Identification and Determination of the Relationship between *ccr* Alleles and Antibiotic Resistance in Clinical Isolates of Methicillin Resistant *Staphylococcus aureus*

M. Vafaeefar (Msc)¹, M. Yousef Alikhani (PhD)¹, H. Tahmasebi (Msc)², M.R. Arabestani (PhD)³

¹Department of Microbiology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, I.R.Iran
²Department of Microbiology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, I.R.Iran
³Brucellosis Research Center, Hamadan University of Medical Sciences, Hamadan, I.R.Iran

ABSTRACT

BACKGROUND AND OBJECTIVE: Resistance to methicillin and the presence of the *ccr* gene in *Staphylococcus aureus* have provided the basis for the emergence of methicillin resistant strains. The aim of this study was to identify the *ccr* cassette alleles in methicillin-resistant *S.aureus* strains and to determine the relationship between the presence of these casts with a multivariate process.

METHODS: In this study, 135 clinical isolates of methicillin-resistant *S.aureus* was isolated by genotypic methods. *ccr* gene cassette was evaluated qualitatively by multiplex PCR method. Data was analyzed using SPSS version 16 and also, the chi - square test was used.

FINDINGS: Out of 135 strains of *S.aureus* resistant to methicillin, penicillin and erythromycin antibiotic resistance were the most frequent, more than 90%, respectively. Also, *ccr* gene cassette in the study on genes *ccrA*/B1, *ccrA*/B2, *ccrA*/B3, *ccrA*/B4, *ccrA2*/B, *ccrC* had taken place, respectively, in 2 isolates (1.3%) for gene *ccrA*/B1, 12 isolates (8.2%) *ccrA*/B2, 15 isolates (10.34) %) *ccrA*/B3, 2 isolates (1.3%) *ccrA*/B4, 4 isolates (8.2 percent) *ccrA2*/B and 22 isolates (15.87 %) were positive for the gene *ccrC*. A significant correlation between the presence of these genes and antibiotic distribution was observed (p=0.05).

CONCLUSION: The *ccr* gene cassette can provide a background of resistance to various antibiotics in methicillin-resistant *S.aureus* strains.

KEY WORDS: Antibiotic Resistance, Methicillin Resistance Staphylococcus Aureus, Ccr Cassettes.

Please cite this article as follows:


*Corresponding author: M.R. Arabestani (PhD)*

**Address:** Brucellosis Research Center, Hamadan University of Medical Sciences, Hamadan, I.R.Iran

**Tel:** +98 81 3838077

**E-mail:** mohammad.arabestani@gmail.com
Introduction

In 1961, resistance to penicillin was fully developed and went as far as in addition to penicillin this bacteria resistance to other drugs such as methicillin, nafcillin and oxacillin, and a wide range of other antibiotic groups such as fluoroquinolones (1, 2). Methicillin was one of the most commonly used antibiotics to treat it, and underlie the emergence of a new age in resistance to methicillin (3, 4).

Methicillin is a beta-lactam antibiotic that by binding to PBPs, inhibits trans-peptidases, prevents the formation of bacterial peptidoglycans, and subsequently destroys the cell wall, eventually leading to bacterial death (5). Staphylococcal aureus has been resistant to Methicillin after a while and has led to the emergence of a new generation of Staphylococcus aureus called Methicillin Resistant Staphylococcus aureus (MRSA). The presence of the mecA gene results in the development of methicillin-resistant Staphylococcus aureus (7).

The presence of some genetic cassettes in Staphylococcus aureus can, in addition to making beta-lactam resistances (such as methicillin), cause the emergence of some strains that resist a wide range of antibiotics. One of the most important staphylococcal aureus gene cassettes is the ccr gene cassette (8). Ccr is a recombinase encoder which is coded by a moving specimen in methicillin-resistant Staphylococcus aureus strains, which has been the basis for the classification of SCCmec. The joint presence of these sequences on this genetically modified element that was identified by Ito and colleagues in 1991 led to the consensus of SCCmec and ccr collections in a moving sequence (9).

The same has led to the formation of various types of ccr and SCCmec in different strains of Staphylococcus aureus. The recombinase produced by ccr can cause the cassette to move in the Staphylococcus aureus genome, and the enzyme also activates the entry/exit of the orfX site from region 5 (10). This massive gene complex is not fully recognized at present, but generally consists of three different groups, including ccrA, ccrB and ccrC. One of the properties of ccr sequences is the Grouping of Staphylococcus aureus bacteria based on different or similar allotypes. ccA and ccrB, which have the most allotypes (4 allotypes), have differences in nucleotide sequence and identity (11). Differences in nucleotide similarities cause differences or similarities in Allotype in different Staphylococcus species. The existence of more than 85% of the nucleotide similarity results in the classification of ccr based on similar allotypes, and a similarity of 60 to 80% leads to the classification of ccr by non-similar allotypes in one genus (12). CcrC in Staphylococcus aureus has more than 87% nucleotide similarity, resulting in similar allotypes in strains of this species. To classify resistant to methicillin strains, Staphylococcus aureus based on ccr should use common naming based on the presence or absence of one or more ccr in the studied strains (13).

