Original Article

J Babol Univ Med Sci
Vol 19, Issu 3; Mar 2017. P:38-46

Molecular Identification of Genes Responsible for Resistance to Aminoglycosides and Methicillin in Clinical Samples of *Staphylococcus Aureus*

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J Babol Univ Med Sci; 19(3); Mar 2017; PP: 38-46
Received: May 22th 2016, Revised: Jul 27th 2016, Accepted: Nov 26th 2016.

ABSTRACT

BACKGROUND AND OBJECTIVE: *Staphylococcus aureus* is one of the most important causes of infection in the hospital and the community. Resistance to aminoglycosides is caused by certain enzymes, which are coded by the genes with the ability of intrastrain circulation by mobile genetic elements such as transposons. This study aims to to determine the frequency of aac (6 ') Ie / aph (2 "), aph (3') - IIIa1, ant (4 ') - Ia1 genes along with mecA gene.

METHODS: In this cross sectional study, 113 *Staphylococcus aureus* strains were isolated from 579 various clinical samples. The Method of determining the minimum inhibitory concentration was used to identify the genes resistant to aminoglycosides by Oxacillin Etest strips. PCR method was used to identify aac (6 ') Ie / aph (2 "), aph (3') - IIIa1, ant (4 ') - Ia1and mecA. The relationship between aminoglycosides and mecA genes was also investigated.

FINDINGS: Of 113 clinical isolates of *Staphylococcus aureus* confirmed by phenotypic tests, 53 isolates (46.91%) had mecA gene, 43 isolates (38.05%) had aac (6') Ie / aph (2") gene, 19 isolate (16.81%) had aph (3') - IIIa1 gene and 22 isolates (19.47%) had ant (4') - Ia1 gene. In addition, there was a significant relationship between the presence of Methicillin-resistant gene and genes responsible for resistance to aminoglycosides. Meanwhile, there was also a significant relationship between the type of isolated samples and the presence of resistance genes in some cases ($p \leq 0/005$).

CONCLUSION: Results of the study demonstrated that frequent use of the aminoglycoside antibiotics along with beta-lactam antibiotics might provide the context for the emergence of multi-drug-resistant *Staphylococcus aureus* (MDR) strains. By appropriate and controlled administration of antibiotics, we can decrease such resistances and prevent the emergence of multi-drug-resistant *Staphylococcus aureus* strains.

KEY WORDS: *Staphylococcus aurous, Aminoglycosides, Drug Resistance, Methicillin Resistance*.

Please cite this article as follows:

Bokaeian M, Tahmasebi H. Molecular Identification of Genes Responsible for Resistance to Aminoglycosides and Methicillin in Clinical Samples of Staphylococcus Aureus. J Babol Univ Med Sci. 2017; 19(3):38-46.

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Introduction

Staphylococcus aureus is one of the most important nosocomial pathogens with the capability to create a wide range of infections (1). The diseases associated with this bacterium may range from a simple skin infection to fatal necrotic pneumonia, skin diseases such as staphylococcal scalded skin syndrome (SSSS) and toxic shock syndrome (TSS), urinary tract infections and even human food poisoning (2–5). Treatment of infections caused by *Staphylococcus aureus* and extensive use of various antibiotics have created resistant strains in this bacterium (6,7). Methicillin–resistant *Staphylococcus aureus* (MRSA) is one of the strains that was quite widespread in the 1980s and Centers for Disease Control and Prevention (CDC) reported that more than 120,000 hospitals from 1990 to 2000 were infected by this bacterium (8).

In 1961, the first methicillin–resistant *Staphylococcus aureus* strain was identified and it quickly spread in the whole world and after a short period, it became resistant to several other antibiotics in addition to methicillin (9,10). Healthcare-acquired methicillin-resistant *Staphylococcus aureus* (HA – MRSA) entered the society quickly and strains of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) appeared (11).

