Each set was carried out in a total volume of 25 µl containing 12.5 µl master mix (Amplicon, Denmark), 20 pmol of each primer and 300 ng of template DNA. Also, two series of single PCR were carried out for the *mecA* and *nucA* genes in 25 µl reaction consisting of 12.5 µl master mix (Amplicon, Denmark), 20 pmol of each primer, and 200 ng of template DNA. The amplification conditions were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of amplification at 94 °C for 45 sec, annealing at (55 °C for *tet* genes, 56 °C for AME genes, *mecA* and *nucA* genes) for 45 sec and extension at 72°C for 45 sec. A final extension step was performed at 72°C for 7 min. The PCR products were separated on 1.5% agarose gel containing ethidium bromide and photographed with a digital camera (Canon, Japan) under UV transilluminator (Major science, Taiwan).

Coagulase gene typing

PCR amplification of the 3'-end region of the *coa* gene was performed according to the primers designed previously (22). PCR was performed in a 50 µl reaction mixture, containing 25 µl of master mix

Table 1. Oligonucleotide sequences of the primers used for detection of aminoglycosides resistance genes, tetracycline resistance genes, and *mecA* gene

Target genes	Oligonucleotide sequences of primers	Size (bp)	Reference
<i>aac(6')-Ie-aph(2'')-I</i>	F-CAGGAATTTATCGAAAATGGTAGAAAAAG R- CACAATCGACTAAAGAGTACCAATC	369	(23)
<i>aph(3')-IIIa</i>	F-GGCTAAAATGAGAATATCACCGG R-CTTTAAAAAATCATACAGCTCGCG	523	(23)
<i>ant(4')-Ia</i>	F-CAAAGTCTAAATCGGTAGAAGCC R-GGAAAGTTGACCAGACATTACGAACT	294	(23)
<i>tetK</i>	F- GTAGCGACAATAGGTAATAGT R- GTAGTGACAATAAACCTCCTA	360	(24)
<i>tetM</i>	F- AGTGGAGCGATTACAGAA R- CATATGTCCTGGCGTGTCTA	158	(24)
<i>tetL</i>	F-ATAAATTGTTTCGGGTCGGTAAT R- AACCAAGCACTAATGACAATGAT	1077	(10)
<i>tetO</i>	F-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA	514	(25)
<i>mecA</i>	F-GTG AAG ATA TAC CAA GTG ATT R-ATG CGC TAT AGA TTG AAA GGA T	147	(26)

(Amplicon, Denmark), 20 pmol of each primer, and 200 ng of DNA template. PCR reactions were carried out in a thermocycler (Biorad T100, USA) with the following program: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 40sec, annealing at 56°C for 40 sec, extension at 72°C for 40 sec, followed by final extension at 72 °C for 7 min. The PCR products were detected by electrophoresis of 15 µl of each amplification mixture in 2% agarose gels containing ethidium bromide and then visualized by UV light illumination. After observation of the expected PCR product bands on the gel agarose, the amount of 10 µl of the PCR products was digested with *AluI* and *HaeIII* enzymes (Fermentas) in a separate reaction at 37 °C for 1.5 hr and was analyzed by gel electrophoresis on a 2.5% agarose gel containing ethidium bromide and was visualized under UV light. Each reaction was performed in 25 µl final volume consisting of 10 µl of PCR product, 2.5 µl of (*HaeIII* or *AluI*, buffer 10x) 1.5 µl of *HaeIII* or *AluI* enzyme and 11 µl of deionized distilled water.

