Frequency of $aac \,(6')\,-le\,-aph\,(2')$ gene and resistance to Aminoglycoside antibiotics in Staphylococcus aureus isolates


ABSTRACT

Staphylococcus aureus is one of the most common infectious bacterial species and one of the agents of community-acquired infections (CAIs) and hospital-acquired infections (HAIs). Aminoglycosides are potent bactericidal agents often used together with Beta Lactams or Glycopeptides, especially in treating Staphylococcal endocarditis. The present research aimed to determine the frequency of the $aac \,(6')\,-le\,-aph\,(2')$ gene that encodes Aminoglycoside modifying enzymes using PCR on clinical isolates of S. aureus. 115 clinical isolates of S. aureus were collected at educational hospitals in Karaj during 12 months. They were first identified by using standard biochemical and laboratory methods and, following CLSI principles and procedures, antibiotic sensitivity patterns of all isolates were obtained using the disc diffusion method. Moreover, using agar dilution, the minimum inhibitory concentration was determined using the antibiotic powder Gentamycin. Finally, gene frequency was measured by employing PCR. The highest levels of resistance to Aminoglycosides were observed in Kanamycin (47.8%), Gentamycin (46.9%), and Tobramycin (46.9%), and Doxycycline and Ciprofloxacin with 50.4 and 49.5 percent respectively, were the non-Aminoglycoside antibiotics to which the highest levels of resistance were exhibited. The frequency of the $aac \,(6')\,-le\,-aph\,(2')$ gene was 39.1 percent. Rapid and timely detection of resistant strains seems to be necessary in selecting suitable treatment options and in preventing the spread of resistance. Furthermore, rapid identification of genes that encode AME enzymes using PCR enjoys special advantages such as high levels of precision and speed.

Keywords:
AME Enzymes, Beta Lactams, Deep Abscesses, Endocarditis, Osteomyelitis, Pneumonia, Sepsis

1. Introduction

Staphylococcus aureus is one of the hospital- and community-acquired infection agents. It can also cause various types of diseases from relatively benign skin infections...
such as folliculitis and furuncle to life-threatening illnesses such as cellulitis, deep abscesses, osteomyelitis, pneumonia, sepsis, and endocarditis. At present, *S. aureus* shows resistance to a broad spectrum of antibiotics including Aminoglycosides, Beta Lactams, Tetracyclines, Fluoroquinolones, and Macrolides. Therefore, there is only a limited number of available antibiotics such as Vancomycin and Teicoplanin that can be used as anti-staphylococcal drugs to treat these types of infections [1, 2].

The appearance of staphylococcal infections has been increasing in recent years due to the spread of resistant strains, the increased number of patients with weakened immune systems, and excessive use of medical devices such as catheters [3, 4]. The reason for the prevalence of antibiotic resistance among *S. aureus* strains is the acquisition of many resistance factors by them. During the past few decades, there has been a considerable increase in the appearance of *S. aureus* strains resistant to Methicillin (MRSA) and to other antibiotics caused by their resistance to penicillinas, especially in HAIs [4].

Since strains resistant to Methicillin are also resistant to other Beta-Lactam antibiotics and to Cephalosporins, therapeutic regimens consisting of Vancomycin, Aminoglycosides, and other non-beta lactam antibiotics are employed to treat patients infected with these strains [5, 6]. Despite Aminoglycoside-induced nephrotoxicity and otoxicity and other problems related to increased resistance of microorganisms to these drugs, Aminoglycosides are still valuable in treating various staphylococcal infections and play an important role in treating and preventing infections caused by Staphylococci. Aminoglycosides have many features and that is why they are considered useful and valuable antimicrobial agents. Among these features are concentration-dependent bactericidal activity, post-antibiotic effect (PAE), and synergistic effects when used with other antibiotics such as Beta Lactams and Glycopeptides [7, 8].

Aminoglycosides are often used together with Beta Lactams and Glycopeptides to treat bacterial endocarditis caused by *S. aureus* [9]. These antibiotics interfere with protein synthesis in bacterial cells through binding to the 30S subunits of ribosomes [10]. The four mechanisms of antibiotic resistance are alterations at the ribosomal target sites of drug attachment, efflux systems, reduced drug permeability and uptake, and enzymatic drug inactivation. Among them, enzymatic drug inactivation is the main resistance mechanism both in Gram-positive and Gram-negative bacteria. This type of resistance, which is caused by Aminoglycoside-modifying enzymes (AMEs), is the main resistance mechanism in *Staphylococcus* spp. to Aminoglycosides. These enzymes are divided into five different classes the three more important ones of which are aminoglycoside acetyl transferases (AACs), aminoglycoside phosphoryl transferases (APHs), and aminoglycoside nucleotidyltransferases (ANTs) [11-13]. These three classes of enzymes are encoded by the *aph*(3')-IIIa, *ant* (4')-Ia , and *aac* (6') - *le-aph* (2”) genes [14-16].

