

# Antimicrobial Resistance and Molecular Characteristics of Methicillin-resistant *Staphylococcus aureus* Isolates from Children Patients in Iran

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**Introduction:** Methicillin-resistant *Staphylococcus aureus* (MRSA) causes high rates of mortality and a substantial burden to health systems worldwide. Here, we investigated the antimicrobial susceptibility and molecular characteristics of MRSA isolated from children referred to Children's Medical Center in Tehran.

**Materials and methods:** A total of 98 MRSA isolates were collected from children. Antimicrobial resistance patterns were determined using the disk diffusion and E-test methods. The presence of biofilm encoding genes and the *pvl* gene were determined by PCR. We used the microtiter plate method to assess the ability of biofilm formation. The MRSA isolates were further analyzed using PFGE and SCCmec typing.

**Results:** Antibiotic susceptibility testing showed that the highest and the lowest antibiotic resistance percentage were related to erythromycin (62%) and minocycline (10%), respectively. Overall, 63% of MRSA isolates were biofilm producers. Resistance to two antibiotics such as erythromycin (72% vs 28%,  $P=0.01$ ) and clindamycin (71% vs 29%,  $P=0.04$ ) was higher among biofilm producers than non-biofilm producers. All strains had biofilm-forming genes and the prevalence of *pvl* gene was 41%. Most MRSA isolates belonged to SCCmec IVa (75%) and SCCmec III (18%). In PFGE technique, 5 common types and 2 single types were identified; Common type 1 with 37 isolates was dominant clone.

**Conclusion:** We thus report preliminary data on the prevalence and distribution of MRSA genotypes in Tehran Children's Hospital. These findings characterize the MRSA colonization dynamics in child patients in Iran and may aid the design of strategies to prevent MRSA infection and dissemination.

**Keywords:** MRSA, biofilm formation, antibiotic resistance, SCCmec typing, PFGE

## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common pathogen causing various forms of infectious disease in humans.<sup>1</sup> Children colonized with MRSA are potential reservoirs for the spread of MRSA in the community.<sup>2</sup> Furthermore, immunologically immature infants and newborns, especially those born prematurely or requiring specialized care, are most susceptible to MRSA infections.<sup>3</sup>

MRSA biofilm formation is regulated by the expression of polysaccharide intracellular adhesion (PIA), which mediates cell to cell adhesion and is encoded by the *icaADBC* operon.<sup>4</sup> Moreover, surface-associated proteinaceous adhesins can contribute to the adherence, colonization and biofilm formation of MRSA. This pathogen

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can express a variety of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), such as fibronectin-binding proteins A and B (FnbA, FnbB), clumping factors A and B (ClfA, ClfB), collagen-binding protein (Cna) and enolase protein (eno). Biofilm formation interferes with bacterial recognition and killing mechanisms of the innate immune system.<sup>5,6</sup>

A number of methods have been created for the detection of biofilm formation ability. Currently, several different methods are used, such as tube test, microtiter plate test, radiolabeling, microscopy and Congo red agar plate test (CRA).<sup>7,8</sup> However, the microtiter plate method (Mtp) is a quantitative and reliable method to detect biofilm-forming bacteria. Compared to tube and CRA methods, it can be recommended as a general screening method for the detection of biofilm-producing bacteria in laboratories.<sup>9,10</sup>

Molecular typing methods have been applied to help researchers map the spread and evolution of MRSA clones, including pulsed-field gel electrophoresis (PFGE) and staphylococcal cassette chromosome mec typing (SCCmec typing).<sup>11,12</sup>

PFGE is still considered a standard reference molecular technique for analyzing the dissemination of hospital and community-acquired MRSA and has proved to be one of the most discriminatory methods in the total sequencing of the MRSA strain.<sup>13</sup> It has been an excellent laboratory tool for emergency identification of new clones.<sup>14</sup>

