



# Distribution of *aac(6′)/aph(2″)*, *aph(3′)-IIIa*, and *ant(4′)-Ia* Genes Among Clinical Nasal Sources for *Staphylococcus aureus* Strains Isolated in Korramabad, Iran

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## Abstract

**Objectives:** The principal mechanism of resistance in many clinical isolates of *Staphylococcus aureus* involves the inactivation of aminoglycoside antibiotics by aminoglycoside-modifying enzymes (AMEs). The present study identified the antimicrobial susceptibility pattern and the prevalence of AMEs among *S. aureus* strains.

**Materials and Methods:** To carry out this cross-sectional research, a total of 100 *S. aureus* were gathered from the university hospitals in Khorramabad, Iran, from January to November 2017. The antibiotic susceptibility pattern of the isolates was determined using the disk diffusion method according to the guidelines suggested by the Clinical Laboratory Standards Institute. The samples were assayed to detect the presence of three AME genes by the use of a triplex polymerase chain reaction (PCR) method.

**Results:** The prevalence of *S. aureus* nasal carriage was 14.7% (50/340). In addition, 15%, 10%, 15%, and 8% of the total isolates were found to be resistant to gentamicin, amikacin, kanamycin, and netilmicin, respectively. Further, *aac(6′)/aph(2″)*, *aph(3′)-IIIa*, and *ant(4′)-Ia* genes were present in 17%, 12%, and 0% of the isolates, respectively. Based on the results, the double combination of *aac(6′)/aph(2″)* and *aph(3′)-IIIa* genes were only observed among clinical-isolated strains (12/50, 24%), which predominantly were resistant to oxacillin (10/12, 83.3%). Eventually, the *aac(6′)/aph(2″)* gene was found in all isolates that were phenotypically resistant to gentamicin and kanamycin.

**Conclusions:** These findings indicated that resistance to aminoglycosides is significantly related to methicillin-resistance ( $P < 0.001$ ). Due to the relatively high occurrence of the main genes modifying aminoglycosides in our region, it is recommended that clinicians combine aminoglycosides synergistically with other antibiotics such as beta-lactams in cases of empirical treatments.

**Keywords:** *Staphylococcus aureus*, Aminoglycoside-modifying enzymes, Multiplex-PCR

## Introduction

*Staphylococcus aureus* is known as one of the human commensal bacteria although it has a high potential for causing various infections in different hosts. The anterior part of the nose is the main reservoir for the colonization of this Gram-positive bacterium. In addition, the horizontal transmission of *S. aureus* from hospital employees to hospitalized patients, particularly immunocompromised individuals, causes life-threatening infections (1).

Aminoglycoside antibiotics target the 30S ribosomal subunit of bacteria and interfere with protein synthesis (2,3). The clinical indication of these antibiotics in curing infections, which are caused by gram-positive bacteria such as staphylococci, is primarily limited to their potent synergistic effects with the other classes of antibiotics such as beta-lactams (2-4). Staphylococci have a long history of being resistant to different antibiotics. This potential is due to their inherent characteristic and the acquisition of mobile genetic elements (5-7). The main mechanism of resistance to these drugs in the *Staphylococcus* genus is the enzymatic modification of aminoglycosides via

aminoglycoside-modifying enzymes (AMEs) (8,9). According to (10), these enzymes fall into three distinct classes, including aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases (APHs), and aminoglycoside nucleotidyl transferases (ANTs). The genes that encode these enzymes are found on chromosomes and plasmids, which can be transposable (11). AAC(6′)/APH(2″), ANT(4′)-I, and APH(3′)-III enzymes encoded by *aac(6′)/aph(2″)*, *ant(4′)-I*, and *aph(3′)-III* vary, because they are the most common antibiotic-modifying enzymes in different species of *S. aureus* (12-15).

The bifunctional enzyme AAC(6′)/APH(2″) is known as the most common enzyme among *S. aureus* isolates. Further, these enzymes cause resistance to gentamicin, tobramycin, kanamycin, netilmicin, and amikacin. Furthermore, ANT(4′)-I enzyme is responsible for mediating the resistance to tobramycin, amikacin, kanamycin, and dibekacin in staphylococci (15,16). Considering the above-mentioned explanations, the aim of the present research was to find out the frequency of *aac(6′)-I*/

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*aph(2'')*, *ant(4')-Ia*, and *aph(3')-IIIa* genes among *S. aureus* using the multiplex-polymerase chain reaction (PCR).

