

Characteristics of Virulence Factors in Methicillin-Resistant *Staphylococcus aureus* Strains Isolated From a Referral Hospital in Tehran, Iran

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) has been known as one of the most important nosocomial pathogens that able to produce a variety of virulence factors.

Objectives: In this study, we aimed to describe the prevalence, presence of different virulence factors, staphylococcal cassette chromosome *mec* (SCC*mec*) and prophage typing of MRSA strains isolated from a referral hospital in Tehran, Iran.

Materials and Methods: A total of 279 *S. aureus* strains were collected from a referral hospital in Tehran during August to December of 2013. All isolates were confirmed using species specific primers and were tested for susceptibility to oxacillin and cefoxitin disks by the recommendations of clinical and laboratory standards institute (CLSI). The staphylococcal enterotoxin (*sea-seq*) and *pvl*, *hly* and *sak* genes were detected and typed using prophage typing and SCC*mec* typing methods.

Results: Out of the 279 *S. aureus* isolates, 91 (32.6%) strains were confirmed as MRSA. Totally, 6 enterotoxin and 2 virulence factor genes were detected in MRSA strains. The *sea*, *sek*, *seq* and *hly* genes were present in all MRSA and *sak*, *seg*, *sei* and *sel* were detected in 85%, 35%, 23% and 44% of the strains. Only SCC*mec* type III and type 3 *ccr* and 2 different prophage patterns were identified among the strains.

Conclusions: Our results show the presence of clonal groups of enterotoxin-producing MRSA strains in this hospital in Tehran. The presence of bacteriophage encoded virulence factors and resistance to oxacillin enable bacteria to produce a broad spectrum range of diseases.

Keywords: Enterotoxins, Prophages, Bacterial Typing Techniques, Methicillin-Resistant *Staphylococcus aureus*

1. Background

Staphylococcus aureus is a facultative anaerobe, gram-positive opportunistic pathogen which is known as a major cause of hospital-acquired (HA) and community-acquired (CA) infections and is able to produce a variety of virulence factors such as enterotoxins, staphylokinase, toxic shock syndrome toxin-1 (TSST-1), microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), β -lysin, capsular polysaccharides, Panton-Valentine leukocidin (PVL), lipase and exfoliative toxin (1). Some of these factors are encoded by bacteriophages through lysogenic phage conversion (1, 2). Staphylococcal enterotoxins (SEs) are members of superantigen family (3, 4), which are involved in modulating the host immune response and also may play a role in evasion of host defenses and bacterial persistence (4). At the moment, 23 known major types of SEs (SEA to SEV) were identified and the production of SEs in MRSA strains may cause food poisoning that is characterized by nausea,

vomiting, abdominal cramps and diarrhea, and it is one of the most common food-borne diseases in the world (4-6). Different enterotoxins are classified into 5 different groups based on the comparisons of amino acid sequences, in which group 1 comprises the SEA, SED, SEE, SEJ, SEN, SEO, SEP and SES, group 2 comprises the SEB, SEC, SEG, SER and SEU, group 3 comprises the SEI, SEK, SEL, SEM, and SEQ and groups 4 and 5 comprise only the SEV and SEH, respectively. Staphylococcal enterotoxins A-I and SER-SET can display the emetic activity (6).

Bacteriophages are a class of mobile genetic elements which convert non-lysogenic staphylococci to lysogenic and virulent one through horizontal gene transfer (7). Incorporation of different classes of prophages into the chromosome of *S. aureus* strains enables them to produce broad spectrum of virulence factors such as PVL (SGA encoded) TSST, exfoliative toxin, lipase (SGB encoded), enterotoxin A, E, G, K and P, β -lysin and staphy-

lokinase (SGF encoded) (4). *S. aureus* bacteriophages are members of *Siphoviridae* (temperate bacteriophages) and *Myoviridae* (lytic bacteriophages) families (1).