Staphylococcal cassette chromosome mec (SCCmec) typing accompanied with overall genotyping has already provided strong evidence for the independent origins of health-care associated MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA).<sup>15</sup> To date, eight different types of *SCCmec* (I–VIII) have been defined on the basis of the combination of *ccr* and *mec* complexes, but only types I–V are globally distributed, while others appear to exist as local strains in the country of origin.<sup>16–18</sup> *PVL* is a two-component *S. aureus* spore-forming protein encoded by the *lukF-PV* and *lukS-PV* genes.<sup>19</sup> *PVL* toxin is responsible for the increased virulence of CA-MRSA, since the gene is responsible for many of the severe clinical syndromes of MRSA such as severe necrotizing pneumonia.<sup>20,21</sup>

However, epidemiological analysis among clinical MRSA isolates from children has rarely been performed. The aim of this study was to investigate the antimicrobial resistance pattern, biofilm formation and molecular characteristics of MRSA strains in children.

## Materials and Methods

### Bacterial Strains

In this cross-sectional study, 98 suspected staphylococcal infection samples were routinely collected from patients referred to the pediatric medical center and then specimens infected with Methicillin-resistant *S. aureus* were included in our study for a specified period (from September 2016 to October 2017). *S. aureus* isolates were confirmed using conventional microbiological methods (Gram's stain, catalase, coagulase, DNase tests and mannitol fermentation on mannitol salt agar (Merck, Germany)). To definitively identify positive *S. aureus* isolates, they were subjected to polymerase chain reaction (PCR) for *nucA* gene. MRSA strains were identified phenotypically using cefoxitin disk-diffusion method (30 µg; MAST, UK). This method was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.<sup>22</sup> Resistance to methicillin in *S. aureus* isolates was confirmed by the amplification of *mec* gene by PCR method.

### Antimicrobial Susceptibility Testing of MRSA Isolates

The antibiotic susceptibility patterns of MRSA isolates were determined by the Kirby-Bauer disk-diffusion method, and the results were interpreted according to CLSI guidelines.<sup>22</sup> The antimicrobial agents (Rosco, Denmark) tested in this study included clindamycin (2 µg), linezolid (30 µg), penicillin (10 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (25 µg), minocycline (30 µg) and erythromycin (15 µg). *S. aureus* ATCC 25923 was used as a standard strain. The minimum inhibitory concentration (MIC) for vancomycin was determined with E-test strips (Liofilchem, Italy) according to manufacturer's instructions. The standard reference strain *S. aureus* ATCC 25923 was used as a quality control strain in every test run.

### Detection of Biofilm Formation by Microtiter Plate Assay (Mtp)

Biofilm production was determined quantitatively using microtiter plate method as described previously.<sup>23</sup> Briefly, bacterial isolates were grown in Brain Heart Infusion (BHI) with 1% glucose (Merck, Germany) and incubated at 37°C for overnight.<sup>24</sup> Cultures were diluted 1:20 in fresh BHI-0.1% glucose. Then, 200 µL of the diluted solution was added to wells of a flat-bottomed polystyrene microtitre plate and incubated for 48 hrs at 37°C. The negative control wells contained 200 µL of BHI-0.1% glucose.

Wells were gently washed 3 times with phosphate-buffered saline (PBS; pH 7.2), fixed with sodium acetate (2%) for 10 mins, dried at room temperature and then stained with 0.1% crystal violet. After removing the crystal violet solution, wells were washed with PBS to remove unbound dye. The optical densities (ODs) of the plates were observed at 630 nm using a microtiter plate reader. Each assay was performed in duplicate. As a negative control, brain heart infusion broth with 1% glucose medium was used to determine the background OD. OD cut-off was then determined as an average OD of negative control + 3× standard deviation of negative control. The OD cut-off value was separately calculated for each microtiter plate. Biofilm formation by isolates was calculated and categorized according to the absorbance of the crystal violet-stained attached cells (Table 1). *Staphylococcus epidermidis* ATCC 35984 was used as the biofilm producer control strain.<sup>25,26</sup>

## Extraction of Genomic DNA

Genomic DNA was extracted from pure cultures using the High Pure PCR Template Preparation Kit (Roche, Germany), according to the manufacturer's guidelines. The concentration of DNA was assessed using a spectrophotometer.