Results

A total of 121 *S. aureus* isolates from various sources including wound and skin lesions (n=54, 44.62%), blood (n=17, 14.05%), catheter (n=20, 16.53%), tracheal aspirates (n=8, 6.62%), urine (n=3,

2.48%), femur lesions (9, 7.44%), abscess (5, 4.13%) and other clinical specimens (n=5, 4.13%) were analyzed. Considering the positive PCR results for the *mecA* gene, 38 (31.4%) of isolates were MRSA and 83 (68.6%) were MSSA. Among all of the isolates, 36 (29.75%) strains were resistant to at least one of the tested aminoglycoside antibiotics in this study, while 32 were resistant to all of the tested amino-antibiotics (tobramycin, gentamicin, glycoside amikacin, and kanamycin) and 4 were resistant against tobramycin and kanamycin. The isolates that were resistant to 4 aminoglycoside antibiotics were also MRSA, while only one of the four isolates that showed resistance to tobramycin and kanamycin were MRSA. Five (6%) of the MRSA strains were sensitive to aminoglycoside antibiotics. There was a significant association between MRSA and resistance to aminoglycoside antibiotics *P*=0.0001. Of 121 *S. aureus* isolates, 39 were resistant to erythromycin, of which 33 isolates also showed resistance to aminoglycoside antibiotics.

The most prevalent AME gene was *aac(6')-Ie-aph(2'')* with the frequency of 35 (97.22%) of the 36 resistant isolates. The *aph (3')-IIIa* and *ant (4')-Ia* genes were detected in 22 and 4 isolates, respectively. Detection profile of AME genes in resistant isolates is shown in Table 2. The most frequent

Table 2. Aminoglycosie resistance pattern and profile of AME genes in resistant isolates of *Staphylococcus aureus*

Resistance phenotype	AME genes				Isolates number
	<i>aac(6')-Ie-ph(2'')</i>	<i>aph (3')-IIIa</i>	<i>ant (4')-Ia</i>	<i>mecA</i>	
AK, GM,K, TN	+	+	+	+	1
AK, GM,K, TN	+	+	-	+	19
AK, GM,K, TN	+	-	-	+	12
K, TN	+	+	-	+	2
K, TN	+	-	+	-	1
K, TN	-	-	+	-	1
Total	35	22	3	34	36

AK;amikacin, GM; gentamicin, K; kanamycin, TN; tobramycin

Table 3. The distribution of tetracycline resistance (*tet*) genes and resistance phenotype in clinical isolates of *Staphylococcus aureus*

Resistance phenotype	Tetracycline resistance gene			
	tetK	tetM	mecA	
TE	+	-	+	3
TE	+	-	-	20
TE	-	+	+	8
TE	+	+	+	1
TE	+	+	-	1
TE, DXT	+	-	+	1
TE, DXT	+	-	-	1
TE, DXT	-	+	+	2
TE, DXT	+	+	+	17
TE, DXT, MN	+	+	+	4
Total	48	33	36	58

profile was *aac(6')-Ie-aph(2'')+ aph (3')-IIIa*, which was detected in 21 (58.33%) isolates. Co-existence of *aac(6')-Ie-aph(2'')*, *aph (3')-IIIa* and *ant (4')-Ia* genes was observed in one isolate. A significant association was observed between amino-glycoside resistance in *S. aureus* isolates and presence of the *aac(6')-Ie-aph(2'')* ($P=0.0001$) and *aph (3')-IIIa* genes ($P=0.001$). Antimicrobial resistance pattern of tetracycline group showed that 58 (47.93%) of *S. aureus* isolates were resistant to tetracycline, 25 (20.66%) to doxycycline, and 4 (3.3%) to minocycline. Seven resistant patterns were observed against these antibiotics groups, and co-resistance to tetracycline and doxycycline was the most prevalent. Of 58 tetracycline resistance isolates, 36 isolates were MRSA, whilst only two MRSA strain isolates were sensitive to tetracycline. The *tetK* and *tetM* genes were detected in 48 (82.75%) and 33 (56.9%) of 58 tetracycline resistant isolates, respectively, in which 23 (39.65%) isolates harbored *tetK* and *tetM* simultaneously. None of the isolates were positive for *tetL* and *tetO* genes. The pattern of tetracycline resistance genes in accordance with the antibiogram profile is presented in Table 3. The entire of 121 studied isolates were confirmed as *S. aureus* by detection of the *nucA* gene using PCR, and all of them gave positive results for amplification of the *coa* gene with a single band but

