Molecular Characterization and Antimicrobial Susceptibility of Methicillin-Resistant *Staphylococcus aureus* Bloodstream Isolated from Alkharj, KSA


1Kayyali Chair for Pharmaceutical Industries, King Saud University, P. O. Box 2457, Riyadh, Saudi Arabia.
2Microbiology and Immunology Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.
3Medicine Department, Salman Bin Abdul Aziz College of Medicine, Al Khairj, Saudi Arabia.
4Department of Tropical Medicine, Al Azhar Faculty of Medicine, Cairo, Egypt.
5Biomarkers Research Program, Biochemistry Department, College of Science, King Saud University, PO Box, 2455, Riyadh, 11451, Saudi Arabia.
6Department of Pharmaceutics, College of Pharmacy, King Saud University, P. O. Box 2457, Riyadh, Saudi Arabia.

(Received: 23 March 2014; accepted: 02 May 2014)

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of hospital-acquired infections in most hospitals worldwide. The aim of this study is to investigate the antimicrobial susceptibility, Panton Valentine Leucocidine (PVL) *mecA* and toxin genes, *agr* types and staphylococcal cassette chromosome *mec* (*SCCmec*) types of MRSA bloodstream isolates collected from Salman University Hospital, Alkharj, KSA. Antimicrobial susceptibilities were investigated by agar diffusion method; PVL, *mecA* and toxin genes by polymerase chain reaction (PCR) and *SCCmec* and *agr* typing were performed by multiplex PCR. Totally MRSA isolates were susceptible to linezolid and glycopeptide vancomycin. *mecA* gene was detected in totally of the isolates, on the other hand, PVL positive isolate was not detected. *sea* was the most frequently (76%) detected enterotoxin gene. *SCCmec* typing revealed type III in 85% and *agr* typing revealed type I in 87% of the isolates.

**Key words:** Antimicrobial susceptibility, MRSA, Molecular epidemiology, PCR, Toxin gene, *mecA*, *SCCmec*.

Infectious diseases are the second most common cause of death worldwide and the third leading cause of death in developed countries. *S. aureus* is the most prevalent pathogens isolated from blood cultures and responsible for a wide range of hospital acquired (HA-MRSA) infections worldwide. Methicillin-resistant *staphylococcus aureus* (MRSA) or toxin-mediated invasive infections is one of the major causes of nosocomial blood stream infection associated with increased prolonged hospitalization and common cause of morbidity and mortality.

*S. aureus*, staphylococcal enterotoxins (SE), A-J, K, L, M and O, toxic shock syndrome toxin I (TSST-1), exfoliative toxins (ET) A, B and D as well as various toxins, hemolysins (α, β, γ) and skin infections, necrotizing pneumonia and community-acquired MRSA (CA-MRSA) infections are closely associated with Panton-Valentine leukocidin (PVL), including some of the arises leukocidin secretion. *S. aureus* as a more...
virulence factor expression, encoding a two-component signal path accessory regulator gene (AGR) locus that are controlled by the signal path of the two-component, the density-sensitive activator peptide ligand is still encoded by the agr gene.

The mecA gene is the main mechanism responsible for MRSA. This gene is carried by the cassette chromosome mec (SCCmec) type. Different SCCmec types are associated with hospital or community isolates and if a bacterial cell acquires such SCCmec, the cell will concomitantly acquire a multidrug resistance phenotype. MRSA with type IV or type V SCCmec is usually associated with severe community infections, however types I, II and III SCCmec commonly occur in the health-care setting.

The epidemiology of invasive S. aureus infections (ISA) has only been defined in few studies of population-based study design. A population-based surveillance study of all invasive S. aureus infections occurring in the Calgary Health Region in Canada from 1999-2001, and estimated an incidence of 28.4 cases/100,000 population (17.9/100,000 population, for bacteraemia), of which 46% were classified as nosocomial. Other studies have been limited either by inclusion of only selected patients with ISA or by failure to include clinical information. In Saudi Arabia, an increasing number of MRSA cases has been recognized. The MRSA prevalence in patients in different regions has increased. The aim of the current study is to determine the antimicrobial susceptibility pattern, Panton Valentine leucocidine (PVL) including molecular clone to investigate the presence of various toxins and pulsed field gel electrophoresis (PFGE) profiles of MRSA strains isolated from blood samples.

