

# Characterization of biofilm formation, antimicrobial resistance, and staphylococcal cassette chromosome *mec* analysis of methicillin resistant *Staphylococcus hominis* from blood cultures of children

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## Abstract

**Introduction:** Methicillin resistant *Staphylococcus hominis* (MRSHo) has been recognized as an important human pathogen, particularly in immunocompromised patients. **Methods:** A total of 19 *S. hominis* isolates were collected from children at the Children's Medical Centre, Tehran, Iran, from March 2012 to February 2013. MRSHo susceptibility against 13 antimicrobial and 3 antiseptic agents was determined using disk diffusion (DAD) and minimum inhibitory concentration (MIC), respectively. All isolates were subjected to polymerase chain reaction (PCR) assay for 15 distinct resistance genes, staphylococcal cassette chromosome *mec* (SCC*mec*), and arginine catabolic mobile elements (ACMEs). Biofilm production of the isolates was determined using a colorimetric microtiter plate assay. **Results:** Of the 19 isolates, 16 were resistant to oxacillin and harbored *mecA*. High resistance was also observed against trimethoprim/sulfamethoxazole (81.2%). All MRSHo isolates were susceptible to the three disinfectants tested (Septicidine-PC, Septi turbo, and Sayaccept-HP). In total, 15 (78.9%) isolates produced biofilms. Three isolates had SCC*mec* types (V and VIII), 13 were untypable (UT), and 5 had ACME type II. **Conclusions:** The results indicate that MRSHo with high antibiotic resistance and unknown SCC*mec* might become a serious problem in the future for the treatment of patients such as children.

**Keywords:** MRSHo. SCC*mec*. ACME. Biofilm.

## INTRODUCTION

*Staphylococcus hominis*, a member of coagulase-negative staphylococci (CoNS) found asymptotically on the skin, arms, legs, and surfaces of the axilla, is an opportunistic pathogen capable of causing a wide variety of diseases including bacteremia, septicemia, and endocarditis especially in immunocompromised patients<sup>1,2</sup>. A growing concern is the emergence of methicillin resistant *Staphylococcus hominis* (MRSHo), which is the third most common pathogen among methicillin resistant CoNS (MRCoNS) in clinical isolates<sup>3,4</sup>. Resistance to methicillin is due to the acquisition of *mecA*, which encodes PBP2a — a transpeptidase with a low affinity for beta-lactam antibiotics such as penicillin. *mecA* is located

on the staphylococcal cassette chromosome *mec* (SCC*mec*). Eleven main types of SCC*mec* differing in size and genetic composition have been assigned for staphylococcal isolates<sup>5</sup>. The capability of CoNS, including *S. hominis*, to produce disease depends on their ability to create a wide variety of virulence factors that contribute to colonization and disease incidence in hosts, such as biofilm production and the arginine catabolic mobile element (ACME)<sup>6</sup>. Arginine catabolic mobile elements includes two gene clusters, *arc* encoding a secondary arginine deiminase system and *opp-3* encoding an ABC transporter<sup>6</sup>. In recent years, the widespread use of chemical disinfectants (Septicidine-PC, Sayaccept-HP, and Septi turbo) in hospital settings played a critical role in the emergence of disinfectant-resistant microorganisms. Despite the incidence of methicillin resistance among staphylococci, there is currently little information available on the incidence and types of these resistances in many countries, such as Iran. The aim of this study was to investigate the antibiotic and antiseptic resistance patterns and prevalence of different SCC*mec* and ACME types among *S. hominis* isolates obtained from Iranian children.

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## METHODS

### Bacterial isolates

From March 2012 to February 2013, 256 CoNS strains were isolated from children at the Children's Medical Center, Tehran. Only one strain for each patient was selected. Isolates were identified as *S. hominis*, using conventional methods confirmed by the targeting of *nuc*<sup>7,8</sup>. In these isolates, the gene encoding methicillin resistance (*mecA*) was targeted by polymerase chain reaction (PCR)<sup>9</sup>.