in five different sizes ranging approximately from 530 to 860 base pair, each size corresponding to one pattern. The *coa* gene with 700 bp size was seen in 72 isolates and was considered a predominant type. The second common type with 17 isolates had a 530 bp band. Fifteen, eleven, and six isolates had a single 610, 780 and 860 bp band for the *coa* gene, respectively. Considering the RFLP patterns of the *coa* gene with *HaeIII* and *AluI* enzymes, the *HaeIII*-RFLP presented higher discriminatory power rather than *AluI*-RFLP and thereby the *HaeIII*-RFLP pattern was used for the further analysis. All of the PCR products for the *coa* gene were digested by the *HaeIII* enzyme. Totally 17 distinct *coa* gene RFLP patterns, numbered C1 to C17, were observed. The C5 was the most frequent *coa* type with 31 isolates (Table 4). It is interesting that aminoglycoside resistant isolates were belonging to C5, C6, C13, and C14 *coa* types. MRSA strains were also found in C3, C5, C7, C13, and C17 *coa* types. Resistance to the tetracycline group was observed in eight *coa* types (C1, C3, C5, C7, C8, C13, C14, and C16). All of the 31 *S. aureus* isolates with C5 *coa* type were resistant against at least one of the aminoglycoside plus tetracycline antibiotics group simultaneously and except for one, all of them were MRSA. Similar to the C5 *coa* type, all of the three isolates with C13 *coa* type were MRSA and also

Table 4. Distribution of *HaeIII* restriction patterns of *coa* gene in according with aminoglycoside and tetracycline resistance in MRSA and MSSA isolates

<i>coa</i> amplification pattern (bp)	RFLP pattern (bp) with <i>HaeIII</i>	<i>coa</i> type	<i>S. aureus</i> with <i>coa</i> type (no)	MRSA N=38	MSSA N=83	Resistant to aminoglycosides (no)	Resistant to tetracyclines (no)
860	140-170-210-340	C1	3	-	3	-	3
	80-210-330	C2	3	-	3	-	-
780	140-260-380	C3	9	2	7	-	5
	320-460	C4	2	-	2	-	-
700	170-210-320	C5	31	30	1	31	31
	80-170-210-240	C6	5	-	5	1	-
	100-140-460	C7	6	2	4	-	1
	130-140-180-250	C8	19	-	19	-	4
	140-180-380	C9	1	-	1	-	-
	80-100-140-380	C10	8	-	8	-	-
	140-170-180-210	C11	1	-	1	-	-
	80-100-130-140-240	C12	1	-	1	-	-
	170-210-240	C13	3	3	-	3	3
	140-470	C14	8	-	8	1	2
610	130-140-170-180	C15	2	-	2	-	-
	140-180-290	C16	2	-	2	-	-
	210-320	C17	17	1	16	-	9

simultaneously were resistant to aminoglycoside and tetracycline antibiotics groups. In resistant isolates, totally 11 different antibiotics resistant patterns were observed, among which, the pattern including 19 isolates that were resistant to the tobramycin, gentamicin, amikacin, kanamycin, tetracycline, and doxycycline was the most prevalent pattern. It is interesting that all of these isolates belong to the C5 *coa* type.

Discussion

In the present study 26.44% of isolates were resistant to tobramycin, kanamycin, amikacin, and gentamycin simultaneously and in addition, co-resistance to tobramycin and kanamycin was observed in 3.22% of the isolates. Although our results are similar to those by Emameini *et al.* from Iran that was conducted on clinical isolates other than burn patients (27), they are lower than the resistance rate of other studies from Iran (28) on different clinical isolates and Emameini *et al.* in burn patients (23). These differences may be related to the type of clinical samples and different policies for infection control in different hospitals. In comparison with other countries, the resistance rates to gentamycin and amikacin were similar to Hauschild *et al.* (29) and Schmitz *et al.* (4) but lower than the results by Ida *et al.* (5). Our results showed that 86.1% of aminoglycoside resistant isolates were MRSA, which was 8 times higher than MSSA strains. Like many previous reports, the resistance rate to aminoglycoside antibiotics was higher in MRSA strains than MSSA strains. It has been documented that there is a correlation between resistance against aminoglycoside and methicillin thereby different resistance rates to aminoglycoside in various studies are contributed to the different number of MRSA isolates (30).

