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Original Article

Molecular investigation of methicillin-resistant *staphylococcus aureus* isolates from blood: USA600 emerges as the major type

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Abstract

Introduction: The widespread emergence of methicillin-resistant *Staphylococcus aureus* is turning into a real worry in public health. The goals of the present study were to identify resistance and virulence encoding genes and molecular characteristics of methicillin-resistant *S. aureus* bloodstream isolates.

Methodology: A cross-sectional study was conducted on 84 *S. aureus* bloodstream isolates during a 10-month period. To evaluate antibiotic susceptibility of the isolates, we used Kirby-Bauer disk diffusion method. In addition, the prevalence of antimicrobial resistance and toxins genes was assessed using polymerase chain reaction. Isolates were typed according to polymorphisms seven housekeeping genes by MLST.

Results: All the isolates were resistant to methicillin. The most prevalent resistance gene was *mecA* gene (100%) followed by *tetM* (57.1%), *aac (6′)-Ie/aph (2″)* (53.6%), *ant (4′)-Ia* (46.4%), *ermA* (45.2%), *msrA* (35.7%), *msrB* (33.3%), *aph (3′)-IIIa* (33.3%), *ermB* (31%), *ermC* (16.7%), and *mupA* (14.3%) genes. The presence of toxin encoding genes *tst*, *pvl*, *eta*, and *etb* were detected in 25%, 14.3%, 3.6% and 3.6%, respectively. The isolates were classified into five different sequence types: ST45 (29.8%), ST239 (27.4%), ST858 (21.4%), ST22 (17.8%), and ST59 (3.6%). All the high-level mupirocin-resistant (HLMUPR) strains belonged to ST239, while the low-level mupirocin resistant (LLMUPR) strains belonged to ST22 (13%) and ST239 (6%).

Conclusions: To the best of our knowledge, the present study is the first report of ST59 in MRSA bloodstream isolates in Iran. Our data demonstrated the need for thorough epidemiological monitoring to detect emergence and dissemination of MDR- MRSA types in our hospitals.

Key words: *S. aureus*; MLST; MRSA; Iran.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), which is a major global cause of infection in both community and hospital, accounts for a broad range of serious human infections ranging from skin infections to sever infections, including bacteremia [1]. According to the literature, compared with non-invasive MRSA infections, higher mortality rate is reported in cases with invasive infections due to MRSA [2]. Although different STs have been found to predominate in a variety of geographical regions, one of the most prevalent sequence types (STs) is USA600 or ST45. The first USA600 strain was reported in some European countries such as Germany and the Netherlands. Since then, numerous surveillance studies have revealed a steady increase in the incidence of this type in the United States and Canada [2, 3]. According to the published data, reported mortality rates among patients with MRSA bloodstream infections (BSIs) were found to be 20 to 30%. Recent studies have reported mortality rates of 60% for patients with MRSA BSIs caused by the USA600, suggesting remarkable ability of this pathogen to produce virulence factors [2-4]. The increasing prevalence of MRSA infections and its ability to spread in hospitals and health care facilities is becoming a major challenge for infection control. During the past years, the widespread use of antibiotics has led to the emergence and dissemination of invasive MRSA strains that systematically harbor multi-resistance genes and cause failure treatment [5-7]. In addition to *mecA* gene, the emergence of co-existence of genes encoding resistance to other antibiotics is the new concern, along with the particular concern of invasive bloodstream infections [8-11]. A major concern with managing MRSA infections in patients with blood stream infection is the lack of awareness

about the molecular types and antibiotic resistance pattern of this bacterium either phenotypically or genotypically [1]. Molecular typing methods have been developed to track MRSA isolates among which Multi-Locus Sequence Typing (MLST) technique, with its high discriminatory abilities, has been introduced as an effective method for typing MRSA strains [12,13]. With regards to the lack of sufficient information about molecular characterization of MRSA strains isolated from blood infection, the present study was conducted to understand phenotypic and genotypic resistance pattern and to identify STs of MRSA obtained from patients with bacteremia in Tehran, Iran.