Resistance to methicillin is because of the presence of mecA gene, which is part of a mobile genetic element called Staphylococcal cassette chromosome mec (SCCmec) (12). This unit includes a set of regulatory genes (mecI, mecR and mecC) and cassette chromosome (ccr) recombinase complex (13). Today, aminoglycosides are among the best antibiotics to cure MRSA strains and although they have several complications, they have favorable effects for treatment of staphylococcal infections (14,15). To improve the effectiveness of these antibiotics, sometimes β-Lactam and aminoglycoside antibiotics are used in addition to aminoglycoside antibiotics (16). Aminoglycosides stop the translation mechanism of the bacterium by binding to the 30S subunit of ribosomes and induce their antibacterial effects (16). Resistance to aminoglycosides is because of the presence of enzymes that cause changes in aminoglycoside antibiotics (17).

These enzymes are coded by several genes including aac (6 ') Ie / aph (2 "), aph (3') - IIIa1 and ant (4 ') - Ia1 with the ability of intra-strain circulation by mobile genetic elements such as transposons (14). The three enzymes AAC(6´) / APH(2″), APH(3´)-III and ANT(4´), which are respectively coded by genes aac (6 ') Ie / aph (2 "), aph (3') - IIIa1 and ant (4 ') - Ia1, are among the most common modifying enzymes in various species of *Staphylococcus aureus* and medically and clinically important (14, 18).

Each of these enzymes code the resistance to a specific antibiotic; resistance to gentamicin, kanamycin and tobramycin is due to the activity of aac (6 ') Ie / aph (2 ") enzyme, resistance to neomycin, tobramycin, amikacin is due to the presence of ant (4 ') - Ia1 gene and resistance to kanamycin and tobramycin is because of aph (3')-IIIa1 enzyme (16, 19, 20). For phenotypic identification of strains that are resistant to aminoglycoside antibiotics, we need to use an appropriate range of aminoglycoside antibiotics including gentamicin, kanamycin, amikacin, tobramycin (21).

The reasons for failure to treat resistant infectious strains include lack of using proper antibiotic disks for initial identification of strains resistant to aminoglycosides, low sensitivity of phenotypic methods, and lack of accurate and fast identification (22, 23). One of the best and most sensitive ways for identification of resistant strains is detecting genes that distribute enzymes responsible for antibiotic resistance using polymerase chain reaction (PCR) technique. This study was conducted for molecular identification of genes responsible for resistance to aminoglycosides and methicillin in *Staphylococcus aureus* clinical samples as well as phenotypic identification of resistant strains. In addition, the frequency of aac (6 ') Ie / aph (2 "), aph (3') - IIIa1, ant (4 ') - Ia1 genes as well as mecA gene was assessed.

Methods

Collecting samples and isolating the bacteria: In this cross – sectional study (Ethics Committee Code: ir.zaums.rec.1394.250) 597 samples of blood, urine, cerebrospinal fluid, nasal swabs, wound, secretions, trachea, phlegm, saliva, etc. were collected from patients hospitalized in different sections of Medical Centers of Zahedan University of Medical Sciences during an 11-month period from May 2015 to December 2015 using convenience sampling method. Patients hospitalized for a long period (based on patient's profile) and suspected of bacterial infections were included in the study and in cases where the disease was not infectious and had no bacterial base, the patient was excluded from the study. The genus

and species were specified by biochemical experiments after primary culture. Finally, 113 *Staphylococcus aureus* isolates were obtained after differential experiments.

Determination of *Staphylococcus aureus* strains resistant to methicillin and aminoglycoside using MIC and Etest: Resistance to methicillin was determined using Minimum Inhibitory Concentration (MIC) and Oxacillin Etest strips (Himedia, India). Gentamicin and amikacin Etest strips were used to determine the resistance to aminoglycoside based the above-mentioned method. Results were analyzed using the latest version of Clinical & Laboratory Standards Institute (CLSI). *Staphylococcus aureus* (ATCC25923) and *Staphylococcus aureus* (ATCC43300) were used as control strains for quality control and evaluation of results (24).

