



SNP Scanning in *MecA* Gene for Methicillin-Resistant *Staphylococcus aureus*

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Background: *Staphylococcus aureus* (SA) is known as an important human pathogen, which is responsible for many cases of both hospital and community-acquired infections all over the world. Studying on drug resistance is regarded as an important prevention strategy regarding these types of infections.

Objectives: The current study is aimed to assess the association between the single-nucleotide polymorphism (SNP) and resistance to antibiotics in the methicillin-resistant *Staphylococcus aureus* (MRSA) strains as well as the molecular typing of isolates, collected from the clinical samples.

Materials and Methods: We used the disc-diffusion method to test the isolates antibiotic resistance. In addition, the genotypes of staphylococcal cassette chromosome *mec* (SCC*mec*) in the Methicillin-resistant *Staphylococcus aureus* isolates were determined by multiplex -polymerase chain reaction (PCR). SNP was identified in the *mecA* gene using sequencing and amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method.

Results: The highest resistance was shown against oxacillin, and erythromycin and cephalexin. The most sensitive antibiotic was vancomycin (97%) and resistance to at least three antibiotic classes were identified in all isolates. Eighty six percent of isolates were positive for *mecA* gene and more than 50% of which were healthcare-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA). Moreover, SCC*mec* type 3, 1 were the predominant strains of the identified MRSA. Also, 23 isolates (23%) were non-typable. By using the ARMS-PCR method, it was found that 10% of the clinical specimens had SNP in the *mecA* gene.

Conclusion: According to the Chi-square test (χ^2), it reveals that the association between SNP in the *mecA* gene and oxacillin, cefoxitin, and erythromycin resistance was confirmed among clinical MRSA. Furthermore, there is a 95% probability of association between SNP and resistance to more than three antibiotics in MRSA strains.

Keywords: Antibiotic resistance, ARMS-PCR, Methicillin-resistant *Staphylococcus aureus*, Molecular Typing, SNP

1. Background

S. aureus is known as an important pathogen with clinical significance which is responsible for various diseases, such as skin infections and other life-threatening infectious diseases including pneumonia, bacteremia, endocarditis, and toxic shock syndrome (1). Introduced in 1959, Methicillin was a semisynthetic beta-lactamase resistant penicillin. Soon after that methicillin resistant SA and coagulase-negative staphylococci were isolated. In early 1960s, MRSA infection outbreaks happened in the Europe (2). Thereafter, MRSA is known as a main caused for both nosocomial and community-acquired infections. It is found that three pandemic MRSA strains originated from the MRSA which were isolated in 1959 in Denmark and England (3). Multi-resistant *S. aureus* isolates emerged and

turned to the responsible pathogen for both nosocomial and community-acquired infections because of the excessive antibiotic therapy (4). The MRSA genome, containing 21 to the 67-kb heterologous mobile genetic element, known as staphylococcal cassette chromosome *mec* (SCC*mec*) carrying the *mecA* gene besides the other resistance determinants. Synthesis of an altered penicillin-binding protein, namely PBP-2a, which is expressed by *mecA* gene mediates methicillin resistance (5, 6, 7, 8, 9). SCC*mec* description relies on disparities of the *mec* gene complex, cassette chromosome recombinase (*ccr*) complex, in addition to the junkyard regions. Five SCC*mec* types (1 to 5) have been established in *S. aureus* (10, 11, 12, 13). Also, SCC*mec* 6 and 7 types have been identified, despite being rare (14).

SNP is defined as variations found in a single nucleotide at a specific site of the genome, in which each variation is found to some considerable degree in a population. SNPs are found in coding, non-coding, and intergenic regions. Note that SNPs in the coding sequence may not inevitably modify the sequence of amino acids of the synthesized protein, because of the genetic code degeneracy. SNPs of the coding region fall into two categories: non-synonymous and synonymous SNPs. Contrary to non-synonymous SNPs, the amino acid sequence of proteins is not affected in the synonymous SNPs do not affect the protein sequence. Of note, the non-synonymous SNPs may be nonsense or missense (15).