## Detection of Biofilm Encoding Genes and *pvl* Gene

All 98 *MRSA* isolates were tested for the presence of *pvl* gene and biofilm encoding genes (*icaA*, *icaD*, *fnbA*, *fnbB*, *clfA*, *clfB*, *cna*, *eno*) with the degenerate primers as listed in Table 2.

## SCCmec Typing

SCCmec typing was performed for all *MRSA* isolates by multiplex PCR according to the works of Zhang et al.<sup>27</sup> Primer sequences are showed in Table 1. SCCmec types

were identified by comparing the banding patterns of *MRSA* to ATCC 10442 (*SCCmec type I*), N315 (*SCCmec type II*), 85/2082 (*SCCmec type III*), CA05 (*SCCmec type IVa*), 8/6-3P (*SCCmec type IVb*), MR108 (*SCCmec type IVc*), JCSC4469 (*SCCmec type IVd*) and JCSC3624 (*SCCmec type V*) as reference strains.

## Pulsed-Field Gel Electrophoresis

PFGE based on *Sma*I macrorestriction analysis was performed using the CDC laboratory protocol for *S. aureus*.<sup>28</sup> The PFGE was run on a CHEF DR III system (Bio-Rad, CA, USA) with optimum settings as follows: initial switch 5 s, final switch 40 s, run time 21 hrs, voltage 6 V/cm and a SeaKem Gold agarose (Lonza, Rockland, USA) gel concentration of 1%. Analysis of PFGE clusters was performed using the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium), using the Dice coefficient, and visualized as a dendrogram by the unweighted pair group method.

## Statistical Analysis

The relationship between biofilm formation and antibiotic resistance among *MRSA* isolates was evaluated by the Pearson Chi-Square test using SPSS version 21. *P*-values less than 0.05 were considered to be significant.

## Results

A total of 98 *MRSA* isolates were collected from children referred to pediatric hospital during a 2014–2015 years. Of these patients, 51 (52%) were girls and 47 (48%) were boys. The median age of the patients was 45 ± 5 months (1 month to 14 years). The *MRSA* isolates were recovered from respiratory secretions (57%), blood (15%), wounds (10%), the ear (8%), as skin abscesses (5%), the eye (5%). All isolates were susceptible to linezolid and vancomycin and resistant to penicillin and cefoxitin. The rates of resistance to the majority of antibiotics tested varied from 10% to 62% (Figure 1). Linezolid and vancomycin showed good activity against *MRSA* isolates. The rate of the susceptibility of vancomycin is shown in Table 3.

The results of Mtp assay of *MRSA* strains are presented in Table 4. Of the 98 *MRSA* isolates, 62 (63%) strains were biofilm producers. Statistical analysis showed a significant relationship between biofilm formation and antibiotic resistance. Resistance to two antibiotics such as erythromycin (72% vs 28%, *P*=0.01) and clindamycin (71% vs 29%, *P*=0.04) was higher among biofilm producers than non-biofilm producers (Table 5). All *MRSA* strains had biofilm-forming genes and the prevalence of *pvl* gene was 41%.

**Table 1** Classification of Biofilm Formation Abilities by Microtiter Plate Method

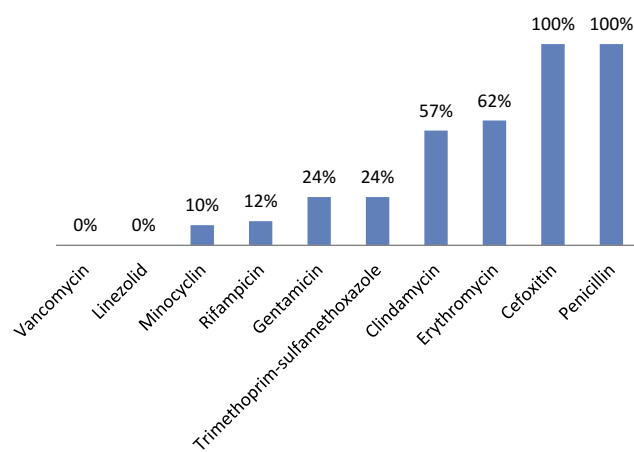
Cut-Off Value Calculation	Mean of OD Values Results	Biofilm Formation Abilities
OD > 4×OD <sub>c</sub>	OD > 0.2	Strong
2×OD <sub>c</sub> < OD ≤ 4×OD <sub>c</sub>	0.1 < OD ≤ 0.2	Moderate
OD <sub>c</sub> < OD ≤ 2×OD <sub>c</sub>	0.05 < OD ≤ 0.1	Weak
OD ≤ 0.05	OD ≤ 0.05	None