It is likely that Staphylococcus aureus MDR strains that, in addition to penicillin and methicillin, have been resistant to some broad-spectrum antibiotics, transmit the genetic elements by the SCCmec genetic cassette. Meanwhile, the emergence of resistance to methicillin, in addition to the presence of SCCmec gene cassette, requires the presence of ccr as well as the J regions. In the meantime, ccr cassette may be observed in more resistant strains due to its structural and genetic diversity. In addition, the placement of SCCmec and ccr locus in the SCC cassette can form the basis for the emergence of multi resistant strains (12, 13). Therefore, the study on resistance profiles and an antibiotic resistance pattern in the presence or absence of ccr cassette can accelerate the identification of multidrug resistance strains.

Therefore, the aim of this study was to detect ccr cassette alleles in methicillin-resistant Staphylococcus aureus strains and to determine the association of these alleles with the multi resistant process.

Methods

This descriptive-analytic study was carried out after approval in the Ethics Committee of Hamedan University of Medical Sciences with 9510075757 code in 1395 by easy and accessible sampling. 510 clinical samples were collected from patients admitted to selected therapeutic centers of Hamedan University of Medical Sciences (Sina Hospitals, Behesht hospital) during 9 months. Patients admitted for more than two weeks and suspected of being infected with bacterial infections were included. Biochemical tests of catalase, coagulase, mannitol fermentation and DNA were isolated from Staphylococcus aureus.

NucA gene was used to confirm the genus and isolate species (Table 1). Finally, out of 510 clinical isolates, 269 isolates of Staphylococcus aureus were obtained (8). To determine the susceptibility of clinical isolates the antibiotic discs of erythromycin 15
micrograms, 10-unit penicillin, 10 micrograms of ciprofloxacin, 30 microgram of tetracycline, 30 microgram of amikacin, 30 micrograms of cefazolin, 10 micrograms of gentamycin, 10 microgram of norfloxacin and 15 micrograms of ciprofloxacin by Kirby- Bauer Disk Diffusion (Mast UK) method were used (17). First, the isolated colonies of the bacteria were cultured in a 5 mm thick Muller Hinton Agar (Merck Germany) medium after dissolving in physiological serum and preparing 0.5 MacFarland's dilution.

Antibiotic disks were then placed on the medium using a dispenser (Mast Germany). Afterwards, it was incubated for 24 hours at 35±2 °C. Resistance to methicillin was determined using disc diffusion method of Cefoxitin disc (30 micrograms). The results were evaluated using the latest CLSI version. To control the quality and evaluate the results, staphylococcus aureus ATCC25923 was used as positive control and Staphylococcus aureus ATCC43300 was used as positive control (11). For genomic DNA extraction, we used Sina-Gene extraction kit. To prepare the isolates for extraction, at first, confirmed isolates were sub cultured on Blood Agar with 5% sheep blood, and incubated for 24 hours at 37 °C. Then the genomic DNA extraction steps were performed based on the protocol of the manufacturing company. (18).

Prepared primers (Table 1) from the Macrogene company ordered by Pishgam Iran were used for the identification of ccrA/B1, ccrA/B2, ccrA/B3, ccrA/B4, ccrA2/B, ccrC and mecA genes. The reaction volume was 25 μL, containing 2 μL of the template DNA, 1 μL of each 25 picomol primer and 12.5 μL of MasterMixe (Ampliqon Germany) (containing Tris-HCl PH8.5, (NH4)2SO4, 3mM MgCl2 12.0.2% Tween 20, 0.4M mNTP, 0.2 unit Ampliqon polymers, Insert red dye and stabilizer). The residual volume with deionized distilled water was brought to the desired volume. For replication of the studied genes, the Eppendorf 5331 (American build) Thermocycler was used. Thermal cycles were as below: for initial denaturation at 95°C for 5 minutes, then 30 thermal cycles including secondary denaturation at 95 °C for 60 seconds, primer coupling for 95 seconds, initial amplification for 60 seconds at 72 °C, and for final replication for 10 minutes at 72 °C. In this study, the standard strain of Staphylococcus aureus ATCC43300 was used as a positive control. Staphylococcus aureus strain ATCC25923 was also used as negative control. For analyzing the obtained results, descriptive statistics (frequency, percentage and mean) were analyzed using SPSS software version 16. Also, Chi-square test was used to compare qualitative results and independent positive test to compare quantitative results and p≤0.05 was considered significant.