Methodology

Study design and population

In the current cross-sectional study, we investigated 84 MRSA isolates, recovered over a 10-month period from November 2016 to August 2017, from patients referring to 12 medical centers located in different areas of Tehran (capital city of Iran). The frequency of MRSA isolated from studied hospitals in Tehran was as following; 12 isolates (14.3%) from hospital A, 12 isolates from hospital C (14.3%), 11 isolates from hospital G (13.1%), 10 isolates from hospital I (11.9%), 9 isolates from hospital E (10.7%), 8 isolates (9.5%) from hospital B, 6 isolates (7.1%) from hospital D, 5 isolates (5.9%) from hospital H, 4 isolates (4.8%) from hospital J, 3 isolates (3.6%) from hospital F, 3 isolates (3.6%) from hospital K and one isolate (1.2%) from hospital L. One isolate per patient was included in the study and duplicate samples were excluded. The study was approved by the Ethics Committee at Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU. MSP.REC.1396.2). Blood cultures obtained from patients with suspected sepsis so as to order to identify *S. aureus* were processed using standard microbiological procedures including colony morphology on blood agar, Gram-stain microscopy, b-hemolysis on 5% sheep blood agar, growth on mannitol salt agar, and giving positive results for catalase, coagulase, and DNase tests. Isolates were confirmed as *S. aureus* via detecting the presence of the *femA* and *nucA* genes (encoding peptidyl transferase and thermostable nuclease, respectively) using PCR [5,14].

Antimicrobial susceptibility testing (AST), DNA extraction, and MRSA screening

Antibiotic susceptibility to ampicillin (AP 10 µg), ciprofloxacin (CIP 5 µg), tetracyclin (T 30 µg), erythromycin (E 15 µg), clindamycin (CD 2 µg), linezolid (LZD 30 µg), penicillin (PG 10 µg), teicoplanin (TEC 30 µg), gentamicin (GM 10 µg), and trimethoprim- sulfamethoxazole (TS 2.5 µg) was determined using Kirby Bauer disk diffusion. The results were interpreted according to the recommendations of the Clinical Laboratory Standard Institute (CLSI) [15]. The Minimum inhibitory concentration (MIC) for vancomycin (VA), daptomycin (DPC), rifampin (RI), and mupirocin (MU) (Sigma-Aldrich, St. Louis, Mo, USA) were determined using micro-broth dilution method. According to the CLSI guidelines the MIC breakpoints for vancomycin were defined as follows: susceptible, ≤ 2 µg/mL; intermediate, 4–8 µg/mL; and resistant, ≥ 16 µg/mL. Strains were considered to demonstrate low-level mupirocin resistance (LLMUPR) if the MIC value was between 8-256 µg/mL and high-level mupirocin resistance (HLMUPR) if the MIC value was ≥ 512 µg/mL. For daptomycin, culture media was supplemented with Ca^{2+} to a final concentration of 50 µg/mL and strains were reported as sensitive to daptomycin if the MIC value was ≤ 1 µg/mL. For rifampin, the MIC values ≤ 1 µg/mL was considered as susceptible and MIC values > 4 µg/mL was considered as resistant. The ranges of MIC value used for antimicrobial agents were including: vancomycin 0.125 to 16 µg/mL; daptomycin 0.125 to 2.0 µg/mL; rifampin 0.004 to 16 µg/mL and mupirocin 0.5 to ≥ 512 µg/mL. All antibiotic disks used in the present study were supplied by Mast, UK. *S. aureus* ATCC25923 and ATCC 29213, as standard reference strains, were employed in every test run. Genomic DNA was extracted using the commercial kit InstaGene Matrix (BioRad, Hercules co., CA, USA). MRSA isolates were screened with cefoxitin disc (30 µg) and oxacillin disc (1µg) and confirmed to presence the *mecA* gene using PCR [7,15].

Detection of resistance and toxin encoding genes

The genes encoding the resistance (*vanA, vanB, mecA, mupA, ermA, ermB, ermC, msrA, msrB, tetM, ant (4′)-Ia, aac (6′)-Ie/aph (2″), aph (3′)-IIIa*), and toxin (*etb*, *eta*, *pvl*, *tst*) were targeted via PCR using specific primers [7,9].

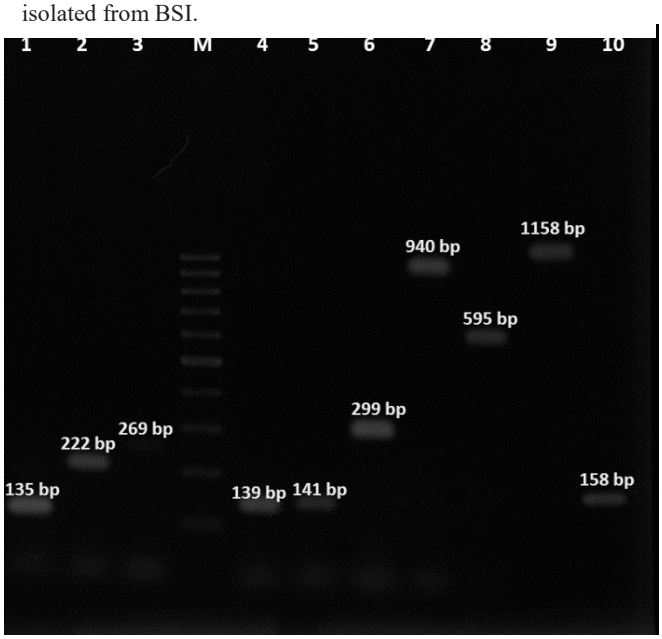
Multilocus Sequence Typing (MLST)

