

SCCmec typing and Panton-Valentine leukocidin occurrence in methicillin resistant *Staphylococcus aureus* isolates from clinical samples of Ahvaz, southwest of Iran

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Summary

Resistance to methicillin in methicillin resistant *Staphylococcus aureus* (MRSA) is dependent on *mecA* gene located on staphylococcal cassette chromosome (SCC). Both SCCmec type and Panton-Valentine leukocidin (PVL) affect *S. aureus* pathogenicity. Aim of this study was to investigate the prevalence of SCCmec types and *pvl* genes among MRSA isolates from inpatients. During this cross-sectional study on 100 clinical isolates, following antibiotic susceptibility test, screening of *mecA* and *pvl* genes, as well as SCCmec typing, was done in a multiplex PCR technique. From the studied samples, 58 isolates were recognized as MRSA. The frequency of *mecA* and *pvl* was 58% and 4%, respectively. All of the MRSA were resistant to cefoxitin and had the highest sensitivity to chloramphenicol. The majority (77.5%) of MRSA was originated from wound samples. The SCCmec III was the most frequent type (22.4%) in these samples. The *pvl* positive isolates were from SCCmec IVb and V, thus meaning they

are from CA-MRSA. These results show a high prevalence of MRSA in the studied region and a widespread prevalence of SCCmec I-V types. Furthermore, high prevalence of SCCmec III indicates the prevalence of multidrug resistant MRSA. This finding is a serious alarm for medical health care practitioners for the correct use of antibiotics in order to limit the spread of multidrug resistant strains. In addition, with regard to life threatening infections caused by *pvl* harbouring strains, early diagnosis and treatment of infections caused by these isolates should be mandatory.

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most important agents of hospital acquired infections that causes self-limiting to life-threatening opportunistic infections (1,2). The methicillin resistant *S. aureus* (MRSA) is one of the main causes of community and hospital acquired infections that can lead to treatment failure (1) and mortality (3). Following appearance of penicillin resistant strains, methicillin was introduced for treatment of *S. aureus* infections but in 1961, just one year following its introduction in the clinical practice, MRSA strains were reported to be present in European hospitals. The emergence of such strains was then reported from other countries such as Canada, North America and Australia. This resistance is due to the acquisition of *mecA* gene (2.1 kbp) that encodes PBP_{2a} and can move between bacterial strains through a large movable chromosomal cassette, namely SCCmec and inserted at a specific site as *Orfx*. PBP_{2a} has a low affinity for β -lactam antibiotics (1). The SCCmec have two essential element cassettes including *ccr* gene complex and *mec* gene complex (4). Until today, 11 main types of SCCmec (I-XI) have been identified and the difference of SCCmec type is applied as a clue for differentiating MRSA clones. Hospital acquired MRSA (HA-MRSA) is mainly found in old patients with predisposing factors such as catheter use, while community acquired MRSA (CA-MRSA) mainly affects younger people without predisposing factors. CA-MRSA is usually sensitive to β -lactam antibiotics and harbour SCCmec type IV, V or VI while HA-MRSA strains show multidrug resistance with SCCmec type I, II or III (5). Therefore, typing of MRSA isolates can reveal the genetic origin of them and provides suggestions for controlling hospital infections in order to prevent cross contaminations (6). Nearly, all *S. aureus* isolates produce different toxins and enzymes that are essential in their pathogenicity. Panton-Valentine leukocidin (PVL) is a cytotoxin and a virulent factor of *S. aureus*. This cytotoxin is specific for leukocytes and two ORFs (Luks-PV and Lukf-PV) encode it. The *pvl* positive strains are commonly involved in skin and mucosal membrane infections, necrotizing pneumonia, urinary infections and endocarditis (7,8,9). The presence of *pvl* gene is

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regarded as a marker for MRSA (4). The *pvl* harboring MRSA usually has SCCmec types IV and V and is related with CA-MRSA (10). Regarding the role that SCCmec plays in resistance and pathogenicity and the relation between virulent factors and SCCmec type, typing of MRSA based on SCCmec can be a useful and practical method for epidemiological study, and a key element for revealing their origin and distribution in different regions. The aim of present study was to investigate the prevalence of *pvl* gene and SCCmec type among clinical *S. aureus* isolates from Razi Hospital of Ahvaz, Iran.

