New update on molecular diversity of clinical Staphylococcus aureus isolates in Iran: Antimicrobial resistance, adhesion and virulence factors, biofilm formation and SCCmec typing

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Abstract

Staphylococcus. aureus is often considered as a potential pathogen and resistant to a wide range of antibiotics. The pathogenicity of this bacterium is due to the presence of multiple virulence factors and ability to form biofilm. SCC*mec* types I, II and III are mainly attributed to HA-MRSA, while SCCmec types IV and V have usually been reported in CA-MRSA infections. In this study, we performed a cross-sectional study in order to determine the antimicrobial resistance, adhesion and virulence factors, biofilm formation and SCC*mec* typing of clinical *S. aureus* isolates in Iran. *S. aureus* isolate was identified using microbiological standard methods and antibiotic susceptibility test was performed as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines. Inducible resistance phenotype and biofilm formation were determined using D-test and tissue culture plate methods, respectively. Multiplex-PCRs were performed to detect adhesion and virulence factors, antibiotic resistance genes, biofilm formation and SCC*mec* typing by specific primers. Among 143 clinical samples, 67.8% were identified as MRSA. All isolates were susceptible to vancomycin. The prevalence of $cMLS_B$, $iMLS_B$ and MS phenotypes were 61.1%, 22.2% and 14.8%, respectively. The TCP method revealed that 71.3% of isolates were able to form biofilm. The predominant virulence and inducible resistance genes in both MRSA and MSSA isolates were related to *sea* and *ermC* respectively. SCC*mec* type III was the predominant type. Data show the high prevalence rates of virulence elements among *S. aureus* isolates, especially MRSA strains. This result might be attributed to antibiotic pressure, facilitating clonal selection.

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most common cause of health-care and community acquired infections and so responsible for a wide variety of illnesses, from soft and skin tissue infections (SSTIs) to life-threatening infections such as septicemia, toxic shock, hospital- and community- acquired pneumonia (HAP and CAP) and endocarditis [1]. *S. aureus* clinical isolates often promote infections by expressing of various exotoxins such as heat-stable staphylococcal enterotoxins (SEs), staphylokinase (SAK), toxic shock syndrome toxin-1 (TSST-1), capsular polysaccharides, lipase, exfoliative toxins (ETA and ETB), hemolysins (α, β, γ, δ) and leukocidins (Panton-Valentine leukocidin; PVL, LukE/D) [2]. From the clinical point of view, indwelling medical devices or catheter–related infections such as central venous catheters (CVC), are at risk of *S. aureus* related infection. The ability to form a stable biofilms is one of the most crucial factors in *S. aureus* pathogenicity and biofilm-associated *S. aureus* infections are often resistant to antibiotic therapy and innate host immune system [3]. Biofilm formation requires polysaccharide intercellular adhesin (PIA), which is encoded and regulated by the intercellular adhesion (*ica*ADCB) operon. This operon includes an N-acetylglucosamine transferase (*ica*A and *ica*B), a predicted exporter (*ica*C), and a deacetylase (*ica*D) [1, 4].

Multidrug-resistant *S. aureus* (MDRSA), is becoming a serious global concern, as a commen cause of nosocomial- and community acquired infections [5]. In recent years, methicillin-resistant *S. aureus* (MRSA), which is now the most common MDR, have emerged with the acquisition of Staphylococcal Cassette Chromosome *mec* (SCC*mec*) elements, which carry a *mecA* gene that encodes a penicillin binding protein (PBP2a or PBP2′) with a low affinity to β-lactams [6]. MRSA are spread worldwide and are common causes of health care (HAIs)-and community-acquired (CAIs) infections. SCC*mec* determinants are classified into various types based on the combination of *ccr* and *mec* genes complexes, which includes 5 and 8 *mec* and *ccr* classes, respectively. To date, at least 13 types of SCC*mec* elements have been recognized and all SCC*mec* types have individual

characteristics. In general, SCC*mec* type I, II, and III are distributed in the hospital-associated MRSA (HA-MRSA) and type IV and V are present in the community-acquired MRSA (CA-MRSA) [7].

