

## Multiple-Locus Variable Number of Tandem Repeats Fingerprinting (MLVF) and Virulence Factor Analysis of Methicillin Resistant *Staphylococcus aureus* SCCmec Type III

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### Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA), particularly strains with type III staphylococcal cassette chromosome *mec* (SCCmec), represent a serious human pathogen in Tehran, Iran. The disease-causing capability depends on their ability to produce a wide variety of virulent factors. The prevalence of exotoxin genes and multiple-locus variable number of tandem repeats fingerprinting (MLVF) profile among MRSA isolates, from patients in Tehran, was evaluated by PCR and Multiplex-PCR. The MLVF typing of 144 MRSA isolates with type III SCCmec produced 5 different MLVF types. Generally, 97.2% (140/144) of all the isolates were positive for at least one of the tested exotoxin genes. The most prevalent genes were *hld*, found in 87.5% (126/144) of the isolates followed by *lukE-lukD* and *hla* found in 72.9% (105/144) and 70.1% (101/144) of the isolates, respectively. The *tst* gene, belonging to MLVF types I, IV and V, was found among three of the isolates from blood and wound samples. The *sea* gene was detected in 58.3% (84/144) of the isolates and the *sed* and *see* genes were found in one isolate with MLVF type V. The coexistence of genes was observed in the 87.5% (126/144) of the isolates. The rate of coexistence of *hld* with *lukE-lukD*, *hla* with *lukE-lukD* and *sea* with *lukE-lukD* were 66.7% (96/144), 44.4% (64/144) and 44.4% (64/144), respectively. The present study demonstrated that MRSA strains with type III SCCmec show different MLVF patterns and exotoxin profiles.

**Key words:** methicillin-resistant *Staphylococcus aureus*, MLVF, exotoxin

### Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) is a nosocomial and community pathogen causing various infections, ranging from simple superficial skin infections to more life-threatening ones, including endocarditis, pneumonia, and toxic shock syndrome (Jarraud *et al.*, 2002; Emaneini *et al.*, 2007; Fatholahzadeh *et al.*, 2008). Hospital-associated MRSA is usually accompanied by specific risk factors such as recent hospitalization, surgery, dialysis and long-term care. Risk factors for community-acquired infections include intravenous drugs, prior antimicrobial usage and underlying illnesses such as pulmonary disease, diabetes and chronic skin diseases (Moreno *et al.*, 1995; Chambers, 1997; Baba *et al.*, 2002). However, the capability of *S. aureus* strains to produce disease depends on its ability to create a wide variety of virulence factors

that contribute to colonization and disease incidence in hosts (Dinges *et al.*, 2000). Some *S. aureus* strains produce a variety of exotoxins such as staphylococcal enterotoxins (SEs), toxic shock syndrome toxin 1 (TSST-1), exfoliative (epidermolytic) toxins (ETs), several haemolysins (alpha, beta, gamma and delta) and Panton-Valentine leukocidin (PVL). Staphylococcal enterotoxins are responsible for staphylococcal food poisoning, whereas TSST-1 and ETs are the causative toxins of toxic shock syndrome and staphylococcal scalded-skin syndrome, respectively (Baba *et al.*, 2002; Kim *et al.*, 2006). PVL is involved in severe diseases among children and young adults with no apparent experience of reference to healthcare establishments (Holmes *et al.*, 2005).

Many molecular methods have been used to determine the genotypic background of *S. aureus* isolates, including Pulsed-Field Gel Electrophoresis (PFGE)

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considered as a "gold standard" to Multi-Locus Sequence Typing (MLST) and Spa typing. However, some limiting factors, such as financial expenses, have caused these methods to be used only in some laboratories. During recent years, PCR-based typing methods have been developed which are cheaper and less time consuming than PFGE or sequence-based typing. Multiple-Locus Variable Number of Tandem Repeats Fingerprinting (MLVF) is a new method used for characterization of *S. aureus* isolates which is based upon the detection of the number variation in short sequence repeats in seven individual genes (*sspA*, *spa*, *clfA*, *clfB*, *sdrC*, *sdrD* and *sdrE*). There are various reports suggesting that the production of staphylococcal toxins does vary according to genotype. Moreover, it has also been suggested that the staphylococcal toxin profiles of particular isolates may be influenced by their geographic origins (Moore *et al.*, 2001; Baba *et al.*, 2002; Banks *et al.*, 2003).