### Susceptibility testing and amplification of resistance genes

Antimicrobial susceptibility was determined using the disk diffusion method on Mueller–Hinton agar based on the Clinical and Laboratory Standards Institute (CLSI) guidelines<sup>10</sup>. The following antibiotics (Mast Co., UK) were tested: ciprofloxacin (5µg), clindamycin (2µg), erythromycin (15µg), gatifloxacin (5µg), gentamicin (10µg), linezolid (30µg), mupirocin (200µg), quinupristin/dalfopristin (15µg), rifampin (5µg), tetracycline (30µg), tigecycline (15µg), and trimethoprim/sulfamethoxazole (1.25/23.75µg). In addition, inducible clindamycin resistance was checked by placing clindamycin and erythromycin disks (D-shaped) on the agar, 15-26mm apart. The minimum inhibitory concentrations (MICs) of oxacillin and vancomycin were determined using the broth micro-dilution method according to CLSI guidelines<sup>10</sup>. Strains that were resistant to three or more antimicrobial classes were considered to possess multiple drug resistance (MDR)<sup>11</sup>.

### Minimum inhibitory concentrations of disinfectants

Commonly used disinfectants in the Children's Medical Centre were obtained in commercial preparations: Septicidine-PC contains 0.5% (w/w) chlorhexidine digluconate and 75% (w/w) alcohol; Sayaccept-HP contains 0.25% (w/w) quaternary ammonium compounds (QACs) and biguanide; and Septi turbo contains 70% (w/w) isopropanol and 0.25% (w/w) QACs (all supplied by Behban Shimi Pharmaceutical Co., Gorgan, Iran). Although these disinfectants should be used without dilution, their MICs were determined by serial 2-fold micro-dilution (1- to 16-fold) in Mueller-Hinton broth (Conda, Spain), and the addition of the reducing dye triphenyl tetrazolium chloride (TTC) (Sigma Aldrich, USA)<sup>12</sup>. Each microdilution was inoculated with 50µL of  $1.5 \times 10^6$  colony-forming units per milliliter (CFU/mL) of the overnight cultures in a brain heart infusion (BHI) blood agar base. The microplates were then incubated at 37°C for 24h, and the lowest concentration of the disinfectant that completely inhibited the growth of the microorganisms was recorded as the MIC. *Staphylococcus aureus* ATCC 29213 was used as a control. Antibiotic-resistance genes, namely, *blaZ*, *ermA*, *ermB*, *ermC*, *msrA*, *msrB*, *tetM*, *tetK*, *aac(6)-Ie-aph(2'')-Ia*, *aph(3)-IIIa*, *ant(4)-Ia*, *mupA*, and *vanA*, and antiseptic-resistance genes, namely, *smr* and *qacA/B*, were screened by PCR and multiplex-PCR methods<sup>13,14</sup>.

### Biofilm production

The capacity of isolates for biofilm production was determined using a colorimetric microtiter plate assay<sup>15</sup>. Briefly,

MRSHo isolates were grown on Trypticase Soy Broth (TSB) overnight at 37°C (Merck, Darmstadt, Germany), and the cultures were then diluted 1:100 in TSB medium. Consequently, 150µL of this cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtiter plates. After 24 h incubation at 37°C, without shaking, the wells were gently washed three times with 200µL of phosphate buffered saline (PBS; Sigma-Aldrich, USA) and dried in an inverted position. For fixation of biofilms, 100µL of 99% methanol was added and after 15 min, supernatants were removed and the plate was air-dried. Next, 100µL of 1% crystal violet (CV; HiMedia, India) was added to all the wells. Excess CV was removed after 20 min by washing the plate under running tap water. Bound CV was released by adding 150µL of 33% acetic acid. The optical density (OD) of each well was measured at 590nm using a microtiter plate reader. All the tests were repeated three times. Uninoculated medium was used as a control to determine the background OD. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control, and the final OD value of a tested strain was defined as the average OD of the strain reduced by the ODc value. The adherence ability of the tested strain was classified into four categories based on the OD<sup>15</sup>: non-adherent (OD < ODc), weakly adherent (ODc < OD < 2XODc), moderately adherent (2XODc < OD < 4XODc), and strongly adherent (4XODc < OD). The genes encoding intracellular adhesion (*icaA* and *icaD*) were targeted by PCR using the primers as previously described<sup>9</sup>.