Yet, non-coding SNPs may still modify splicing of gene, binding of the transcription factor, degradation of the mRNA, as well as the noncoding RNA sequences. This type of gene expression modification is called eSNP (expression SNP) which occurs both and upstream or downstream to the gene (16).

2. Objectives

The major objective of the current study was scanning and finding SNP(s) in the *mecA* gene using sequencing technique and to select the best one for association study. Finding the association between SNP and resistance to the antibiotic can help to evaluate bacterial antibiotic resistance pathway.

3. Materials and Methods

Collectively, we isolated 115 *S. aureus* strains from admitted patients in different hospitals of Tehran, Iran during January, 2016 to September, 2016. These strains were isolated from various clinical samples, such as blood samples, urine samples, wounds, CVP, and sputum. Demographic data of the enrolled patients were collected via medical records and sometimes by interview. Informed written consents have been received from all patients who enrolled in the study and approved by the authorized board of ethics at Iran University of Medical Sciences. To identify the *S. aureus* isolates, culture studies were performed in addition to the conventional biochemical and morphological tests, such as catalase test, Gram staining, tube coagulase test, mannitol fermentation, slide coagulase test, and DNase test. The samples were inoculated on to mannitol salt agar (Merck, Germany) and incubated at the temperature of 37 °C for 48 hours. *S. aureus* was identified considering the morphology of the colony morphology, as well as Gram staining and the biochemical tests including coagulase and catalase tests.

Isolates were kept at the temperature of -70 °C in tryptic soy broth (Merck, Germany) which was supplemented with 15% glycerol. The disk diffusion method was

used to identify antimicrobial agent susceptibility of *S. aureus* on Mueller-Hinton agar medium, based on the guidelines of the clinical and laboratory standards institute (CLSI) (17).

The following antibiotics were tested in this study: gentamicin (10 µg), vancomycin (30 µg), oxacillin (1µg) , amikacin (30µg), cephalexin (2µg), cefoxitin (30µg), clindamycin (2µg), and erythromycin (15µg) and methicillin (30 µg) (HiMedia, India). To control the quality of antibiotic susceptibility tests, strain ATCC25923 of *S. aureus* was used.

3.1. Genotypic Detection of *mecA* Gene

In order to genotype *mecA* gene locus, DNA was extracted from all the isolates. We inoculated a single colony on 15 mL of the brain heart infusion broth. It was incubated overnight, at the temperature of 37 °C using the DNA extraction kit (CinnaGen, Iran) according to the provided instructions by the manufacturer. ATCC 33591 and ATCC 25923 Typing Culture Collections were used as *mecA* positive and negative, respectively. The 147 bp fragment of the *mecA* gene was amplified through PCR using the specific primers presented in **Table 1**. The mixture of amplification reaction (25 µL) content included 4 µL of DNA template (100 ng), 2.5 µL of PCR buffer (10X), 1 µL of Forward primers (10 PmoL), 1 µL Reverse Primers (10 PmoL), 0.75 µL of MgCl₂ (50 mM), 0.5 µL of deoxynucleotide triphosphates (dNTPs, 10 mM), 0.25 µL of Ex-Taq DNA polymerase (5 u.µL⁻¹), and 15µL of distilled water. The PCR conditions were set as follows: primary denaturation was performed at the temperature of 94°C for 5 minutes. Next, 30 cycles of denaturation were conducted at the temperature of 94 °C for 30 seconds. Thereafter, the annealing process was done at the temperature of 55 °C for 90 seconds, followed by extension at the temperature of 72 °C for 90 seconds. Lastly, the final extension was done at the temperature 72 °C for one minute.

3.2. Multiplex PCR for SCC_{mec} Typing

We performed SCC_{mec} typing for one hundred MRSA isolates using multiplex PCR method, for which we used the primers listed in **Table 1**. Each round of PCR was conducted with a final volume of 15 µL, composed of 2 µL of DNA template, 1.5 µL of PCR buffer (10X), 0.75 µL of MgCl₂ (50 mM), 0.6 µL of dNTPs (10 mM), 0.3 µM of F, R from each nine primers, 0.25 µL of Ex-Taq DNA polymerase (5 u.µL⁻¹), and 7.3µL of distilled water. Multiplex PCR was done in the conditions: primary denaturation was done at the temperature of 94 °C for 15 minutes, followed by 10 cycles of denaturation at the temperature of 94 °C for 45seconds. Next, annealing

Table 1. Nucleotide sequences of the primers used for the Multiplex PCR for the typing and subtyping of MRSA SCCmec [19].