The Mupirocin -a topical ointment that broadly used for SSTIs and nasal decolonization of MRSA- is effective on the isoleucyl-tRNA synthetase (IleRS) which is encodes by *ileS* gene, interfering with protein synthesis. According to the minimal inhibitory concentration (MIC), two mupirocin-resistant phenotypes have been identified; MIC 8–256 µg/ml (low-level resistant-LLR or LMR) and MIC ≥ 512 µg/ml (high-level resistant-HLR or HMR). A point mutation in *ileS-1* gene (*mupL*) led to LLR isolates, while HLR usually mediated by a conjugate plasmid-borne *ileS-2* (*mupA*) gene which encodes a new IleRS that is not bound by mupirocin [8]. Aminoglycosides are a class of bactericidal broad spectrum antimicrobials that bind to the A-position of 16S rRNA in 30S ribosomal small subunit and inhibit protein synthesis. Aminoglycoside-modifying enzymes (AMEs) and 16S ribosomal RNA (16S rRNA) methylation are two important mechanisms for antibiotic inactivation in the *S. aureus* [9]. Based on their functions, AMEs are generally categorized to three types: AAC (aminoglycoside acetyltransferase), APS (aminoglycoside phosphotransferase), and ANT (aminoglycoside nucleotidyltransferase). Numerous AMEs, counting variants of acetyltransferases AAC(3)-I, AAC(3)-II, AAC(3)-III, AAC(6')-I, AAC(6')-II, and AAC(6')-III, the phosphotransferases APH(3')-I, APH(3')-II, and APH(3')-VI, and the nucleotidyltransferases ANT(3)-I, ANT(4')-I, and ANT(2'')-I have been known so far in *S. aureus* [10, 11].

The aim of this study was new update on molecular diversity of antimicrobial resistance, adhesion and virulence factors, biofilm formation and SCC*mec* typing of clinical *S. aureus* isolates in Iran.

Material And Methods

Clinical sampling and laboratory identification

A total of 143 non-duplicative clinical samples were collected from admission patient referred to teaching therapeutic hospitals (Shahid Beheshti & Ruhani Hospitals, Babol, Iran) in a period of 8 months from September 2019 to April 2020. The samples were transported to the microbiology laboratory in Brain-Heart Infusion Broth (Merck Co., Germany). Each sample was cultured on Mannitol Salt Agar (supplemented with 7.5% sodium chloride) (Merck Co., Germany) and incubated in 37˚C for 24 h. All *S. aureus* colonies were identified based on routine biochemical and microbiological standard tests [12].

Antimicrobial susceptibility test

Antimicrobial susceptibility was determined using the agar disk diffusion method on Mueller-Hinton agar plates (Merck Co., Germany) as described by the Clinical and Laboratory Standards Institute (CLSI document M100-S14) [13]. The test antimicrobials were used as follows; Clindamycin (CD; 2 µg), Erythromycin (ERY; 15 µg), Gentamicin (GM; 10 µg), Vancomycin (VAN; 30 µg), Ciprofloxacin (CIP; 5 µg), Tetracycline (TET; 30 µg), Mupirocin (MUP; 5 µg), Rifampicin (RIF; 2 µg), Cefoxitin (FOX; 30 µg), and Co-trimoxazole (SXT; 5 µg) (MAST Diagnostics, Merseyside, UK). Mupirocin MIC was determined by the E-test strip method (AB Biodisk, Solna, Sweden) on the Mueller-Hinton agar Petri dish according to the manufacturers' guidelines. *S. aureus* ATCC 29213 was used as positive quality control.

Inducible resistance phenotype

The Inducible resistance phenotype was recognized using the double disk test including, Clindamycin (CD; 2 µg) and Erythromycin (ERY; 15 µg) disks applied 20 mm separately [10,11]. After an incubation time of 24 h at 35°C, a flattening inhibition zone adjacent to the ERY disk representing an inducible type (D-shaped zone) of MLS_B resistance (IR), whereas no-susceptibility to both ERY and CD was mentioned to as a constitutive type (CR). The nonappearances of a D-shaped zone in ERY-resistant and CD-susceptible isolates were interpreted as the M/MS_B efflux phenotype [14].