Aminoglycoside modifying enzymes in *S. aureus* isolates are encoded by *aac(6')-Ie-aph(2'')*, *aph(3')-IIIa*, and *ant(4')-Ia* genes and confer resistance to aminoglycosides. In this study 97.22% of aminoglycoside resistance isolates, harbored the *aac(6')-Ie-aph(2'')* gene as the most prevalent gene, which is similar to findings of the majority of studies from Iran as well as different European countries, South Korea, and Poland in which the *aac(6')-Ie-aph(2'')* has been reported as a most prevalent gene ranging from 28.9 to 93.7% of their isolates (2, 4, 23, 29, 30). However, in some of these studies the prevalence of the *aac(6')-Ie-aph(2'')* positive strains was lower than what we found. The *aph(3')-IIIa* was the second common AMEs gene (61.11%) among our studied strain, which is higher than other reports from Iran as well as Japan, South Korea, Poland, and 19 European countries in which this gene has been identified in 8.9%-46% of their isolates (4, 5, 28-30). In contrast to our results, the *aph(3')-IIIa* was the

most prevalent AMEs gene in two different studies conducted by Emameini *et al.* (82.78% and 93.7%) and Mahdiyoun *et al.* (77%) from Iran (23, 27, 31) and Liakopoulos from Greece (73.7%) (7). Different policies for prescription of antibiotics, infection control program, and monitoring among hospitals in different regions and countries result in different rates of antibiotic resistant strains reflecting the diversity in the distribution of resistant genes. The lowest frequency among AMEs genes belonged to *ant(4')-Ia* gene which was detected in 11.11% of *S. aureus* isolates. Although our results are approximately similar to those of Liakopoulos *et al.* (13.6%) and Perumal *et al.* (9%) (7, 32), they are in contrast with many studies in which the *ant(4')-Ia* gene has been identified in 26.7% to 89.24% of isolates (4, 5, 23, 27-30). This gene was not detected in two other studies (33, 34). It is quite interesting that 2 of the 3 isolates that harbored *ant(4')-Ia* were MSSA. It is probable that the *ant(4')-Ia* gene has a role in resistance to aminoglycosides antibiotics in MSSA strains although further studies are needed to analyze this matter. Regarding the comparison between various studies, different aminoglycoside resistance rates and also different prevalence of AME encoded genes were observed between countries and even in hospitals and cities of one country. We inferred that these differences may be related to the source of clinical isolates, geographical location, bacterial genotype, prescription of aminoglycosides, and the rate of MRSA isolates. Since *aac(6')-Ie-aph(2'')* causes resistance to most of the aminoglycosides (5), in our study this gene was found in all of the aminoglycoside resistance isolates except for one. In the present study two out of 3 isolates with the *ant(4')-Ia* gene, showed resistance to tobramycin and kanamycin and the other isolate in addition to these antibiotics was also resistant to gentamicin and tobramycin. It is interesting that all of the isolates with the *ant(4')-Ia* gene were resistant to tobramycin. These results are in accordance with other studies that reported the *ant(4')-Ia* gene inactivates tobramycin (5, 7). The *aph(3')-IIIa* gene modifies kanamycin and amikacin; in our study all 22 isolates that harbored *aph(3')-IIIa* were resistant to kanamycin and 90.9% were also resistant to amikacin.