Primers	Oligonucleotide sequences (50–30)	Concentration (mM)	Amplicon size (bp)	Specificity
Type 1-F Type 1 -R	5'-GCTTTAAAGAGTGTGCGTTACAGG-3' 3'-GTTCTCTCATAGTATGACGTCC-5'	0.2	613	SCCmec 1
Type 2-F Type 2-R	5'-CGTTGAAGATGATGAAGCG-3' 3'-CGAAATCAATGGTTAATGGACC-5'	0.2	398	SCCmec 2
Type 3-F Type 3-R	5'-CCATATTGTGTACGATGCG-3' 3'-CCTTAGTTGTCGTAACAGATCG-5'	0.2	280	SCCmec 3
Type 4a-F Type 4a-R	5'-GCCTTATTCGAAGAAACCG-3' 3'-CTACTCTTCTGAAAAGCGTCG-5'	0.2	776	SCCmec 4a
Type 4b-F Type 4b-R	5'-TCTGGAATTACTTCAGCTGC-3' 3'-AAACAATATTGCTCTCCCTC-5'	0.2	493	SCCmec 4b
Type 4c-F Type 4c-R	5'-ACAATATTTGTATTATCGGAGAGC-3' 3'-TTGGTATGAGGTATTGCTGG-5'	0.2	200	SCCmec 4c
Type 4d-F Type 4d-R	5'-CTCAAATACGGACCCCAATACA-3' 3'-TGCTCCAGTAATTGCTAAAG-5'	0.2	881	SCCmec 4d
Type 5-F Type 5-R	5'-GAACATTGTTACTTAAATGAGCG-3' 3'-TGAAAGTTGTACCCTTGACACC-5'	0.2	335	SCCmec 5
MecA-F MecA-R	5'-GTGAAGATATACCAAGTGATT-3' 3'-ATGCGCTATAGATTGAAAGGAT-5'	0.2	147	mecA

was done at the temperature of 55 °C for 45seconds. Thereafter, extension was done at the temperature of 72 °C for 75 seconds, followed by 25 cycles of denaturation at the temperature of 94 °C for 45 seconds. Afterward, annealing was performed at the temperature 50 °C for 45 seconds, and extension at the temperature of 72 °C for 75 seconds. The final extension was done at the temperature of 72 °C for 10 minutes (18).

The PCR products (3 µl) were conducted on a 1.5% agarose gel in 0.5X TBE buffer (110 mM Tris; 90 mM Borate; 2.5 mM EDTA; pH 8.3). Staining was done in ethidium bromid.

PCR products were sequenced (Macrogen company in South Korea) in order to scan new SNP(s). The most sensitive and the most resistant samples were chosen for sequencing (sample 51, 72).

3.3. ARMS PCR

SNP detection in other MRSA samples was performed by the ARMS PCR method. After detecting SNP

in the *mecA* gene, suitable primers for ARMS PCR reaction were designed by Oligo software. The mixture of amplification reaction (25 µL) consisted of 4 µL of DNA template, 2.5 µL of PCR buffer (10X), 0.75 µL of MgCl₂ (50 mM), 0.5 µL of deoxynucleotide triphosphates (dNTPs, 10 mM), 1 µL of each primer (2 µL collectively), 0.25 µL of Ex-Taq DNA polymerase (5 u.µL⁻¹), and 15µL of distilled water. The following PCR conditions were set: primary denaturation was done at the temperature of 94 °C for 5 minutes, followed by 30 cycles of denaturation at the temperature of 94 °C for 30 seconds. Next, annealing was conducted at the temperature of 58.9 °C for 90 seconds, and extension at the temperature of 72 °C for 90 seconds. The final extension was performed at the temperature of 72 °C for 60 seconds. For this reaction, 2 forward primers and 1 reverse primer were used (**Table 2**). Thus, two reactions were performed for each sample with two forward primers in two separate microtubes. ARMS PCR was used to amplify the 250 bp fragment gene.