Results

Of 269 isolates of Staphylococcus aureus, 135 clinical isolates (50.18%) were identified as methicillin-resistant Staphylococcus aureus. Of these, 22 isolates (16.29%) of the wounds, 32 isolates (23.71%) of the blood, 41 isolates (31.6%) of the urine, 9 isolates (6.6%) of the chips, 6 isolates (42.4%) of the catheter, 10 isolates (7.5%) of the swab and 15 isolates (11.9%) were isolated from outpatients. Among the 350 clinical isolates of Staphylococcus aureus isolated from different clinical specimens, 59 isolates (43.3%) had a halo diameter of 14 and resistant to cefazidime; 87 isolates (75.1%) had a 25 mm halo diameter and resistant to 5 μg of Erythromycin, 115 isolates (83.3%) had a 29-mm halo diameter and resistant to10-unit penicillin, 105 isolates (73.53%) had a halo diameter of less than 18 and resistant to 15 μg of norfloxacin, 65 isolates 43.53%) had a halo diameter of less than 18 and resistant to 15 μg of gatifloxacin and 99 isolates (70.63%) with a halo diameter of 18 and resistant to 5 mg of fluxacin(Fig 1).

Also, ccr gene cassette was performed in ccrA/B1, ccrA/B2, ccrA/B3, ccrA/B4, ccrA2/B, ccrC genes in the present study 2 isolates (1.3%) for the ccrA/B1 gene, 12 isolates (8.2%) for the ccrA/B2 gene, 15 isolates (10 34.3%) for the ccrA/B3 gene, 2 isolates (3.1%) For the ccrA/B4 gene, 4 isolates (8.2%) were positive for the ccrA2/B gene and 22 isolates (15.8%) for the ccrC gene were positive (Fig 2).

There was a significant correlation between the presence of these genes and the distribution of antibiotics (p=0.05). Meanwhile, the highest frequency of studied genes was from urine and blood samples. There was also a significant relationship between multiple resistance strains and the presence of ccr caste genes. So that the values of P.value obtained for the ccrA/B1 gene was 0.039, for the ccrA/B2 gene 0.11, for the ccrA/B3 gene 0.026, for the ccrA/B4 gene 0.039 for the ccrA2/B was 0.041 and ccrC was 0.05 (Table 2).
Table 1. List of primers used to detect methicillin-resistant Staphylococcus strains and strains which have adhesion factors

<table>
<thead>
<tr>
<th>Reference</th>
<th>Amplicon length</th>
<th>Nucleotide sequence</th>
<th>Studied genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(14)</td>
<td>1022</td>
<td>CTT TCA CGA TAG ACA CAG TAA AAG AAG TTC ATA GCC GTT AAA TTG G</td>
<td>ccrA/B1</td>
</tr>
<tr>
<td>(14)</td>
<td>962</td>
<td>GCA TTC ATC ATC CAA AAT G CTA TAA CCT TCT GTG CTT TGC A</td>
<td>ccrA/B2</td>
</tr>
<tr>
<td>(14)</td>
<td>706</td>
<td>TCC GTA ATA AGA AGC AAC TTC AC ACT ATA GCC TTC AGT ACT TTG GA</td>
<td>ccrA/B3</td>
</tr>
<tr>
<td>(14)</td>
<td>1555</td>
<td>TGA AGA AGC ACA AGA GCG GC CTG CAC CAC ATT TTG GGC AC</td>
<td>ccrA/B4</td>
</tr>
<tr>
<td>(14)</td>
<td>518</td>
<td>CGTCTATTACAAGATGTTAAGGATAAT CCTTTATAGACTGGATTATTCAAAATA</td>
<td>ccrC</td>
</tr>
<tr>
<td>(14)</td>
<td>460</td>
<td>ATTCCTTGTATAATAGGCCYCTT TAAAGGCATCAATGCAAAACACT</td>
<td>ccrA2-B</td>
</tr>
<tr>
<td>(15)</td>
<td>583</td>
<td>AGAAGATGGTATGGAAGTTAG ATGTATGTGCGATTGTATTGC</td>
<td>mecA</td>
</tr>
<tr>
<td>(16)</td>
<td>270</td>
<td>AGCCAAGCGCTTGACGAACTAAAGC CGGATTGATGTTGAT ACGGT</td>
<td>nucA</td>
</tr>
</tbody>
</table>