Materials and Methods

During a 9-month period in a cross-sectional study, *S. aureus* isolates from blood, wound, tracheal secretions, urine and pus samples were collected and identified according to the phenotypic methods (11). The antibiotic susceptibility of isolates against 13 common antibiotics was investigated using Kirby-Bauer standard disc diffusion method according to CLSI 2011 and 2013 Guidelines. Resistance to methicillin was assessed using cefoxitin disk (30 µg) based on CLSI criteria (2013). Furthermore, the presence of *mecA* gene was also investigated. DNA of all isolates were extracted using boiling method.

A *S. aureus* strain containing *mecA* gene was also extracted and used as positive control. In a multiplex PCR reaction *mecA* and *pvl* gene screening was performed; *mecA1*-F (GTAGAAATGACTGAACGTCCGATAA) and *mecA2*-R (CCAATTCACATTGTTTCGGTCTAA) primer pairs and Luk-PV-1F (ATCATTAGGTAAAATGTCTGGACATGATCCA) and Luk-PV-2R (GCATCAAGTGTATTGGATAGCAAAAAGC) primer pairs were used for amplification of *mecA* and *pvl* gene, respectively (12).

The PCR reaction was performed at 25 µL final volume containing 12.5 µL of 2× master mix (Master Mix Ampliqon, Denmark), 0.24 µM of *mecA* primers, 0.2 µM of Luk-PV primers and 2 µL of extracted DNA. The amplification program was: initial denaturation (94°C, 10 min), 10 cycles each containing denaturation (94°C, 45s), annealing (55°C, 45s) and extension (72°C, 75s), then 25 cycles were performed as previously mentioned except 50°C was used as annealing temperature and a final extension (72°C, 10 min). A negative control (distilled water) and a positive

control (*mecA* positive strain) were also used in parallel. Finally, amplification of *mecA* gene (310bp) and *pvl* gene (433bp) was confirmed through electrophoresis in 1% agarose containing DNA safe stain. MRSA strains were subjected to SCCmec typing according to the multiplex PCR method described by Zhang *et al.* (2005) with minor modifications (13).

In order to prevent non-specific reactions, 3 different sets of multiplex PCR reactions were adjusted, each containing 3 primer pairs including set 1: SCCmec type I, IVa and IVb primer pairs; set 2: SCCmec type II, III and V primer pairs, and set 3: SCCmec type I, IVc and IVd. Each 25 µL reaction consists in 12.5 µL of 2× Master Mix, 1.5 U Taq DNA polymerase enzyme, 1.5 mM MgCl₂, 0.4 µM of each primer and 5 µL of extracted DNA. Amplification program was as follows: initial denaturation (94°C, 15 min), 10 cycles consisting in denaturation (94°C, 30s), annealing (55°C, 90s) and extension (72°C, 90s), then 25 cycles including denaturation (94°C, 45s), annealing (50°C, 90s) and extension (72°C, 90s) and a final extension (72°C, 10 min).

The successful amplification was confirmed through electrophoresis on 1.3% agarose gel. The data were analysed using SPSS V.17 and Fisher and Chi-square at 0.05 confidence interval.

Results

Out of the 100 *S. aureus* strains, 56 were from male patients and 44 from female. The distribution of isolates among clinical specimens was as follows: wound (78), blood (6), tracheal secretions (8), urine (7) and knee abscess (1). In antibiotic susceptibility analysis of MRSA, 58% of isolates were resistant to cefoxitin, while 97% and 82% of strains were resistant to methicillin and oxacillin, respectively. According to the CLSI 2013 Guidelines, the resistance to cefoxitin was regarded as MRSA. In MRSA screening based on *mecA* amplification, 58 samples were also positive. Therefore, based on the phenotypic and genotypic analysis, the frequency of MRSA was 58%; 60 and 40% of MRSA were isolated from male and female patients, respectively. The frequency of MRSA based on clinical samples was as follows: wound: 77.5%; tracheal secretions: 5.1%; urine: 6.7%; blood: 8.6% and knee abscess: 1.7%.

The antibiotic susceptibility profile of all *S. aureus* isolates has been described in Table 1. As it reported, the highest sensitivity

Table 1. Antibiotic susceptibility testing of *S. aureus* isolates.