Table 2. Nucleotide sequences of the primers used for the ARMS-PCR

Primers	Oligonucleotide sequence(5'-3')	Concentration(mM)	Amplicon size(bp)
Forward 1	5'-CAGATCCTTTCAATCTATAGCGCATT -3'	0.2	250
Forward 2	5-' CAGATCCTTTCAATCTATAGCGCATA-3'	0.2	250
Reverse	5-' GTCTGCCACTTTCTCCTTGTTTC-3'	0.2	250

3.4. Statistical Analysis

SPSS software version 18 (SPSS Inc., Chicago, IL, USA) was used to calculate the rate of resistance to various and the analyze the association between SNP and antibiotic resistance was evaluated by Fisher's exact test or Chi-square.

If any expected frequency is below 1 or 20% of the expected frequencies are below or equal to 5, we should use the Fisher's exact treatment of the fourfold table instead of the chi-square test.

After ensuring the association between the SNP found in the *mecA* gene and the antibiotic resistance by Chi-square test (χ^2), by the Odds ratio, the strength of this SNP relationship with antibiotic resistance was studied. To this end, (CI) was calculated according to the data provided by www.medcalc.org.

4. Results

4.1. Characterization and Susceptibility of Clinical Isolates of *Staphylococcus aureus* to Antimicrobial Agents
During nine months, 115 *S. aureus* isolates from different clinical samples were gathered from hospitals of Tehran. These isolates were gathered from urinary samples (n =15, 13%), blood samples (n =4, 3.4%),

wound samples (n=81, 70.4%), CVP samples (n=9, 7.8 %), and sputum samples (6, 5.2%). Collectively, 37 (32.1%) samples were obtained from women and 78 (67.8%) samples from men. Patients mean age was 50 years ranging from 1 year to 89 years).

Out of 115 samples, 100 samples having *mecA* gene were detected by PCR. The disk diffusion method was used for the detected samples. The results showed that *S. aureus* resistance to vancomycin (2%), gentamicin (81%), amikacin (61%), cephalexin (79%), ceftiofur (67%), oxacillin (72%), clindamycin (73%) and erythromycin (80%) (**Table 3**).

4.2. PCR for SCCmec Typing

The *mecA* gene was identified in 86% (n=100) of the 115 strains which were tested by the PCR method. Lastly, *S. aureus* isolates were assessed to determine the SCCmec type of MRSA isolates. According to the results, 24 (24%), 10(10%), 26(26%), 7(7%) and 2(2%), 4(4%), 0(0%), 4(4%) of MRSA isolates were positive for SCCmec, 1, 2, 3, 4a, 4b, 4c, 4d, and 5, respectively. Furthermore, 23 isolates (23%) were non-typable (**Fig. 1, 2**). The HA-MRSA prevalence of was 57.7% (n = 55) in this study, which is above CA-MRSA.

Table 3. Antibiotic resistance in the 100 MRSA isolated were positive for *mecA* gene

Antibiotic	clindamycin	cephalexin	vancomycin	gentamicin	erythromycin	amikacin	ceftiofur	oxacillin
Sensitive(n)	15	10	97	11	10	32	26	19
Resistance(n)	85	90	3	89	90	68	74	81