**Table 2** Oligonucleotide Primers Used in This Study

Genes	Primers Sequence (5'-3')	Product Size (bp)	Reference
<i>nuc</i>	F-GCGATTGATGGTGATACGGTT R-AGCCAAGCCTTGACGAACTAAAGC	270	29
<i>mecA</i>	F-GTAGAAATGACTGAACGTCCGATAA R-CCAATCCACATTGTTTCGCTCTAA	310	30
<i>icaA</i>	F-GGAGGTCTTTGGAAGCAAC R-TGCGACAAGAACTACTGCTG	390	In this study
<i>icaD</i>	F-GGAGGTCTTTGGAAGCAAC R-TGCGACAAGAACTACTGCTG	229	In this study
<i>clfA</i>	F-ATTGGCGTGGCTTCAGTGCT R-CGTTTCTCCGTAGTTCATTG	280	In this study
<i>clfB</i>	F-GCAGCATTACTACCGGTTT R-CTACAACAGAGCCAGCTTCA	301	In this study
<i>fnbA</i>	F-CACTGCGCCAGTTACAATAC R-GATGGTGGAGGTGGATATGT	306	In this study
<i>fnbB</i>	F-TCTCTGCAACTGCTGTAACG R-GGAAAGTGGGAGTTCAGCTA	320	In this study
<i>cna</i>	F-CGATTAAAGTGGCATGGCCG R-CTGGCAGCATATCTCCCGTT	333	In this study
<i>eno</i>	F-ACGTGCAGCAGCTGACT R-CAACAGCATCTTCAGTACCTTC	301	31
<i>pvl</i>	F-CTCTAGCCGATGTCGCTCAA R-ATACCTGAGGCTCGCCACTG	433	32
<i>SCCmec I</i>	F: GCTTTAAAGAGTGTGTTACAGG R: GTTCTCTCATAGTATGACGTCC	613	33
<i>SCCmec II</i>	F: CGTTGAAGATGATGAAGCG R: CGAAATCAATGGTTAATGGACC	398	33
<i>SCCmec III</i>	F: CCATATTGTGTACGATGCG R: CCTTAGTTGTCGTAACAGATCG	280	33
<i>SCCmec IVa</i>	F: GCCTTATTCGAAGAAACCG R: CTACTCTTCTGAAAAGCGTCG	776	33
<i>SCCmec IVb</i>	F: TCTGGAATTACTTCAGCTGC R: AAACAATATTGCTCTCCCTC	493	33
<i>SCCmec IVc</i>	F: ACAATATTTGTATTATCGGAGAGC R: TTGGTATGAGGTATTGCTGG	200	33
<i>SCCmec IVd</i>	F: CTCAAATACGGACCCCAATACA R: TGCTCCAGTAATTGCTAAAG	881	33
<i>SCCmec V</i>	F: GAACATTGTTACTTAAATGAGCG R: TGAAAGTTGTACCCTTGACACC	325	33

Total distribution of SCCmec types in the MRSA strains of various clinical infections is shown in Table 6. Our results showed that the most commonly

detected SCCmec types in the MRSA strains were *SCCmec IVa* (74 strains, 75%) and *SCCmec III* (18 strains, 18%).



**Figure 1** Antimicrobial resistance patterns of MRSA isolates.

PFGE of 98 MRSA strains generated 7 pulsotypes, consisted of five common types (CT1–CT5) and 2 single types (ST1 and ST2). The majority of the strains (38%) were CT1, followed by CT2 which contained 27 strains (28%), CT3 which contained 14 strains (14%), CT4 which contained 12 strains (12%) and CT5 which contained 6 strains (6%) (Figure 2).