Considering the high prevalence of antibiotic resistance and the potential of this bacterial species to cause serious diseases, the need for extensive and comprehensive research on *S. aureus* is clearly necessary. Therefore, the present research attempted to determine the frequencies of the genes encoding the enzymes that modify the Aminoglycoside antibiotics used against clinical isolates of *S. aureus* in this region. For this purpose, clinical isolates of *S. aureus* were collected from educational hospitals in Karaj and the frequency of the *aac* (6')-*le-aph* (2”) gene was determined using the molecular method.

2. Materials and Methods

2.1. Clinical isolates

One hundred and fifteen *S. aureus* isolates were isolated and identified in clinical samples taken from patients hospitalized in the educational hospitals of Karaj. All the *S. aureus* isolates were confirmed first using biochemical and laboratory tests including the catalase, DNase, slide/ tube coagulase, and mannitol fermentation tests [17]. In the current study, the PCR method was also employed besides the biochemical tests to
determine the identity of *S. aureus* isolates more accurately with respect to the presence of the *femA* gene, which is the proprietary gene for this bacterial species [18].

### 2.2. DNA extraction

To perform the PCR test, the DNA in each isolate was extracted first using the extraction method in which the Bioneer extraction kits (manufactured in South Korea) were used. 0.5 ml of each isolate was cultured in Luria broth using a shaking incubator at 37°C for 24 hours. The culture media containing the bacterial strains were then centrifuged at 5000 rpm for 20 minutes. 185 and 15 μl of TE buffer and recombinant Lysostaphin (produced by the Sigma Company) were added to the precipitate, respectively.

The mixture was put in the incubator at 37°C for 30 minutes. Next, as was mentioned above, Bioneer extraction kits (Genomic DNA kit extraction, Bioneer Inc., South Korea) were used as instructed by the manufacturing company (Figure 1). All the obtained DNA samples were measured employing a NanoDrop instrument and using the A260 to A280 ratio. Finally, all the isolates were confirmed using the PCR test with respect to the presence of the *femA* gene (which is the proprietary gene of *S. aureus* isolates) and employing the primers listed in (Table 1) [19, 20].

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**Table 1. The primers used in the present research**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5’-3’)</th>
<th>Size of amplified product (bp)</th>
</tr>
</thead>
</table>
| *aac (6)·le-aph (2)* | F-CAGGAAATTATCGAAAATGGTAGAAAAG  
R-CACACGACTAAAGAGTACCAATC | 369                           |
| *femA*     | F-GAAAAAAGCACATAACAAGGG  
R-GATAAAAGAAGAACGCCAGCAG | 132                           |

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### 2.3. The PCR test

The following conditions were used to confirm the presence of *aac (6)·le-aph (2)* gene. Twenty-five μl of the sample was used to perform the reaction. 200μmol of dNTP, 10 picomols of each primer, 1.5 mmol/l of MgCl2, 0.5 unit of the Taq polymerase, and 50 ng of the DNA template were used in each PCR test. Finally, the gene mentioned above was amplified employing a thermal cycler (Applied
Biosystems, USA) under the conditions mentioned in (Table 2) [19].

**Table 2.** Conditions under which the PCR test was performed

<table>
<thead>
<tr>
<th>gene</th>
<th>femA (Tm (ºC); Time(min))</th>
<th>aac (6')-le-aph (2') (Tm (ºC); Time(min))</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94:5</td>
<td>94:5</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94:2</td>
<td>94:2</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>58:2</td>
<td>45:5</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72:2</td>
<td>72:2</td>
<td>1</td>
</tr>
</tbody>
</table>

The PCR products were then studied using an electrophoresis instrument on 1% agarose gel and stained with SYBR Green to confirm the presence of aac (6')-le-aph (2') gene.

### 2.4. Phenotypic characterization of antibiotic resistance

The phenotypic method involving the disc agar diffusion (DAD) test was used to study resistance to Aminoglycoside antibiotics [21].

