



# Molecular typing and investigation of virulence factors of methicillin-resistant *Staphylococcus aureus* strains isolated from patients hospitalized in an Isfahan teaching hospital

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## Abstract

The increase in the incidence of *Staphylococcus aureus* infections in nosocomial settings each year is a significant concern for the public health sector, as it is one of the most common nosocomial infections. Furthermore, it produces a variety of toxins that aggravate the disease that the host is contracting. It was our aim in this study to detect and identify Sccmec typing of MRSA strains isolated from a teaching hospital in Isfahan, Iran, as part of a research project. The presence of different virulence factors was also investigated. To conduct this study, 50 strains were collected from different samples of Shariati Hospital in Isfahan between September 2018 and July 2020. Different types of SCCmec were investigated using the multiplex PCR method. Conventional PCR was performed to identify genes of virulence factors including *pvl*, *tst*, *hly*, *sak*, *eta*, and *etb*. The MRSA strains detected included Sccmec type III in 96% of the cases and Sccmec type IV in the remaining 4% of the cases. The frequencies of *hly*, *sak*, *eta*, *tst*, *pvl*, and *etb* genes in clinical isolates of MRSA were 82%, 50%, 42%, 6%, 4%, and 0%, respectively. Ten toxin patterns were observed in the studied MRSA strains, and six MRSA strains did not have the studied toxins' gene. Considering the production of different toxins in MRSA strains and the circulation of one type in the studied hospital, implementing an appropriate infection control policy is imperative to prevent the spread of MRSA types across a hospital.

## 1. Introduction

*Staphylococcus aureus* is known as an important pathogenic and opportunistic agent in hospitals, and one of the problems currently endangering human health is antibiotic resistance. Only one year after the first use of methicillin to treat penicillin-resistant *S.aureus* infections in England, the first methicillin-resistant strain was discovered as a result of its use, with more

methicillin-resistant strains appearing over the last two decades. Today, *S. aureus* strains that are resistant to methicillin have spread throughout the world. They are among the most prominent causes of fatal infections and deaths among healthy people. It is reported that this bacterium is widespread in hospitals, communities, and hospitalized individuals. The horizontally transmissible mobile genetic element *mecA*, also

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known as the staphylococcal cassette chromosome *mec* (SCC*mec*), mediates methicillin resistance. A penicillin-binding protein, PBP2a, is encoded by the *mecA* gene. Because of its low affinity for beta-lactamase an entire class of antibiotics are resistant to this protein (Gunawardena et al., 2012; Perez-Roth, Claverie-Martin, Villar, & Mendez-Alvarez, 2001). All MRSA strains possess the *mecA* gene in addition to the *mecR1*, *ccr AB*, *ccr C*, and *j* connective sections in SCC*mec* areas. The *ccr* and *mec* gene complexes are two crucial components of staphylococci's SCC*mec* elements. The SCC*mec* area may also contain genes that provide antibiotic resistance (Gill et al., 2005).

*S. aureus* has many virulence factors that cause various infections. Some of these factors allow bacteria to adhere to surfaces, while others interfere with the functioning of the immune system. All of these factors can harm the host. *S. aureus* produces various extracellular and toxic proteins that are convenient for the development of bacterial pathogenesis. Concurrently, skin and soft tissue infections are commonly caused by *S. aureus*, which are being transmitted through hospitals and communities (MH & Hamid, 2013).

Some of these factors include beta hemolysins, which are among the membrane-damaging peptides produced by *S. aureus*. While beta hemolysin does not create a hole in the cell membrane, it does create hydrolyzes sphingomyelin in the cell membrane, with the enzyme activity of sphingomyelinase (Vandenesch, Lina, & Henry, 2012). Another, toxic shock syndrome toxin (TSST-1), is one of the most significant virulence factors and one of the superantigens that severely affect the host. This *S. aureus* toxin is associated with toxic shock syndrome (TSS), a severe illness that includes shock and multiple organ failure. This disease causes symptoms such as fever, diarrhea, vomiting, muscle pain, velvety skin rashes, and in severe cases, hypotension, lymphadenopathy, and