S. aureus strains have the ability to acquire antibiotic resistance genes via mobile genetic elements which make them resistant to a broad spectrum of antibiotics such as methicillin. Methicillin-resistant *S. aureus* (MRSA) strains were appeared first in 1961 and spread worldwide during decades (7). Infections caused by hospital-acquired MRSA (HA-MRSA) strains have increased during the past decades and exhibited increased antimicrobial resistance. On the other hand, community-associated MRSA (CA-MRSA) strains occur in people who have not been hospitalized or recently had invasive procedures. CA-MRSA strains are susceptible to different antibiotics other than beta-lactams and harbor *pvl* gene as a marker and infection occur typically as skin or soft tissue infections (7). Resistance to methicillin is due to the presence of staphylococcal cassette chromosome *mec* (SCC*mec*), which eleven types have been recognized and MRSA strains are classified as type's I - XI (8).

2. Objectives

In this experimental study, we aimed to describe the prevalence, presence of different virulence factors, SCC*mec* and prophage typing of MRSA strains isolated from a referral hospital in Tehran, Iran.

3. Materials and Methods

3.1. Samples Collection

A total of 279 *S. aureus* strains were collected from patients (outpatients and inpatients) who exhibited clinical symptoms of infections in a referral hospital in Tehran during August to December of 2013. Patients were hospitalized for at least 72 hours classified as inpatients. Strains were collected from wound (106), urine (83), trachea (45), blood (20), sputum (18) and cerebrospinal fluid (CSF) (7).

All isolates were identified as *S. aureus* using species specific *nucA* gene primers as described previously (9). For DNA extraction from all strains, the boiling method was employed, according to the protocol introduced by Rahimi et al. (10). Moreover, DNA of the MRSA strains was extracted using a High Pure PCR Template Preparation kit (Roche, Germany). Also, the polymerase chain reaction (PCR) and multiplex-PCR reactions were mixed in a volume of 25 μ L consisting of 1 μ L of template DNA, 10X PCR buffer, dNTP mix (100 μ M), MgCl₂ (0.8 μ M), each primer (0.4 μ M) and *taq* DNA polymerase (1 U).

3.2. Screening of Methicillin-Resistant *Staphylococcus aureus* Strains

Susceptibility of all *S. aureus* strains to oxacillin (1 μ g) and ceftiofloxacin (30 μ g) as a surrogate indicator for

detection of MRSA strains (Mast diagnostics, Merseyside, United Kingdom) was examined according to the guidelines of clinical and laboratory standards institute (CLSI) (11).

3.3. Detection of Virulence Genes

All MRSA strains were examined for the presence of enterotoxin genes (*sea-seq*) according to the protocols introduced previously (*sea-sej* and *sel*: cycle conditions: 94°C for 5 minutes; 30 cycles at 94°C for 45 seconds, 62°C for 45 seconds, and 72°C for 105 seconds; and a final extension at 72°C for 10 minutes) (9) (*sek* and *sem-seq*: cycle conditions: 95°C for 15 minutes; 40 cycles at 94°C for 30 seconds, 55°C for 40 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 7 minutes) (12). Moreover, PCR assays were performed to detect genes *sak* (staphylokinase toxin), *hly* (haemolysin) by specific primers described by Goerke et al. (13) (cycle conditions: 94°C for 4 minutes; 35 cycles at 94°C for 30 seconds, 55°C for 2 minutes, and 72°C for 60 seconds; and a final extension at 72°C for 7 minutes). The *pvl* gene was detected in a PCR assay according to the specific primers introduced by McClure et al. (14) (cycle conditions: initial activation at 94°C for 10 minutes; 10 cycles at 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 75 seconds; 25 cycles at 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 75 seconds; and a final extension at 72°C for 10 minutes). The list of primers used for identification of virulence genes has been shown in Table 1.