Quantitative biofilm production assay

In Brief, pure colonies were inoculated in 10 mL of 1% glucose-rich tryptic soy broth (TSBglu), incubated at 37°C for 24h in stationary growth phase and diluted 1:100 with fresh medium. Each well of sterile 96 well-flat bottom polystyrene tissue culture microtiter plates (Falcon® 3046, Lincoln Park, NJ) was full with 200 µl aliquots of diluted cultures. Sterile TSBglu broth was used as a negative control. All plates were incubated at 37°C for 24 h and then, substances of all wells were gradually removed by tapping the petri. The wells were washed three times with 0.3 mL of phosphate buffer saline (PBS, pH 7.2) to remove loosely attached and floating “planktonic” microorganisms. Biofilm formed by adherent “sessile” isolates in petri were immobile with sodium acetate (NaA) and stained with crystal violet (0.1% w/v). Extra dye was removed by washing with sterile dideionized water and plates were kept for drying. Adherent *S. aureus* cells frequently formed biofilm on the sides of the wells and were regularly stained with crystal violet (CV; 1%). The investigation of biofilm formation was assessed by adding the 200 µl of 95% CH₃−CH₂−OH (ethanol) to decolorize the wells. Optical density (OD) of stained adherent isolates were measured with a micro ELISA auto-reader (Bio-Tek Instruments, USA) at wavelength of 570 nm (OD570 nm). Biofilm formation was recorded as follows: non-biofilm forming (A570 <1); weak (1 <A570 <2); ++, moderate (2 <A570 < 3); +++, strong (A570 >3) [1, 15].

Multiplex-Polymerase Chain Reactions (M-PCRs)

M-PCRs reactions were performed for detection of virulence, resistance and biofilm corresponding genes. Chromosomal DNA was extracted from the pure colonies using the Bacterial Genomic DNA Extraction kit (TaKaRa Biotechnology Co., Ltd, Dalien, China). The DNA concentration and purity were evaluated using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, UK) and then kept at -20 °C until further use. The details of the primers used in this study are shown in Table 1. The process of M-PCR reactions in the final volume of 25 µl was performed according to Table 2 in an Eppendorf MasterCycle Gradient Thermocycler (Eppendorf, Hamburg, Germany). M-PCRs products were electrophoresed in a 1% agarose/0.5 × TBE (45 mM-Tris-borate, 1 mM-EDTA) gel stained with 0.1 µl/ml Gel Red™ (Biotium, USA), then photographed under an UV trans-illuminator (Tanon, China).

Data analysis

SPSS version 18.0 for Windows (SPSS Inc., Chicago, USA) was used for statistical analysis. $P \leq 0.05$ was considered as a statistical significance.

Results

In this cross-sectional study, clinical samples were collected from 143 patients, 51.7% (n = 74) female and 48.3% (n = 96) male with the mean age of 61.4 ± 1.1 years (range from 14 to 98 years). *S. aureus* isolates were collected