MLST was performed on isolates based on seven housekeeping genes (*arc*C, *aro*E, *glp*F, *gm*K, *pta*, *tpi*A, and *yqi*L) as described by Enright *et al*. [13] Sequence type (ST) of each isolate was determined by comparing the sequences at each locus with those of the known alleles in the *S. aureus* MLST database (http://www.mlst.net/).

Results

A total of 84 *S. aureus* strains isolated from BSI were investigated during the 10-month study period. All the isolates were found to be MRSA. The results of AST showed resistance to most of the antibiotics, including ampicillin (89.3%), ciprofloxacin (67.9%), tetracycline (77.4%), gentamicin (58.3%), erythromycin (53.6%), clindamycin (35.7%), mupirocin (33.3%), trimethoprim-sulfamethoxazole (31%), and rifampin (21.4%). Resistance to linezolid, teicoplanin, vancomycin, and daptomycin was not found among the tested isolates. Based on the micro-broth dilution method, isolates were inhibited by vancomycin at similar MIC_{50} and MIC_{90} 1 µg/ml. The MIC_{50} and MIC_{90} values for daptomycin were found to be 0.25 µg/mL and 0.5 µg/mL, respectively. Of the 28 mupirocin resistant MRSA isolates, 12 (14.3%) isolated carry *mupA* gene and were confirmed as HLMUPR strains. The predominant resistance profile among MDR isolates included a resistance profile to six antibiotics (56%) followed by seven antibiotics (33.3%) and five antibiotics (2.4%), simultaneously. Seven isolates were resistant to one antibiotic (8.3%). With regards to the resistance genes, the most prevalent was

Figure 1. Detection of resistance genes of MRSA strains isolated from BSI.



Lane M, 100-bp DNA ladder (Fermentas, London, UK); lane 1 *ant(4′)-Ia*, lane 2 *aac(6′)-Ie/aph(2″)*, lane 3 *aph(3′)-IIIa,* lane 4 *ermA,* lane 5 *ermB,* lane 6 *ermC,* lane 7 *msrA,* lane 8 *msrB,* lan 9 *mupA,* lane 10 *tetM.*

Table 1. Molecular characterization of MRSA strains isolated from BSI.

Molecular types	Toxin profile (No;%)	Antibiotic resistance gene profile (No;%)	Hospitals (No;%)	Antibiotic resistance profile (No;%)	No (%)
ST45	-	*mecA* (25; 100), *aac(6′)-Ie/aph(2″)* (20; 80), *ant(4′)-Ia* (18; 72), *aph(3′)-IIIa* (12; 48), *tetM* (12; 48), *ermA* (10; 40), *ermB* (9; 36), *ermC* (8; 32), *msrA* (7; 28), *msrB* (5; 20),	A (4; 16), B (2; 8), C (5; 20), D (3; 12), E (2; 8), G (4; 16), I (3; 12), J (2; 8)	PG, AP, CIP, GM, T, TS (10; 40) PG, AP, T, E, CIP, GM (8; 32) PG, AP, CIP, T, GM, RI (5; 20) PG, E, CD, GM, RI (2; 8)	25 (29.8)
ST239	*tst* (12; 52.2), *eta* (3; 13), *etb* (2; 8.7)	*mecA* (23; 100), *msrA* (18; 78.3), *ermA* (15; 65.2), *msrB*(14; 60.9), *ant(4′)-Ia* (12; 52.2), *mupA* (12; 52.2), *tetM* (12; 52.2), *aph(3′)-IIIa* (7; 30.4), *ermB* (7; 30.4), *aac(6′)-Ie/aph(2″)*(6; 26.1)	B (1; 4.3), C (3; 13.1), D (2; 8.7), E (3; 13.1), F (2; 8.7), G (3; 13.1), H (2; 8.7), I (5; 21.7), J (1; 4.3), K (1; 4.3)	PG, AP, T, E, CD, TS, MUP (12; 52.2) PG, AP, CIP, GM, T, TS (4; 17.4) PG, AP, CIP, E, CD, MUP, RI (5; 21.7) PG, AP, CIP, T, E, GM (2; 8.7)	23 (27.4)
ST585	*tst* (9; 50), *etb* (1; 5.6)	*mecA*(18; 100), *tetM* (15; 83.3), *aac(6′)-Ie/aph(2″)* (14; 77.8), *aph(3′)-IIIa* (9; 50), *ant(4′)-Ia* (8; 44.4), *ermB* (6; 33.3), *ermA* (5; 27.8), *msrA*(5; 29.4), *msrB*(4; 22.2), *ermC* (3; 16.7)	A (3; 16.6), B (3; 16.6), C (2; 11.1), E (2; 11.1), F (1; 5.6), G (2; 11.1), H (1; 5.6), I (2; 11.1), J (1; 5.6), L (1; 5.6)	PG, AP, CIP, T, E, GM (5; 27.8) PG, AP, CIP, GM, T, TS (10; 55.6) PG (3; 16.6)	18 (21.4)
ST22	*pvl* (9; 60)	*mecA* (15; 100), *tetM* (7; 46.7), *ermA* (6; 40), *msrB* (5; 33.3), *ermB* (4;26.7), *ermC* (3; 20), *ant(4′)-Ia* (1; 6.7)	A (4; 26.7), B (2; 13.3), C (2; 13.3), D (1; 6.7), E (2; 13.3), G (2; 13.3), K (2; 13.3)	PG, AP, T, E, CD, TS, MUP (6; 40) PG, AP, CIP, E, CD, MUP, RI (5; 33.3) P (4; 26.7)	15 (17.8)
ST59	*pvl* (3; 100)	*mecA*(3; 100), *tetM* (3; 100), *ermA* (2; 66.7)	A (1; 33.3), H (2. 66.7)	PG, AP, CIP, GM, T, TS (2; 66.7) PG, AP, CIP, T, GM, RI (1; 33.3)	3 (3.6)