**MATERIALS AND METHODS**

**Bacterial Isolates**

During October 2011 to January 2013, different clinics of 100 MRSA strains were isolated from blood samples. The strains were isolated from inpatients at Salman University Hospital (SUY), Alkharj, Saudi Arabia. No duplicate isolates from a single patient were included. As a result, the strains were stored in simple broth with 15% glycerol at -20 °C. This study was approved by the Ethics Committee of SUH.

**Culture and Identification**

The identification of S. aureus was depended on morphology and biochemical reactions according to standard laboratory criteria. Phenotypic characterization to the species level was performed using the API system for the identification of S. aureus (Biomerieux, France). Additional screened for methicillin resistance following the Clinical Laboratory Standards Institute (CLSI) guidelines.

**Oxacillin agar screening**

For oxacillin agar screening, samples were subjected to subculture using Mueller-Hinton agar (Difco Laboratories, Detroit, Michigan) containing 4% NaCl and 6µg/ml oxacillin. Standard inocula were prepared from overnight culture in Mueller Hinton Broth (Difco Laboratories, Detroit, Michigan) after matching the 0.5 McFarland (10⁵ CFU/ml) and further dilution so as final inoculums is approximately 10⁶ CFU/ml. Oxacillin agar plates were inoculated with 10 µL of the tested broth. The plates were incubated at 35±2 °C for 48 h. The MRSA positive and negative standard reference controls were concurrently used. The growth of one or more colonies per spot indicates a positive test result. In this study, as a positive control, HPV107 (Iberian clone), BK2464 (New York / Japan clone), HUSA 304 (Hungarian clone), HSJ216 (Brazilian clone), HDE288 (Pediatric clone), GRE14 (PVL-positive), 10, NCTC 10442 (SCCmec type I), N315 (SCCmec type II), 8572082 (SCCmec type III) and JSCS 4744 (SCCmec type IVA) strains were used.

**Antibiotic Susceptibility Testing**

Antimicrobial susceptibility testing was carried out using the disk diffusion method as described by the CLSI guidelines with different antimicrobial disks include, rifampin (5 µg), erythromycin (15 µg), trimethoprim sulfamethoxazole (1.25/23.75 µg), ciprofloxacin (5 µg), gentamicin (10 µg), tetracycline (30 µg), cefoxitin (10 µg) Linezolid (10 µg) and vancomycin (30 µg). The diameter of the clear zone of growth inhibition was measured after incubation at 35±2 °C for 24 h. For the samples showed that halos sensitivity ≥ 13 mm and ≥ 18 mm for oxacillin disk and ≥ 20mm and ≥ 25mm for the cefoxitin disk were classified as sensitive, respectively. The samples were classified as resistant when presented halos sensitivity is smaller than mentioned.
DNA extraction

Bacterial DNA was extracted according to the method previously described by Ida et al. 2001[20]. Briefly, colonies obtained from overnight S. aureus cultures from sheep blood agar were harvested and suspended in 100 ml of lysis solution (20 mM Tris HCl, 140 mM NaCl, 5 mM EDTA [pH 8.0]). Lysostaphine (2 mg/mL) were added and the suspension was incubated at 37°C for 3 hours. 200 ml of distilled water was added and incubated at 95°C for five minutes. Phenol chloroform extraction and ethanol precipitation steps were then performed for DNA extraction which was stored at -20 °C until analysis.