from several clinical samples including, wound (n = 38, 26.6%), pus (n = 31; 21.7%), blood (n = 27; 18.9%), skin lesion (n = 19; 13.3%), bronchoalveolar lavage (n = 11; 7.7%), sputum (n = 9; 6.3%), intratracheal tube (n = 6; 4.2%), joint fluid (n = 1; 0.7%), and cerebrospinal fluid (CSF) (n = 1; 0.7%). The majority of *S. aureus* was isolated from different wards of the hospital as follows: ICU (n = 38; 26.6%), NICU (n = 26; 18.2%), internal medicine (n = 19; 13.3%), surgery (n = 15; 10.5%), urology (n = 14; 9.9%), hemodialysis (n = 14; 9.9%), ENT (Ear, Nose and Throat) (n = 7; 4.9%) hematology-oncology (n = 6; 4.2%), gynecology (n = 2; 1.4%), neurosurgery (n = 1; 0.7%) and orthopedics (n = 1; 0.7%). Of the 143 *S. aureus* strains, 67.8% (n = 97) were resistant to 30 µg-FOX disk phenotypically considered as MRSA isolates and confirmed by *mecA*-gene amplification using PCR. As shown in Table 3, the resistance rate in MRSA strains was higher than MSSA (P-value ≤ 0.05) (Table 3). The resistance rate on MRSA strains showed that 91.7%, 87.6%, 84.5%, 83.5%, 76.3%, 62.8%, 55.7% and 31.9% of isolates were resistant to CIP, GM, SXT, ERY, TET, RIF, CD and MUP, respectively. MUP MIC E-test showed that, of 31 MUP-resistant MRSA strains, 16.1% (n = 5) and 83.9% (n = 26) were MuL and MuR respectively. Also, all MSSA isolates were susceptible to RIF and MUP. Among the 143 *S. aureus* isolates, 78.3% (n = 112) of the isolates were resistant to at least 3 different antibiotic classes and therefore considered as MDR. The frequency of MDR strains in MRSA and MSSA isolates was 75.3% (n = 73/97) and 45.6% (n = 21/46), respectively. The result of inducible resistance test showed that 39.2% (n = 56) of the isolates were resistant to both CD and ERY. Indeed, 61.1% (n = 33) strains had resistant phenotype to $cMLS_B$ (resistant to both ERY and CD), 22.2% (n = 12) were inducible resistance $iMLS_B$ (resistant to ERY but susceptible to CD), 14.8% (n = 8) isolates had the MS phenotype (susceptible to ERY and resistant to CD) and lastly, 5.5% (n = 3) were susceptible to ERY and resistant to CD. All $iMLS_B$ strains, except two isolates, belonged to the MRSA. The prevalence of $cMLS_B$, $iMLS_B$ and MS phenotypes in the MRSA isolates were 81.8% (n = 27), 83.3% (n = 10) and 62.5% (n = 5), respectively. So, 11.1% (n = 6), 3.7% (n = 2) and 5.5% (n = 3) of MSSA isolates have $cMLS_B$, $iMLS_B$ and MS phenotypes, respectively. The TCP method revealed that 71.3% (n = 102/143) of isolates were able to form biofilm, including strong (n = 69/102, 67.6%), moderate (n = 21/102, 20.6%) and weak (n = 12/102, 11.7%). Blood and wound isolates have the highest proportion for strong biofilm phenotype (n = 21/27; 77.7%, and n = 27/38; 71.1%). Biofilm formation in MRSA isolates was far greater than MSSA (P value ≤ 0.05). In MRSA isolates, 84.1% (n = 58/69), 80.9% (n = 17/21) and 58.3% (n = 7/12) of isolates had a strong, moderate and weak phenotype, respectively. But, in the MSSA strains 84.1% (n = 11/69), 19% (n = 4/21) and 41.6% (n = 5/12) had strong, moderate and weak biofilm formation phenotype, respectively.

Table 3
Antimicrobial resistance profile in MRSA and MSSA strains

S. aureus isolates		No, (%) of antimicrobial resistance pattern								
		ERY	CD	GM	CIP	TET	MUP	RIF	SXT	VAN
MRSA (n = 97, 67.8%)	S	16 (16.5)	42 (43.3)	12 (12.4)	7 (7.2)	23 (23.7)	83 (85.6)	36 (37.1)	15 (15.5)	97 (100%)
	I	0 (0.0)	1 (1)	0 (0.0)	1 (1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	R	81 (83.5)	54 (55.7)	85 (87.6)	89 (91.7)	74 (76.3)	14 (14.4)	61 (62.8)	82 (84.5)	0 (0.0)
MSSA (n = 46, 32.2%)	S	19 (41.3)	13 (28.3)	33 (71.7)	36 (78.3)	29 (63)	46 (100)	46 (100)	40 (86.9)	46 (100%)
	I	0 (0.0)	1 (2.2)	0 (0.0)	1 (2.2)	2 (4.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	R	27 (58.7)	32 (69.6)	13 (28.3)	9 (19.6)	15 (32.6)	0 (0.0)	0 (0.0)	6 (13)	0 (0.0)