### Staphylococcal cassette chromosome *mec* and arginine catabolic mobile element typing

The primers used to detect SCCmec types and their variants, the conditions of PCR, and the size of the amplified products were each used as described by Ito et al.<sup>16</sup> The ACME typing PCR was performed with template deoxyribonucleic acid (DNA) from each isolate to determine if they harbored parts of ACME genes I to III<sup>17</sup>.

## RESULTS

Of the 256 CoNS strains, 19 (7.4%) were *S. hominis*; 16 (84.2%) of which were resistant to oxacillin (MIC<sub>90</sub> = 32µg/mL) and contained *mecA*. These 16 MRSHo isolates were obtained from blood cultures from the following wards: emergency (5), oncology (2), ophthalmology (2), neonatal intensive care unit (2), interior (2), nephrology (1), infection (1), and unknown (1). The phenotypic and genotypic characterizations of MRSHo isolates are shown in **Table 1**. All isolates were susceptible to linezolid, quinupristin/dalfopristin, and rifampin and had intermediate susceptibility to vancomycin (MIC<sub>90</sub> = 4µg/mL); no *vanA* was detected. High resistance rates were found against trimethoprim/sulfamethoxazole (81.2%), erythromycin (75%), and tetracycline (62.5%). Isolates were also resistant to gentamicin (25%), ciprofloxacin (25%), clindamycin (25%), mupirocin (18.7%), gatifloxacin (12.5%), and tigecycline (12.2%). All isolates were susceptible to the tested disinfectants, and the MICs of Septicidine-PC, Septi turbo, and Sayaccept-HP were 8-, 8-, and 14-fold lower than the concentration recommended for use by the manufacturers (**Table 1**).

TABLE1  
Antibiotic and disinfectant resistance and biofilm production of MRSHo.

Isolates	Ward	MIC (µg/mL)		MIC (dilution fold) of antiseptics			Biofilm	Genes
		OXA*	VAN	SEP <sup>5</sup>	SYA	TOR		
1	UN	64	4	9	15	8	W <sup>#</sup>	<i>Ie-aph(2)-Ia, ant(4)-Ia, tetk, blaZ, mup, smr,</i>
2	NICU <sup>8</sup>	32	2	10	16	10	W	<i>Ie-aph(2)-Ia, ant(4)-Ia, tetk, blaZ, mup, msrA</i>
3	ONC	8	4	11	16	10	W	<i>Ie-aph(2)-Ia, tetk, blaZ</i>
4	NEPH	8	4	9	15	8	M	<i>tetk, blaZ</i>
5	INT	8	4	9	15	8	W	<i>tetk</i>
6	OP	16	4	11	15	9	W	<i>tetm, blaZ</i>
7	NICU	32	2	9	15	8	W	<i>Ie-aph(2)-Ia, tetk, blaZ</i>
8	INF	2	4	9	15	8	W	<i>tetk, ermA, blaZ, msrA</i>
9	EMR	2	2	9	15	9	M	<i>aph(3)-IIIa, ant(4)-Ia tetk, blaZ</i>
10	OP	32	4	9	16	10	M	<i>aac(6)-Ie-aph(2)-Ia, aph(3)-IIIa, tetk, blaZ, smr, icaA&amp;D</i>
11	EMR	64	4	9	16	10	W	<i>aac(6)-Ie-aph(2)-Ia, aph(3)-IIIa, blaZ, msrA</i>
12	EMR	128	4	8	15	8	W	<i>Ie-aph(2)-Ia, ant(4)-Ia, tetk, blaZ, mup, msrA, qacAB</i>
13	EMR	4	2	9	15	10	W	<i>tetk, msrA</i>
14	ONC	16	2	9	14	8	W	<i>tetk, blaZ, msrA</i>
15	EMR	16	4	10	15	8	W	<i>blaZ</i>
16	INT	4	8	9	15	9	N	<i>blaZ, msrA</i>