Antimicrobial Agent	Sensitive, n (%)	Intermediate, n (%)	Resistance, n (%)
Penicillin (P)	5 (5)	-	95 (95)
Oxacillin (OX)	17 (17)	1 (1)	82 (82)
Methicillin (Me)	2 (2)	1 (1)	97 (97)
Co-Trimoxazol (SXT)	31 (31)	2 (2)	67 (67)
Vancomycin (V)	48 (48)	-	52 (52)
Ciprofloxacin (CP)	22 (22)	9 (9)	69 (69)
Erythromycin (E)	9 (9)	7 (7)	84 (84)
Chloramphenicol (C)	59 (59)	7 (7)	34 (34)
Clindamycin (CC)	20 (20)	-	80 (80)
Gentamycin (GM)	28 (28)	4 (4)	68 (68)
Rifampicin (RA)	34 (34)	1 (1)	65 (65)
Ceftazidime (CAZ)	7 (7)	-	93 (93)
Imipenem (IPM)	31 (31)	9 (9)	60 (60)

was to chloramphenicol (59%), and rifampicin (34%) and the highest resistance was to methicillin (97% and penicillin (95%). Table 2 reports the antibiotic susceptibility of MRSA strains. The highest resistance was to methicillin (98.27%), penicillin (95%) and the highest sensitivity was to chloramphenicol (36.2%).

In this study, 82% of *S. aureus* isolates showed multiple drug resistance (MDR) *i.e.* non-susceptible to oxacillin and at least other 2 antibiotics. Precisely, eight isolates from 10 CA-MRSA and 25 isolates from 27 HA-MRSA had multiple drug resistance. Furthermore, 18 isolates of 21 multiband and non-typeable MRSA showed MDR pattern. Therefore, in total 87.9% of MRSA had multiple drug resistance. The frequency of MDR in clinical sam-

ples was as follows: wound (76.9%), tracheal secretions (62.5%) and blood, urine and pus (100%).

The result of multiplex PCR for *mecA* and *pvl* revealed that their frequency was 58% and 4%, respectively. The SCCmec typing of 58 MRSA showed that 5 isolates, including 5,9,13,4 and 6, belonged to SCCmec type I, II, III, IVb and V, respectively (Figure 1). The SCCmec type III was the most frequent type, followed by type II, V, I and IVb.

Three isolates had produced several bands, while SCCmec typing of 18 isolates was impossible, thus indicating that typing was inconclusive and that likely they belong to types VI -XI (14). According to the typing results, we have identified 27 HA-MRSA

Table 2. Antibiotic susceptibility pattern of MRSA.

Antimicrobial Agent	Sensitive, n (%)	Intermediate, n (%)	Resistance, n (%)
Penicillin (P)	3 (5)	-	55 (95)
Oxacillin (OX)	6 (10.34)	1 (1.72)	51 (87.93)
Methicillin (Me)	1 (1.7)	-	57 (98.27)
Co-Trimoxazol (SXT)	15 (25.86)	-	43 (74.13)
Vancomycin (V)	28 (48.27)	-	30 (51.72)
Ciprofloxacin (CP)	15 (25.86)	1 (1.72)	42 (72.41)
Erythromycin (E)	5 (8.62)	3 (5.17)	50 (86.2)
Chloramphenicol (C)	21 (36.2)	3 (5.17)	34 (58.62)
Clindamycin (CC)	10 (17.24)	-	48 (82.75)
Gentamycin (GM)	13 (22.41)	1 (1.72)	44 (75.86)
Rifampicin (RA)	15 (25.86)	1 (1.72)	42 (72.41)
Ceftazidime (CAZ)	5 (8.62)	-	53 (91.37)
Imipenem (IPM)	17 (29.31)	3 (5.17)	38 (65.51)

