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Original Article

Characterization of *Staphylococcal* Cassette Chromosome Mec Elements in Biofilm-producing *Staphylococcus Aureus*, Isolated from Hospital Infections in Isfahan

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Background and Aims: *Staphylococcus aureus* is one of the important pathogens around the world. The present investigation was carried out to study the distribution of *Staphylococcal* Cassette Chromosome Mec (SCCmec) types and antibiotic resistance properties in methicillin-resistant *Staphylococcus aureus* isolated from Isfahan hospitals.

Materials and Methods: A total of 250 clinical specimens were collected from three major Isfahan hospitals. The samples were cultured, and biofilm producer isolates were subjected to several polymerase chain reaction methods. The patterns of antibiotic resistance were studied using the disk diffusion method.

Results: In the present study, 110 out of 250 samples (44%) were found to be positive for *Staphylococcus aureus*, and all the isolates produced the biofilm in different levels. The most commonly infected samples were collected from wounds (44.5%). The incidence of *mecA*, *tetK*, *ermA*, *ermC*, *tetM*, *aacA-D*, *linA*, *msrA*, *vatA*, *vatC* and *vatB* antibiotic resistance genes were 93.6%, 34.84%, 28.20%, 29.30%, 21.87%, 18.71%, 9.48%, 8.65%, 7.18%, 4.43% and 3.71%, respectively. The distribution of *SCCmec* III (42) was found to be the most type out of 103 *mec* positive strains.

Conclusions: In the prsent study, the highest resistance belonged to methicillin (90.2%), erythromycin (89.7%), ciprofloxacin (89.5%) and penicillin (88%) and the lowest resistance was reported for vancomycin (10%) and nitrofurantoin (8%). These infections with these strains require more advanced hospital care with an emerging demand for the novel antibiotics.

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Introduction

The increasing prevalence of bacterial resistance to commonly used antibiotics may result in an insufficient array of substances to combat some bacterial infections, demonstrating importance of antibiotic resistance pattern in *Staphylococcus aureus* (*S. aureus*) that has long been recognized as an important pathogen of hospital acquired infections. *S. aureus* can be colonized both in the community and hospital settings [1]. Infections caused by this bacterium are mainly treated with methicillin or aminoglycosides that its resistance pattern increased every day. Methicillin resistance in S. aureus (MRSA) is mediated by a penicillin binding protein (PBP)2a encoded by the mecA gene [2-4]. Normally, PBPs show a high affinity for binding to the beta lactam ring. MRSA strains present *mecA* gene and another kind of protein (PBP2a) that generate lower affinity for binding to methicillin. Subsequently, some antibiotics like methicillin will lose their ability for cell wall destruction as well as their efficiency on bacteria. According to other studies, 50% to 90% of *S. aureus* strains isolated from hospital infections were resistant to methicillin [5, 6]. *S. aureus* is introduced as the most common cause of skin and soft-tissue infections (e.g. impetigo, furunculosis, superficial and surgical wounds, and abscess), as well as systemic infections (e.g. pneumonia, urinary tract infections-UTIs and endocarditis) [7-13]. Biofilm producing is regarded as one of the important factors in order to increase antibiotic resistance pattern. The pathogenicity of *S. aureus* depends on various bacterial surface components and extracellular proteins. The pathogenesis of a particular *S. aureus* strain is attributed to combination of extracellular factors and such properties as adherence and biofilm formation. Different proteins of the family of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) which involved in S. aureus adhesion, and affected on important locus that involved biofilm producing genes [9].

The mecA genes, specially called Staphylococcal Cassette Chromosome Mec (SCCmec) elements, are conducted from a special region which are currently classified into types I, II, III, IV and V based on the nature of the *mec* and *ccr* gene complexes, and are further classified into subtypes according to differences in their J region DNA [14-18]. Complete identification of bacterial genetic background and the *SCCmec* element is considered extremely important in regard with molecular typing especially in MRSA [19-21]. It is noticeable that *mecA* gene is transferred by *SCCmec* as a mobile genetic element with the size of 21–67 k bp, that is exclusively found in the *Staphylococcus* genus. A complex of the *SCCmec* gene contains *mec* complex, ccr complex and the Junkyard area. Due to different allotypes and complex classes, five (I–V) main types of *SCCmec* elements were classified in the resent study [22]. *SCCmec* type I was determined in United Kingdom for the first time in 1961, type II was identified in Japan and types III, IV and V were recognized in New Zealand. According to different types of *SCCmec*

elements, hospital acquired-MRSA (HA-MRSA) and community acquired-MRSA (CA-MRSA) are known as independent deviations. CA-MRSA strains are susceptible to most widespread antibiotics in comparison with HA-MRSA and more antibiotic resistances can also be detected in *SCCmec* type III. Therefore, in the present study aimed to investigate antimicrobial resistance pattern as well as to determine frequency of the *mecA* gene and different *SCCmec* types in an epidemiologic research [23].