Molecular distribution of virulence-related genes was significantly higher in MRSA strains, especially in the isolates collected from ICU (P-value ≤ 0.05). The most prevalent virulence-related gene was *sea* in both MRSA and MSSA isolates. *tsst-1*, *sec* and *sed* genes were present only in MRSA strains. 8.2% and 2.3% of MRSA and MSSA isolates were positive for *pvl* gene, respectively. The frequencies of inducible-resistance encoding genes in the MRSA strains were 21.6%, 16.5%, 44.3% and 9.3%, respectively. Such as MRSA strains, in MSSA isolates *ermC* was the predominant gene. *vanA* gene was not detected in both MRSA and MSSA isolates. So, distribution of AMEs genes in MRSA strains were 33%, 62.8%, 24.7% and 85.6% for *APH(3')-I*, *APH(3')-IIIa*, *aac(6')/aph(2")* and *ANT(4')-Ia*, respectively. All MRSA biofilm-producing isolates carried the *icaA* gene. The prevalence of the *ica* genes in the MRSA strains was as follows: *icaB* (70.1%), *icaC* (74.2%) and *icaD* (81.4%). Of 97 MRSA isolates, 55.7% (n = 54) and 44.3% (n = 43) were HA-MRSA and CA-MRSA, respectively. SCC*mec* type III was the most predominant type. In general, among 97 MRSA isolates, 34% (n = 33), 23.7% (n = 23), 18.5% (n = 18), 11.3% (n = 11) and 6.2% (n = 6) were belonged to the SCC*mec* type III, I, IV, II, and V, respectively; though, 6.4% (n = 6) isolates were nontypable. SCC*mec* type I was only found in the blood strains, types IV and V were mostly observed from wound, BAL and sputum and type III was found in all clinical samples (Table 4).

Table 4
Distribution of virulence, resistance and biofilm genes in MRSA and MSSA isolates

Gene type	gene	MRSA (n = 97)	MSSA (n = 46)
virulence-related genes	*cna*	51 (52.3%)	28 (60.7%)
	clfA	55 (56.7%)	19 (41.3%)
	clfB	64 (66%)	31 (67.4%)
	fnbA	15 (15.5%)	17 (37%)
	fnbB	13 (13.4%)	21 (45.7%)
	fib	73 (75.3%)	25 (54.3%)
	eno	82 (84.5%)	33 (71.3%)
	hla	88 (90.7%)	39 (84.7%)
	hlb	43 (44.3%)	6 (13%)
	hld	80 (82.5%)	39 (84.7%)
	hlg	6 (6.2%)	13 (28.3%)
	tsst-1	5 (5.2%)	0 (0.0%)
	Pvl	8 (8.2%)	1 (2.3%)
	sea	97 (100%)	41 (89.1%)
	seb	17 (17.5%)	2 (4.3%)
	sec	5 (5.2%)	0 (0.0%)
	sed	3 (3.1%)	0 (0.0%)
	eta	11 (11.3%)	2 (4.3%)
	etb	6 (6.2%)	4 (6.5%)
Resistance encoding genes	*ileS-2*	8 (8.2%)	0 (0.0%)
	APH(3')-I	32 (33%)	17 (36.9%)
	APH(3')-IIIa	61 (62.8%)	21 (45.6%)
	aac(6')/aph(2")	24 (24.7%)	6 (13%)
	ANT(4')-Ia	83 (85.6%)	12 (26.1%)
	vanA	0 (0.0%)	0 (0.0%)
	ermA	21 (21.6%)	12 (26.1%)
	ermB	16 (16.5%)	7 (15.2%)
	ermC	43 (44.3%)	23 (50%)
	ereA	9 (9.3%)	0 (0.0%)

Gene type	gene	MRSA (n = 97)	MSSA (n = 46)
Biofilm genes	*icaA*	82 (84.5%)	36 (78.3%)
	icaB	68 (70.1%)	12 (26.1%)
	icaC	72 (74.2%)	18 (39.1%)
	icaD	79 (81.4%)	42 (91.3%)

Discussion

In the current study, a high prevalence of MRSA (67.3%) was found, especially in samples obtained from ICU and NICU wards. These data are in agreement with Mir et al (2019) [16], but do not agree with Darban-Sarokhalil et al (2016) [30]. In a study directed by Kateete et al (2011), all isolates were found to be MRSA [31]. These conflicts could be attributed to the sample types (burn vs. other samples), year of study, geographic location (Uganda vs Iran), level of hygiene, different protocols in infection control, irrational antibiotic administration, and laboratory method for determination of methicillin-resistant isolates. In line with our study, Guardabassi et al (2007) indicated that the 30 µg Fox disk diffusion method is preferred to most of the other recommended tests such as; oxacillin disc diffusion and oxacillin screen agar tests and it is currently an accepted method for recognition of MRSA isolates by Clinical and Laboratory Standards Institute strategies [32].

