Molecular characterization of methicillin-resistant *Staphylococcus aureus*: characterization of major clones and emergence of epidemic clones of sequence type (ST) 36 and ST 121 in Tehran, Iran

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One sentence summary: Molecular characterization of MRSA.

ABSTRACT

Information about the molecular structure of MRSA strains provides significant insights into the epidemiology of this important pathogen. To investigate the molecular characteristics of MRSA isolates, MRSA isolates were subjected to molecular typing by means of spa typing, multilocus sequence typing, Staphylococcal Cassette Chromosome mec (SCCmec) grouping and to phenotypic antimicrobial susceptibility testing by means of disk diffusion assay. Then the presence of *pvl* genes was evaluated. Cluster analysis by eBURSTv3 showed that MRSA isolates belonged to two major clonal complexes (CC); CC8 (ST239, ST585, ST2732, ST1294) and CC30 (ST30, ST36, ST1163) and four singletons. Subsequent analysis of MRSA isolates revealed that the most prevalent SCCmec type was type III (55.8%) followed by type IV (34.9%) and type II (2.3%). Totally 11 different *spa* types were discriminated among which types t037 and t030 were predominant. The prevalence of Panton-Valentine leukocidin (PVL)-positive MRSA strains was high (20%), which is a matter of great concern, because the PVL is frequently associated with severe and recurrent SSTIs. ST239-III- t037 represented the most predominant MRSA clone. The presence of sequence type (ST) 36 and ST 121 are being reported for the first time in Iran.

Keywords: molecular typing; multilocus sequence typing; antibacterial drug resistance; methicillin-resistant *Staphylococcus aureus*; Panton-Valentine leukocidin

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the major drug-resistant pathogens worldwide and has evolved in a quite few lineages which are ecologically successful (Vindel et al. 2009). Five major pandemic clones, designated the Iberian, Brazilian, Hungarian, New York/Japan and pediatric clones, have been recognized and some other clones have reported from...
certain regions (D’Souza, Rodrigues and Mehta 2010). The prevalence rates of MRSA in hospitals of some Asian countries were reported 70–80% (Chuang and Huang 2013). A systematic review showed that the rate of MRSA in Iran is high and varies between 20.4 and 90% in different parts of the country (Askari et al. 2012). Having information about predominant MRSA clones circulating in hospital with their antibiotic resistance profile is a requisite to choose an appropriate antibiotic for treatment and to find the source of infection as well (Atshan et al. 2012). Therefore, tracking and limiting the intra- and interhospital transmission of MRSA strains is very important and requires the use of an accurate and effective epidemiological typing system to differentiate unrelated isolates which are derived from a primary ancestor (Aires de Sousa and de Lencastre 2004).

Nowadays, the use of Staphylococcal Cassette Chromosome mec (SCCmec) typing, multilocus sequence typing (MLST) and spa typing have created a significant progress in MRSA typing (Liu et al. 2009). In surveillance and evolutionary studies, using a method alone is insufficient and strains should be evaluated by combination of different typing systems (Aires de Sousa and de Lencastre 2004). Previous studies showed that CC8 (ST239), CC5 (ST5) and CC22 (ST22) are the major reported Asian MRSA clones (Stefani et al. 2012). There are only a few reports regarding molecular epidemiology of MRSA in Iran, and there is no study in Tehran. We conducted this study to determine the composition of MRSA genotypes to compare them with the worldwide data. To achieve this goal, the isolates were subjected to molecular typing by means of spa-typing, MLST, SCCmec grouping and to phenotypic antimicrobial susceptibility testing by means of disk diffusion assay.

MATERIALS AND METHODS

Hospital setting

The study was conducted in Motahari hospital, one of the major teaching hospitals in Tehran, Iran. This center is the first leading burn center in Iran and a specialized provider of medical services to more than 3000 patients and more than 10 000 outpatients each year.

Bacterial isolates

In this cross-sectional survey, a total of 135 pus/wound swabs (cotton) from skin and soft tissue infections (SSTIs) were collected from burn patients during eight months (January–August 2013). All patients gave written informed consent. Swab samples transferred to Tehran University of Medical Sciences molecular laboratory on appropriate transport medium and subcultured on blood agar and incubated for 24 h at 37 °C. Then, S. aureus were identified morphologically and biochemically by standard laboratory procedures (Gram’s stain, catalase, coagulase and DNase activities and mannitol fermentation on mannitol salt agar). Only one isolate per patient was involved in this study.