In the present study 47.93%, 20.65%, and 3.3% of *S. aureus* isolates were resistant to tetracycline, doxycycline, and minocycline, respectively. Approximately 62% of tetracycline resistant isolates were MRSA, which is similar to the results of Schmitz *et al.* (4) and Emameini *et al.* (27) studies in which they found 57.1% and 61% of MRSA strains were tetracycline resistant. Based on studies from the United States and Canada the rates of occurrence of tetracycline resistance among MRSA isolates has been reported as 15.6% and 14.8%, respectively,

whereas in Latin America and the Western Pacific regions the rates were considerably higher, exceeding 60% (35). Two main resistance mechanisms against tetracycline have been explained in *S. aureus*; the first active efflux which is mediated by plasmid encoded *tetK* and *tetL* genes and the second ribosomal protection which is mediated by transposon or chromosomal *tetM* and *tetO* determinants (36). In 58 tetracycline resistant isolates, *tetK* and *tetM* were detected in 82.75% and 56.9% isolates, respectively, while none of the isolates were positive for *tetL* and *tetO* genes. Although except for one, all of the *tetM* positive isolates were MRSA, the *tetK* gene was detected in 45% of MSSA strains. In accordance with our results, Schmitz *et al.* have shown that *tetM* was more frequent in MRSA isolates (9). In a multicenter study (37) the *tetK* gene was detected in higher numbers in MRSA isolates. They showed that all of the tetracycline resistant MRSA isolates from North America harbored solely the *tetK* gene and in Eastern and Western European countries, 86% of isolates encoded only the *tetK* gene (37). Although in the present study the *tetK* gene was the predominant gene, 54.16% of them were MRSA, while this rate for the *tetM* gene was 97%. In another study *tetK* and *tetM* genes were detected in 14.3% and 50% of MRSA isolates, respectively (33). Thereby the *tetM* gene may have an important role in resistance to tetracycline antibiotics in MRSA in comparison with MSSA strains. One study showed that MRSA isolates harbored *tetK* and *tetM* in 31.8% and 36.4%, respectively (10). Co-existence of *tetK* and *tetM* genes was observed in 23 isolates, and except for one, all of them were MRSA. On the other hand the combination of the *tetM* and *tetK* was approximately 22 times more prevalent in tetracycline-resistant MRSA isolates rather than in tetracycline-resistant MSSA isolates; this is in agreement with the previous report of Schmitz *et al.* as their MRSA strains also harbored both *tetK* and *tetM* simultaneously 10 times more than MSSA isolates (9). Similar to many reports, *tetL* and *tetO* were not detected in any of the isolates in the present study, indicating that these genes have no role in tetracycline resistance in our isolates (9, 38-40).

The coagulase gene amplification has been considered as a simple and accurate method for typing of *S. aureus* (17). In the current study 17 distinct *coa* gene RFLP patterns (*coa*-type) were identified, among which, the C5 as a prevalent *coa*-type was identified in 31 (25.61%) isolates. All of the isolates with C5 *coa*-type were resistant to at least one of the aminoglycoside antibiotics and also all of them showed resistance to tetracycline antibiotics. In addition 30 out of 31 isolates were MRSA. This finding all together showed the importance of *S. aureus* with C5 *coa* type in isolates that were

resistant to aminoglycosides and tetracycline and also acquisition of resistance to methicilin (MRSA). On the other hand, the majority of MRSA (78.94%) belonged to the C5 *coa* type indicating the spread of a specific type of MRSA in these hospitals. Similar to our results, another study found that 77% of MRSA strains belonged to the specific *coa* type, the L21 *coa* type (14). From the 17 different *coa* types, only 4 types were found in aminoglycoside resistant isolates, of which most of the isolates belonged to the C5 and C13 types representing the relationship among specific *coa* types and resistance to aminoglycoside antibiotics. Hence, it may be specific *coa* types (genotype) of *S. aureus* that acquire resistance to aminoglycosides and cause spreading antibiotic resistance in a hospital setting. Among aminoglycoside resistance isolates the *aph(3')-IIIa* gene was identified only in the C5 *coa* type, while *aac(6')-Ie-aph(2'')* and *ant(4')-Ia* were identified in other *coa* types. It is possible that there is a relationship between *aph(3')-IIIa* and certain *coa* types, nonetheless, further research is needed. Tetracycline resistance isolates were found in 8 different *coa* types, where the *tetM* gene was found in only three *coa*-types, while *tetK* was found in 7 *coa* types. Probably in spite of the *tetK* gene which can be present in tetracycline resistant isolates with any *coa* type, the *tetM* genes prefer certain *coa* types.