Detection of mecA, Panton-Valentine Leukocidin and Toxin Genes

PCR was performed to detect the mecA gene using the methodology previously described by Kearns et al.,21. The existence of the lukF-PV and lukS-PV genes encoding PVL components was determined as described by Lina et al., 22 using PCR. To evaluate the specificity of lukF-PV and lukS-PV amplification, the PCR products were exposed to DNA sequencing (Genome Express, France) using S. aureus ATCC 49775 as positive amplification controls and N65 as a negative amplification control. Staphylococcal enterotoxin genes (sea, seb, sec, sed, see, seg, city, sea and sej); toxic shock syndrome toxin-1 (TSST-1), and exfoliative toxins (ETA and ETB) were performed as method previously described by Johnson et al.,23.

Evaluation of the pattern of DNA amplicons

After amplification, 17 µl of PCR sample was loaded on a 1 % (w/v) agarose gel (Bioline, London, UK) containing 0.5 gr/ml ethidium bromide and run in a horizontal gel electrophoresis unit (Mini-Sub DNA cell, BioRad). The stained bands were visualized with UV light (309 nm) using a trans-illuminator and gels were recorded as digital TIFF images using a gel documentation system (UVImager). The positive result for the presence of the mecA gene was demonstrated by the amplification of the fragment of 214 pair base. This was confirmed by the positive control and marker molecular weight.

Typing

Staphylococcal cassette chromosome mec (SCCmec) types (I, IA, II, III, IIIA, IIIB and IV) was performed using a multiplex PCR technique with sets of region specific primers, as described by Oliveira et al. Multiplex PCR; The accessory gene regulator (agr) types groups that were described in the literature were specific multiplex PCR assay.27

PFGE

Pulsed Field Gel Electrophoresis was performed as a specific method described by McDougal et al.24. In Brief, A single colony of the test isolate was inoculated into 5 ml of brain heart infusion broth and incubated at 37°C for 24 h with vigorous shaking. The concentrations of the cell suspensions were adjusted using a spectrophotometer to an absorbance of 0.9 to 1.1 at 610 nm. Aliquot of 200 µl of the adjusted cell suspension was centrifuged at 12,000 × g for 3 min. The pellet was resuspended in 300 µl of Tris-EDTA (TE) buffer (10 mM Tris HCl, 1 mM EDTA [pH 8]) and equilibrated in a 37°C water bath for 10 min. Four microliters of recombinant (no. L-0761; Sigma, St. Louis, Mo.) lysostaphin stock solution (1 mg/ml in 20 mM sodium acetate [pH 4.5]) and 300 µl of 1.8% (wt/vol) SeaKem Gold agarose (FMC, Rockland, Maine) in TE buffer were added to the cell suspension, gently mixed, and dispensed into the wells of either a large-plug mold (~250 µl volume of each well). The plugs were allowed to solidify for 15 min at room temperature. The plugs were removed and placed into a tube containing at least 3 ml of EC lysis buffer (6 mM Tris HCl, 100 mM EDTA, 1 M NaCl, 0.5% Brij-58, 0.5% sodium lauroylsarcosine, 0.2% sodium deoxycholate) and incubated for 5 h at 37°C. The EC lysis buffer was decanted and 4 ml of TE buffer was supplemented. The TE washings were repeated three times and the plugs were stored at 4°C.

Multilocus sequence typing

MLST was performed by PCR amplification using primers designed by Enright et al.25. Sequences, using the ABI Prism 310 Genetic Analyzer with Big Dye fluorescence were detected. Each sequence was submitted to the MLST database Web site (http://www.mlst.net) for assignment of the allelic profile and sequence type (ST). Clonal isolates belonging to the same group as determined by PFGE, for the same or similar to previous studies that range from 4.17 to 19 of the type, suggesting that PFGE MLST analysis after each selected samples were used for the group.
RESULTS

In the study, isolates of β-lactam antibiotics are not included according to the results of the in vitro susceptibility to glycopeptide vancomycin in Table 1. Investigation the presence and distribution of the genes studied are shown in Table 2. The most frequently detected toxin gene sea (76%) were 13 (13%) did not isolate the toxin gene are shown in table 2 and Fig. 1.