Determination of resistance pattern to aminoglycoside antibiotics in *Staphylococcus aureus*: To determine the resistance pattern to aminoglycosides, antibiotic disks of gentamycin (10 micrograms), amikacin (30 micrograms), kanamycin (30 micrograms), tobramycin (10 micrograms), and netilmicin (10 micrograms) were used through disk diffusion method (all from Himedia, India). For this purpose, after preparing MHA – containing (Merck, Germany) plates, the disks were placed on the medium using sterile forceps and were incubated at 37 ˚C for 24 hours. The created inhibitory zone diameter was measured using CLSI (25).

Genomic extraction using extraction kit: The colonies obtained from 24–hour culture were inoculated in 5 ml Luria-Bertani (LB) broth medium (Merck, Germany) that were previously divided and numbered in lidded glass tubes based on the number of isolates and were incubated at 37 ˚C for 20 hours. 1.5 cc of the culture medium was poured into 1.5 lidded plastic microtubes and all steps of DNA extraction were taken using Extraction Kit (CinnaGen Co., Iran) based on the protocols of the manufacturer. The obtained DNA was stored at -20 ˚C for molecular experiments.

Preparation of primers and PCR: 25 µl of the final solution containing 2 µl DNA templates, 1 µl of each primer with a concentration of 25 pM and 25 µl of PCR Master Mix (Ampliqon, Germany) (containing Tris-Hcl PH8.5, (NH4) SO4, 3 mMMgcl 2, 0.2% Tween 20, 0.4MmdNTP, 0.2 unit Ampliqon polymeras, Insert red dye and stabilizer) was used for PCR reaction. The remaining volume was compensated by distilled water.

The 451 bp long 5"-GAAGTACGCAGAAGAG-3" and 5"-GAAGTACGCAGAAGAG-3 primers were used for proliferation of aac (6') Ie / aph, 242 bp long 5"-GAAGTACGCAGAAGAG-3" and 5"-GAAGTACGCAGAAGAG-3 primers were used for proliferation of aph (3') - IIIa1 gene, 195 bp long 5"-GAAGTACGCAGAAGAG-3" and 5"-GAAGTACGCAGAAGAG-3 primers were used for ant (4') - Ia1 gene and 544 bp long 5"-GAAGTACGCAGAAGAG-3" and 5"-GAAGTACGCAGAAGAG-3 primers were used for proliferation of mecA.

BioRad MJ Mini thermocycler (USA) was used for proliferation of the aforementioned genes. The temperature cycles for primary denaturation were considered 95˚C for 3 minutes, 35 thermal cycles including secondary denaturation at 95 ˚C for 40 seconds, primers connection was at 56 ˚C for 45 seconds for aac(6')Ie/aph(2") gene and at 52 ˚C for 40 seconds for ant(4')-Ia1 and aph(3')-IIIa1 genes. The primary proliferation was done at 72 ˚C for 40 seconds. The final proliferation was done at 72 ˚C for 10 seconds. The standard strain of *Staphylococcus aureus* (ATCC43300) was used as positive control and *Staphylococcus aureus* (ATCC25923) was used as negative control.

Electrophoresis on 1.5% agarose gel: 5 µl of the final PCR product was electrophoresed on 3% agarose gel in 0.5 X Buffer. 100 bp Fermentas marker (Thermofisher, USA) was used for the identification of the target bond. PCR test was used as standard gold to compare the results with other results.

Data analysis: The results of determining antibiotic resistances by phenotypic method were analyzed using WHONET Ver. 5.5. For this purpose, descriptive statistical methods (determining the frequency, percentage and mean) were used. SPSS ver. 16 and X2 statistical test were used to analyze the relationship between variables and $p<0.05$ was considered significant.