Materials and Methods

Bacterial isolates

Required samples or all samples required and *Staphylococcus* identification were collected during one year from three hospitals of Isfahan in Iran. A total of 250 clinical samples from various types of infections were selected, among which 110 isolates were Staphylococcus aureus, including blood (n=17), bed sore (n=6), wound (n=49), abscess (n=3), tracheal secretion (n=25), catheter (n=4), joint fluid (n=3) and cerebrospinal fluid (CSF) (n=3). All the samples were directly cultured into 7% sheep blood agar (Merck, Darmstadt, Germany) and incubated aerobically at 37°C for 48 h. After incubation, suspicious colonies were examined using techniques appropriate for diagnosing *Staphylococcus spp*.(microscopical morphology, catalase and coagulase production). The 110 isolates were distinguished by the conventional microbiological methods too, such as growth on blood agar, mannitol salt agar, DNase tests and coagulase. The API-20-*Staph* system kit (bio Merieux, France) was used for the final confirmation. After growth, *staphylococci* were identified on the basis of colony characteristics, Gram staining, pigment production, hemolytic and the following biochemical reactions: catalyses activity, coagulated test (rabbit plasma), Oxidase test, glucose O/F test, resistance to bacitracin (0.04 U), mannitol fermentation on Mannitol Salt Agar (MSA) (Merck, Darmstadt, Germany), urease activity, nitrate reduction, novobiocin resistance, phosphatase, Deoxyribonuclease (DNase) test and carbohydrate fermentation tests [24]. Five MRSA strains were used for the standard strains, consisting of NCTC10442, N315, 85/2082, CA05, and WIS (WGB8318) [25].

Phenotypic assay on slime production

The slime production assay was performed by cultivation of the *S. aureus* strains on Congo Red Agar (CRA) plates as described in the different studies. The CRA plates were incubated at 37ºC in aerobic conditions for 24 h, followed by storage at room temperature for 48 h. The formation of reddish black colonies with a rough, dry, crystalline consistency on CRA plates was considered to be indicative of slime production. Nonsmile-producing strains produced pinkish red, smooth colonies with a darkening at the center. To complete this method, microtiter plate assay was used. In this way, polysterne plate was applied, 20 µL of isolates were added, and then incubated for 48 h at 37ºC, washing with Phosphate-buffered saline. Safranin was utilized for staining the isolates in the polysterne plates, and finally ethanol was used to release biofilm producer isolates. Absorbance was read in 490 nm. Analysis of biofilm producing is shown in

different levels in table 1. These isolates were selected to determine the antibiotic resistance pattern [9-11].

Table 1. Biofilm analysis in *S.aureus* isolates

Biofilm producing	Optical Density (OD)	Cut-off
Strong	OD>0.332	OD>4*ODC[2]
Average	0.166<OD<=0.332	2*ODC<OD<=4*ODC
Weak	0.083<OD<=0.166	ODC<OD<=2*ODC
Anny attachment	OD<=0.083	OD<=0.083

Antibiotic susceptibility testing

Biofilm-producing *S.aureus* isolates were selected and the antibiotic resistance pattern was performed by disc diffusion method on Mueller–Hinton agar. *S. aureus* isolates were tested for susceptibility to penicillin (10 u/disk); imipenem (10 µg/disk); cefazoline (30 µg/disk); cefalotin (30 µg/disk); ceftriaxone (30 µg/disk); gentamicin (10 µg/disk); ciprofloxacin (5 µg/disk); clindamycin (2 µg/disk); azithromycin (15 µg/disk); erythromycin (15 µg/disk); mupirocin (30 µg/disk); rifampicin (5 µg/disk); tetracycline (30 µg/disk); trimethoprim (5 µg/disk); vancomycin (30 µg/disk) and nitrofurantoin (300 µg/disk) by the Kirby-Bauer disk diffusion method (MAST, Merseyside, England). According to Clinical and Laboratory Standards Institute (CLSI) 2011, *S. aureus* ATCC25923 was used as the control strain [26].