A recent study has reported a high incidence of MRSA with SCC*mec* type III in Tehran (Fatholahzadeh *et al.*, 2008). However, the properties of their virulence factors are unknown. This study was designed to determine the prevalence of different exotoxins and genotypes among the MRSA isolates, obtained from patients in Tehran.

## Experimental

### Material and Methods

**MRSA Isolates.** A total of 144 clinical isolates of MRSA with SCC*mec* type III were involved in the present study. These isolates were selected from a collection of 178 MRSA strains obtained from various nosocomial infection sites (wound, blood, respiratory, cerebrospinal fluid, pleural fluid, osteomyelitis, catheter-related infection, septicemia and urine) from patients who developed infections within the first 48 hr of their admission to a hospital in Tehran. The collection process was carried out during April 2008 to March 2009 from different wards of the hospital. Only one isolate was investigated per patient. To confirm the identification of the isolate as MRSA, the *femA* and *mecA* gene were amplified by a PCR-based method, using primers *femA*-F-5'-AAA AAG CAC ATA ACA AGC G-3', *femA*-R-5'-GAT AAA GAA GAA ACC AGC AG-3', *mecA*-F 5'-TGG CTA TCG TGT CAC AAT CG-3' and *mecA*-R 5'-CTG GAA CTT GTT GAG CAG AG-3' (Mehrotra *et al.*, 2000). The MRSA isolates were confirmed as type III SCC*mec* using the method described by Oliveira *et al.* (Oliveira and de Lencastre, 2002). The WBG525 (EMRSA-1, SCC*mec* type III, *ccr 3*) strain were used as control.

**Toxin gene detection.** All the isolates were tested for the presence of genes encoding haemolysins (alpha,

beta, gamma and delta), TST, ETA, ETB, SEA, SEB, SEC, SED, SEE, LUKE and LUKF by PCR and Multiplex – PCR using previously described primers (Mehrotra *et al.*, 2000; Jarraud *et al.*, 2002). *S. aureus* ATCC 14458, NCTC 7428 and ATCC 49775 were used as controls.

**MLVF typing.** The primers used to detect *clfA*, *clfB*, *sdrCDE*, *spa*, and *sspA* were as described by Sabat *et al.* (Sabat *et al.*, 2003). The conditions of multiplex PCR were as described by Tenover *et al.* (Tenover *et al.*, 2007) with some modifications, which are as follows; the PCR was performed in a final volume of 25  $\mu$ L containing 2 units of *Taq* DNA polymerase, 1X of PCR buffer, 0.2 mM of each deoxynucleoside triphosphate, 2.5 mM MgCl<sub>2</sub>, 1  $\mu$ mol/ml of ClfA-F/R, 0.6  $\mu$ mol/ml of ClfB-F/R, CdrCDE-F/R, Spa-F/R, SspA-F/R and 6  $\mu$ l of DNA template. The cycles included 94°C for 5 min for the first cycle followed by 30 cycles of 94°C for 40 s, 53°C for 40 s, 72°C for 1 min and a final cycle of 72°C for 5 min. Amplified products were analyzed by electrophoresis on 2% agarose gel. DNA bands were visualized by staining with ethidium bromide and photographed under UV illumination. MLVA profiles were analyzed visually using the criteria of Sabat *et al.* (Sabat *et al.*, 2003). Any pattern differing by one or more bands was considered as a distinct type.

## Results and Discussion

MRSA is a pathogen of serious notice in Tehran for being a cause of a wide variety of nosocomial infections. A high prevalence rate of MRSA with SCC*mec* type III was previously reported in this region (Fatholahzadeh *et al.*, 2008). In order to identify the ability of MRSA strains to produce virulence factors, a population of 144 clinical MRSA isolates, causing a broad spectrum of infections, was investigated.