Results

In this study on various clinical samples of *Staphylococcus aureus*, 36 urine isolates (23%), 5 cerebrospinal fluid isolates (4.4%), 14 nasal swab isolates (12.38%), 19 wound isolates (16.81%), 10 blood isolates (11.3%), 8 phlegm isolates (7.07%), 11 catheter isolates (9.7%), 3 secretion isolates (2.6%) and 17 isolates (15.40%) were collected from other clinical samples, while most samples were collected

from female patients. Of 113 obtained isolates, 61 isolates (53.98%) were identified to be resistant to methicillin using Oxacillin Etest strips. In addition, according to the results of determining minimum inhibitory concentrations (MIC) of Etest strips to identify isolates resistant to aminoglycoside antibiotics, 55 isolates (48.67%) were resistant to gentamicin and 49 isolates (43.36%) were resistant to amikacin. Based on Antibiotic resistance pattern of these 113 isolates, highest resistance belonged to gentamicin and amikacin and lowest resistance belonged to tobramycin(Fig 1).

Based on PCR test, of all *Staphylococcus aureus* isolates resistant to aminoglycosides in primary screening, 53 isolates (46.91%) contained mecA gene. In addition, 43 isolates (38.51%) had aac(6')Ie/aph(2''), 19 isolates (16.81%) had aph(3')-IIIa1 gene and 22 isolates (19.46%) had ant(4')-Ia1 gene (Fig 2). In terms of gene frequency based on clinical samples, the highest frequency of genes was observed in samples of wound, urine and blood isolated from patients in Pediatrics Section, ICU and outpatient referral (table 1). There was a significant relationship between the presence of genes responsible for resistance to aminoglycoside and mecA gene. In addition, there was a significant relationship between the presence of some samples and the presence of genes responsible for resistance to β-Lactam and aminoglycoside antibiotics. A significant relationship was observed between isolates of blood, wound and urine and the frequency of aminoglycoside genes ($p \leq 0.011$), ($p \leq 0.041$), ($p \leq 0.029$).

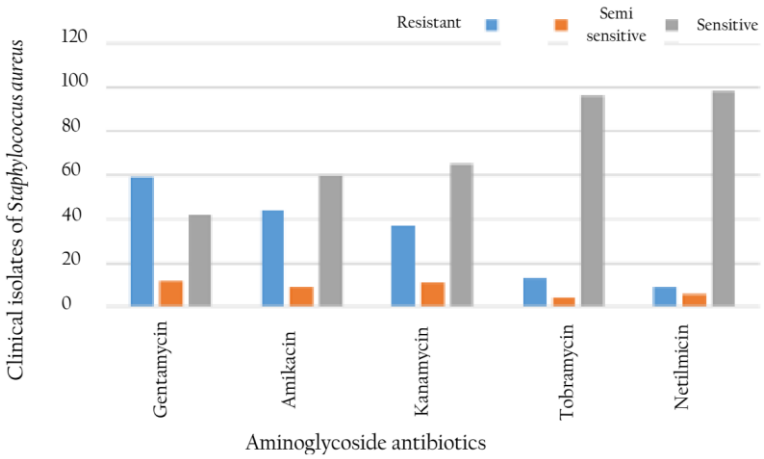

Figure 1. Frequency of antibiotic resistance of *Staphylococcus aureus* isolates to aminoglycoside