DNA extraction and *Staphylococcus* confirmation

A typical colony of the biochemically identified *S. aureus* was cultivated in 1 mL TSB for 24 h at 37°C. The bacterial genomic DNA of *S. aureus* strains was extracted via a QIAGEN plasmid Minikit (Fermentas, Germany) as recommended.

Antibiotic resistance genes amplification

The presence of mecA (encoding methicillin resistance), tetK, tetM (tetracycline resistance), ermA, ermC (macrolide-lincosamide-streptogramin B resistance), aacA-D (aminoglycoside resistance), linA, msrA, vatA, vatC and vatB (streptogramin A resistance) genes were analyzed using the Kumar technique [27]. List of primers are demonstrated in table 2. The multiplex polymerase chain reaction (PCR) were performed in a total volume of 25 µL, including 2 mM of MgCl2, 50 mM of KCl, 10 mM of Tris-HCl (pH 9.0), 0.1% of Triton X-100, 150 µM of dNTPs each (Fermentas, Germany), 2.5 µL of PCR buffer (10X), 25 pmoL of each primers, 2 U of Taq DNA polymerase (Fermentas, Germany), and 4 µL (40-260 ng/µL) of the extracted DNA template of the Staphylococcus isolates. The four set of primer pairs were used in each reaction mixture. The thermal cycler was adjusted as follows: 94°C 5 min., 30 cycles of 1 min. at 95°C for the denaturation step and 1 min. at 55°C for the annealing-extension step followed by the final extension at 72°C for 90 seconds. This study was approved by Ethics Committee of Islamic Azad University of Shahrekord

branch. All ethical issues were considered, according to which this study was performed obtaining the permission of hospitals.

Table 2. Oligonucleotide primers for amplification of antibiotic resistance genes

Genes	Primer Sequence (5'-3')	Length(bp)	Reference
***aac*A-D**	F : TAATCCAAGAGCAATAAGGGC R : GCCACACTATCATAACCACTA	227	**(35)**
***tet* K**	F : GTAGCGACAATAGGTAATAGT R : GTAGTGACAATAAACCTCCTA	360	**(32)**
***tet* M**	F : AGTGGAGCGATTACAGAA R : CATATGTCCTGGCGTGTCTA	158	**(25)**
***msr*A**	F : GGCACAATAAGAGTGTTTAAAGG R: AAGTTATATCATGAATAGATTGTCCTGTT	940	**(24)**
***erm*A**	F: AAGCGGTAAACCCCTCTGA R: TTCGCAAATCCCTTCTCAAC	190	**(35)**
***erm*C**	F: AATCGTCAATTCCTGCATGT R: TAATCGTGGAATACGGGTTTG	299	**(35)**
***vat*A**	F: TGGTCCCGGAACAACATTTAT R: TCCACCGACAATAGAATAGGG	268	**(35)**
***vat*B**	F: GCTGCGAATTCAGTTGTTACA R: CTGACCAATCCCACCATTTTA	136	**(35)**
***vat*C**	F: AAGGCCCCAATCCAGAAGAA R: TCAACGTTCTTTGTCACAACC	467	**(34)**
***lin*A**	F:GGTGGCTGGGGGGTAGATGTATTAACTGG R:GCTTCTTTTGAAATACATGGTATTTTTCGA	323	**(34)**

Multiplex PCR amplification for *SCCmec* typing

S.aureus that were resistant to *mecA* were selected to continue other methods according to the aim of study. Different *SCCmec* types were determined by the specific primers, which were previously described, and listed in Table 2. PCR amplification was performed in a volume of 50 ml by Emerald Amp MAX PCR Master Mix (Takara, Japan) for all the PCR reactions. The DNA Thermal Cycler 480 (Applied Bio systems, Foster City, CA, USA) was programmed as follows: the first denaturation at 94 °C for 5 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and an extension at 72 °C for 60 s for 40 cycles in the final extension at 72°C for 4 min. PCR products were analyzed by electrophoresis on agarose 1.5% via SYBR Safe staining [28]. Method lists of primers are shown in table 3. Fifteen microliters of PCR products were resolved on a 1.5% agarose gel containing 0.5 mg/mL of ethidium bromide in Tris–borate–EDTA buffer at 90 V for 1 h, using suitable molecular weight markers. Ultimately, the products were examined under ultraviolet illumination.