Detection of MRSA

Resistance to methicillin was determined by the agar disk diffusion method using Muller–Hinton agar medium containing 4% NaCl and oxacillin disk (1 μg oxacillin; MAST Diagnostics, Merseyside, UK). All plates were incubated at 35 °C overnight and interpreted according to Clinical and Laboratory Standards Institute (2011) guidelines. Then mecA gene was detected in DNA extracts by PCR assay (Rastegar Lari et al. 2011).

Antibiotic susceptibility test

Antimicrobial resistance patterns were determined using a panel of 17 antibiotic disks included: amikacin (30 μg), ciprofloxacin (5 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), erythromycin (30 μg), fusidic acid (5 μg), gentamicin (10 μg) linezolid (30 μg), mupirocin (5 μg), quinupristin-dalfopristin (15 μg), rifampin (5 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), tetracycline (30 μg), teicoplanin (30 μg), tigecycline (15 μg), tobramycin (10 μg) and vancomycin (30 μg) (MAST Diagnostics, Merseyside, UK). This was carried out in accordance with the Kirby–Bauer method and Clinical and Laboratory Standards Institute guidelines guidelines (Stefani et al. 2012). Staphylococcus aureus ATCC29213 was used as standard strain.

DNA extraction

Genomic DNA of MRSA isolates was extracted using a Dneasy kit (Qiagen, Valencia, CA) as recommended by the manufacturer, with the modification that at the first stage, 1.5 μl lysostaphin (5 mM) was added to the bacterial suspension and incubated for 30 min at 37 °C. Finally, the purified DNA was used for molecular analyses.

Multiplex PCR for SCCmec typing

Multiplex PCR assay conducted for characterization of mec gene and cr gene complexes, according to the method of Zhang et al. (2005).

spa typing

The short sequence repeat X region of the spa gene was amplified by PCR assay. Primers and program were used as described previously (Moodley et al. 2006). Staphylococcus aureus ATCC 25923 and sterile deionized water were used as positive and negative control, respectively. PCR products were visualized on agarose gel electrophoresis by GelRed. Purified PCR products were sequenced (DNA Sequencer ABI, model 3730-XL) commercially (Takapouzist, Iran) using the spa primers. The sequences obtained were subjected to spa repeat analysis and spa typing using the SpaServer (http://www.spaserver.ridom.de).

Multilocus sequence typing

MLST was performed by the methodology described by Enright et al. (2000). Seven housekeeping genes (arcC, aroE, glp, gmk, pta, tpi and yqiL) were amplified by PCR and sequenced. Then their allelic profile (allele numbers) and STs were determined via the S. aureus MLST database (http://www.mlst.net) hosted by Imperial College in London, UK.

Detection of PVL genes

cpl genes ( lukS-PV and lukF-PV) detection was carried out as previously described (Lina et al. 1999). A standard strain NCTC 13300 and distilled water were used as a positive and negative control, respectively.

RESULTS

MRSA distribution

Through 135 pus/wound swabs recovered from SSTIs, 66 (48.88%) S. aureus were isolated. Using phenotypic (disk diffusion
method) and genotypic (PCR for detection of meca gene) methods, 45(68.18%) isolates were confirmed as MRSA. The presence of the meca gene was verified by PCR only for phenotypically oxacillin resistant isolates.

**SCCmec typing**

Multiplex PCR results for SCCmec typing revealed that the most prevalent SCCmec type was type III (55.8%) followed by type IV (34.9%) and type II (2.3%). Most of SCCmec III MRSA strains were ST239.

**spa typing**

Totally 11 different spa types were discriminated among the 45 MRSA isolates. The two predominant spa types among the MRSA isolates (t037 and t030) represented more than 50% of all isolates. The remaining nine spa types were scattered more homogeneously among MRSA isolates. Three spa types (t5598, t0199 and t159) were represented by a single strain.