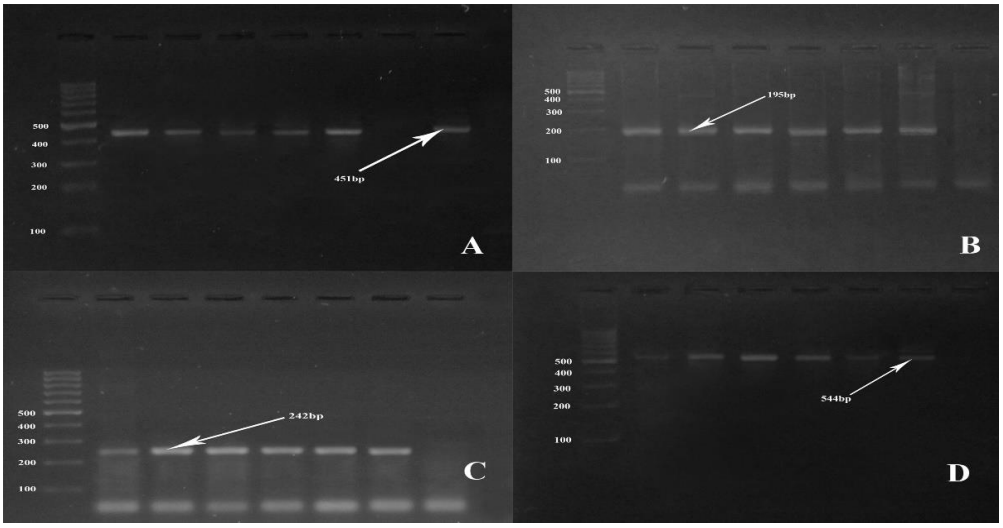

Figure 2. A: PCR test on 451 bp long aac(6') Ie/aph(2''), B: PCR test on 195 bp long ant (4')-Ia1, C: PCR test on 242 bp long aph(3')-IIIa1, D: PCR test on 544 bp long mecA. Well 6=*Staphylococcus aureus* (ATCC43300) as positive control. Well 5=*Staphylococcus aureus* (ATCC25923) as negative control. M: 100 bp marker. Wells 1 to 4: positive samples in terms of gene presence.

Table 1. The frequency of aac (6 ') Ie / aph (2 "), aph (3') - IIIa1, ant (4 ') - Ia1 genes and mecA in *Staphylococcus aureus* isolates based on the isolated clinical samples

Staphylococcus aureus Type of sample	ant(4')-Ia1(n=22) N(%)	aph(3')-IIIa1(n=19) N(%)	aac(6')Ie/aph(2")(n=43) N(%)	mecA(n=53) N(%)
Blood	0	2(10.52)	3(6.9)	5(9.4)
Urine	7(31.81)	5(31.26)	9(20.93)	13(24.52)
Catheter	0	5(31.26)	1(2.3)	5(9.4)
Wound	4(18.18)	3(15.78)	7(16.27)	8(15.09)
Secretions	0	0	0	3(5.06)
Phlegm	0	0	1(2.3)	2(3.7)
Nasal swab	4(18.18)	1(5.26)	9(20.93)	11(20.74)
Cerebrospinal fluid	1(4.03)	0	0	4(7.5)
Other cases	6(27.27)	3(15.78)	13(30.23)	5(9.4)

Discussion

A wide range of clinical samples in this study were obtained from urine culture, indicating the prevalence of this bacterium in the occurrence of urinary tract infections. After urine culture, blood culture and nasal swab culture constituted most cases. Of 113 Staphylococcus aureus isolates obtained from various samples, 61 isolates (53.98%) were identified to be resistant to methicillin using Oxacillin Etest strips in primary screening.

In addition, 55 *Staphylococcus aureus* isolates (48.67%) were resistant to aminoglycosides. Of these 55 isolates, 53 isolates were resistant to methicillin and this result is consistent with the study of Shokravi et al. (21). Ardic et al. in Turkey also demonstrated that the highest prevalence of resistant to aminoglycosides was found in methicillin – resistant *Staphylococcus aureus* (25). On the other hand, determination of resistance pattern to aminoglycoside antibiotics demonstrated that highest antibiotic resistance was found in gentamicin, which included 59 isolates (52.21%); this was consistent with the studies of Glad et al. in Kuwait (26), Abdal et al. in Qom (16), and Ghotaslou et al. in Tabriz (27).