### 2.5. Disc agar diffusion (DAD)

To perform this test, Mueller-Hinton agar (MHA) medium was first spread to the depth of 4 mm in 12 cm plates and, after coagulation, the plates were put in an incubator at 37°C (according to CLSI instructions) to control contamination. The bacterial strains of interest were then cultured in nutrient agar culture and placed in an incubator at 37°C for 24 hours. Suspensions were prepared to equal the turbidity of a 0.5 McFarland standard using the bacteria that had grown in the nutrient agar environment [22]. Following that, sterilized swabs were used to inoculate the surface of Mueller-Hinton agar media with "lawns" of bacteria from the 0.5 McFarland suspensions in three different directions. After several minutes, antibiotic discs including 10 μg Gentamicin (GM), 30 μg Amikacin (AN), 30 μg Kanamycin (K), 10 μg Tobramycin (TOB) and 30 μg Netilmicin (NET) were placed on the medium 22 mm apart from each other and at a distance of 6 mm from the plate wall. The plates were then placed in an incubator at 37°C, and inhibition zones were read for all of the antibiotics after 18-24 hours using a ruler. The standard strain of *Staphylococcus aureus* ATCC25923 was used to control experiments for determining sensitivity to the antibiotics (Figure 2) [21, 23].

### 3. Results

One hundred and fifteen *S. aureus* isolates were taken for identification, and all of them were reported positive for the presence of the femA gene. Based on gender, age, and type of clinical samples, the isolates were distributed as follows:

Of the 115 *Staphylococcus aureus* isolates, 65, 40, and 10 isolates (56.5, 34.7, and 8.6%) were taken from male, female, and newborn patients, respectively. The average age of the hospitalized patients was 48.2 years. Sample separation based on clinical types is presented in Table 3. As shown in Table 4, the highest antibiotic resistance was shown to Kanamycin (47.8%). The prevalence rate of aac (6')-le-aph (2') gene (the cause of resistance to Aminoglycosides) among the 115 isolates of *S. aureus* was 39.1 percent.

**Table 3.** Distribution of the *S. aureus* isolates based on sample type

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>28</td>
<td>24.3</td>
</tr>
<tr>
<td>Phlegm</td>
<td>27</td>
<td>23.4</td>
</tr>
<tr>
<td>Wound</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Catheter</td>
<td>11</td>
<td>9.5</td>
</tr>
<tr>
<td>Urine</td>
<td>10</td>
<td>8.6</td>
</tr>
<tr>
<td>Abscess</td>
<td>9</td>
<td>7.8</td>
</tr>
<tr>
<td>Respiratory secretions</td>
<td>5</td>
<td>4.3</td>
</tr>
<tr>
<td>Joint</td>
<td>5</td>
<td>4.3</td>
</tr>
<tr>
<td>Nose</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Throat</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Pimple</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Tissue</td>
<td>2</td>
<td>1.7</td>
</tr>
<tr>
<td>Total</td>
<td>115</td>
<td>100</td>
</tr>
</tbody>
</table>
### Table 4. Antibiotic Sensitivity

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Sensitive (%)</th>
<th>Intermediate (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>(39.1) 45</td>
<td>(13.2) 29</td>
<td>(47.8) 55</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>(49.5) 57</td>
<td>(3.4) 4</td>
<td>(46.9) 54</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>(49.5) 57</td>
<td>(3.4) 4</td>
<td>(46.5) 54</td>
</tr>
<tr>
<td>Amikacin</td>
<td>(47.8)55</td>
<td>(6) 7</td>
<td>(46) 53</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>(62.6) 72</td>
<td>(11.3) 13</td>
<td>(26.1) 30</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>(43.4) 50</td>
<td>(6) 7</td>
<td>(50.4) 58</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>(43.4) 50</td>
<td>(6.9) 8</td>
<td>(49.5) 57</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>(61.7) 71</td>
<td>(1.7) 2</td>
<td>(36.5) 42</td>
</tr>
<tr>
<td>Mupirocin</td>
<td>(86.9) 100</td>
<td>(4.3) 5</td>
<td>(8.6) 10</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>(78.2) 90</td>
<td>(17.3) 20</td>
<td>(4.3) 5</td>
</tr>
</tbody>
</table>

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4. Discussion

The bacterial species *Staphylococcus aureus* is clearly a potent pathogenic agent that causes many infections. It is also one of the main agents of hospital- and community-acquired infections and has acquired multi-resistance to a broad spectrum of antibiotics including Beta Lactams, Aminoglycosides, Tetracyclines, Fluoroquinolones, and Macrolides. Moreover, it is the causal agent of various diseases and conditions including toxic shock syndrome, endocarditis, osteomyelitis, pneumonia, empyema, etc [24]. Despite their nephrotoxicity, ear toxicity, and problems associated with bacterial resistance, aminoglycosides continue to be effective, particularly in the treatment of staphylococcal infections [8].