Table 3. Oligonucleotide primers for amplification of *SCCmec* types in *S. aureus* strains isolated from Isfahan hospitals

Types	Primer Sequence (5'-3')	Length(bp)	Reference
***SCCmec* I**	F:GCTTTAAAGAGTGTCGTTACAGG R: GTTCTCTCATAGTATGACGTCC	613	**(35)**
***SCCmec* II**	F: CGTTGAAGATGATGAAGCG R: CGAAATCAATGGTTAATGGACC	398	**(34)**
***SCCmec* III**	F: CCATATTGTGTACGATGCG R: CCTTAGTTGTCGTAACAGATCG	280	**(31)**
***SCCmec* Iva**	F: GCCTTATTCGAAGAAACCG R: CTACTCTTCTGAAAAGCGTCG	776	**(35)**
***SCCmec* IVb**	F: TCTGGAATTACTTCAGCTGC R: AAACAATATTGCTCTCCCTC	493	**(35)**
***SCCmec* IVc**	F:ACAATATTTGTATTATCGGAGAGC R: TTGGTATGAGGTATTGCTGG	200	**(35)**
***SCCmec* IVd**	F: CTCAAAATACGGACCCCAATACA R: TGCTCCAGTAATTGCTAAAG	881	**(35)**
***SCCmec* V**	F: GAACATTGTTACTTAAATGAGCG R: TGAAAGTTGTACCCTTGACACC	325	**(35)**

Results

Out of 250 clinical samples, 110 were positive for *S. aureus*. All the isolates were biofilm producers in different levels. Superficial and surgical wounds had the highest incidence of *S. aureus* (44.5%), while CSF and joint fluid were the samples that had the lowest incidence (2.8%). Frequency of biofilm producing was arranged as below: wound (78.2%), abscess (10.9%), Tracheal secretion (65.4%), blood (24%), catheter (63%), bed sore (85%), joint fluid (17%), cerebrospinal fluid (13.3%). *MecA* (93.6%) involved the most commonly detected antibiotic resistance genes and the lowest antibiotic resistance pattern belonged to *vatC (*4.43%) and *vatB* (3.71%) genes. Isolates with *mecA* genes were selected for *SCCmec* typing. *S. aureus* isolates of superficial, post-surgical wounds, and respiratory infections had the highest incidence of antibiotic resistance genes. *S. aureus* frequency in different parts of hospitals is demonstrated in table 4.

Table 4. Frequency of *S.aureus* isolates in different sections of hospitals

Selected area	Number and Percent of isolates
Orthopedics	35 (35.3)
Internal	33 (33.3)
Intensive care unit	20 (20.2)
Special intensive care unit	11 (11.2)

In the present study, the highest incidence of *SCCmec* types were *SCCmec* type III and II. The incidence of IVa, IVc and IVb *SCCmec* types were proved to be the lowest in *S.aureus* isolates. As a matter of fact, positive results were reported for all of the types. The study results revealed that most of the *S. aureus* isolates were resistant to methicillin, penicillin, tetracycline. The highest levels of antibiotic resistance in *S. aureus* belonged to methicillin (90.2%), erythromycin (89.7%), ciprofloxacin (89.5%) and penicillin (88%). The lowest resistance was observed for vancomycin (10%) and nitrofurantoin (8%). A high incidence of multi-drug resistant (MDR) *S. aureus* strains was observed in patients' clinical samples. All of the specimens such as tracheal secretion, bed sore, superficial and surgical wounds and abscesses of the patients were infected with these strains or other isolates were found as high incidence of multi-drug resistant *S. aureus* strains. It should be all the 110 isolates revealed a high virulence. Moreover, frequency of *S.aureus* isolates we detected from different patients, most of which aged 61-70 with 41(37.3%) prevalence that were hospitalized in 3 major hospitals of Isfahan in Iran. In the current study, 73% of patients were women and 27% were the men. Frequency and recognition procedures of *SCCmec* typing is shown in table 5.