## Materials and Methods

### The Tested Bacterial Isolates

In our previous research (a cross-sectional study) from July 2011 to January 2012, 340 swab samples were obtained via rotating sterile cotton-tipped swabs into both anterior nares of the staff (males and females) working in distinct wards of four referral and university-affiliated hospitals in Khorramabad, Iran (17). Individuals who had consumed antibiotics for a one-week period before sampling were excluded from the study. To enrichment, swabs were inoculated and incubated in a trypticase soy broth (TSB) for 24 hours at 37°C. Subsequently, a loopful of broth tubes was subcultured on 10% sheep blood agar, mannitol salt agar, and nutrient agar plates (Merck, Germany). Following the overnight incubation at 37°C, presumptive staphylococcal colonies were further identified by traditional microbiology and biochemical tests, including gram-staining, catalase, clumping factor, coagulase, DNase, and mannitol fermentation (18). Gram-positive cocci were confirmed based on a positive reaction for catalase, DNase, coagulase, and mannitol fermentation.

On the other hand, 50 *S. aureus* isolates were also collected in an interval from January to November 2017 from various clinical specimens (i.e., wound, abscess, blood and, urine) and were included in this study. Overall, the tested sample size was 100 *S. aureus* strains.

### Antibiotic Susceptibility Testing

First, the suspension of bacteria equivalent to 0.5 McFarland turbidity standard was prepared on the TSB medium. Subsequently, the susceptibility assay was performed via the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Merck, Germany) based on the approaches suggested by the Clinical and Laboratory Standards Institute (20). The applied antibiotic-impregnated discs were penicillin G (10U), cefoxitin (30 µg), vancomycin (30 µg), gentamicin (10 µg), kanamycin (30 µg), amikacin (30 µg), and netilmicin (30 µg) provided from Mast, UK.

### DNA Extraction and Polymerase Chain Reaction

DNA from all strains was extracted via AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea) after making certain alterations. Table 1 presents the applied amplifying primers for *aac(6')-Ie/aph(2'')*, *ant(4')-Ia*, and *aph(3')-IIIa* genes, which were previously designed and applied by Choi et al (19). Multiplex PCR was carried out in a total volume of 25 µL, including 2.5 µL buffer 10x, 0.4 mM dNTP mix, 3 mM MgCl<sub>2</sub>, 0.2 µM every one of forward and reverse primers, a 200 ng DNA template, 1.5U Taq DNA polymerase, and up to 25 µL DNase free water. PCR amplification for the desired genes was optimized under the following circumstances: the primary denaturation at

95°C for 5 minutes, 30 cycles with an initial denaturation at 95°C for 2 minutes, the annealing step at 58°C for 1 minute, extension at 72°C for 1 minute and the final extension at 72°C for 10 minutes (19). Eventually, 5 µL of PCR amplicons and the 100 bp DNA ladder (Fermentas, Lithuania) were exposed to electrophoresis on the agarose gel (1.5% w/v in Tris-acetate-EDTA buffer). In addition, the standard strain DNA of *S. aureus* ATCC 25923 was used as the negative control and *S. aureus* containing genes *aac(6')*, *Ie/aph(2'')*, *ant(4')-Ia*, and *aph(3')-IIIa* (Bacteriology Department, Tarbiat Modares University, Tehran, Iran) were used as the positive control.

### Data Analysis

The difference in the resistance pattern to the tested aminoglycosides between methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) strains was analyzed using the chi-square test via SPSS software, version 16.  $P < 0.05$  was regarded statistically remarkable.

