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

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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 vancomycine. *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[1]. *S. aureus* is the most prevalent pathogens isolated from blood cultures and responsible for a wide range of hospital acquired (HA-MRSA) infections worldwide[2] 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[3,4].

S. aureus, staphylococcal enterotoxins (SE), A-J, K, L, M and O, toxic shock syndrome toxin 1 (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[5-6]. *S. aureus* as a more

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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*[7].

The *mec*A gene is the main mechanism responsible for MRSA. This gene is carried by the cassette chromosome *mec* (SCC*mec*)[8]. Different SCC*mec* types are associated with hospital or community isolates and if a bacterial cell acquires such SCC*mec*, the cell will concomitantly acquire a multidrug resistance phenotype[9]. MRSA with type IV or type V SCC*mec* is usually associated with severe community infections, however types I, II and III SCC*mec* commonly occur in the health-care setting[10].

The epidemiology of invasive *S. aureus* infections (ISA) has only been defined in few studies of population-based study design[11]. 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[6]. Other studies have been limited either by inclusion of only selected patients with ISA or by failure to include clinical information[12,13]. In Saudi Arabia, an increasing number of MRSA cases has been recognized[14,15]. The MRSA prevalence in patients in different regions has increased[16,17]. 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 (SUH), 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[18]. 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 (10^5 CFU/ml) and further dilution so as final inoculums is approximately 10^4 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, HPV 107 (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[19] 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 ^{0}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 37ºC for 3 hours. 200 ml of distilled water was added and incubated at 95ºC 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 (UVI-Tech). 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[7].

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 200µl 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 1. Distribution of the isolates studied by clinical diagnosis with bloodstream infection and susceptibility (%) against various antimicrobial agents

Clinical diagnosis	Antibiotic Number of resistant isolates (resistance %)							
	Rifampin	Erythromycin	Trimethoprim-Sulfamethoxazole	Ciprofloxacin	Gentamicin	Tetracycline	linezolid	Vancomycin
Cirrhosis (23)	26 (96.2)	18 (66.6)	1 (3.7)	26 (96.2)	26 (96.2)	26 (96.2)	-	-
Diabetes mellitus (16)	30 (88.2)	31 (91.1)	31 (91.1)	5 (14.7)	32 (94.1)	29 (85.2)	-	-
Chronic obstructive pulmonary disease (34)	16 (100)	14 (87.5)	-	16 (100)	14 (87.5)	14 (87.5)	-	-
Others (27)	20 (86.9)	16 (69.5)	2 (8.6)	19 (82.6)	19 (82.6)	22 (95.6)	-	-
Total (100)	92 (92)	79 (79)	8 (8)	93 (93)	91 (91)	91 (91)	-	-

Table 2. Results of PFGE patterns and toxin genes of the isolates studied by Clinical diagnosis with bloodstream infection

			Toxin genes, n (%)								
Clinical diagnosis	mecAn%	PVLn%	Sea	sea+ seg	Sea, seg, sec	Sea, sei	*tst*	Sea, seg, sei	Seg	Gene no	PFGE
Cirrhosis (23)	23 (100)	0	19 (82.6)	2 (8.6)	1 (4.3)	0	0	0	0	1(4.3)	A
Diabetes mellitus (16)	16 (100)	0	13 (81.2)	0	0	1 (6.2)	1 (6.2)	0	0	1 (6.2)	A , B
Chronic obstructive pulmonary disease (34)	34 (100)	0	25 (73.5)	2 (5.8)	0	0	0	1 (2.9)	1 (2.9)	5 (14.7)	A , C , D
Others (27)	27 (100)	0	20 (74)	0	0	1 (3.7)	0	0	0	6 (22.2)	A
Total (100)	100 (100)	0	77 (77)	4 (4)	1 (1)	2 (2)	1 (1)	1 (1)	1 (1)	13 (13)	

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

	SCCmec type N (%)						*agr* type N (%)				
Clinical diagnosis	IA	II	III	IIIA	IIIB	NT	*I*	II	III	IV	NT
Cirrhosis (23)	0	0	21 (91.3)	0	1 (4.3)	1 (4.3)	22 (95.6)	1 (4.3)	0	0	0
Diabetes mellitus (16)	1 (6.2)	1 (6.2)	13 (81.2)	0	1 (6.2)	0	15 (93.7)	0	0	0	1 (6.2)
Chronic obstructive pulmonary disease (34)	0	0	24 (70.5)	1 (2.9)	1 (2.9)	8 (23.5)	27 (79.4)	0	1 (2.9)	0	6 (17.6)
Others (27)	0	0	26 (96.2)	0	0	1 (3.7)	27 (100)	0	0	0	0
Total (100)	1 (1)	1 (1)	84 (84)	1 (1)	3 (3)	10 (10)	91 (91)	1 (1)	1 (1)	0	7 (7)