3.4. Prophage Typing

A multiplex PCR assay was performed for prophage typing of MRSA strains using specific primers (2) for prophage types SGA, SGB, SGF, SGL and SGD and also 2 subtypes SGFa and SGFb (cycle conditions: 94°C for 5 minutes; 30 cycles at 94°C for 60 seconds, 57°C for 1.5 minutes, and 70°C for 1.5 minutes; and a final extension at 70°C for 3 minutes).

3.5. SCC*mec* Typing

The presence of *mecA* gene among MRSA strains was tested according to the protocol published previously (14) (cycle conditions: 94°C for 10 minutes; 25 cycles at 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 75 seconds; and a final extension at 72°C for 10 minutes). SCC*mec* typing was performed using a multiplex-PCR assay contained specific primers for types I - V as reported previously (15). A multiplex-PCR assay was employed for *ccr* typing of MRSA strains according to the protocol of Zhang et al. (15) (cycle conditions: initial activation at 94°C for 5 minutes; 10 cycles at 94°C for 45 seconds, 65°C for 45 seconds, and 72°C for 1.5 minutes; 25 cycles at 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1.5 minutes; and a final extension at 72°C for 10 minutes)

Table 1. Primers Used for Polymerase Chain Reaction Identification of Virulence Genes

Genes	Sequence	Reference
sea		(9)
F	5'-TAAGGAGGTGGTGCCTATGG	
R	5'-CATCGAAACCAGCCAAAGTT	
seb		(9)
F	5'-TCGCATCAAACGACAAACG	
R	5'-GCAGGTACTCTATAAGTGCC	
sec		(9)
F	5'-ACCAGACCCTATGCCAGATG	
R	5'-TCCCATTATCAAAGTGGTTCC	
sed		(9)
F	5'-TCAATTCAAAAGAAATGGCTCA	
R	5'-TTTTCCGCGCTGTATTTTT	
see		(9)
F	5'-TACCAATTAACITGTGGATAGAC	
R	5'-CTCITTCACCTTACC GC	
seg		(9)
F	5'-CCACCTGTTGAAGGAAGAGG	
R	5'-TGCAGAACCATCAAACCTCGT	
seh		(9)
F	5'-TCACATCATATGCGAAAGCAG	
R	5'-TCGGACAATATTTTTCTGATCTTT	
sei		(9)
F	5'-CTCAAGGTGATATTGGGTAGG	
R	5'-CAGGCAGTCCATCTCCTGTA	
sej		(9)
F	5'-GGTTTTCAATGTCTGGTGGT	
R	5'-AACCAACGGTCTTTTTGAGG	
sek		(12)
F	5'-ATGAATCTTATGATTAATTCAGAATCAA	
R	5'-ATTATATCGTTCTTTATAAGAAATATCG	
sel		(9)
F	5'-CACCAGAATCACACCGCTTA	
R	5'-CTGTTTGATGCTTGCCATTG	
sem		(12)
F	5'-ATGAAAAGAATACTTATCATTGTTGTTTTAATG	
R	5'-CTTCAACTTTCGTCCTTATAAGATAITTC	
sen		(12)
F	5'-ATAAAAAATATTA AAAAGCTTATGAGATTGTTTC	
R	5'-ACTTAATCTTTATATAAAAAATACATCAATATG	
seo		(12)
F	5'-TATGTAGTGTAACAATGCATATGCA	
R	5'-TCTATTGTTTTATTATCAITATAAAATTGCAAAT	
sep		(12)
F	5'-TTAGACAAACCTATTATCATAATGGAAGT	
R	5'-TATATAAATATATATCAATATGCATATTTTTAGACT	
seq		(12)
F	5'-GGAAAATACACTTTATATTCACAGTTTCA	
R	5'-AITTATTCAGTTTCTCATATGAAATCTC	
h1b		(13)
F	5'-AGCTTCAAACCTAAATGTCA	
R	5'-CCGAGTACAGGTGTTGGTA	
sak		(13)
F	5'-GTGCATCAAGTTCATTGAC	
R	5'-TAAGTTGAATCCAGGGTTTT	
pvl		(14)
F	5'-ATCATTAGGTAAAAATGCTGGACATGATCCA	
R	5'-GCATCAAGTGTATTGGATAGCAAAGC	