liver and kidney failure. The *tst* gene, which is the cause of the disease, can be easily transmitted among *S. aureus* strains in the community (Dinges, Orwin, & Schlievert, 2000). Also, the Panton Valentin leucocidin (PVL) toxin is a hemolytic exotoxin that increases the permeability of the cell membrane and thus causes the lysis of leukocytes and tissue necrosis. PVL is active against cells of the immune system, including neutrophils, monocytes, and macrophages. It has been found to be active in humans and rabbits, but not in mouse and monkey phagocytic cells. *S. aureus* strains that produce PVL toxin are commonly isolated from human CA-MRSA infections. However, little is known about the isolation of PVL-producing strains from animals, and there are few published reports dealing with the isolation of PVL-positive MRSA from veterinary sources (Yoong & Torres, 2013).

As indicated, SCC*mec* is divided into eight different types (I-VIII). A literature review indicated that SCC*mec* type I was designated in the United Kingdom in 1961, type II in Japan, and types III, IV, and V in New Zealand. Several studies related to the SCC*mec* element have shown that nosocomial-acquired MRSA (HA-MRSA) is detected in SCC*mec* type III, whereas community-acquired MRSA (CA-MRSA) is found in a variety of SCC*mec* types. Extracellular proteins called epidermolytic staphylococcal exfoliative toxins (ETs), which separate the skin's epidermal layer, are specifically produced by some *S. aureus* strains. These bacteria primarily affect children and produce staphylococcal scalded skin syndrome, a dangerous but uncommon illness. Among Staphylococci, there are two ETs: ETA, which is encoded by the gene *eta* located on the chromosome, and ETB, which is encoded by the *etb* gene located on a large plasmid, both of which are present on the same cell surface (Smith et al., 2009).

In a previous study, we examined 125 strains of *S. aureus* and the frequency of MRSA strains and antibiotic resistance of MRSA strains (Zarkesh-

Esfahani, Ghandehari, Nasr-Esfahani, & Beheshti-Maal, 2021). This study examined the *Sccmec* type and virulence factors that are present in MRSA strains based on the same isolates of bacteria that were used in the previous study.

## 2. Material and methods:

### 2.1. Isolation and detection of MRSA

The sampling process is similar to the one described in the previous study. In brief, between September 2018 and July 2020, suspected strains of *S. aureus* were isolated from clinical samples from the Shariati hospital in Esfahan, Iran, then identified using *nucA* primers, finally, using cefoxitin (30 g) and the *mecA* gene, all 50 MRSA strains were confirmed to be resistant against cefoxitin.

### 2.2. DNA extraction:

All MRSA isolates were grown at 37°C on nutrient agar, and DNA was extracted using the phenol/chloroform method (Cheng & Jiang, 2006).

### 2.3. SCCmec and ccr typing:

As previously mentioned, the multiplex PCR typing test was used to identify SCCmec types that included four pairs of primers, specifically

the distinct and specific primers for SCCmec types (I, II, III, IV, and V) (Boye, Bartels, Andersen, Møller, & Westh, 2007). Multiplex PCR conditions were as follows: Denaturation at 94°C for 4 minutes, proceeded by 35 cycles of 30 seconds at 94°C, annealing at 55°C for 30 seconds, with an extension at 72°C for 60 seconds, and a final extension at 72°C for 10 minutes. Four sets of primers, one for each of the *ccr* genes, were utilized in a second multiplex PCR test to characterize *ccr* gene complexes. (Zhang, et al., 2005).

### 2.4. Toxin genes detection:

The PCR was used to identify the important genes with specific primers such as *hly* (Goerke, Wirtz, Flückiger, & Wolz, 2006), *sak* (Goerke et al., 2006), *pvl* (McClure et al., 2006), *tst* (Johnson et al., 1991), *eta* and *etb* (Jarraud et al., 1999) (Table 1). PCR was performed based on previous protocols and kit instructions (Master Mix Red Amplicon, A-180301).