**Multilocus sequence typing**

MLST results revealed 11 different STs for MRSA isolates (ST239, ST291, ST121, ST30, ST1163, ST36, ST25, ST585, ST2732, ST1294 and ST22). Cluster analysis by eBURST v3 showed that the MRSA strains belonged to two major clonal complexes (CC, CC8 (ST239, ST585, ST2732, ST1294) and CC30 (ST30, ST36, ST1163) and four singletons. The four non-CC8 non-CC30 STs, ST121, ST25, ST22 and ST291 belonged to global MLST clonal complexes CC121, CC25, CC22 and CC398, respectively. MRSA clone, ST239-MRSA-III was dominant among the isolates.

**Distribution of pvl genes**

The presence of pvl genes was examined in all MRSA isolates, and nine (20%) MRSA were harbored pvl genes mostly distributed among CC30 (Table 1).

**Antibiotic susceptibility pattern**

The highest resistance rate of MRSA isolates was for tetracycline (97.77%) and after that for both gentamicin and erythromycin (84.44%). The majority of these isolates were also resistant to other aminoglycosides including amikacin (64.44%) and tobramycin (57.77%). Contrary to the gentamicin and tetracycline resistance to some other non-β-lactam antibiotics tested, including chloramphenicol (8.88%) and rifampin (15.55%) was low. None of the MRSA isolates were resistant to teicoplanin, tigecycline, quinupristin-dalfopristin, linezolid, fusidic acid and vancomycin. Overall, using 17 antibiotics (excluding oxacillin), 11 different antibiotic types were found as shown in Table 2. Antibiotyp 4 was the most prevalent. MRSA with antibiotype 11 were susceptible to all antibiotics except oxacillin while isolates with antibiotype 1 except fusidic acid, teicoplanin, linezolid, tigecycline, quinupristin-dalfopristin and vancomycin were resistant to other antibiotics.

**DISCUSSION**

There are limited data on the MRSA population structure in Iran. Accordingly, we examined MRSA isolates by using multiple genotyping methods and analysis of data outlined molecular features of MRSA in Iran. Isolates were grouped by using spa typing and were assigned to MRSA clones on the basis of MLST and SCCmec typing.

In current study, CC8 was shown as the predominant clone. CC8 is one of the most prevalent CCs worldwide which contains major epidemic nosocomial MRSA isolates (Liu et al. 2009) and encompasses various STs disseminated differently in many countries (Stefani et al. 2012). Our results indicated that epidemic MRSA clones, ST239-MRSA-III (Brazil/Hungary) and ST291-IV were dominant among the isolates under study. This is in accordance with previous studies reported from other parts of Iran (Havaei et al. 2011; Japoni et al. 2011). ST291 has been supposed to be double locus variant of CC398. ST291 isolates have been reported from areas related with the presence of CC398 such as Italy, France, Switzerland and the USA, but currently have broader geographical distribution (Stegger et al. 2013).

### Table 1. Molecular characteristics of MRSA isolates recovered from burn patients in Tehran, Iran.

<table>
<thead>
<tr>
<th>CC type (n) based on cluster analysis by eBURST v3</th>
<th>ST</th>
<th>No. (%)</th>
<th>spa typing</th>
<th>SCCmec typing</th>
<th>Pvl gene (no.)</th>
<th>Antibiotic type(no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC8 (26)</td>
<td>239</td>
<td>18 (40)</td>
<td>t037 (10)</td>
<td>III</td>
<td>–</td>
<td>1(3), 2(1), 3(3), 5(3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t030 (6)</td>
<td>III</td>
<td>–</td>
<td>5(1), 4(6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t388 (2)</td>
<td>III</td>
<td>–</td>
<td>4(2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t387 (5)</td>
<td>III</td>
<td>–</td>
<td>4(4), 7(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2732</td>
<td>2 (4.44)</td>
<td>t037 (2)</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1294</td>
<td>1 (2.22)</td>
<td>t037 (1)</td>
<td>III</td>
</tr>
<tr>
<td>CC30 (7)</td>
<td>30</td>
<td>4 (8.9)</td>
<td>t021 (3)</td>
<td>IV</td>
<td>+ (3)</td>
<td>7(2), 10(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t019 (1)</td>
<td>II</td>
<td>+ (1)</td>
<td>6(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t018 (2)</td>
<td>IV</td>
<td>+ (2)</td>
<td>9(2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36</td>
<td>2 (4.44)</td>
<td>t019 (1)</td>
<td>IV</td>
<td>+ (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1163</td>
<td>1 (2.22)</td>
<td>t021 (1)</td>
<td>IV</td>
</tr>
<tr>
<td>Singleton (12)</td>
<td>121</td>
<td>1 (2.22)</td>
<td>t159 (1)</td>
<td>IV</td>
<td>+ (1)</td>
<td>8(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22</td>
<td>3 (6.66)</td>
<td>t032 (3)</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>291</td>
<td>6 (13.33)</td>
<td>t1149 (5)</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5598 (1)</td>
<td>IV</td>
<td>–</td>
<td>11(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25</td>
<td>2 (4.44)</td>
<td>t081 (2)</td>
<td>II</td>
</tr>
</tbody>
</table>

CC, clonal complex.
ST, sequence type.