**MRSHo:** methicillin resistant *Staphylococcus hominis*; **MIC:** minimum inhibitory concentration; \***OXA:** oxacillin; **VAN:** vancomycin; <sup>5</sup>**SEP:** Septicidine-PC; **SYA:** Sayaccept-HP; **TOR:** Septi turbo; **UN:** unknown; <sup>8</sup>**NICU:** neonatal intensive care unit; **ONC:** oncology; **NEPH:** nephrology; **INT:** interior; **OP:** ophthalmology; **INF:** infection wards; **EMR:** emergency; <sup>#</sup>**W:** weak; **M:** moderate; **N:** no biofilm.

MIC<sub>90</sub> of strains to Septicidine-PC, Septi turbo, and Sayaccept-HP were 8-, 8-, and 15-fold diluted, respectively. The prevalence of resistance genes were as follow: *blaZ* (81.2%), *tetK* (75%), *tetM* (6.2%), *aac(6)-Ie-aph(2)-Ia* (43.7%), *ant(4)-Ia* (18.7%), *aph(3)-IIIa* (25%), *msrA* (43.7%), *ermA* (6.2%), *mupA* (18.7%), *Smr* (12.2%), and *qacA/B* (6.2%). Approximately 15 (78.9%) of the isolates produced biofilms. Among these, 12 (80%) were weakly adherent biofilm producers, and 3 (20%) produced moderately adherent biofilms. *icaA* and *icaD* were found in only one of moderate biofilm producers.

Of the 16 oxacillin-resistant MRSHo isolates, only 3 were typed by SCCmec typing: 2 were type VIII and 1 was type V (Table 2). Analysis of the *mec* and *ccr* complexes among the 13 untypable (UT) isolates revealed that four had *mec* complex A + *ccrC* (UT1); two, *mec* complex A + *ccrAB1* and *ccrC* (UT2); one, *mec* complex B + *ccrC* (UT3); one, *mec* complex A and *c1* + *ccrAB1* (UT4); one, *mec* complex A and no *ccr* (UT5); one, only *ccrAB1* and *ccrC* (UT6); three, neither *ccr* nor *mec* (UT7). Some of the isolates within the same UT group had different junk yard motifs. In ACME typing, only *arcC* was positive in 5 (31.2%) isolates, which were considered ACME type II. All isolates were negative for the *opp-3* gene.

## DISCUSSION

In this study, the methicillin resistance rate was slightly higher than that reported in other studies conducted in the USA (80-82%), Mexico (81%), and Tunis (75%)<sup>2-4,18</sup>, and the MDR rate was higher than that reported in the Tunis study (94%)<sup>2</sup>. The different MRSHo rates in different countries may reflect differences in the doses and dosing schedules used and in the spread of particular clones. In the current study, the reduced vancomycin susceptibility observed in some clinical isolates could preclude full resistance, which is in contrast to the findings of Palazzo et al.<sup>19</sup>, who observed 83.3% of isolates with resistance to vancomycin. In this study, we also observed high levels of resistance to gatifloxacin and tigecycline. Unfortunately, fluoroquinolones, especially ciprofloxacin and gatifloxacin, are widely used for the treatment of various bacterial infections in Iran, which may contribute to the emergence of resistant strains<sup>20</sup>. Moreover, several studies that used MIC methods revealed that 100% of CoNS were susceptible to tigecycline<sup>21-23</sup>. Given that we used disk diffusion to determine the tigecycline susceptibility of our MRSHo isolates, MIC-based methods might indicate that all of our isolates were susceptible.