The MLVF typing of the 144 isolates produced 5 different MLVF types (Fig. 1). Ninety three isolates (64.6%) were identified as type I, eighteen (12.5%) as type II, fourteen (9.7%) as type III, twelve (8.3%) as type IV and seven isolates (4.9%) as type V. The observation of different MLVF types among a single SCC*mec* type has not been previously reported. However, different MLVF types with identical PFGE patterns have been shown in the MRSA isolates (Moser *et al.*, 2009). In the current study, the MRSA isolates belonged to a type of MLVF pattern that is widely distributed across various wards as well as among all the age groups of patients. This fact suggests that one major MRSA clone was circulated in the hospitals under assay. This was in agreement with the previous reports of other authors who had indicated that a few MRSA clones were predominant in nosocomial infections (Teixeira *et al.*, 1995; Stefani and Varaldo, 2003).

Table I  
Distribution of toxin genes among methicillin-resistant *Staphylococcus aureus* isolates with different MLVF types

Toxin Genes	MLVF type															Total MRSA n=144
	I			II			III			IV			V			
	W† n=45	B n=19	O n=29	W n=9	B n=2	O n=7	W n=7	B n=2	O n=5	W n=2	B n=4	O n=6	W n=3	B n=2	O n=2	
<i>sea</i>	2		1													3
<i>hla</i>	-													1		1
<i>hld</i>	1	1		1			1				1					5
<i>lukE-lukD</i>	1	1	1			1			1							5
<i>sea + hla</i>			1													1
<i>sea + hld</i>	1	1	2													4
<i>sea + lukE-lukD</i>		1	1													2
<i>hla + hld</i>	1		1			1										3
<i>hld + hld</i>	2															2
<i>hld + lukE-lukD</i>	1	1														2
<i>hlg + lukE-lukD</i>	-			1												1
<i>hla + hld + lukE-lukD</i>	3	2	2	1	1	1	1	1				1				13
<i>hld + hld + lukE-lukD</i>	1		3													4
<i>sea + hld + lukE-lukD</i>	1	2		1			1					1	1			7
<i>sea + hla + hld</i>	3	1	1		1						1	1				9
<i>sea + hla + lukE-lukD</i>		1														1
<i>hla + hld + hld</i>		1	1			1							1			4
<i>hla + hld + lukE-lukD</i>	1	2	1			1										6
<i>sea + seb + hld + tst</i>													1			1
<i>sea + hla + hld + lukE-lukD</i>	20	2	10	4		1	3	1	1	1	3		1			45
<i>sea + hld + hld + lukE-lukD</i>	2	1														3
<i>sea + hla + hld + hld</i>	1		1													2
<i>hla + hld + hld + lukE-lukD</i>	2	2	2			1						1	1			10
<i>sea + hla + hld + hlg + tst + lukE-lukD</i>		1														1
<i>sea + hla + hld + hld + lukE-lukD</i>	2			1			1									4
<i>sea + sed + sec + tst + hla + hld + hld + hlg + lukE-lukD</i>							1							1		1

† W – wound, B – blood, O – others

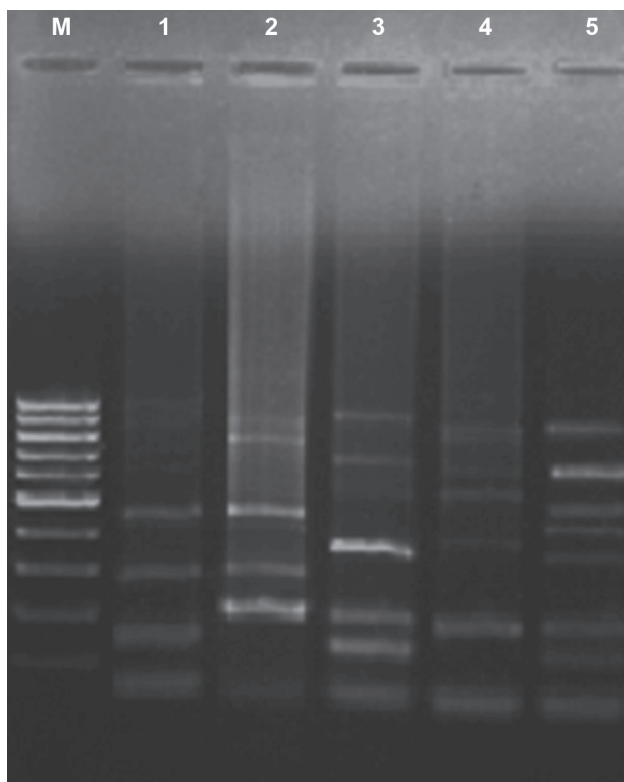


Fig. 1. Multiplex PCR MLVA typing of MRSA isolates. Lanes 1–5: tested MRSA isolates, identified as MLVA type I to V (lane 1; type II, lane 2; type III, lane 3; type IV, lane 4; type V, lane 5; type I).