As a result, SCCmec typing of the isolates, the most common type III (85%) was detected, following the 10 isolates could not be typed by the method used (Table 3) (Fig. 2). At the end of agr typing of the isolates, the most common type IA (87%) were found, seven isolates could not be typed (Table 3) (Fig. 3).

Up to six group differences in isolates of the same type within the 100 isolates showed that 97 were evaluated according to their opinion, six subtypes (A1-A6) with a pattern formed by the remaining three isolates, unrelated to each other with a difference of 3 different PFGE pattern and six-band pattern (B, C, D) form was observed (Table 2) (Fig. 4). Gene directory with visual assessment with the same results was obtained in the program. Patterns A from 2; B, C and D were selected to be a pattern 5 of the MLST analysis result of the isolate clones involved in this isolate was found that the ST 239.

DISCUSSION

In this study, we determined the molecular characteristics of nosocomial MRSA blood isolates from Salman University Hospital, Alkharj, KSA. All MRSA isolates showed different resistant value to rifampin, erythromycin, trimethoprim-sulfamethoxazole, tetracycline, ciprofloxacin and gentamicin and were susceptible to linezolid and vancomycin. Most of the strains (93%) were resistant to ciprofloxacin. In addition, 92% of the strains were resistant to rifampin, 91% were resistant to gentamicin and tetracycline, 79% were resistant to erythromycin and 8% were resistant to trimethoprim/sulfamethoxazole.

Previous studies have analyzed the distribution of SCCmec types of MRSA isolates; five different SCCmec types have been identified. SCCmec types I, II and III in hospital-acquired
### Table 2. Results of PFGE patterns and toxin genes of the isolates studied by Clinical diagnosis with bloodstream infection

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>mecA%</th>
<th>PVL%</th>
<th>Sea</th>
<th>sea+seg</th>
<th>Sea, seg, sec</th>
<th>Sea, sei</th>
<th>tst</th>
<th>Sea, seg, sei</th>
<th>Seg</th>
<th>Gene no</th>
<th>PFGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cirrhosis (23)</td>
<td>23 (100)</td>
<td>0</td>
<td>19 (82.6)</td>
<td>2 (8.6)</td>
<td>1 (4.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>i(4.3)</td>
</tr>
<tr>
<td>Diabetes mellitus (16)</td>
<td>16 (100)</td>
<td>0</td>
<td>13 (81.2)</td>
<td>0</td>
<td>0</td>
<td>1 (6.2)</td>
<td>1 (6.2)</td>
<td>0</td>
<td>0</td>
<td>1 (6.2)</td>
<td>A, B</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease (34)</td>
<td>34 (100)</td>
<td>0</td>
<td>25 (73.5)</td>
<td>2 (5.8)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (2.9)</td>
<td>1 (2.9)</td>
<td>5 (14.7)</td>
<td>A, C, D</td>
</tr>
<tr>
<td>Others (27)</td>
<td>27 (100)</td>
<td>0</td>
<td>20 (74)</td>
<td>0</td>
<td>0</td>
<td>1 (3.7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6 (22.2)</td>
</tr>
<tr>
<td>Total (100)</td>
<td>100 (100)</td>
<td>0</td>
<td>77 (77)</td>
<td>4 (4)</td>
<td>1 (1)</td>
<td>2 (2)</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>13 (13)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Results of SCCmec and agr strains typing by Clinical diagnosis with bloodstream infection

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>SCCmec type N (%)</th>
<th>agr type N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IA</td>
<td>II</td>
</tr>
<tr>
<td>Cirrhosis (23)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diabetes mellitus (16)</td>
<td>1 (6.2)</td>
<td>1 (6.2)</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease (34)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Others (27)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total (100)</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
</tbody>
</table>

NT: Non-typeable
MSRA (HA-MRSA) are the vast majority, however SCCmec types IV and V are mainly associated with community-acquired MRSA (CA-MRSA) [26,24]. In our study, 100 MRSA isolates, 84 of the SCCmec type III, one of the type IA strain, respectively, were found to be type II and type IIIA, type IV does not contain any of the hospital isolates were determined. Our findings were similar to other studies [27,28].