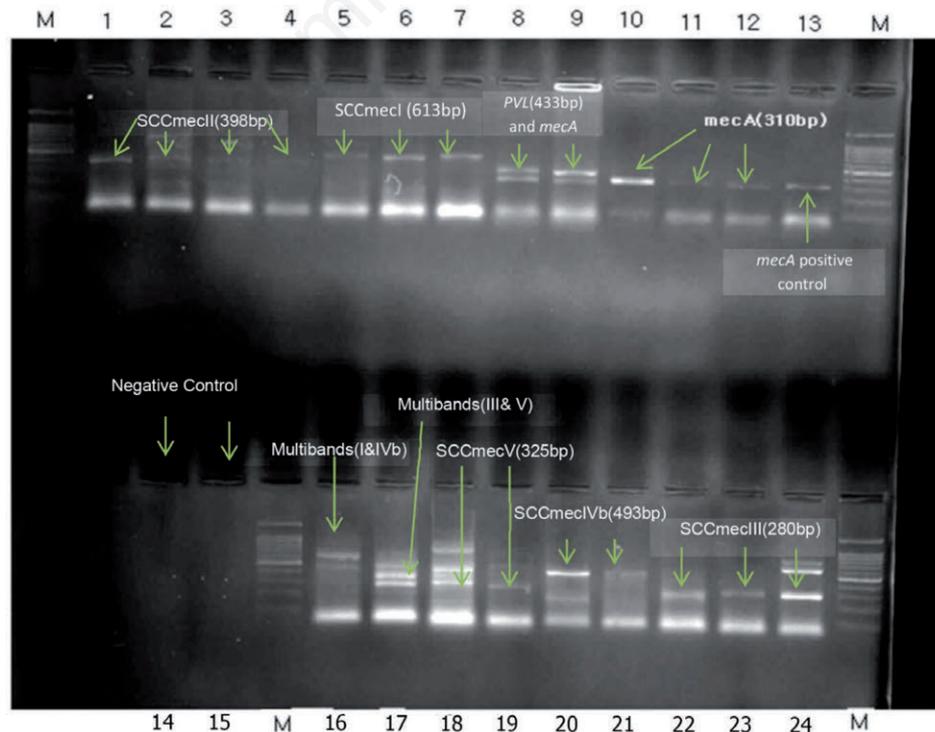


Figure 1. The electrophoresis of PCR products from SCCmec typing and *mecA* and *pvl* screening.

and 10 CA-MRSA isolates. All 4 *pvl* positive isolates were categorized as CA-MRSA; more precisely, 3 isolates of type V and 1 isolate of type IVb. These results are in agreement with previous findings indicating that *pvl* positive MRSA belong to type IV and V and are related to CA-MRSA. Therefore, the presence of *pvl* can be regarded as a genetic marker for CA-MRSA (4). Table 3 presents the frequency of SCCmec types among MRSA.

The frequency of SCCmec types based on the clinical samples is reported in Table 4. The most common type, *i.e.* type III, was isolated from wound specimens.

Statistical analysis shows that there is a significant ($\chi^2 < 0.001$) correlation between phenotypic methicillin resistance and *mecA* in these isolates. Furthermore, the multiple drug resistance is in correlation ($\chi^2 = 0.020$) with the presence of *mecA*. A significant relation was found between the CA-MRSA and *pvl* gene ($P < 0.001$), and also between the urine samples with the presence of HA-MRSA ($P = 0.041$).

Discussion

With regard to the constant increase of antibiotic resistance in pathogenic bacteria and the consequent problems in health care systems, monitoring of antibiotic resistance pattern of local pathogens is of great importance in order to prevent antibiotic treatment failure of infectious diseases. Furthermore, using suitable genetic markers for finding the source of infections can help clinicians to diminish or even prevent infections. Therefore, the present study has evaluated antibiotic resistance pattern of *S. aureus* isolates from a Hospital in Ahvaz. The results showed that resistance to methicillin is the high-

est resistance pattern while the least resistance was to chloramphenicol, as reported by Rezazadeh *et al.* (15). In the study of Moradi *et al.*, the majority of isolates was sensitive to vancomycin, chloramphenicol and rifampicin, and the highest resistance was found against cefoxitin (16).

In this study, the frequency of MRSA was 58%, of which 46.5% categorized as HA-MRSA and 17.2% as CA-MRSA, highlighting the prevalence of HA-MRSA. This finding is in agreement with similar studies carried out in China, Australia and Africa (5-25% range), Portugal (49%), Greece (40%), Italy (37%) and Romania (34%) (17). Fathollah Zadeh *et al.*, in their study carried out in Tehran have reported a MRSA frequency of 36% (18), while Khosravi *et al.* showed a prevalence of 87.3% (19), Zeinali *et al.* 58% (6), and Abdollahi *et al.* 47.5% (20), respectively. The differences between the reported data from different regions can be a result of the different infection control programs, antibiotic therapy regimens and other factors that can affect resistance spread among bacterial species.