**TABLE 2**  
SCCmec and ACME types of MRSHo isolates.

Isolates	mec complex	SCCmec			ACME	
		ccr complex	junkyard	type	gene	type
1	A	A1B1/A4B4	-	VIII+AB1	-	-
2	A/C1	AB1	-	UT4	-	-
3	B	C	Iva	UT3	-	-
4	A	C	-	UT1	<i>arcA</i>	II
5	A	C	-	UT1	-	-
6	A/C2	C	Ia/Iia	V+ A	<i>arcA</i>	II
7	A	A1B1/C	-	UT2	-	-
8	A	A1B1/C	Ia/Iia	UT2	<i>arcA</i>	II
9	-	A1B1/C	Iva/Iia/Ivc	UT6	-	-
10	A	C	-	UT1	-	-
11	-	-	Ia	UT7	-	-
12	A	A1B1/A4B4/C	-	VIII+A1B1+Ccr C	-	-
13	-	-	-	UT7	-	-
14	-	-	-	UT7	<i>arcA</i>	II
15	A	-	-	UT5	-	-
16	A	C	Ia	UT1	<i>arcA</i>	II

**SCCmec:** Staphylococcal cassette chromosome *mec*; **ACME:** arginine catabolic mobile element; **MRSHo:** methicillin resistant *Staphylococcus hominis*; **untypable (UT) 1:** *mec* complex A + *ccrC*; **UT2:** *mec* complex A + *ccrAB1* and *ccrC*; **UT3:** *mec* complex B + *ccrC*; **UT4:** *mec* complex A and *c1* + *ccrAB1*; **UT5:** *mec* complex A - *ccr*; **UT6:** only *ccrAB1* and *ccrC*; **UT7:** neither *ccr* nor *mec* genes.

In the current study, some strains with or without resistance genes (*smr* and *qacA/B*) had the same MIC, which may be owing to the presence of other resistance genes. In a Norway study that worked on cattle and goats, *smr* was detected in one of two *S. hominis* isolates, which is higher than our results in humans<sup>24</sup>. With regards to biofilm production, our results were similar to those obtained in certain studies conducted in Argentina and Mexico<sup>25,26</sup>; however, they contrasted with that of a different Mexico study<sup>4</sup>, which reported high biofilm production in most strains. Therefore, the low and moderate biofilm production may be related to factors other than the presence of *ica*. Given that different ACME types help to maintain *S. epidermidis* in the environment, the presence of ACME type II in some of these strains may improve their ability to remain and colonize. The SCCmec types observed in our study have previously been found in the USA (type VIII), China (type VIII and V), and Tunis (type VIII); nevertheless, none of these studies reported additional segments with these types<sup>2,3,27</sup>. The two strains in our study with SCCmec type VIII (strain 1 and 12) exhibited approximately the same antibiotic resistance (MDR) and biofilm production (weak), but strain 12 had *qacA/B* and an extra *ccrC* gene; these two strains were identified with an interval of 10 months. A combination of *mec* complex class A with *ccr* type 5 and *mec* complex class A with *ccr* type 1 has been reported before<sup>2,4,27</sup>. We also found isolates harboring *ccrC*, which is similar to that in the USA study<sup>3</sup>. A limitation of our study is that Multiplex

PCR could not able to detect new SCCmec elements due to wide variation in *ccr* genes. Therefore, sequence-based methods are recommended.

In conclusion, the horizontal transfer of genes between species leads to increases in antibiotic- and disinfectant-resistant genes and the emergence of new types of SCCmec, all of which can influence the clinical features of MRSA in the future. In addition, MRSHo with high antibiotic resistance and unknown SCCmec type might become a serious problem for the treatment of patients with immune deficiencies, such as children.

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#### Conflict of interest

The authors declare that have no conflicts of interest.

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