Conclusion

According to our study, there were significant associations between MRSA strains and resistance to aminoglycoside and tetracycline antibiotics. The *aac(6')-Ie-aph(2'')* and *aph(3')-IIIa* genes have an important role in resistance to aminoglycoside antibiotics and *tetK* and *tetM* confer tetracycline resistance in *S. aureus*. The *coa* gene RFLP patterns showed 17 distinct *coa* types, among which limited *coa* types were found to be aminoglycoside resistant or MRSA. It is interesting that all of the *S. aurei* with C5 as the most prevalent *coa*-type were resistant to at least one of the aminoglycoside antibiotics and also all of them showed resistance to tetracycline antibiotics. Moreover, nearly all of the isolates with this *coa* type were MRSA indicating the importance of the C5 *coa*-type in MRSA strains and also in isolates that were resistant to aminoglycosides and tetracycline.

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Conflict of interest

Authors declare no conflict of interest in this works.

References

- Pérez-Vázquez M, Vindel A, Marcos C, Oteo J, Cuevas O, Trincado P, *et al.* Spread of invasive Spanish *Staphylococcus aureus* spa-type t067 associated with a high prevalence of the aminoglycoside-modifying enzyme gene *ant* (4')-Ia and the efflux pump genes *msrA/msrB*. *J Antimicrob Chemother* 2009; 63:21-31.
- Mohammadi S, Sekawi Z, Monjezi A, Maleki MH, Soroush S, Sadeghifard N, *et al.* Emergence of SCCmec type III with variable antimicrobial resistance profiles and spa types among methicillin-resistant *Staphylococcus aureus* isolated from healthcare-and community-acquired infections in the west of Iran. *Int J Infect Dis* 2014; 25:152-158.
- Gorwitz RJ, Kruszon-Moran D, McAllister SK, McQuillan G, McDougal LK, Fosheim GE, *et al.* Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001-2004. *J Infect Dis* 2008; 197:1226-1234.
- Schmitz FJ, Fluit AC, Gondolf M, Beyrau R, Lindenlauf E, Verhoef J, *et al.* The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococci from 19 European hospitals. *J Antimicrob Chemother* 1999; 43:253-259.
- Ida T, Okamoto R, Shimauchi C, Okubo T, Kuga A, Inoue M. Identification of aminoglycoside-modifying enzymes by susceptibility testing: epidemiology of methicillin-resistant *Staphylococcus aureus* in Japan. *J Clin Microbiol* 2001; 39:3115-3121.
- Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev* 1993; 57:138-163.
- Liakopoulos A, Foka A, Vourli S, Zerva L, Tsiapara F, Protonotariou E, *et al.* Aminoglycoside-resistant staphylococci in Greece: prevalence and resistance mechanisms. *Eur J Clin Microbiol Infect Dis* 2011; 30:701-705.
- Esposito S, Leone S, Petta E, Noviello S, Ianniello F. Treatment options for skin and soft tissue infections caused by methicillin-resistant *Staphylococcus aureus*: oral vs. parenteral; home vs. hospital. *Int J Antimicrob Agents* 2009; 34:S30-S35.
- Schmitz FJ, Krey A, Sadurski R, Verhoef J, Milatovic D, Fluit AC. Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. *J Antimicrob Chemother* 2001; 47:239-240.
- Trzcinski K, Cooper BS, Hryniewicz W, Dowson CG. Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* 2000; 45:763-770.
- Li QT, Zhu YZ, Dong K, Liu C, Zhou YH, Ni YX, *et al.* A novel sequence-based *coa* genotyping method to discriminate nosocomial methicillin-resistant *Staphylococcus aureus* isolates. *Irish J Med Sci* 2011; 180:463-8.