Table 2. Virulence Patterns for Methicillin-Resistant *Staphylococcus aureus* Strains

No. of Virulence Genes	Frequency (%)	Pattern
Four virulence genes	4 (4.4)	
<i>sea, sek, seq, hlb</i>	4 (4.4)	1
Five virulence genes	45 (49.5)	
<i>sea, sek, seq, hlb, sak</i>	45 (49.5)	2
Six virulence genes	6 (6.6)	
<i>sea, seg, sek, sel, seq, hlb</i>	2 (2.2)	3
<i>sea, sei, sek, sel, seq, hlb</i>	4 (4.4)	4
Seven virulence genes	31 (34)	
<i>sea, seg, sek, sel, seq, hlb, sak</i>	19 (20.8)	5
<i>sea, sei, sek, sel, seq, hlb, sak</i>	6 (6.6)	6
<i>sea, seg, sei, sek, seq, hlb, sak</i>	2 (2.2)	7
<i>sea, seg, sei, sek, sel, seq, hlb</i>	4 (4.4)	8
Eight virulence genes	5 (5.5)	
<i>sea, seg, sei, sek, sel, seq, hlb, sak</i>	5 (5.5)	9

Table 3. The Frequency of Prophage Patterns, SCCmec and *ccr* Types Among Methicillin-Resistant *Staphylococcus aureus* Isolates

Patterns	Prophage Types				SCCmec	<i>ccr</i>	<i>pvl</i>	Frequency (%)
	SGB	SGF	SGFa	SGFb				
1	+	+	+	+	III	3	-	72 (79.1)
2	-	+	+	+	III	3	-	19 (20.9)

4. Results

4.1. Identification and Screening of Methicillin-Resistant *Staphylococcus aureus* Strains

All 279 strains isolated in this study were confirmed as *S. aureus* using the genotyping method. Among these, 91 strains (32.6%) that showed resistance to oxacillin and cefoxitin and also were positive for the *mecA* gene were selected as MRSA.

4.2. Identification of Virulence Genes

As shown in Table 2, six different enterotoxin genes were successfully detected among MRSA strains. Among these, *sea, sek* and *seq* were present in all strains and *seg, sei* and *sel* were detected in 35%, 23% and 44% of the strains. On the other hand, the PCR for detection of *hlb* and *sak* genes were positive among 100% and 85% of the MRSA strains, respectively.

According to the presence of different virulence genes, a total of 9 different patterns were identified. Among these, 5 isolates harbored 8 different virulence genes together. Also, 4 isolates were also positive for genes encoding, SEA, SEK, SEQ and HLB, and the presence of virulence factors among isolates was limited to at least 4 virulence genes. Moreover, nineteen strains (20.8%) were classified in the virulence pattern 5 and harbored seven genes.

4.3. Prophage Typing and SCCmec Typing

Among all 91 MRSA strains, we could detect SGB, SGF, SGFa and SGFb prophage types (Table 3), and all isolates at least contained 1 prophage type and 2 subtypes. Moreover, 2 prophage patterns were also identified among all isolates.

Only SCCmec type (III) and type 3 *ccr* were detected among all 91 MRSA strains and none of the isolates were found to be positive for *pvl* gene (Table 3).

5. Discussion

The prevalence of MRSA in this study was 32.6%. The rate of MRSA in Iran varies in different cities (19 - 90%) (1, 7, 10, 16-21). The variation in different reports in Iran could be due in part to different populations, geographical locations and the quality of hospitals samplings which were carried out.