## Discussion

*S. aureus* is included in the group of “ESKAPE” bacteria, which comprise the MDR pathogens that are currently

considered as the biggest concern for humanity.<sup>34,35</sup> There is a relative abundance of the different antibiotic-groups for the treatment of MRSA.<sup>36,37</sup> This is underlined by the recent WHO report, urging drug companies to invest and target various drug-resistant bacteria during antibiotics research, which also includes MRSA.<sup>38</sup> The pathogenicity of *S. aureus* is related to the ability to produce toxins and extracellular factors such as biofilms that enable the bacterial adhesion and resistance to phagocytosis.<sup>39,40</sup> It is now estimated that biofilms are responsible for more than 65% of nosocomial infections and 80% of all microbial infections.<sup>41</sup> In biofilm formation of different bacterial species, the transmission of antimicrobial resistance markers occurs more frequently, and the transfer of antibiotic resistance from *Enterococcus* to more pathogenic bacteria such as *aureus* is a major threat.<sup>41</sup> In this study, 63% of isolates were capable of biofilm formation by microtiter plate method, of which 1% were strongly adherent, 8% moderately adherent, 54% weakly adherent and 37% non-adherent, which were matched with the researches conducted by Lotfi et al and Yousefi et al.<sup>25,42</sup> Studies show that the microtiter plate method is more sensitive and specific than other methods and has been introduced as a gold standard in biofilm identification.<sup>10,23,24</sup> In this study, all isolates were susceptible to vancomycin and linezolid, while (62%) isolates showed resistance to erythromycin, (57%) to clindamycin, (24%) to trimethoprim-sulfamethoxazole, (24%) to gentamicin, (12%) to rifampin and (10%) to minocycline. Although antibiotic resistance was higher in biofilm-producing strains than other strains, there was a statistically significant relationship between antibiotic resistance of erythromycin and clindamycin and biofilm formation. All genes involved in biofilm formation including *clfA*, *clfB*, *fnbA*, *fnbB*, *cna*, *eno*, *icaD*, *icaA* were identified in all *S. aureus* isolates. In the study by Yousefi et al in Iran, the prevalence of biofilm-related genes was 100%,<sup>42</sup> while in the study by Mohamed et al in Iraq, the prevalence of *fnbA*, *clfA* and *cna* genes was, respectively, 56%, 56% and 81%.<sup>43</sup> The results of this study and other studies indicate that biofilm formation in *Staphylococcus* strains is dependent on environmental conditions and is influenced by environmental signals that can respond to external stress and inhibitory concentrations of antibiotics.<sup>24</sup> Failure in biofilm formation despite the presence of *ica* genes can be due to the inactivation of *ica* operons by activation of *icaR* repressor.<sup>44</sup>

In the present study, frequency of SCCmec typing was, respectively, *SCCmec I* (1%), *SCCmec III* (18%), *SCCmec IVa* (75%), *SCCmecIVb* (4%) and *SCCmec IVc* (2%).

**Table 3** The MIC Range of Vancomycin in MRSA Isolates

MIC (µg/mL)	n (%)
0.19	1 (1%)
0.25	8 (8%)
0.38	2 (2%)
0.5	28 (29%)
0.75	15 (15%)
1	23 (23.5%)
1.5	18 (18.5%)
2	2 (2%)
2.5	1 (1%)
Total	98 (100%)

**Table 4** Percentage of Biofilm Formation in Microtiter Plate Method

Biofilm Formation	Number	Percent
Strong	1	1%
Moderate	8	8%
Weak	53	54%
None	36	37%
Total	98	100%