The prevalence of the recognized genes among the studied isolates and the coexistence frequency of various virulence factors are summarized in Table I. Generally, 97.2% (140/144) of all the isolates were positive for at least one of the exotoxin genes tested. This was higher than the data reported by Kim *et al.* (Kim *et al.*, 2006), who had found that 85.5% of the MRSA isolates produced one or more toxins.

*S. aureus* produces a number of cytotoxins including alpha, beta, delta and gamma hemolysins and Leukocidal toxin encoded by *hla*, *hnb*, *hld*, *hlg*, and *lukE-lukD* genes respectively. These toxins are important in several staphylococcal diseases (Dinges *et al.*, 2000). In the present study, the prevalence of these genes were 59%, 15.2%, 87.5%, 2.1% and 72.2%, respectively. This contrasted the results obtained by Kocsis *et al.* (Kocsis *et al.*, 2009) who reported a high prevalence rate of such genes among MRSA strains, isolated from Austria and Hungary. Although the genes encoding haemolysin toxins are located on chromosomes, the prevalence of these genes can be reduced by acquiring movable genes on genetic elements.

The prevalence of *lukS-PV-lukF-PV* gene among MRSA isolates differs noticeably in different countries (Jarraud *et al.*, 2002; Karahan *et al.*, 2008). In this study, all the isolates were negative for the *lukS-PV-lukF-PV* gene. This may be due to the fact that

the tested isolates only carried the SCCmec type III. Other studies have shown that the gene encoding PVL is the molecular marker for community acquired-MRSA which usually carries type IV SCCmec element (Jarraud *et al.*, 2002; Karahan *et al.*, 2008).

The genes encoding SEs are carried on various genetic elements including phages (*sea*, *see*), plasmids (*sed*) and pathogenicity island (*seb* and *sec*) (Novick *et al.*, 2001; Lawryniewicz-Paciorek *et al.*, 2007). The present study also detected enterotoxin genes among 58.3% of the isolates. This shows a 16% lower rate than the data reported by Kim *et al.* (Kim *et al.*, 2006) who reported that 74.4% of the MRSA isolates with SCCmec type III were positive for enterotoxin genes and that the *sea* genes were the most prevalent (58.3%). However, the distribution pattern of the enterotoxin genes was different in the current study showing 74.4% *sea* and 48.8% *see*. In this study, the *sec* gene was not detected in any of the isolates and the *seb* gene was amplified in one isolate belonging to MLVA type IV from the wound associated with *sea* and *tst* genes. The *sed* and *see* genes associated with *sea* and *tst*, were observed in one isolate obtained from the blood belonging to the MLVA type V.

Coexistence of genes was observed in 87.5% (126/144) of the isolates. The rate of coexistence of *hld* with *lukE-lukD*, *hla* with *lukE-lukD* and *sea* with *lukE-lukD* were 66.7% (96/144), 44.4% (64/144) and 44.4% (64/144), respectively. The coexistence of *sea* with *lukE-lukD*, observed in this study, has also been reported by other studies (Schmitz *et al.*, 1997; Gravet *et al.*, 1999)

None of the MRSA isolates, in this study, were positive for the genes encoding the exfoliative toxin. This result is in agreement with other investigations showing a low prevalence rate of this gene among the tested isolates (Becker *et al.*, 2003; Cha *et al.*, 2006). As reported previously, only some strains of the *S. aureus* produce exfoliative toxins, whilst approximately all the isolates produce enzymes and hemolysins that contribute to their pathogenicity (Becker *et al.*, 2003).

The *tst* gene encodes TSST-1, which is associated with staphylococcal toxic shock syndrome (TSS) and is considered to be the cause of nearly all cases of menstrual TSS and at least 50% of non-menstrual cases (Cha *et al.*, 2006). Our results revealed that 3 isolates from blood and wound expressed the *tst* gene, in combination with other toxin genes. These isolates belonged to MLVF types I, IV and V.

Finally, it is concluded that the MRSA strains with type III SCCmec indicate different MLVF patterns and staphylococcal toxin profiles.

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