Unlike other studies in Iran (1, 7, 20), in this study, the prevalence of prophage types among MRSA strains was limited to SGB, SGF, SGa and SGFb, and none of the isolates harbored SGA and SGL prophage types. As we showed previously (7), the absence of SGA prophage type among MRSA strains results in the lack of the *pvl* gene and consequently no CA-MRSA strains. Moreover, according to our previous claim that the presence of SGA prophage type is related to the low minimum inhibitory concentra-

tion (MIC) of oxacillin, none of the isolates had MIC \geq 4 μ g/mL (data not shown). Also, 2 prophage patterns were identified among isolates and type 1 was the dominant type and 80% of the strains were classified in this pattern. We previously showed that, this type was the dominant type among MRSA strains in Tehran (1, 7, 20). Presence of 2 different prophage patterns among MRSA strains indicated the potential of our strains to produce broad spectrum of virulence factors such as exfoliative toxin A, toxic shock syndrome toxin-1 (TSST1), lipase, enterotoxins (A, E, G, K and P), staphylokinase and beta-lysin (1). Similar prophage patterns in previous studies further suggest the circulation of some MRSA clonal types in Tehran.

Similar to this study, reports from Iran and other countries indicated the dominant SCCmec type III among different hospitals (7, 16, 17, 19, 22-24), environment (25) and poultry farms (26). Moreover, all isolates were negative for the *pvl* gene and the hospital origin of all isolates was demonstrated. In this study, the distribution of SCCmec type III was consistent with the fact that all strains were isolated from patients after 72 hours of admission to the hospital, and were HA-MRSA.

In this study, 6 different enterotoxin and also 2 virulence genes were detected among all MRSA strains. Genes encoding enterotoxins A, K and Q and also hemolysin B were present in all MRSA strains. SEA, SEK and HLB are prophage (SGF) -encoded virulence factors and SEQ is encoded by SaPI. Interestingly, as we showed all MRSA strains harbored SGF, SGFa and SGFb prophage types; so, the presence of *sea*, *sek* and *hnb* was not surprising for us. The prevalence of *sea*, *sek* and *seq* genes among these isolates was higher than other reports (27-29), indicating the pathogenic potential of these isolates. These variations could be due to the origin of these strains and also the location and geographical situation of different countries (30). Like other reports (9, 12, 31-35), here all strains were grouped into different patterns consisting of multiple virulence genes. One explanation for the presence of multiple toxins in most strains is that these genes are often structurally linked. The absence of different enterotoxin genes among the isolates in this study could be due to the lack of plasmids and pathogenicity islands encoding these enterotoxins (4, 36). For example, the *sed* and *sej* genes are located at the same plasmids; so, it was not surprising we could not detect both genes. At the moment, the enterotoxin producing strains are spread extensively in the world and have become a challenge for public health and involved in colonization and a broad spectrum diseases (37, 38).

This study demonstrated that the *hnb* gene is widely distributed among MRSA strains in this hospital. In this study all MRSA isolates were positive for the *hnb* gene. Although the role of beta hemolysin in diseases is not clearly understood (39), Katayama et al. (40) illustrated that hemolysin also has an important role in skin colonization by damaging keratinocytes, in addition to its well-known hemolytic activity for erythrocytes. Staphy-

lokinase also is another prophage- encoded virulence factor, which favors symbiosis of staphylococci with the host that makes it an important colonization factor (41).

In conclusion, our findings illustrated the presence of highly virulent HA-MRSA strains in a referral hospital in Tehran. The presence of bacteriophage encoded virulence factors and resistance to oxacillin enable bacteria to produce broad spectrum range of diseases, which highlight the potential risk for hospitalized patients in this hospital. Persistence of such strains in hospitals in Tehran could be a challenge for public health.

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Footnote

Authors' Contribution: Fateh Rahimi researched, supervised and developed the study concept, designed and revised the manuscript. Sharmin Karimi researched, developed the study and prepared the draft of the manuscript.

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