Results of the present study indicated that 45 of the 115 isolates (39.1%) had the *aac(6′)-le-aph (2″)* gene. Studies conducted in other countries showed that *aac(6′)-le-aph (2″)* gene was the most prevalent gene encoding the Aminoglycoside Modifying Enzymes (AMEs) in clinical isolates collected in European countries [25, 26]. Moreover, similar results were obtained in previous study that *Staphylococcus aureus* isolates were resistant against kanamycin (%80), tobramycin (%71), amikacin (%53) and gentamicin (%31). The *aac(6′)-le-aph(2″)*-la gene at %44/24 of isolates of methicillin-resistant *Staphylococcus aureus* was detected. In the present study the highest antibiotic resistance was shown to Kanamycin which is consistent with previous study [27]. In a previous study, the obtained results showed that the *aac (6′)/aph (2″)-la* was the predominant gene. In this study, 78.3% and 72.2% of MRSA isolates carried the *aac (6′)/aph (2″)-la* gene in Tehran and Sari, respectively [28].

It has been used the disc diffusion method to study the resistance of 109 MRSA isolates to Aminoglycoside antibiotics in Iran. They found that 97, 96, 87, 93, and 80 percent of the isolates were resistant to Kanamycin, Tobramycin, Gentamycin, Amikacin, and Netilmicin, respectively. They also used the PCR method to determine the frequencies of the AME genes and they reported that *aac (6′)-le-aph (2″)* gene with 83 percent was the most frequent one followed by the *aph-(3)-IIIa* and the *ant-(4′)-la* genes with 71 and 26 percent of the total number of isolates, respectively [29]. In another study, it has been conducted, the results demonstrate that the *aac (6′)-le-aph (2″)* gene was dominant among the gentamicin-resistant strains of MRSA, and the isolates that were positive for this gene showed a high-level resistance to gentamicin [30].

In a study [31], *aac (6′)-le-aph (2″)* gene was determined in 64% of MRSA isolates (103/161), and interpreted as the most frequently held responsible for aminoglycoside resistance, followed by the *aph-(3)-IIIa* and *ant-(4′)-la* genes with 42.2% (68/161), and 11.8% (19/161) respectively. The resistance pattern of MRSA strains to aminoglycoside antibiotics was: gentamicin 136 (84.5%); amikacin 125 (77.6%); kanamycin 139 (86.3%); tobramycin 132 (82%); and neomycin 155 (96.3%) which is consistent with the results of the present study [31].

One of the reasons for the high level of resistance in the study mentioned above compared to the present research was that it has been studied and reported only the resistance of MRSA isolates to Aminoglycosides, whereas the present study investigated antibiotic resistance among 115 *S. aureus* isolates. With regard to the high rate of aminoglycoside-resistant *Staphylococcus aureus* isolates in our study, we recommend that the use of aminoglycosides against *S. aureus* infections must be limited in Iranian hospitals [32-35]. Previous studies revealed that the detection of resistance genes in antibiotic-susceptible strains is due to the...
amplification of repressed antibiotic resistance genes or AMEs of these strains display lower enzymatic activity [32-36]. Considering the increased prevalence of resistance to Aminoglycoside antibiotics combined with the excessive and uncontrolled clinical use of these drugs, rapid and timely detection of resistant strains seems to be necessary in order to select suitable treatment options and prevent the spread of resistance. Rapid and accurate detection of genes encoding AMEs using the PCR method enjoys special advantages because the use of the PCR method, which is a rapid and reliable technique, enables us to identify the gene causing the resistance in less than three hours.

5. Conclusion

Results of the present research and their comparison with other similar studies show that the frequency and prevalence of antibiotic resistance vary in different geographical regions. Therefore, this may be one of the reasons for the differences in the results of the various studies. Considering the increased prevalence of resistance to Aminoglycoside antibiotics combined with the excessive and uncontrolled clinical use of these drugs, rapid and timely detection of resistant strains seems to be necessary in order to select suitable treatment options and prevent the spread of resistance. Rapid identification of genes that encode AMEs using the PCR method enjoys special advantages such as high accuracy and speed.

Conflict of Interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No human or animals were used in the present research.

Consent for publications

All authors read and approved the final manuscript for publication.

Informed Consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors’ contributions

All authors had equal role in study design, work, statistical analysis and manuscript writing.

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