### 2.5. Statistical analysis:

SPSS (v.22) statistics software was used for the statistical analysis. P values  $p \leq 0.05$ ,  $\leq 0.01$ , and  $\leq 0.001$  were judged statistically significant.

**Table1:** Toxin primers used in this research

Primers	Oligonucleotide sequences	Amplicon size (bp)
<i>hly</i>	hly-F AGCTTCAAACCTTAAATGTCA	525
	hly-R CCGAGTACAGGTGTTTGTA	
<i>sak</i>	sak-F GTGCATCAAGTTCATTCGAC	383
	sak-R TAAGTTGAATCCAGGGTTTT	
<i>pvl</i>	F: 5' -ATCATTAGGTAAAATGTCTGGACATGATCCA	433
	R: 5' -GCATCAAGTGTATTGGATAGCAAAGC	
<i>eta</i>	ETA-1 (forward) CTAGTGCATTTGTTATTCAA	119
	ETA-2 (Reverse) TGCATTGACACCATAGTACT	
<i>etb</i>	ETB-1 (forward) ACGGCTATATACATTCAATT	200
	ETB-2 (reverse) TCCATCGATAATATACCTAA	
<i>tsst</i>	TSST-1 (forward): ATGGCAGCATCAGCTTGATA	350
	TSST-2 (reverse): TTCCAATAACCACCCGTTT	

### 3. Results and Discussion:

#### 3.1. Prevalence of MRSA:

The MRSA strains were identified based on resistance to cefoxitin in 50 of the 125 (41%) strains. Prior studies showed that all 50 strains contained the *mecA* gene, indicating that they were MRSA.

#### 3.2. SCCmec and ccr typing

The Multiplex-PCR analysis found the majority of MRSA isolates (96%) contained SCCmec type III and were PCR-positive with type 3 *ccr*. Furthermore, 2% had SCCmec type IV with type 2 *ccr*, 96% were HA-MRSA, and 2% CA-MRSA.

#### 3.3. Detection of toxin genes:

The highest distribution (82%) of studied toxins in MRSA strains was related to the *hly* gene. The *sak* gene was found in 50% of MRSA strains, the *tst* gene in 6%, and the *eta* gene in 42% of MRSA strains; however, none of the strains carried the *etb* gene. The *pvl* gene was observed in just 4% of MRSA strains which is less than other virulence factors. MRSA strains with SCCmec type IV were *pvl* gene positive. CA-MRSA strains were the only ones with the *pvl* gene. Regarding the presence of toxins, 10 toxin patterns were observed in the studied MRSA strains; pattern number one with *sak* and *eta* genes had the highest rate (26.0%). Six MRSA strains did not have the studied toxins gene. All genes were reproducibly performed three times. The positive control in each PCR process is shown in the Gel electrophoresis (Fig. 1, Table 2).

Antibiotic resistance and factors involved in pathogenesis influence infections caused by *S. aureus*. Acquiring antibiotic resistance in this bacterium causes changes in the expression and secretion of effective factors in pathogenesis, including bacterial toxins. Several methods have been used to identify *S. aureus* toxins such as the PCR method, enzyme-linked immunosorbent