Figure 1. Frequency of Antibiotic Resistance Pattern for Clinical Isolates of Staphylococcus Aureus

Table 2. Frequency of different types of locus ccR genes in Staphylococcus aureus isolates isolated from different clinical specimens

<table>
<thead>
<tr>
<th>Number of isolates with this gene</th>
<th>Number of different clinical samples</th>
<th>Studied gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Outpatient</td>
<td>Catheter</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>22</td>
<td>1</td>
<td>13</td>
</tr>
</tbody>
</table>
Identification and Determination of the Relationship between ...; M. Vafaeefar, et al

*Figure 2.* Electrophoresis result of successful reproduction of ccrA/B1, ccrA/B2, ccrA/B3, ccrA/B4 and ccrA2/B genes, with amplicon length of 1022 bp for the ccrA/B1 gene, 962 bp for the ccrA/B2, 706 bp for the ccrA/B3 gene and 1550 bp for the ccrA/B4 gene on the 2.5% agarose gel. Wells 1 to 6 positive samples in terms of the presence of genes, Well 7 positive control, Well 8 negative control. M Marker Lobe with a length of 100 bp. Staphylococcus aureus strain ATCC33591 was used as a standard strain of positive control and strain of Staphylococcus aureus ATCC25923 was used for negative control.

**Discussion**

The results of this study showed that the highest antibiotic resistance was to penicillin and erythromycin with an incidence of more than 90%. However, resistance to vancomycin, either intermediate or in full, was not seen in this study. The presence of Staphylococcus aureus as one of the most important bacteria in the occurrence of various types of diseases has been considered continuously. This bacterium placed in the hospital bacteria group that has exhibited a relative and even one hundred percent antibiotic resistance over a wide range of antibiotics over time, since 1950, the emergence of various and dangerous strains and sub-strains have been observed in this bacterium (19).

Methicillin-resistant Staphylococcus aureus (MRSA), which has been identified and introduced since the mid-nineteenth century, has been able to take so many victims. This dangerous strain, seen at the beginning of the emergence only in the hospitals and involved patients, after a short time came to the society and moved into different places as a carrier (20). In studies by Akia et al. in Saveh, it was found that more than 90 percent of the clinical studied isolates had a 100% resistance to penicillin, erythromycin and gentamicin antibiotics (21). Also, in studies by Tafaroji et al. in Qom, the highest resistance to penicillin and clindamycin antibiotics was observed (22). In Hamedan, Arabestani et al showed that the highest antibiotic resistance in Staphylococcus aureus strains is resistant to methicillin, penicillin, gentamicin and ciprofloxacin, which have a pattern with an abundance of more than 90% (23). Antibiotic resistance pattern in the present study was completely consistent with the above studies. Of course, in some studies in different cities of Iran, as well as in other countries, the different prevalence of antibiotic resistance can be seen.

This can be attributed to the culture of drug use, the correct pattern of prescription by physicians, the non-proliferation of mutated strains as well as the weather. The ccr gene cassette in this study was performed on ccrA/B1, ccrA/B2, ccrA/B3, ccrA/B4, ccrA2/B, ccrC genes, with a frequency of 3.1%, 8.2%, 34.10%, 1.3%, 75.2% and 15.87% respectively. In studies conducted by Urushibara et al. and Zhang et al. on the ccr gene cassette, the results were similar to those obtained in this study (24, 25).

Of course, in the study by Ito et al. in Japan, the results differed from those reported in the present study (10). In addition, in studies conducted by Havaei et al. in Isfahan, the ccrC gene was found to be the most frequent among ccr cassette genes (15). Also, Hill-Cawthorne and colleagues in Saudi Arabia showed similar genetic abundance (12). There was a significant relationship between the studied genes and the multidrug resistance strains. However, Petrelli and colleagues in Italy, Murugesan et al in India reported a significant relationship between the presence of ccr gene cassette and the presence of multidrug resistance strains (26, 27).

The probable association between the presence and activity of ccr cassette genes and multiple antibiotic resistance in methicillin-resistant Staphylococcus
aureus, along with resistance to beta-lactam antibiotics, can also be resistant to other antibiotic classes. With this in mind, the need for better and more accurate identification of methicillin-resistant Staphylococcus aureus strains is of particular importance.

Acknowledgments

Hereby, we would like to thank the Vice-Chancellor for Research and Technology of Hamedan University of Medical Sciences for financial support of this research.
References
17. CLSI. M100-S25 performance standards for antimicrobial susceptibility testing; Twenty-fifth informational supplement; 2015.