However, it was not consistent with the study of Rahimi et al. in Isfahan and the level of resistance to gentamicin and kanamycin was higher than the present study (28). After gentamicin, amikacin (38.93%) and kanamycin (32.74%) showed the highest level of resistance. Sattari et al. in Tehran and Malek Hoseyni et al. in Ahwaz demonstrated that antibiotic resistance is far more than what is reported in the present study (29, 30). Such differences in some parts of the reports in different regions can be attributed to changes in the strains based on the geographical region. In a study by Shmitz et al. in Europe, the phenotypic prevalence of resistance to gentamicin and amikacin was reported in more than 30% of cases, which is completely different from the results of this study (31). 13 isolates (11.50%) and 28 isolates (24.77%) were respectively resistant to tobramycin and netilmicin antibiotics. These results were consistence with the results of Malek Hoseyni et al. (30). However, they were not consistent with the results of Sattari et al., reporting 30% resistance to tobramycin and netilmicin, which is higher than the results of our study (29).

Hammerberg et al. in England demonstrated that there is a significant relationship between resistance to aminoglycoside and methicillin (32). Several other studies is different regions of the world confirmed this fact. Other studies have also proved the relationship between resistance to methicillin and resistance to aminoglycoside (31 – 34). In addition, antibiotic resistance was not observed to aminoglycoside antibiotics in this study in MSSA isolates. Results of molecular experiments also demonstrated that aac (6') Ie/aph (2") gene had highest frequency in aminoglycoside – resistant isolates. This indicates the presence of aac (6') Ie /aph (2") gene as the most important mechanism of resistance to aminoglycoside in *Staphylococcus aureus* (35, 36).

Of 113 *Staphylococcus aureus* isolates, 53 isolates (46.91%) had mecA, which was responsible for resistance to methicillin. Of 53 isolates restant to methicillin, 43 isolates (38.05%) had aac (6') Ie / aph (2") gene, 19 isolates (16.81%) had aph (3') - IIIa1 gene and 22 isolates (19.47%) had ant (4') - Ia1 gene. Looking at the studies regarding identification of genes responsible for resistance to aminoglycoside antibiotics, one can find that the highest prevalence belongs to aac (6') Ie / aph (2") gene and then aph (3') - IIIa1 gene. These results have been reported by Shokravi et al. and Malek Hosseini et al. Aac (6') Ie / aph (2") gene has the highest frequency in this study.

However, ant (4 ') - Ia1 with a prevalence of 24.77% is substituted with aph (3') - IIIa1 with a frequency of 11.50%, which is not consistent with the results of the present study (21, 30).

In terms of frequency genes based on clinical samples, most genes were isolated from wound, urine and blood samples from patients in Pediatrics Section, ICU and outpatient referral, which is consistent with the studies of Bhatt et al. in India and Butin et al. in France; in these studies, the frequency of samples from Intensive Care Units was high (37, 38). In a study by Temiz et al. in Turkey, the frequency of aac (6') Ie / aph (2") was about 7% and the frequency of aph (3') - IIIa1 was about 10%, which is lower than the results of the present study (39).

In a study in South Korea, Choi et al. reported the prevalence of aac (6 ') Ie / aph (2 "), aph (3') - IIIa1 and ant (4 ') - Ia1 to be 83, 21 and 42%, respectively, which is higher than the results of the present study (36). These differences in results may be due to different geographic conditions, unique genotypic characteristics of bacteria, the location from which the sample is isolated and the type of samples. One of the most important factors that made the prevalence of antibiotic resistance, particularly aminoglycoside antibiotics, in this study different from other studies was the method, amount and period of using aminoglycoside antibiotics. We can prevent genetic transfers by modifying antibiotic administration pattern and not using various antibiotics in the process of treatment. Moreover, we can conclude that co-administration of different antibiotics from different classes may create multidrug-resistant (MDR) strains.

Acknowledgments

Hereby, we express our deepest sense of gratitude and indebtedness to Deputy of Research and Technology of Zahedan University of Medical Sciences for their financial support, Mr. Javad Adabi for his contribution with diagnostic steps of strains and Technical authorities of the Ali-ibn Abi Talib Hospital Lab in Zahedan, particularly Ms. Rigi and Ms. Mazaheri for their contribution with sample collection.

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