PG, penicillin; AP, ampicillin; CD, clindamycin; E, erythromycin; GM, gentamicin; MUP, mupirocin; RI, rifampicin; T, tetracycline; CIP, ciprofloxacin; TS, trimethoprim- sulfamethoxazole.

mecA gene (84; 100%) followed by *tetM* (48; 57.1%), *aac (6′)-Ie/aph (2″)* (45; 53.6%), *ant (4′)-Ia* (39; 46.4%), *ermA* (38; 45.2%), *msrA* (30; 35.7%), *msrB* (28; 33.3%), *aph (3′)-IIIa* (28; 33.3%), *ermB* (26; 31%), *ermC* (14; 16.7%), and *mupA* (12; 14.3%) genes (Figure 1). Toxin analysis revealed that *tst* gene was the most frequent toxin genes among tested isolates (21; 25%) followed by *pvl* (12; 14.3%), *eta* (3; 3.6%), and *etb* (3; 3.6%), respectively. Using MLST technique, the strains were classified into five different sequence types (STs) as follows: ST45 (25 strains; 29.8%), ST239 (23 strains; 27.4%), ST858 (18 strains; 21.4%), ST22 (15 strains; 17.8%), and ST59 (3 strains; 3.6%). The results of distribution of various STs in 84 MRSA isolated from BSIs among 12 medical centers in Tehran are presented in Table 1. The overall prevalence of mupirocin resistance was found to be 33.3% using MIC out of which 12 (14.3%) and 16 (19%) were HLMUPR and LLMUPR, respectively. All the HLMUPR-MRSA strains belonged to ST239. ST22 was the predominant type observed among LLMUPR-MRSA strains (13 %) followed by ST239 (6%).

Discussion

The present study reports on several important findings in relation to molecular typing of MRSA strains isolated from BSI, including detection of ST45 as the most predominant type and high prevalence of mupirocin resistance (33.3%), which was distributed among ST239 and ST22. Meanwhile, we reported ST59 the first time in Iran for although at a low frequency (3.6%).

In contrast with a study conducted in the Iran, which reported low frequency of ST45 (4%) in MRSA strains isolated from patients with bacteremia [1], in the present study, we observed a high frequency of the ST45 (29.8%) among MRSA strains isolated from BSIs. These findings may reflect a true increase in the prevalence of ST45 in Iran. The study showed that none of the ST45 isolates were susceptible to gentamycin and penicillin while 60%, 40%, and 8% were susceptible to trimethoprim- sulfamethoxazole, erythromycin, and clindamycin respectively. These findings are in contrast to those reported in Moore *et al*'s who reported low frequency of resistance to gentamycin (25%) among ST45 strains while it was found that all the isolates were resistant to erythromycin and clindamycin [2]. Variability in resistance and toxin encoding genes within ST45 strains were previously reported by several investigators [4,16]. The resistance gene profiles of the isolates have shown that the overwhelming majority of these isolates carried *aac(6′)-Ie/aph(2″)*(80%), and *ant(4′)-Ia* (72%). All the isolates were PVL negative. *S. aureus* ST45 was previously identified in Iran [1], Netherlands [17], Switzerland [18], and Croatia [19]. This high prevalence of ST45 isolates in our study may be attributed to high transmissibility and rapidity of its spread within the community and/or increase in the volumes of international travel.