The resistance rate in MRSA isolates is significantly higher than in the MSSA ($P \leq 0.05$), which is consistent with the study of Solgi et al, (2019) [33], Mir et al (2019) [16] and Pournajaf et al (2014) [24]. Among antibiotics used for MRSA strains, CIP showed the least anti-staphylococcal activity and VAN was the most effective. According to the study performed by Solgi et al (2019) [33], VAN is still the best option in the treatment of patients with MRSA infection. In compare with other studies, there has been an increase in resistance to antimicrobial in MRSA isolates. The resistance rates in the MRSA straits were as follows: CIP (91.7%), GM (87.6%), SXT (84.5%), ERY (83.5%), TET (76.3%), RIF (62.8%), CD (55.7%) and VAN (0.0%). So, 78.3% of our isolates were considered MDR. This could be due to the continuous and empirical usage of broad-spectrum antimicrobials and the lack of an appropriate antibiotic treatment strategy. According to our data, although 14.4% of MRSA strains were resistant to MUP, it's still recommended as an option in the removal of MSSA nasal colonization. These data are in agreement with Chaturvedi et al (2014) [34] and Antonov et al (2015) [35] studies. Interestingly, among 14.4% MUP-resistant MRSA strains, only 8.2% were positive for *ileS-2* gene. These data are consistent with Solgi et al (2019) [33] and McNeil et al (2011) [36] studies. Solgi et al (2019) [33] declare that low-level MUP resistance may be occurred due to another responsible genes such as; *mupL/D/W/O/T*. Contrary to our study, Mir et al (2019) [16], showed that 85.6% of the isolates were resistant to MUP. On the other hand, Chen et al (2012) reported high frequency of MUP-resistant MRSA isolates in burn centers [37]. This topical bacteriostatic antimicrobial mainly used for prophylaxis against *S. aureus* nasal carriage and other skin diseases. Its target is the bacterial isoleucyl transfer ribonucleic acid synthetase. The long-term use of MUP, mostly for decolonization of nasal carriage, burn, diabetic foot, bedsores and other skin lesions could be related to development of resistance to MUP [33].

D-test revealed that the prevalence of cMLSB, iMLSB and MS resistance phenotypes were 61.1%, 22.2% and 14.8%, respectively. This data has also been described by Solgi et al (2019) [33], Khodabandeh et al (2019) [14] and Gupta et al (2009) [38], but have conflict with Seifi et al (2012) [39], Adhikari et al (2017) [11], Ruiz-Ripa et al (2019) [40] and Deotale et al (2010) [10] studies. These conflicts may be related to year of study, topographical

locations and surveillance strategies, as well as limitation in drug prescription. The rate of inducible resistance varies from hospital to hospital and even from patient to patient. In agreement with Solgi et al (2019) [33], and Gupta et al (2009) [38], the frequency of $cMLS_B$ phenotype was higher than $iMLS_B$, but in another study, the frequency of $iMLS_B$ phenotype showed to be higher than $cMLS_B$. Therefore, notice of regional frequency of MLS_B resistant isolates is very important for microbiology laboratories to choose to perform D-test regularly. In concordance with Khodabandeh et al (2019) [14], *ermC* was the predominant gene on both MRSA and MSSA isolates. The prevalence of *ermA*, *ermB*, *ermC* and *ereA* in the MRSA isolates were 21.6%, 16.5%, 44.3% and 9.3%, respectively. *ereA* gene was only found in the MRSA isolates which were collected from ICU. The combination of *ermA/ ermB/ ermC* genes was detected in only two MRSA isolates collected from blood samples. So, 26.1%, 15.2% and 23% of MSSA isolates were positive for *ermA*, *ermB* and *ermC*, respectively. Our findings contradicts with the study conducted by Ghanbari et al (2016) [41] and Saribas et al (2006) [42]. This discrepancy could be due to genetic variation and spread of single clone in our area. Distribution of AMEs genes in our samples were as fallows; *aph(3')-I* (33%), *aph(3')-IIIa* (62.8%), *aac(6')/aph(2")* (24.7%) and *ant(4')-Ia* (85.6%) in the MRSA isolates and *aph(3')-I* (36.9%), *aph(3')-IIIa* (45.6%), *aac(6')/aph(2")* (13%) and *ant(4')-Ia* (26.1%) in MSSA. M-PCR showed that 8.2% (n = 8/97), 12.4% (n = 12/97), and 33% (n = 32/97) of MRSA isolates carried simultaneously *aph(3')-IIIa/ aac(6')/aph(2")*, *aph(3')-I/ aph(3')-IIIa*, and *aph(3')-IIIa/ ant(4')-Ia* genes, respectively. Only 2.1% (n = 2/97) isolates were positive for all AMEs tested genes. These results are inconsistent with the studies of Khosravi et al (2017) [43] and Goudarzi et al (2020) [44]. In agreement with our study, the *ant(4')-Ia* was the most prevalent gene in Yadegar et al (2009) [45]. As a result, according to other studies, the AMEs gene in the MRSA strains was higher than MSSA, which could be due to the ability of these strains to acquire resistance genetic elements.