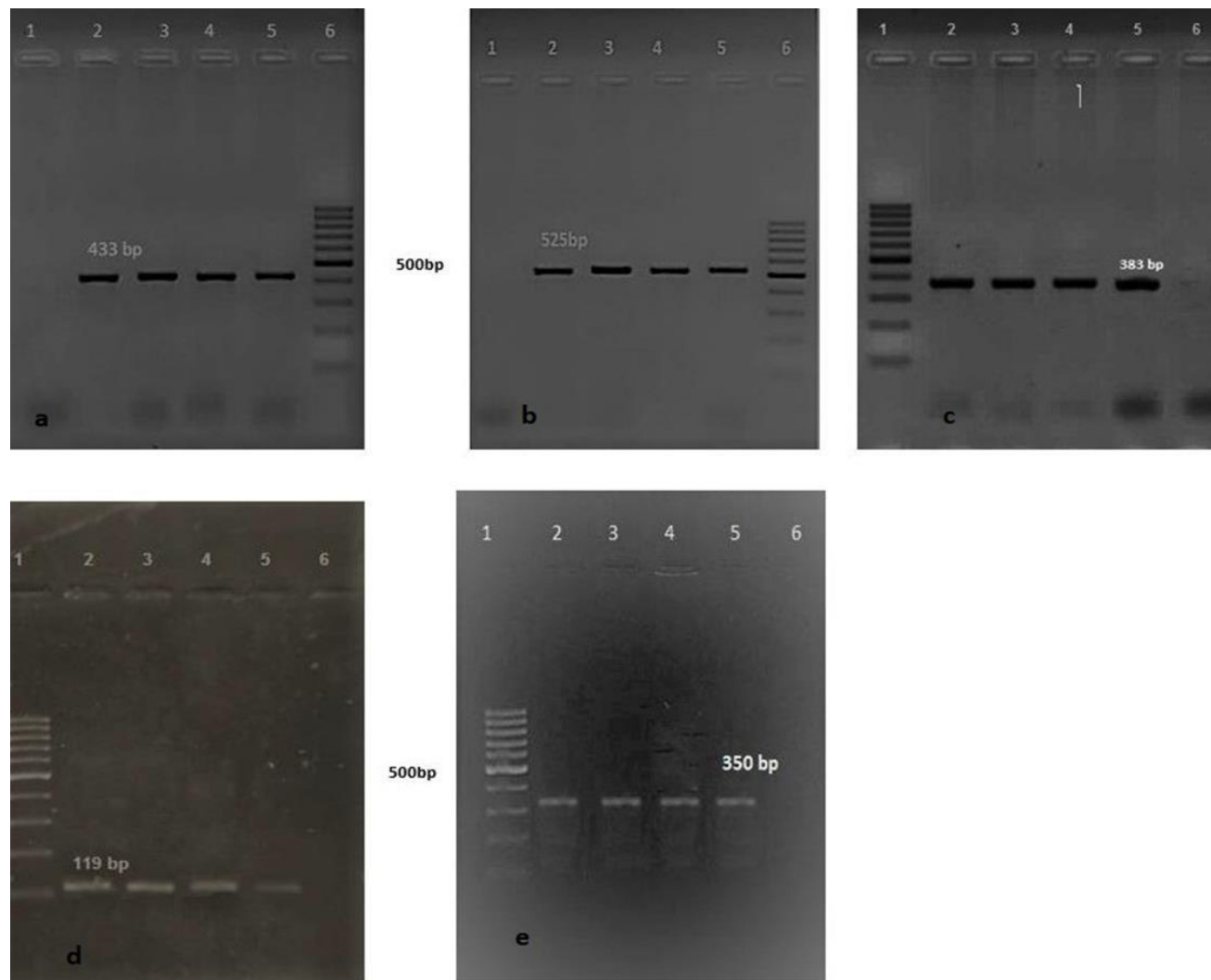
assay, enzyme-linked immuno filtration assay, and radioimmunoassay. The PCR method is a suitable tool for rapid diagnosis and has significantly improved sensitivity and specificity compared to other phenotypical methods for *S. aureus* toxin genes (Aslanimehr, Tavakoli, Peymani, & Javadi, 2013).

In another study, the results of antibiotic resistance testing using the disc diffusion method showed that 40% of the strains were resistant to the antibiotic cefoxitin, which was considered MRSA, and all the strains were positive for *mecA* gene (Zarkesh-Esfahani et al., 2021).

The high prevalence of SCCmec type III in this study shows that the MRSA strains causing the infection are probably of hospital origin. In Abiri and Gudarzi's 2017 study on the typing of *S. aureus* strains resistant to methicillin, the most common SCCmec type was found to be type III with 53.6% (Abiri & Goudarzi, 2018).