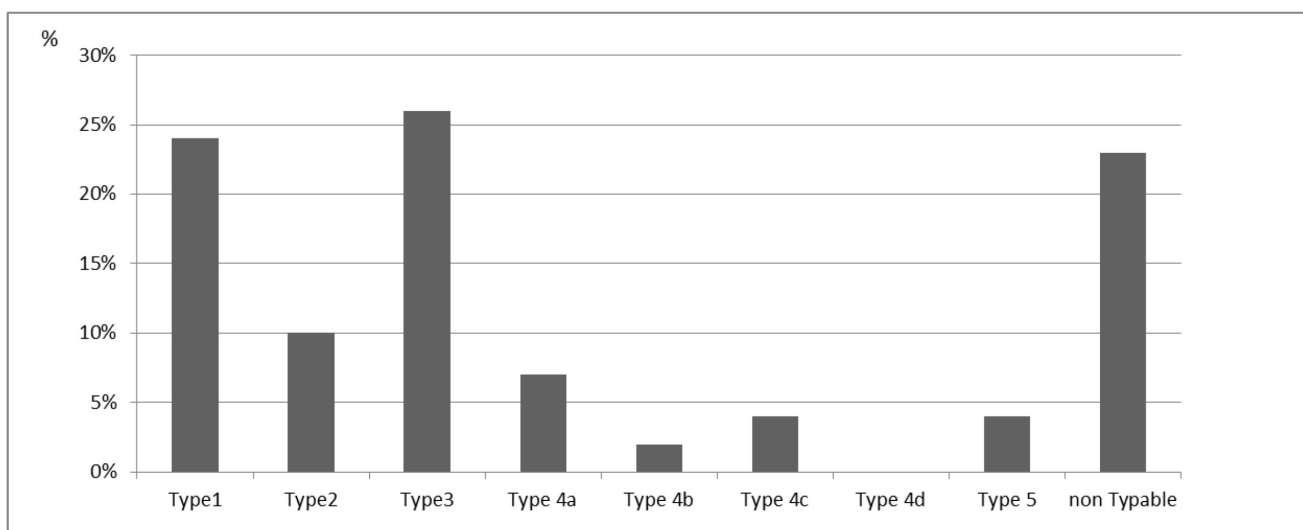


Figure 1. SCCmec types in the clinical samples collected from Tehran hospitals. Scmec type3, 1(26%,24%) were the predominant strains of the identified MRSA. 23(23%) samples were recognized as non-typable and SCCmec 4d was not observed in this study.

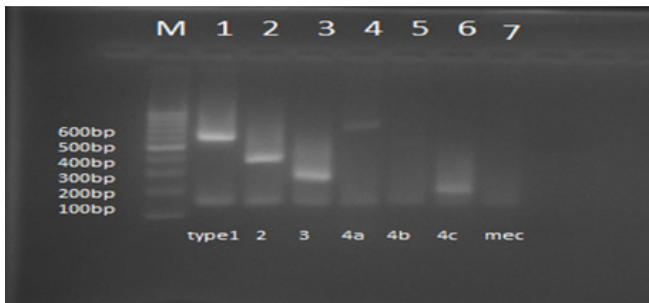


Figure 2. Agarose electrophoresis of multiplex PCR. Lane1, SCC mec type 1 (613 bp), Lane 2, SCC mec type 2 (398 bp), Lane 3, SCC mec type 3(280 bp), Lane 4, SCCmec type 4a (776bp), Lane5, SCC mec type 4b (493bp), Lane 6, SCCmec type 4c(200bp), Lane 7, mecA (147 bp).

4.3. Sequencing and ARMS-PCR

By alignment the results of sequencing with the complete genome of *Staphylococcus aureus* strain 12 MecA(mecA)(GU227428), in the NCBI (<https://www.ncbi.nlm.nih.gov/>) website, it was found that T nucleotide at the position 116 in the *mecA* gene was altered to nucleotide A, in the oxacillin susceptible *mecA* positive *S. aureus* clinical strain No. 51. However, this nucleotide change was not observed in the oxacillin-resistant specimen No. 72.

The results of ARMS-PCR reaction showed that only 9 samples from all clinical specimens, such as sample 51, had SNP in the *mecA* gene or, in the other words, 10% of the clinical specimens had SNP in the *mecA* gene (Fig. 3).