Community-acquired pneumonia, necrotizing skin infections in diabetic patients, such as furunculosis, especially with PVL-positive MRSA strains was founded, while traumatic skin infections, nosocomial pneumonia, infective endocarditis and bacteremia of patients with PVL-negative isolates were detected. PVL-positive, usually accompanied by SCCmec type IV in CA-MRSA often identified as HK PVL positive isolates.
that are rarely mentioned in the literature\textsuperscript{5,29}. Chini \textit{et al}.\textsuperscript{30} found that 23\% of PVL are positive. Wannet \textit{et al}.
\textsuperscript{31} in their study, found that 20\% of isolates were identified as PVL positive strains obtained from hospitals in the Netherlands, were 65\% of PVL-positive strains in that SCC\textit{mec} type IV and type III and type I, respectively. In Taneike \textit{et al}.\textsuperscript{13} study, 1985-1986, 1990-1992 and 2000-2005 periods studied in Japan and found that PVL positive for PVL-positive rates in hospitalized patients, 23.5\% (4/17), 3.4\% (2/59) and 0 (0/379), respectively. In our study, none of the PVL positive blood isolates have been detected and our findings are compatible with the data of Budimir \textit{et al}.\textsuperscript{32}. However, in the future, PVL-positive, SCC\textit{mec} type IV non-hospital environment types, locations, forecast dispensers checks should not be ignored.

A “quorum-sensing” system, \textit{agr}, some surface proteins and secreted out of the cell are involved in the regulation of the transcription of genes encoding enzymes\textsuperscript{33}. \textit{agr} type I and II studies with reduced vancomycin susceptibility, shows the relationship between the development of type III and type IV \textit{Exfoliator} with the production of toxic shock syndrome and necrotizing pneumonia induced by PVL\textsuperscript{34}. In this study, 91\% of the isolates were found to be type II \textit{agr}. Isolates were susceptible to vancomycin was observed, but in \textit{agr} type I, likely to be hetero-VISA isolates should not be disregarded.

MRSA sequence type 239 (ST-239) is widely used in the world and clonal complex 8 (CC8) is a branch of the clinical significance\textsuperscript{11,35,36}. This sequence type EMRSA contains 1, 4, 7, 9, 11 clones in Brazil, Portugal and Vienna\textsuperscript{13}. In this study, the same PFGE pattern indicated group, two samples A and B, C and D group and a copy MLST investigated, and found to be all of the ST 239 respectively. In our study, comprising 87 isolates of PFGE patterns, epidemic MRSA clones are not consistent with the one to one, but many of epidemic MRSA clone ST 239 may have a different PFGE pattern\textsuperscript{36}.

Aires de Sousa and de Lencastre\textsuperscript{37} in their study, have reported that six major pandemic MRSA clones (Iberian, Brazilian, Hungarian, New York / Japan, pediatric or EMRSA 16) show similarities with the presence of four minor or sporadic clones of the assets have been reported. These four clones were isolated in 1995 and 1996, ST-239, CC8, SCC\textit{mec} type II and PVL- negative clones. Our present study is in parallel with said clones.

In Saudi Arabia, there are few publications about investigating the molecular characteristics of isolated MRSA strains. Monecke \textit{et al}., studied the molecular characterization of MRSA in Riyadh, Saudi Arabia and reported that the prevalence of the genes encoding the PVL was surprisingly high (54.21\%) [28]. In conclusion, dominant MRSA clone in our hospital is SCC\textit{mec} type III, \textit{agr} type I, PVL-negative, \textit{sea} and ST-239 features were found to be positive. These findings show that it is the dominant clone, but in Saudi Arabia, the determination of the dominant clone, larger studies are needed to evaluate the properties.

\textbf{ACKNOWLEDGEMENT}

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group project No RGP-VPP-202.

\textbf{REFERENCES}

5. Gillet Y., Issartel B., Vanhems P. Association


26. Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, N., Tienasai, C. Structural comparison of three types of staphylococcal