## Results

### Staphylococcal Isolates and Susceptibility Testing

In total, 51 *S. aureus* strains were isolated from the nostrils of 340 hospital personnel. Therefore, the nasal carriage rate was found to be 15%. Among the nasally-isolated *S. aureus* strains (n=51), 50 isolates were included and tested in this study, from which 22 (44%) were males and 28 (56%) were females. The obtained findings following the use of disk diffusion assay indicated that 100% of 100 nasal and clinical-originated *S. aureus* isolates were sensitive to vancomycin and 100% were resistant to penicillin (Table 2). Moreover, with regard to resistance to aminoglycoside antibiotics, among nasal-isolated strains (NIS), 5 (10%) strains were resistant to kanamycin, as well as gentamicin and others exhibited susceptibility to all tested aminoglycosides. Regarding clinical-isolated strains (CIS), the lowest resistance rate was 16% that was related to netilmicin. Interestingly, 8 (16%) strains were simultaneously resistant to kanamycin, gentamicin, amikacin, and netilmicin.

The data analysis (the chi-square test) showed that the differences in resistance to tested aminoglycosides between MRSA and MSSA strains were significant for both CIS and NIS ( $P < 0.001$ ).

**Table 1.** Primers and Conditions of Polymerase Chain Reaction Used in This Study

Genes	Primers	Size (bp)	Ref.
<i>aac(6')-Ie/aph(2'')</i>	5'-GAAGTACGCAGAAGAGA-3' 5'-ACATGGCAAGCTCTAGGA-3'	491	(19)
<i>ant(4')-Ia</i>	5'-AATCGGTAGAAGCCAA-3' 5'-GCACCTGCCATTGCTA-3'	135	(19)
<i>aph(3')-IIIa</i>	5'-AAATACCGCTGCGTA-3' 5'-CATACTCTTCCGAGCAA-3'	242	(19)

**Table 2.** Resistant Profile of the Tested *Staphylococcus aureus* Strains According to Their Susceptibility to Methicillin

Antibiotics	Total n = 100 (%)	MRSA n = 21 (%)		MSSA n = 79 (%)	
		NIS n = 7	CIS n = 14	NIS n = 43	CIS n = 36
		Penicillin	100 (100)	7 (100)	14 (100)
Cefoxitin	21 (21)	7 (100)	14 (100)	0 (0)	0 (0)
Vancomycin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gentamicin	15 (15)	4 (57.1)	9 (64.2)	1 (2.3)	1 (2.7)
Amikacin	10 (10)	0 (0)	9 (64.2)	0 (0)	1 (2.7)
Netilmicin	8 (8)	0 (0)	8 (57.1)	0 (0)	0 (0)
Kanamycin	15 (15)	4 (57.1)	9 (64.2)	1 (2.3)	1 (2.7)

Note. NIS: Nasal-isolated strains; CIS: Clinical-isolated strains; MSSA: methicillin-susceptible *Staphylococcus aureus*; MRSA: methicillin-resistant *S. aureus*. The differences in the resistance pattern to the tested aminoglycosides between MRSA and MSSA strains were significant ( $P < 0.001$ ).

### Multiplex-PCR

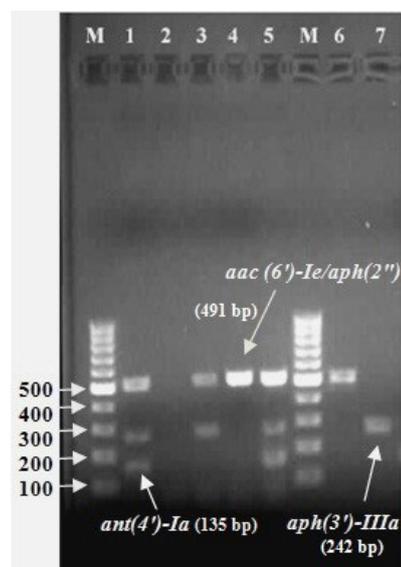
The results of Multiplex-PCR indicated that the frequency of *aac(6')-Ie/aph(2'')*, *aph(3')-IIIa*, and *ant(4')-Ia* genes among 100 isolated strains were 17%, 12% ,and 0%, respectively (Table 3, Figure 1).