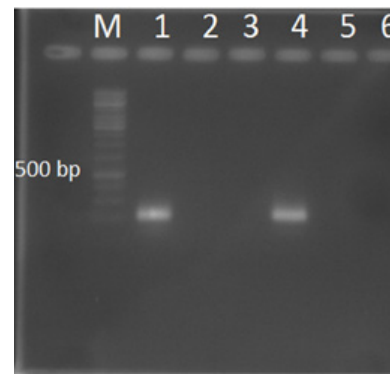


Figure 3. Agarose electrophoresis of ARMS PCR. Lane1, PCR productsample72 with Forward Primer that it has 3' endT. lane2, PCR product sample72 with Forward Primer that it has3' end A. Lane3, PCR product sample 51 with Forward Primer that it has 3' endT. Lane 4, PCR product sample 51 with Forward Primer that it has 3'end A. M, Marker 1kb

4.4. Association Between SNP and Antibiotic Resistance

Based on the Chi-square test, SNP's association with resistance to antibiotics was examined and it was determined whether this SNP identified in this study is related to the antibiotic resistance. Due to the fact that 20% of the strains had numbers less than 5, the Fisher's exact test was used. The result showed that *P* value is less than 0.05, in this means there is an association between SNP and antibiotic resistance. According to **Table 4** CIs in all cases do not cross one, this mean SNP has a major effect on antibiotic resistance pathway.

Table 4. Association between SNP and resistance to antibiotics

Nucleotide	S	R	S	R	S	R	S	R	R	R
	OXA	OXA	FOX	FOX	ERY	ERY	AN	AN	More than3 antibiotic	Less than3 antibiotic
A(n)	6	4	9	1	9	6	6	4	7	3
T(n)	13	77	17	73	6	84	26	64	11	79
P	0.002		0.00		0.008		0.05		0.000	
OR	8.88		38.6		9.33		3.6		16.75	
CI	2.2019-35.84		4.58-325.98		2.5-42.34		0.9622-14.16		3.76-74.53	

S=Sensitive, R=Resistant, OXA= oxacillin, FOX= cefoxitin, ERY= erythromycin, AN= amikacin, OR= Odds ratio, 95% CI = 95% confidence interval, n=Nucleotide, A=Adenine (SNP), T=Thymine,

P= *P* value. *P* value is less than 0.05, in this means there is an association between SNP and antibiotic resistance. If a 95% CI for the odds ratio includes the null value of 1, then there is insufficient evidence to conclude that the groups are statistically significantly different. SNP has a major effect on oxacillin, cefoxitin, and erythromycin resistance pathway. It is 95% probable; this SNP was associated with resistance to more than three antibiotics.

5. Discussion

Staphylococcus aureus is known as an important human pathogen and it is the major cause of both nosocomial and community-acquired infections over the past decades. Identifying the rapid treatment of MRSA infections is among the most important preventive actions against the

infection spread and reduce the risk of mortality in patients. MRSA infections are classified into two categories, HA-MRSA, and Community-Associated-MRSA (CA-MRSA). These classifications are identified based on the mobile genetic elements source, SCCmec (20).

SCCmec is the key factor which defines MRSA infections

source in society. MRSA infections may be managed using the best protocol for treatment if we manage to differentiate and identify HA-MRSA and CA-MRSA (19). Any kind of SCCmec consist of exclusive genetic elements. The *mecA* gene identification using PCR test is more sensitive and accurate compared with the oxacillin disc test. As a result, we identified the *mecA* gene using PCR test. Based on our findings, the *mecA* gene was present in 86% of the 115 studied strains, while 72% of the strains were oxacillin-resistant, as previously stated. In the phenotypic method, 83 strains oxacillin-resistant while the *mecA* gene was found in 81 strains.

This result is comparable to the findings of the Tokue and Choi *et al.* study. Their study showed that among 57 strains of *Staphylococcus aureus* in the phenotypic method, 22 strains were oxacillin-resistant and the *mecA* gene was found in 28 strains (21). In addition, Choi *et al.* study showed that 54 isolates of a total of 92 *S. aureus* isolates contained *mec* gene (22). In another study by Taherikalani *et al.*, all MRSA strains isolated from clinical samples containing the *mecA* gene (23).