According to the literature, ST239 have evolved from ST8 and ST30 parents and accounts for at least 90% of hospital-acquired-MRSA in the South American, Europe, and some other Asian countries, including Kuwait, Saudi Arabia and Malaysia [3,16,20,21]. In the present study, multi resistant ST239 was the second most common type identified (27.4%), although at a low level. Our previous study from Iran, carried out to identify molecular characteristics of MRSA isolated from blood samples, showed that ST239 was the most predominant type (43%). In line with the studies conducted by Abimanyu *et al*. in India [22] and Udo *et al*. in Kuwait [20], who demonstrated that HLMUPR-MRSA strains belonged to ST239 clone, our findings showed that 12 HLMUPR-MRSA strains (14.3%) and 5 HLMUPR-MRSA strains (5.9%) belonged to ST239. Among our ST239 isolates, 12 (14.3%), 3 (3.6%), and 2 isolates (2.4%) were positive for *tst*, *eta* and *etb* genes, respectively. *S. aureus* ST239, harboring *tst, eta*, and *etb* genes, has been detected Iran [1], Korea [23], Taiwan [3], and Kuwait [20], as well.

As our results showed, ST585, as a single-locus variant (SLV) of ST239, was the third most-common type among the MRSA strains isolated from BSI (21.4%). Low frequency of this type has previously been reported [1,24]. In our previous research on MRSA strains isolated from BSI, ST585 was detected in 10.9% of tested isolates [1], which was lower than the rate found in the present study. Ohadian Moghadamet *et al*. [24], in Iran, identified molecular characterization of MRSA isolates obtained from clinical samples. They found that MRSA isolates belonged to two major clonal complexes (CC); CC8 (ST239, ST585, ST2732, ST1294) and CC30 (ST30, ST36, ST1163) and the presence of ST585 was reported in 11.1% of the tested MRSA isolates. With regard to ST858 antibiotic resistance to gentamicin, tetracycline, and ciprofloxacin pattern, our findings support the recent study conducted by Goudarzi *et al.* [1].

Moreover, ST22 was the fourth most-common type identified in the current research (17.8%). This type was associated with a LLMUPR pattern (13.1%), and less than half of these strains carried the resistance genes *tetM* (7; 46.7), *ermA* (6; 40), *msrB* (5; 33.3), *ermB* (4; 26.7), *ermC* [3;20], and *ant(4′)-Ia* (1; 6.7). Variable

virulence markers in ST22-MRSA-IV have reported by investigators [20]. In the present study, 60% of ST22 strains harbored the *pvl* gene. *S. aureus* ST22 harboring *pvl* gene was previously detected in Saudi Arabia [25], Iran [5,9], Kuwait [26], and England [27], too.

Another type found among our strains was ST59. In contrast to a previous study in Taiwan [3], which reported a rate of 17.2% for ST59 strains isolated from blood, in the present study, we found low frequency of ST59 (3.6%), which all were positive for *pvl* encoding genes. Although antimicrobial resistance pattern in ST59 isolates may vary, we found that all the isolates were resistant to ciprofloxacin, tetracycline, and gentamicin. These findings, with regards to resistance to tetracycline, resembled those of the previous reports by Wang *et al*. [3] in Taiwan and Coombs *et al*. in Western Australia [28]. To the best of our knowledge, it is the first report of ST59 in MRSA strains isolated from patients with BSI in Iran. Given the wide dissemination of clonal complex 59 (CC59)/ST59 in the Asia Pacific region, it is guessed that transmission of these isolates may occur, due to the rate of transportation between these countries.

Conclusion

In conclusion, the present survey showed that MRSA isolated from blood has genetic diversity, including Five STs with different toxin and resistance gene profiles which should seriously be taken into consideration. ST59 was reported for the first time in Iran, indicating the emergence of the new ST in our region. Further and periodical studies are needed to delineate the genetic changes in MRSA isolated from blood.

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Authors' Contributions

Conceived and designed the experiments: MG HG. Performed the experiments: MG FS MH. Analyzed the data: MG HG HA. Contributed reagents/materials/analysis tools: MG HG. All authors read and approved the final manuscript.

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