NT: Non-typeable

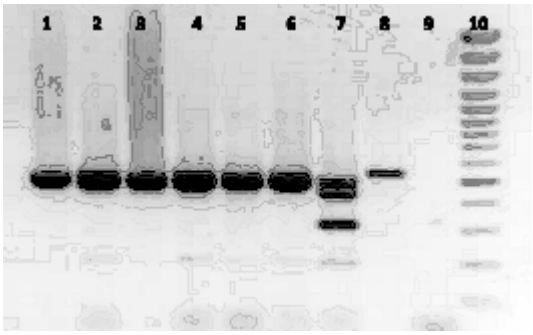

Fig. 1. The presence of toxin genes in MRSA isolates. Lines 1-6: Enterotoxins; Line 7: enterotoxin A + G + I; Line 8: TSST; Line 9: negative control; Line 10: molecular size marker (100bp DNA ladder)

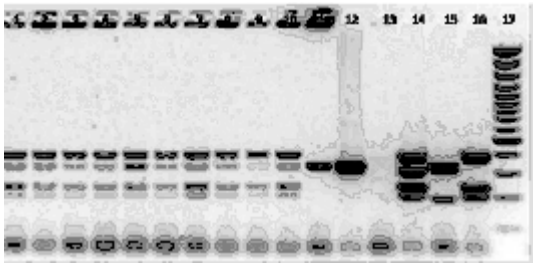

Fig. 2. The most common clone of MRSA isolates cassette chromosome type. Lines 1-10: type III; Line 11: Positive control (type IV) GRE 14; Line 12: Positive control JSCS 4744 (type IV); Line 13: Negative control; Line 14: Positive control 85/2082 (type III); Line 15: Nontypeable; Line 16: Positive control HSJ 216 (type IIIA); Line 17: molecular size marker (100bp DNA ladder).

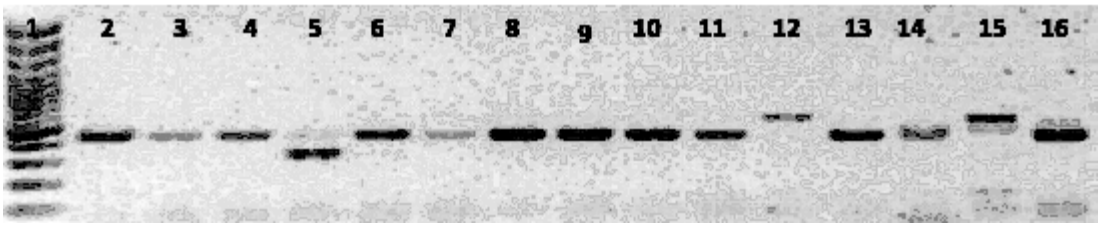

Fig. 3. agr types of MRSA isolates. Line 1: molecular size marker (100bp DNA ladder); Lines 2-3, 6-11, 13, 14, 16: *agr* type I; Line 5: *agr* type III; Lines 12 and 15: *agr* type II.

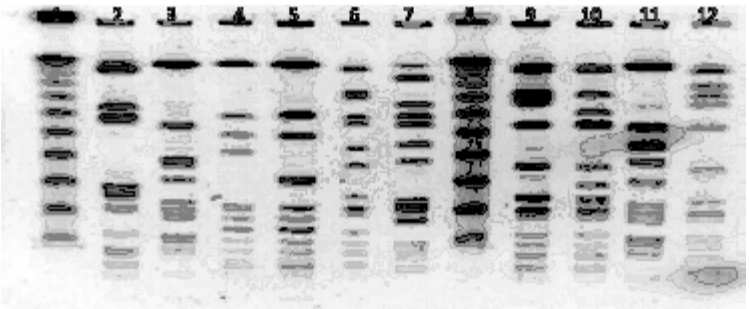

Fig. 4. MRSA isolates obtained by cutting with the SmaI enzyme of the PFGE pattern. From left to right, line 1, and 8: molecular size marker (lambda ladder PFG Marker, New England Biolabs); Line 9: Controlling MRSA clone A, line 2, 3, 6, 7, 10 and 12: A clone subtypes, line 4: B clone, line 5: C clone, line 11: D clone.

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[5,29]. Chini *et al*[30] found that 23% of PVL are positive. Wannet *et al*[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 *SCCmec* type IV and type III and type I, respectively. In Taneike *et al*[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 *et al* [32]. However, in the future, PVL-positive, *SCCmec* type IV non-hospital environment types, locations, forecast dispensers checks should not be ignored.

A "quorum-sensing" system, *agr*, some surface proteins and secreted out of the cell are involved in the regulation of the transcription of genes encoding enzymes[33]. *agr* type I and II studies with reduced vancomycin susceptibility, shows the relationship between the development of type III and type IV Exfoliator with the production of toxic shock syndrome and necrotizing pneumonia induced by PVL[34]. In this study, 91% of the isolates were found to be type II *agr*. Isolates were susceptible to vancomycin was observed, but in *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[11, 35, 36]. This sequence type EMRSA contains 1, 4, 7, 9, 11 clones in Brazil, Portugal and Vienna[35]. 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[36].

Aires de Sousa and de Lencastre[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, *SCCmec* 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 *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 *SCCmec* type III, *agr* type I, PVL-negative, *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.

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