- Sabat A, Malachowa N, Miedzobrodzki J, Hryniewicz W. Comparison of PCR-based methods for typing *Staphylococcus aureus* isolates. *J Clin Microbiol* 2006; 44:3804-3807.
- Harmsen D, Claus H, Witte W, Rothgänger J, Claus H, Turnwald D, *et al.* Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. *J Clin Microbiol* 2003; 41:5442-5448.
- Ishino K, Tsuchizaki N, Ishikawa J, Hotta K. Usefulness of PCR-restriction fragment length polymorphism typing of the coagulase gene to discriminate arbekacin-resistant methicillin-resistant *Staphylococcus aureus* strains. *J Clin Microbiol* 2007; 45:607-609.
- Khoshkhamr-Roodmajani H, Sarvari J, Bazargani A, Kandekar-Ghahraman MR, Nazari-Alam A, Motamedifar M. Molecular typing of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from Shiraz teaching hospitals by PCR-RFLP of coagulase gene. *Iran J Microbiol* 2014; 6:246-252.
- Goh SH, Byrne SK, Zhang JL, Chow AW. Molecular typing of *Staphylococcus aureus* on the basis of coagulase gene polymorphisms. *J Clin Microbiol* 1992; 30:1642-1645.
- Himabindu M, Muthamilselvan DS, Bishi DK, Verma RS. Molecular analysis of coagulase gene polymorphism in clinical isolates of methicillin resistant *Staphylococcus aureus* by restriction fragment length polymorphism based genotyping. *Am J Infect Dis* 2009; 5:170-176.
- Mahon CR, Lehman DC, Manuselis G. Textbook of diagnostic microbiology-E-Book. New York: Elsevier Health Sciences; 2014.
- Sahebkhah N, Nochi Z, Eslampour M, Dabiri H, Bolfion M, Taherikalani M, *et al.* Characterization of *Staphylococcus aureus* strains isolated from raw milk of bovine subclinical mastitis in Tehran and Mashhad. *Acta Microbiol Immunol Hung* 2011; 58:113-121.
- Wayne P. Clinical and laboratory standards institute. Wayne, PA: Performance Standards for Antimicrobial Susceptibility Testing; 2007. P. 17.
- Perez-Hernandez X, Mendez-Alvarez S, Claverie-Martin F. A PCR assay for rapid detection of vancomycin-resistant enterococci. *Diagn Microbiol Infect Dis* 2002; 42:273-277.
- Hookey JV, Edwards V, Cookson BD, Richardson JF. PCR-RFLP analysis of the coagulase gene of *Staphylococcus aureus*: application to the differentiation of epidemic and sporadic methicillin-resistant strains. *J Hosp Infect* 1999; 42:205-212.
- Emanini M, Bigverdi R, Kalantar D, Soroush S, Jabalameli F, Noorazar Khoshnab B, *et al.* Distribution of genes encoding tetracycline resistance and aminoglycoside modifying enzymes in *Staphylococcus aureus* strains isolated from a burn center. *Ann Burns Fire Disasters* 2013; 26:76-80.
- Strommenger B, Kettlitz C, Werner G, Witte W. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. *J Clin Microbiol* 2003; 41:4089-4094.
- Malhotra-Kumar S, Lammens C, Piessens J, Goossens H. Multiplex PCR for simultaneous detection of macrolide and tetracycline resistance determinants in streptococci. *Antimicrob Agents Chemother* 2005; 49:4798-800.
- Zhang K, McClure JA, Elsayed S, Louie T, Conly JM. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol* 2005; 43:5026-5033.
- Emanini M, Taherikalani M, Eslampour MA, Sedaghat H, Aligholi M, Jabalameli F, *et al.* Phenotypic and genotypic evaluation of aminoglycoside resistance in clinical isolates of staphylococci in Tehran, Iran. *Microb Drug Resist* 2009; 15:129-132.
- Yadegar A, Sattari M, Mozafari NA, Goudarzi GR. Prevalence of the genes encoding aminoglycoside-modifying enzymes and methicillin resistance among clinical isolates of *Staphylococcus aureus* in Tehran, Iran. *Microb Drug Resist* 2009; 15:109-113.