**Table 5** Relationship Between Biofilm Formation and Antibiotic Resistance

Antibiotic	Resistance		Susceptible		Total	P-Value
	Biofilm +	Biofilm -	Biofilm +	Biofilm -		
Erythromycin	44 (72%)	17 (28%)	18 (49%)	19 (51%)	98 (100%)	0.01
Clindamycin	40 (71%)	16 (29%)	22 (52%)	20 (48%)	98 (100%)	0.04
Gentamicin	18 (75%)	6 (25%)	44 (59%)	30 (41%)	98 (100%)	0.1
Rifampicin	8 (67%)	4 (33%)	54 (63%)	32 (37%)	98 (100%)	0.7
Minocycline	6 (60%)	4 (40%)	56 (65%)	32 (35%)	98 (100%)	0.8
Trimethoprim sulfamethoxazole	15 (63%)		46 (64%)	27 (36%)	98 (100%)	0.9
Linezolid	–	–	62 (63%)	36 (37%)	98 (100%)	–
Vancomycin	–	–	62 (63%)	36 (37%)	98 (100%)	–
Penicillin	62 (63%)	36 (37%)	–	–	98 (100%)	–
Cefoxitin	62 (63%)	36 (37%)	–	–	98 (100%)	–

**Table 6** Multiplex PCR Results of SCCmec Typing

Genes	n (%)
<i>SCCmec I</i>	1 (1%)
<i>SCCmec II</i>	0
<i>SCCmec III</i>	18 (18%)
<i>SCCmec IVa</i>	73 (75%)
<i>SCCmec IVb</i>	4 (4%)
<i>SCCmec IVc</i>	2 (2%)
<i>SCCmec IVd</i>	0
<i>SCCmec V</i>	0

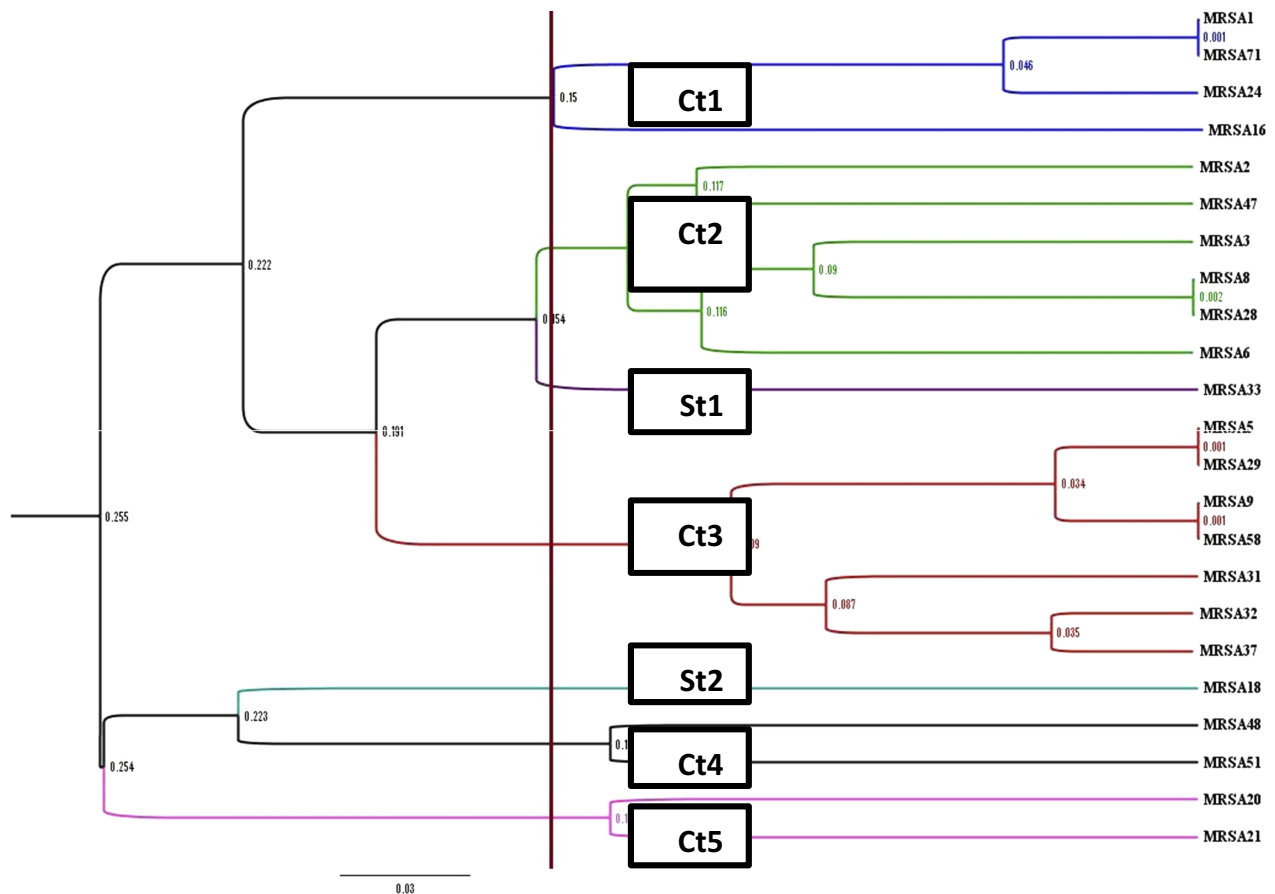
*SCCmec II*, *SCCmec IVd* and *SCCmec V* types were not detected. Results of studies indicated that strains carrying large chromosomal cassettes, such as *SCCmec I-III*, are often resistant to non- $\beta$ -lactam antibiotic classes and rarely carry the *pvl* gene. In contrast, strains carrying smaller chromosomal cassettes, such as *SCCmec IV* and *SCCmec V*, are less resistant to non- $\beta$ -lactam antibiotic classes and often carry the *pvl* gene.<sup>45,46</sup> In this study, the strains showed less resistance to non- $\beta$ -lactam drugs such as gentamicin, minocycline and rifampin, and 41% of the strains carried the *pvl* gene.