### The Association Between the Phenotype of the Resistance to Aminoglycosides and the Presence of the *aac(6')-Ie/aph(2'')* and *aph(3')-IIIa* Genes

Five NIS, which carried the *aac(6')-Ie/aph(2'')* gene were concurrently resistant to gentamicin and kanamycin (Table 4). None of the tested strains, which were phenotypically susceptible to aminoglycosides, harbored the tested genes. Interestingly, 2 (4%) CIS simultaneously harbored the *aac(6')-Ie/aph(2'')* and *aph(3')-IIIa* genes while they were not resistant to any of the tested aminoglycosides as specified by the disk diffusion method. In addition, 10 (20%) other strains concurrently carried the *aac(6')-Ie/aph(2'')* and *aph(3')-IIIa* genes and demonstrated phenotypic resistance to at least three aminoglycosides (i.e., gentamicin, kanamycin, and amikacin), the details of which are provided in Table 4.

### Discussion

Nowadays, the resistance of staphylococci to antibiotics has complicated therapeutic procedures. The results of our research indicated that in the clinical samples, the highest resistance was observed to oxacillin, kanamycin, and gentamicin, respectively, after penicillin. Our results

**Figure 1.** Multiplex-PCR Products Electrophoresis on Agarose Gel.

Note. PCR: Polymerase chain reaction; Lane M: 100 bp DNA size marker (Fermentas, Lithuania); Lanes 1, 5, and 7: *S. aureus* strain containing *acc(6')-Ie/aph(2'')*, *ant(4')-Ia*, and *aph(3')-IIIa* (positive control); Lane 3: Clinical isolated *S. aureus* strain (containing *aac(6')-Ie/aph(2'')* and *aph(3')-IIIa* genes); Lanes 4 and 6: Nasal-isolated *S. aureus* strains (containing *aac(6')-Ie/aph(2'')* gene); Lane 2: *S. aureus* strain ATCC 25923 (negative control). The electrophoresis was run in a 1.5% agarose gel, which was visualized with ethidium bromide.

further demonstrated that NIS were not resistant to amikacin and netilmicin. Yadegar et al showed that among 100 *S. aureus* strains isolated from clinical samples, resistance to penicillin, oxacillin, gentamicin, amikacin, netilmicin, kanamycin, and vancomycin was 100%, 48%, 52%, 48%, 22%, 68%, and 0%, respectively (20). Moreover, their lowest resistance was related to netilmicin, which was similar to our result. In another study carried out by Sadari et al in Iran, 348 samples were collected by swabs from personnel nares in the hospitals. Eighty-seven individuals (25%) were the carriers of *S. aureus*, and 90.8%, 11.8%, 5%, and 0% of isolated strains were resistant to penicillin, oxacillin, gentamicin, and vancomycin, respectively (21). Based on the results, both nasal and clinical originated MRSA strains were more resistant compared to MSSA. Furthermore, the results showed that resistance to aminoglycosides among MRSA strains was greater compared to MSSA strains ( $P < 0.001$ ). These compatible results were reported in the study by Sadari et

**Table 3.** Distribution of Aminoglycosides-modifying Genes in 100 Nasal and Clinical Isolated *Staphylococcus aureus* Strains

Resistance Gene (s)	MSSA n = 79 (%)		MRSA n = 21 (%)		Total (%)
	NIS* (n = 43)	CIS (n = 36)	NIS (n = 7)	CIS (n = 14)	
<i>aac(6')-Ie/aph(2'')</i>	1 (2.3%)	0 (0)	4 (57.1)	0 (0)	5 (5)
<i>aac(6')-Ie/aph(2'')</i> + <i>aph(3')-IIIa</i>	0 (0)	2 (5.5)	0 (0)	10 (71.3)	12 (12)
<i>aph(3')-IIIa</i>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Not detected	42 (97.7)	34 (94.5)	3 (42.9)	4 (58.6)	83 (83)

NIS: Nasal-isolated strains; CIS: Clinical-isolated strains; MSSA: Methicillin-susceptible *Staphylococcus aureus*; MRSA: Methicillin-resistant *S. aureus*.