29. Hauschild T, Sacha P, Wieczorek P, Zalewska M, Kaczyńska K, Tryniszewska E. Aminoglycosides resistance in clinical isolates of *Staphylococcus aureus* from a University Hospital in Bialystok, Poland. *Folia Histochem Cytobiol* 2008; 46:225-228.
30. Choi SM, Kim SH, Kim HJ, Lee DG, Choi JH, Yoo JH, *et al.* Multiplex PCR for the detection of genes encoding aminoglycoside modifying enzymes and methicillin resistance among *Staphylococcus* species. *J Korean Med Sci* 2003; 18:631-636.
31. Mahdiyoun SM, Kazemian H, Ahanjan M, Houri H, Goudarzi M. Frequency of aminoglycoside-resistance genes in methicillin-resistant *staphylococcus aureus* (MRSA) isolates from hospitalized patients. *Jundishapur J Microbiol* 2016; 9:e35052.
32. Perumal N, Murugesan S, Krishnan P. Distribution of genes encoding aminoglycoside-modifying enzymes among clinical isolates of methicillin-resistant *staphylococci*. *Indian J Med Microbiol* 2016; 34:350-352.
33. Ardic N, Ozyurt M, Sareyyupoglu B, Haznedaroglu T. Investigation of erythromycin and tetracycline resistance genes in methicillin-resistant *staphylococci*. *Int J Antimicrob Agents* 2005; 26:213-218.
34. Hosseini M, Asghar A, Khoramrooz S, Marashifard M, Parhizgari N, Mansouri F. Frequency of the genes encoding aminoglycoside modifying enzymes in *staphylococcus aureus* isolated from hospitalized burn patients. *J Mazandaran Univ Med Sci* 2016; 25:147-157 (Persian).
35. Diekema D, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, *et al.* Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. *Clin Infect Dis* 2001; 32:S114-S132.
36. McCallum N, Berger-Bächi B, Senn MM. Regulation of antibiotic resistance in *Staphylococcus aureus*. *Int J Med Microbiol* 2010; 300:118-129.
37. Jones CH, Tuckman M, Howe AY, Orlowski M, Mullen S, Chan K, *et al.* Diagnostic PCR analysis of the occurrence of methicillin and tetracycline resistance genes among *Staphylococcus aureus* isolates from phase 3 clinical trials of tigecycline for complicated skin and skin structure infections. *Antimicrob Agents Chemother* 2006; 50:505-510.
38. Sekiguchi J, Fujino T, Saruta K, Konosaki H, Nishimura H, Kawana A, *et al.* Prevalence of erythromycin-, tetracycline-, and aminoglycoside-resistance genes in methicillin-resistant *Staphylococcus aureus* in hospitals in Tokyo and Kumamoto. *Jap J Infect Dis* 2004; 57:74-77.
39. Lozano C, Porres-Osante N, Crettaz J, Rojo-Bezares B, Benito D, Olarte I, *et al.* Changes in genetic lineages, resistance, and virulence in clinical methicillin-resistant *Staphylococcus aureus* in a Spanish hospital. *J Infect Chemother* 2013; 19:233-242.
40. Lim KT, Hanifah YA, Yusof M, Thong KL. *ermA*, *ermC*, *tetM* and *tetK* are essential for erythromycin and tetracycline resistance among methicillin-resistant *Staphylococcus aureus* strains isolated from a tertiary hospital in Malaysia. *Indian J Med Microbiol* 2012; 30:203-207.