Table 5. Frequency and recognition procedures of *SCCmec* typing

Infection	Type I	Type II	Type III	Type Va1	Type VbI	Type VcI	Type VdI	Type V	Number
Wound	10	16	15	2	1	1	1	3	**49**
Blood	2	7	8	-	-	-	-	-	**17**
Tracheal	2	10	11	1	-	-	1	-	**25**
Abscess	-	1	-	-	-	-	-	2	**3**
Joint fluid	-	1	-	-	-	-	-	-	**1**
CSF	-	-	2	-	-	-	-		**2**
Catheters	1	-	1	-	-	-	-	-	**2**
Bedsore	-	1	5	-	-	-	-	-	**6**

Discussion

The study findings revealed that 90.3% of all *Staphylococcus* strains had the gene coding resistance against methicillin. In addition to methicillin, the *Staphylococcus* strains had the genes coding resistance against some antibiotics such as macrolides, erythromycin, lincosamides, aminoglycosides and tetracycline. *Staphylococcus* strains were held to have the highest levels of antibiotic resistance against methicillin (90.2%), erythromycin (89.7%), ciprofloxacin (89.5%) and penicillin (88%). The lowest resistance was reported for vancomycin (10%) and

nitrofurantoin (8%). The statistical analysis showed significant differences between the incidence of *mecA* and other antibiotic resistance genes. In addition, a statistically significant association was detected between the resistance levels s to tetracycline, penicillin, cefalotin, ciprofloxacin, and ceftriaxone with mupirocin, rifampicin, trimethoprim, imipenem and gentamicin antibiotics. Tocharian's study reported that 72% of *S. aureus* isolates of Lebanon hospitals were methicillin-resistant and 18% of them were resistant to 10-18 antibiotics. Udo also proposed that 1765 (95.6%) inpatients and 81 (4.4%) outpatients of Kuwait hospitals were positive for *S. aureus* with 32% incidence rate of the methicillin-resistant strains [29-31].

Similar studies have indicated incidence rate of MRSA, among which Alghaithy reported 61% in Saudi- Arabia, Młynarczyk stated 40% in Warszawie and Rijals' study showed 56.1% in Bokhara. In a study conducted by Virdis, 56% of *S. aureus* isolates were resistant to one or more antimicrobial agents including kanamycin (28%), ox tetracycline (16%), and ampicillin (12%). The most commonly used antibiotics consisted of oxacillin, nafcillin, cefathiamidine. In fact, vancomycin had the highest resistance to *S. aureus* strains of the Deng's report [31-36]. Nishijima and Kurokawa reported that the incidence of resistance to penicillin, cephalosporin and clindamycin were 20 to 30% and the occurrence of gentamicin, erythromycin, roxithromycin and meticillin resistance were 55.2%,, 39.6%,, 39.1% and 21%, respectively. The *S. aureus* isolates were highly resistant to antibiotics in this study: 36.4% were resistant to streptomycin, 33.6% to ox tetracycline, 29.9% to gentamicin and 26.2% to chloramphenicol, pristinamycin and ciprofloxacin which were in line with the results of the current study [27-34].

Methicillin-resistant *S.aureus* were detected for *SCCmec* typing via M-PCR. The II phenotype (32.83%) and III phenotype (64.52%) were the most commonly detected *SCCmec* types among the *mec* positive strains of *S. aureus* . Significant differences were found between the incidence of *SCCmec* V and *SCCmec* IVc, *SCCmec* IVb and *SCCmec* Iva types. Similar studies have previously been reported, among which D'Souza showed that a total of 97 *mecA*-positive strains were SCC*mec* III (25%), 136 were *SCCmec* IV (34%), and 162 were *SCCmec* V (41%) which were concordant to the present study results. They also proposed that all the *SCCmec* III strains, 73% of *SCCmec* IV and V strains and 72% of *SCCmec* IV and *SCCmec* V strains were multidrug resistant [25].

Moussa reported that the most predominant *SCCmec* types among the examined isolates in Saudi Arabia were type V (42.5%), followed by *SCCmec* type III 39 (38.6%) which were similar to this study findings [35].

Conclusion

The present study findings revealed that both *mecA, tetK, tetM, ermA, ermC, aacA-D, linA, msrA, vatA, vatC* and *vatB* genes, also resistance to penicillin, methicillin, tetracycline, ceftriaxone, azithromycin and

tetracycline were introduced as the most commonly detected characteristics of the biofilm producing strains isolated from the hospitals infections.

All the isolates produced biofilm in different levels that is stated as one of the important reasons to create multidrug resistance in the isolates. The high level of *Staphylococcus aureus* resistance against commonly used antibiotics as well as high presence of *SCCmec* types of meticillin-resistance in virulent strains of *S. aureus* represent that infections with these strains require higher levels of hospital cares with an emerging demand for the novel antibiotics. Hence, the clinicians' role in judicious usage of antibiotics is pivotal.

Conflict of Interest

The authors report no conflicts of interest.

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