**Table 2:** Distribution of toxin patterns among MRSA isolates.

patterns	toxins					Number of MRSA strains
	<i>hly</i>	<i>sak</i>	<i>tst</i>	<i>pvl</i>	<i>eta</i>	
1	✓	✓	-	-	-	13
2	✓	-	-	-	✓	10
3	✓	✓	-	-	✓	8
4	✓	-	-	-	-	5
5	✓	-	✓	-	-	2
6	✓	✓	-	✓	-	2
7	-	✓	-	-	✓	1
8	-	✓	-	-	-	1
9	-	-	-	-	✓	1
10	✓	-	✓	-	✓	1
11	-	-	-	-	-	6



**Figure 1:** Agarose gel electrophoresis of genes as PCR amplification products.

- for *pvl* genes (433 bp): lane 1, negative control, lane 2- 4 positive *pvl* sample, lane 5, positive control, lane 6, 100-bp DNA ladder
- for *hlb* genes (525 bp): lane 1, negative control, lane 2-4 positive *hlb* sample, lane 5, positive control, lane 6, 100-bp DNA ladder
- for *sak* genes (383 bp): lane 1, 100-bp DNA ladder, lane 2, positive control, lane 2-4 positive *sak* sample, lane 6, negative control
- for *eta* genes (119 bp): lane 1, 100-bp DNA ladder, lane 2, positive control, lane 3-5 positive *eta* sample, lane 6 negative control,
- for *tst* genes (350 bp): lane 1, 100-bp DNA ladder, lane 2, positive control, lane 3-5 positive *tst* sample, lane 6, negative control

Another study conducted by Rashidi et al. in 2016 on 116 isolates of *S. aureus* isolated from the ICU ward of several hospitals in Tehran over

a period of 11 months, with results contradicting the findings of the present study, showed that SCC*mec* type IV had the highest frequency, with

57.9% (Nezhad, Meybodi, Rezaee, Goudarzi, & Fazeli, 2017).

Detection of different types of SCCmec depends on the place of the strain's detection, whether in the hospital or for patients because each reports different statistics of resistance in Iran. Most of the studies conducted in this country have also reported SCCmec type III as the most common type among methicillin-resistant *S. aureus* strains isolated from clinical samples, although some studies report types IV as dominant types, which is consistent with some of the previous studies mentioned above. However, some worldwide studies report the spread of *S. aureus* strains resistant to methicillin differently, which are similar to the results of several other studies conducted in Iran.

SCCmec types are usually related to the evolutionary origin of MRSA. SCCmec types I, II, and III are most often found in hospital-acquired MRSA, whereas SCCmec types IV and V are mainly associated with community-acquired MRSA (CA-MRSA) (Chmelnitsky et al., 2008). In a study in China, the frequency of SCCmec type III in the examined samples was reported as 98%, while a study in Korea and Japan reported 2% and 1%, respectively, but in the same study, the prevalence of SCCmec type II was 75% in Korea and 91% in Japan (Ito et al., 2001; Qing et al., 2010).

Among Middle Eastern countries, Iran has the second highest prevalence of MRSA after Iraq. On the other hand, studies in Asian countries showed that the prevalence of HA-MRSA is lower than in some other countries. Argentina and Mexico are similar to Japan in this respect, Australia and the United States have lower and higher prevalence rates than Iran, respectively, and European countries also exhibit heterogeneous prevalence rates (Ghasemian & Mirzaee, 2016). As mentioned in similar studies, every year we see an increase in these strains almost all over the world. In addition to being a serious challenge for doctors and hospital infection control management in countries with low health and care indicators, there continues to be an expectation of an increase in drug

resistance at the hospital level. Moreover, strains resistant to methicillin are highly dependent on the geographical area, and periodic checks of these changes should be done every 4 to 5 years.

In this study, the important toxins of *S. aureus* including *hly*, *sak*, *eta*, *etb*, *pvl*, and *tst* were investigated. Hemolysins are one of the most important pathogenic factors in *S. aureus* strains that cause infection. They were also very abundant in the present study, with 82% of investigated strains carrying the *hly* gene. In the study by Amini et al. (2019), the frequency of the *hly* gene was reported on 57.6% of samples isolated from children's wounds (Abiri & Goudarzi, 2018). This difference in resistance statistics is derived from the diversity of sampling from different places, and the difference of sampling in diverse geographical areas. Therefore, further analysis of different samples of *Staphylococcus* in different regions and hospitals is necessary.