**Table 4.** Relationship Between Phenotypic and Genotypic Aminoglycosides Resistance Nasal and Clinical-isolated *Staphylococcus aureus* Strains

Phenotypic Resistance*	Number of Strains	Resistance Gene (s)
GM, AK, N, & K	8	<i>aac(6')-Ie/aph(2'')</i> & <i>aph(3')-IIIa</i>
GM, AK, & K	1	<i>aac(6')-Ie/aph(2'')</i> & <i>aph(3')-IIIa</i>
GM, K	5	<i>aac(6')-Ie/aph(2'')</i>

al as well (21).

In our research, the *aac(6')-Ie/aph(2'')* gene had the highest frequency (17%) among the tested strains. Interestingly, the NIS only carried the *aac(6')-Ie/aph(2'')* gene (10%) while CIS harbored *aac(6')-Ie/aph(2'')* and *aph(3')-IIIa* genes, simultaneously (24%). In a study conducted by Choi et al in Korea, similar results were obtained, indicating that the *aac(6')-Ie/aph(2'')* gene was the most frequent gene among the studied strains with a frequency of 65%, followed by the *ant(4')-Ia* (41%) and *aph(3')-IIIa* (9%) genes, respectively (19). In another research carried out by Ardic et al, the highest and the lowest frequencies were related to *acc(6')-Ia/aph(2'')* (66%) and *aph(3')-IIIa* (8%) genes, respectively (8). Consistent with our results, Fatholahzadeh et al (14) and Emaneini et al (22) reported the *aac(6')-Ie/aph(2'')* gene as the most prevalent AME encoding gene among various *S. aureus* isolates (coagulase-positive/negative). Similarly, Yadegar et al (20) conducted a study on 100 clinically isolated *S. aureus* and indicated that the *ant(4)-Ia* was the most common gene (58%), followed by *aac(6')-Ie/aph(2'')* (46%) and *aph(3')-IIIa* (6%), which contradicts the results of our research and those of previous studies. Likewise, Khoramrooz et al reported *aac(6')-Ie-aph(2'')*, *aph(3')-IIIa*, and *ant(4')-Ia* genes in 97.22%, 61.11%, and 11.11% of aminoglycoside-resistant isolates, respectively (23). In another study, Seyedi-Marghaki et al found that MSSA and MRSA strains were resistant to kanamycin (41.2% and 83%, respectively), tobramycin (76.2%), gentamicin (71.4%), amikacin (59.5%), and netilmicin (23.8%). The frequencies for *aac(6')-Ie-aph(2'')*, *aph(3')-IIIa*, *ant(4')-Ia*, and *aph(2'')-Id* were 45.2%, 19%, 14.3%, and 4.8% among MRSA isolates, respectively (24). Several studies confirmed the significant association between the resistance to methicillin and aminoglycosides (25,26). Given the development of resistance to aminoglycoside antibiotics, particularly among MRSA strains, the detection of resistant strains for choosing effective therapeutic options is necessary. Multiplex-PCR is known as a swift and versatile *in vitro* method which is used to detect resistant genes. Moreover, this method was developed to provide the opportunity for the selective amplification of a specific target DNA sequence to take place within a heterogeneous collection of DNA sequences (3). To the best of our knowledge, the present study is the first one to detect *aac(6')-Ie/aph(2'')*, *ant(4')-Ia*, and *aph(3')-IIIa* genes among MRSA and MSSA *S. aureus*

in Khorramabad, Iran. Due to the limited period and high costs, we were unable to extend the examination to newer antibiotics and the minimum inhibitory concentration determination for common aminoglycoside antibiotics. Regarding the spread of resistance to aminoglycosides, particularly among MRSA strains, it is recommended that further studies shall be carried out on other mechanisms involved in resistance to aminoglycosides. The molecular typing methods for finding the origin and relatedness of resistant clones should be performed as well. Due to the presence of main genes modifying aminoglycosides in our region, our clinicians are recommended to combine aminoglycosides synergistically with other antibiotics such as beta-lactams in cases of empirical treatments.

### Conflict of Interests

Authors have no conflict of interests.

### Ethical Issues

This study was confirmed by the Ethics Committee of Lorestan University of Medical Sciences, Khorramabad, Iran (No. 200/49017). Further, informed consent was obtained from all hospital employees who were volunteered to participate in this study.

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