In our study, isolates collected from blood and wound from patients hospitalized in ICU had the highest ability in biofilm formation. With this regard, 71.3% of the isolates were able to form biofilm, including strong (67.6%), moderate (20.6%) and weak (11.7%). In MRSA isolates, 84.1%, 80.9% and 58.3% had a strong, moderate and weak phenotype, respectively. So, 84.1%, 19% and 41.6% of MSSA strains had strong, moderate and weak biofilm phenotype, respectively. These data are in contrast with Avila-Novoa et al (2018) [15] and Gowrishankar et al (2016) [28]. One reason for this discrepancy is source of the samples (food and pharyngitis samples vs our clinical samples). Contrary to our study, Ghasemian et al (2015) [27] declare that the prevalence of *icaA*, *icaB*, *icaC* and *icaD* were 73% (n = 16), 63.6% (n = 14), 73% (n = 16) and 73% (n = 16), respectively. So, they showed that there was no significant difference between MRSA and MSSA strains for the presence of *icaADBC* operon. In the present study, biofilm formation in MRSA isolates was far greater than MSSA. In line with Kord et al (2018) study, the highest and lowest *ica* gene was *icaA* and *icaD*, respectively [46].

In our study, all MRSA isolates were harbored *sea* gene. The *seb*, *sec* and *sed* were detected in 17.5%, 5.2% and 3.1% of MRSA isolates. *Sec* and *sed* was not found in MSSA isolates. In a study performed by Mehrotra et al (2000) [22], among 107 strains collected from nasal swabs from healthy humans, 19.6% (n = 21), 24.3% (n = 26), 5.6% (n = 6), 7.5% (n = 8) and 1.9% (n = 2) were positive for *sea*, *tst*, *seb, sec*, and *sed*, respectively. This contrast may be related to source of the sample (anterior nasal swabs vs clinic samples). Sabouni et al (2014) [47] showed that of 133 *S. aureus* isolates 48% (n = 64) were MRSA. The frequency of virulence-encoded genes was 40.6%, 19.6%, 12.8%, 11.3%, 9%, 4.5% and 3% for *sea*, *seb*, *tsst*, *eta*, *etb*, *sed* and *sec*, respectively. In contrast with the present study, among MSSA isolates, *seb* and *tsst* were the more prevalent toxins in comparison with MRSA isolates. In our samples, none of the MSSA isolates were positive for *tsst-1* gene. Mir et al (2019) [16] showed that the frequency of *hla*, *hlb*, *hld*, *hlg*, *tst* and *pvl* genes was 92.8%, 34.7%, 89.8%, 11.9%, 10.7%, and 0.5%

respectively. In line with our study, *hla* gene had the highest frequency among isolates (94.4% for MRSA and 89.8% for MSSA). Exfoliative toxin A and B were detected in the 11.3% and 6.2% of MRSA isolates, respectively. In the MSSA strains, the *etA* gene was present in 4.3% and *etB* in 6.5% of isolates. These data are similar to the results reported by Sila et al (2009) [48]. Also to support Sabouni et al (2014) study [47], *etA* gene was higher among MRSA isolates. However, no significant relationship was observed in the presence of *etB* between MRSA and MSSA strains. The *etA* and *etB* genes are more common in samples collected from Skin and soft tissue lesions [47].