In this study, PFGE technique was used as a powerful discriminative tool to investigate the epidemiological characteristics of MRSA strains. This method has high resolution and reproducibility and is used as the gold standard method for typing this genus. Genotyping techniques such as PFGE are helpful in finding a transferable clone and infection control and prevention measures.

In this study, 5 common types with 4 to 37 subtypes and 2 single types were identified. Common type 1 with 37 isolates was dominant clone and all strains had *SCCmec IV*, most of which were isolated from outpatients with

respiratory infections. Whereas Common type 2 with 27 isolates was often obtained from inpatients in different departments of the hospital. Based on these results, it is likely that Common type 1 colonizes in the respiratory tract of children and circulating in the community, whereas Common type 2 is circulating in hospitals and in different parts of it. Common type 3 with 14 isolates also had the same antibiotic resistance pattern and were only separated from the emergency and surgical departments. A similar study by Ohadian moghadam et al, in 2017, was performed to classify MRSA strains using the PFGE technique in Iran. In this study, 43 MRSA strains were isolated from wound swabs of patients referred to Shahid Motahhari Hospital (specializing in the treatment of burns). After performing PFGE, 5 common types and 31 single types were identified. The investigation indicated that each common type represents an outbreak, because it was taken over an identical time interval and the diversity of strains had been explained by the acquisition of MRSA from various sources.<sup>47</sup> Another study by Hussein et al investigated the typing of 114 strains of *S. aureus* isolated from healthcare workers using the PFGE technique in Iraq. In this study, 8 common types were isolated, more than 50% of isolates belonged to types A and B, indicating infection with the same source.<sup>48</sup>

In this study, since no sampling of hospital personnel and equipment was carried out, it is impossible to investigate the source of infection and its transmission to patients in different departments of the hospital, which requires a large-scale study, but the important point that should be noted is the infection is transmitted from the community to the hospital, which must be prevented by appropriate infection control measures.



**Figure 2** Dendrogram of MRSA patterns generated by an UPGMA clustering method.

## Conclusion

In this study, most of the strains belonged to CA-MRSA because they were mostly carriers of the *SCCmec IVa* gene and were highly sensitive to non-beta-lactam drugs such as minocycline and rifampin. According to the PFGE technique, cross-sectional circulation of clones was observed in the hospital, which requires careful control of infection in different parts of the hospital.

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## Disclosure

The authors report no conflicts of interest in this work.

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