Among MRSA strains isolated from the hospitals presented in this research, the frequency of having the *pvl* gene in MRSA strains with SCCmec type IV was found to be 4%. A Chinese study to investigate if the *pvl* gene is present in MRSA strains isolated from skin infections found that 28.6% of the isolates had the *pvl* gene, which is higher than the results of the present study (Chen, Hiramatsu, Huang, Wang, & Lauderdale, 2009). According to Khosravi et al., 7.2% of MRSA strains contain the *pvl* gene, which differs significantly from our study (Khosravi, Hoveizavi, & Farshadzadeh, 2012). The study of Aung Soe Meiji et al. in 2016 reported the prevalence of *pvl* gene to be 12.5%, which was higher than our results (Aung et al., 2017).

Considering that the *S. aureus* strains with PVL are more virulent and more transmissible than strains without PVL and this toxin increases the aggressive power of *staphylococcus* by creating resistance to phagocytosis, it can be suggested that observing an increase in the strains carrying this toxin should be considered a warning for stricter controls.

Another important toxin is the exfoliative toxins, also called epidermolytic toxins. In this research, the frequency of the *eta* gene among MRSA isolates isolated from the hospital was 42%, but the *etb* gene was not isolated in any of the strains. In the study by Taghizadeh Maleki et al. in 2018, of 59 *S. aureus* isolated from wounds, 100% of the strains had the *eta* gene (Taghizadeh et al., 2019). Similar to the present study, Xie and colleagues did not find the *etb* gene in any of the strains of examined toxin coding gene of 118 strains of *S. aureus* (Xie et al., 2011).

Zeresaz et al.'s study conducted on *S. aureus* strains in 2019 showed that the frequency of the *tst* gene in these strains was 2.32%, and like the present study, these strains carrying the *tst* gene were also isolated from wound samples (Zerehsaz & Najar, 2020). Also, in Benavidi et al.'s research, among one hundred strains of MRSA, the prevalence rate of *tst* gene was reported as 17%. Since this virulence factor can be transmitted to other *S. aureus*, there is a risk of an epidemic infection with such isolates in the hospital and among hospitalized patients. These samples are often the main cause of the spread of high antibiotic resistance (Benvidi et al., 2017).

According to the reports, most of the MRSA isolates in the studied hospitals were positive for these types of genes. This shows the high prevalence of multiple antibiotic resistances. Since this virulence factor can be transmitted to other *Staphylococcus aureus*, there is a risk of an epidemic infection with such isolates in the hospital and among hospitalized patients.

Our findings showed that the *pvl* gene was observed only in SCCmec type IV strains isolated from wounds, but SCCmec type III strains did not have this gene. While the *tst* gene was observed in some SCCmec type III strains, the type IV strains did not have it. With these types of findings, we can conclude that it is possible to determine the likely source of infection in the hospital, which may lead to the prevention of the spread of infection.

#### 4. Conclusion:

The spread of infection caused by *S. aureus* is very common and fast. Also, with the emergence of antibiotic resistance, identification and investigation of these factors seems necessary considering the production of different MRSA strains toxins and the circulation of one type in the studied hospital. In order to prevent the spread of MRSA types across a hospital, it is imperative that an appropriate infection control policy be implemented. Due to the fact that methicillin-resistant strains are placed on the second priority in the list of drug-resistant bacteria in WHO, it is necessary to prevent the self-administration of antibiotics and pay special attention to the disinfection of hospital wards, especially the more sensitive ones such as the ICU, to prevent the increase of resistance to these and other antibiotics. Therefore, it is healthier to correctly diagnose the cause of the infection by using appropriate diagnostic methods. As one of the most important of these methods, future studies should focus on the use of molecular approaches. Monitoring isolates with drug-resistant virulence genes in hospitals can also be effective in caring for people at risk, such as immunocompromised people and sensitive groups.

#### Conflict of Interest

The authors declare that there is no conflict of interest.

#### Ethical approval

This manuscript does not contain any studies with human participants or animals performed by any of the authors.

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