The most frequent SCCmec type in the present study was SCCmec type III. Similarly, among the HA-MRSA, SCCmec type III has been the dominant type in Taiwan, Hong Kong, Thailand, India and Srilanka (21). Conversely, Zeinali *et al.* (2011) reported that SCCmec type II was the most frequent type and none of the MRSA strains had SCCmec type III. Furthermore, 58.6% of their samples were non-typeable (6). Similarly, in the study of Abdollahi *et al.* (2012), SCCmec type II was the most frequent (20). Prevalence of five SCCmec types in the present study proves the high variety of types in the area under study.

The prevalence of *pvl* gene in MRSA isolates was 4%, all of them belong to CA-MRSA. Different studies have reported frequency values from 2 to 35% (22,23), while in Argentina and Iran

Table 3. SCCmec type frequency among MRSA.

SCCmec type	CA-MRSA/HA-MRS	Amplicon size (bp)	N. of isolates (%)
SCCmecI	HA-MRSA	613	5 (8.62)
SCCmecII	HA-MRSA	398	9 (15.51)
SCCmecIII	HA-MRSA	280	13 (22.41)
SCCmecIVa	CA-MRSA	776	-
SCCmecIVb	CA-MRSA	493	4 (6.89)
SCCmecIVc	CA-MRSA	200	-
SCCmecIVd	CA-MRSA	881	-
SCCmecV	CA-MRSA	325	6 (10.34)
Multiple bands SCCmec III and SCCmec IVb	Unidentified	(280+493)	1 (1.72)
Multiple bands SCCmec I and SCCmec IVb	Unidentified	(613+493)	1 (1.72)
Multiple bands SCCmec III and SCCmec V	Unidentified	(280+325)	1 (1.72)
Non typable			18 (31.03)

Table 4. Frequency of SCCmec types in different clinical specimens.

	SCCmecI	SCCmecII	SCCmecIII	SCCmecIVa	SCCmecIVb	SCCmecIVc	SCCmecIVd	SCCmecV	Multibands	Nontypable
Wound (45)	4	6	11	-	3	-	-	5	1	15
Blood (5)	-	1	-	-	-	-	-	1	2	1
Urine (4)	1	2	1	-	-	-	-	-	-	-
Tracheal secretions (3)	-	-	-	-	1	-	-	-	-	2
Knee Abscess (1)	-	-	1	-	-	-	-	-	-	-
Total 58	5	9	13	-	4	-	-	6	3	18

have been observed values of 56% and 61.8%, respectively. These differences can be related to the frequency of this gene, as well as to the different methodologies that have been used.

The isolates from skin, wound and pulmonary infections showed a higher prevalence than other clinical samples (24,25). Interestingly, all four *pvl* positive isolates in this study originated from wound samples. In the report of Khosravi *et al.* in burn patients of Ahvaz, 87% of isolates had *mecA* gene and 7% were positive for *pvl* (19). Abdorrazagh *et al.* (2014), have reported a 27% prevalence of *pvl* in Iraq (26). Shore *et al.* found that the frequency of *pvl* in MRSA isolates during 2002-2011 increased from 2 to 8.8%, while its frequency in MSSA decreased from 20 to 2.5% (27). The cross-sectional study results of Qiwen *et al.* on 259 HA-MRSA showed a *pvl* prevalence of 28.6% (28).

Conclusions

High genotypic diversity of MRSA in the present study in Ahvaz may be a result of population diversity, as well as its borderline geographical location. All SCCmec types in this study showed a multiple drug resistance. This fact can lead to both treatment failure in patients and increases in the costs for infection control. This finding can be a serious threat for the health care management system in order to correctly use antibiotics for infection control. With regard to the dangerous nature of *pvl* positive *S. aureus*, the infections caused by these isolates can be a life-threatening factor. Therefore, early diagnosis and treatment of these infections are recommended. The results of this study suggest that by using PCR technique it is possible to detect multiple resistant strains and their SCCmec types and, thus, control their origin. Finally, it can be hypothesized that, based on the significant correlation between *pvl*, SCCmec and CA-MRSA, it is necessary that screening of *S. aureus* isolates be regarded as a useful approach for the diagnosis of these strains and their control.

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