As previously mentioned, 3% of isolates were vancomycin-sensitive according to the results of antibiotic susceptibility test. Oxacillin-resistance was below 80%, and resistance to gentamicin, amikacin, cephalexin, erythromycin, cefoxitin, and clindamycin were between 60% to 85% (**Table 3**). However, some antibiotics, including vancomycin, still affected MRSA in this study; suggesting that this agent can be applied in the MRSA infections treatment. The highest resistance to oxacillin, methicillin and erythromycin was observed, which was similar to the results of Moon and colleagues in 2007 (24). In this study, 5 types of SCCmec were identified, with type 3 being the most common. This finding is in consistency with earlier reports on the SCCmec type 3 predominance in most countries in Asia (25), especially in Iran (26). Consistent with and supported by the results of other studies. the other predominant types of SCCmec were type 1 (24%) and type 2 (10%) respectively (27). SCCmec 4d was not identified in the current study.

In a study carried out in Spain by Vazquez in 2009 (28) on several samples, SCCmec type 4 was the most common isolate, while SCCmec type 3 was the most frequently identified in a Neela *et al.* study conducted in Malaysia in 2010 (29).

In this study, out of 100 MRSA samples, 23 samples were recognized as non-type able. These strains in this study are significantly more than other studies (28). The non-type able isolates may be due to possibly new types or new sub types. These observations, as explained by other researchers, may be attributed to the

existence of new types of structures and new structures or reconstruction of structures and recombination of the *mec* element (30, 25). Additional studies, including the *mec* element sequencing, is now required to describe these currently non-type able isolates.

Multidrug resistance was more commonly observed in type 3 and 1 strains. In a similar study in 2016 in North of Iran, just SCCmec type 3 had the most multidrug-resistance (26). According to other studies, SCCmec types 1, 2, 3 are identified as HA-MRSA while CA MRSA isolates are associated with SCCmec types 4 and 5 (31). The HA-MRSA prevalence was 57.7% (n=55) in this study, which is greater than CA-MRSA. Strandén *et al.*, and Kumari *et al.*, (32, 33) reported similar findings in this regard. The sequencing of the *mecA* gene was performed to SNP scanning. Then, ARMS-PCR was used to determine SNP in other strains of MRSA. The results of the ARMS-PCR reaction showed that 10% of the clinical specimens had SNP in the *mecA* gene.

According to the Chi-square test, it was found that there is 95% association between the SNP in the *mecA* gene and resistance to cefoxitin, oxacillin, and erythromycin antibiotics among the clinical MRSA strains. It was also found that with 95% probability, this SNP was associated with resistance to more than 3 antibiotics. The *mecA* gene found in MRSA leads to bacterial resistance to antibiotics such as penicillin, methicillin, and the other penicillin-like antibiotics. This gene encodes the PBP2A (penicillin-binding protein 2A) protein. PBP2A affinity for antibiotics of beta-lactam group, including methicillin and penicillin, is low.

6. Conclusion

In the current study, the SNP detected in the *mecA* gene has led to the conversion of leucine 538 to the stop codon. As a result, the mutated protein has 131 amino acids shorter than normal proteins. These depleted amino acids form a significant part of the C-terminal domain of the protein. This domain is responsible for the activity of trans-peptidase. Since a number of amino acids in the active site have been removed and the 3D structure of the C-domain is damaged, therefore, the mutated protein cannot have trans-peptidase properties. This is likely to make strain 51 different from phenotype characteristics. Or, in other words, be sensitive to oxacillin when the *mecA* gene is present.

The current study is aimed to detect new SNP(s) in the *mecA* gene, and assess the association between SNP and antibiotics resistance in the MRSA isolates and the type of SCCmec of MRSA isolates in patients admitted to three hospitals, in Tehran, Iran. From a total of 100 studied MRSA isolates during 2015–2016 using multiplex PCR

assay, the most frequently identified SCCmecs were type 3 (26%) and type 1 (24%). According to findings of this study, MRSA isolates probably originated from clonal diversion of MRSA or HA-MRSA. Also, there was a 95% association between the *mecA* gene SNP and the resistance to cefoxitin, oxacillin, and erythromycin antibiotics among the clinical strains of MRSA.

The findings of this study suggested a high-level of gentamicin, erythromycin and cephalixin resistance among the isolates of *Staphylococcus aureus*. Therefore, in order to select an appropriate antibiotic to manage the infections caused by *Staphylococcus aureus* and to prevent resistance to antibiotics, physicians are recommended to prescribe appropriate antibiotics considering both efficacy and availability.

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