1Number of isolates in each antibiotyp group.
ST239 is the most globally successful MRSA lineage (Uhlemann et al. 2014) and has been reported to be universal (Chongtrakool et al. 2006). This lineages is resistant to multiple antibiotics and is responsible for at least 90% of HA-MRSA in several Asian and South American countries, with recent reports that it is also circulating in Eastern Europe (Harris et al. 2010). In current study, ST239 isolates had SCCmec type III and were multidrug resistant (MDR) and most of them belonged to antibiotic type 4, and some were resistant to rifampin and mupirocin as well.

All of MRSA isolates were sensitive to vancomycin, quinupristin-dalfopristin, linezolid, tigecycline, teicoplanin and fusidic acid. Therefore, these antibiotics can still be used for staphylococcal infections in Iran. The highest antibiotic resistance was to tetracycline (97.77%), gentamicin and erythromycin (84.44% each). It seems that misuse and overuse of these antibiotics could have caused to high prevalence of resistance in our country. We identified PVL-positive ST30-IV and ST36-IV strains among MRSA studied. The PVL-positive ST30 MRSA has been spreading worldwide (Kawaguchiya et al. 2011). ST30-IV generally referred to the South West Pacific clone. Studies have shown that ST30 is prevalent in Asian countries and may spread between countries (Song et al. 2011). ST-36 has been reported from Finland, Spain, Australia (Enright et al. 2002; Cooper and Feil 2006) and Pakistan (Shabir et al. 2010). CC30- ST36 (UK-EMRSA-16, USA200) is common in the USA and the UK (Stefani et al. 2012), and now is an emergent strain in Iran.

Our findings showed the presence of ST-22 (CC22) of SCCmec type IV among our MRSA isolates. However, this result was not consistent with previous study in Isfahan, Iran which reported no strains of ST22 to be methicillin resistant and all were MSSA (Shore et al. 2010). ST22-MRSA-IV (EMRSA-15) is a pandemic CC22-MRSA strain. It was first reported in the UK and then became one of the most dominant HA-MRSA clones worldwide (Japoni-Nejad et al. 2013). A current survey showed that ST22 was the major MRSA lineage across Europe (Grundmann et al. 2010). In current study, this clone accounted for SSTIs among burn patients. There was only one PVL-positive isolate of MRSA with ST121 among the MRSA studied. There are some reports regarding ST121 MRSA which were pvl positive and related to osteomyelitis and soft tissue abscess (Chheng et al. 2009).

Our study had some limitations regarding the MRSA clones depiction such as the limited numbers of isolates that were investigated during this study, but the data gained is significant as it enables us to form a basis against which to monitor the future spread and emergence of strains in our country.

In summary, we described that the major universal MRSA clones, ST239, ST291 and ST30 have spread across Iran and were the causative agents of staphylococcal infections. We found different SCCmec and spa types distributed among MRSA strains. In some cases, a multiple spa types corresponded to a single MLST ST (ST239, ST30 and ST291). This result may indicate either permanent import of novel spa types. SCCmec type II was very frequent in this study, and we did not detect any SCCmec types I and V. Moreover, the existence of ST36 and ST121 clones as the causes of the wound infection in burn patients was documented. This is the first report of these clones from a hospital in Iran, which may pose a new threat in terms of pathogenicity and epidemiology of MRSA in our region. The detection of PVL-positive strains exhibited MDR was an observation of some concern. It is worth to mention that some sort of environmental or anonymous sampling of staff should be explored in future studies in order to understand if strains are circulating in the health care environment.

Additional studies from other regions of the country are required to reach an outlook on clonal dynamics of MRSA in Iranian hospitals.

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Conflict of interest. None declared.

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