Bacterial adherent to target cell is the primary stage of infection. At this stage, attachment of *S. aureus* is facilitated by microbial surface component-recognizing adhesive matrix molecules (MSCRAMMs) including, *fnbA* and *fnbB* (encoding fibronectin–binding proteins A and B), *fib* (encoding fibrinogen-binding proteins), *cna* (encoding collagen binding protein), *clfA* and *clfB* (encoding clumping factors A and B), and *eno* (encoding laminin binding protein) [49]. The prevalence of the MSCRAMMs-encoding genes in the MRSA isolates was as follows: *eno* (84.5%), *fib* (75.3%), *clfB* (66%), *clfA* (56.7%), *cna* (52.3 %), *fnbA* (15.5%) and *fnbB* (13.4%). These data are similar to Mir et al (2019) study [16]. As a result, the frequency of MSCRAMMs genes in MRSA strains was higher than in MSSA, which could be due to the high pathogenicity of the strains. The distribution of these genes in the samples collected from the ICU was much higher than the samples of other units. Also, the strains collected from the wound, sputum, blood, and BAL samples, had the highest frequency of these genes, respectively. Mir et al (2019) reported that various molecules such as collagen, fibrinogen, fibronectin and other factors are present in the burn wound [16]. *S. aureus* encodes many MSCRAMMs that precisely interact with host cells and it enables the microbe to colonize on the burn wounds. One of the most important virulence factors in *S. aureus* infections, particularly in skin and soft-tissue infections is the Panton-Valentine Leucocidin (PVL). This cytotoxin has been known as a virulence factor related to tissue necrosis such as necrotizing pneumonia (NP). M-PCR showed that 6.3% (n = 9/143) of *S. aureus* carried *pvl* gene. In contrast with Mir et al (2019) [16] and Mkrtchyan et al (2017) [50] studies, 8% of MRSA strains were positive for *pvl* gene, all of which were CA-MRSA. In our isolates, 55.7% and 44.3% of *mecA*-positive strains were HA-MRSA and CA-MRSA, respectively. In various studies directed by Rodrigues et al (2013) [51] and Teare et al (2010) [52], the prevalence of *pvl* gene was 14.6% and 2% respectively. Surprisingly, in concordance with Mir et al (2019) [16] only one MSSA isolate (2.3%) was positive for *pvl gene*.

According to our result, the frequency rate of types I, II, III, IV, and V of SCC*mec* was 23.7%, 11.3%, 34%, 18.5% and 6.2%, respectively. In line with Mariem et al (2013) [53] and Ghanbari et al (2016) [41], SCC*mec* typing did not show 100% type ability and had poor discriminatory power, as 6.4% of MRSA strains were nontypable. Overall, 75% (n = 6 of 8) of *pvl*-positive MRSA strains belonged to the SCC*mec* type IV and V. In agreement with Taherirad et al (2016) [54], Ghanbari et al (2016) [41] and Moosavian et al (2017) [55], the most common SCC*mec* type was type III. However Jamshidi et al (2019) [56] and Boye et al (2007) [29] reported type IV as the most predominant type. This contrast may be related to the patients included in the study, multiple sclerosis (MS) cases vs various *Staphylococcus* infections and geographical locations.

Conclusion

We determined the high prevalence of virulence elements and raised rate of antimicrobial resistance in our samples. MRSA strains also have a high ability to form biofilm. In addition, SCC*mec* type III was recognized as

the predominant type. These data recommend that efficient control procedures must to be considered to prevent the transmission of MRSA isolates among patients in hospital units especially in the ICU.

Declarations

Acknowledgments

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Author Contributions

HK and MTA conceived and designed the experiment; AP and MT conducted the study and collected the samples. AP, MT and FPG performed the experiments and analyzed the data. All authors contributed to paper writing. The authors have read and approved the final manuscript.

Compliance with Ethical Standards

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Conflict of interest

The authors declare that they have no conflicts of interest relevant to this study.

Ethical Approval

This study was approved by the Research Ethics Committee of Babol Branch, Islamic Azad University (approved ID: IR.IAU.BABOL.REC.1399.035) and performed in accordance with the Declaration of Helsinki. The participants signed the informed consent forms from each patient, and the Research Ethics Committee of Babol Branch, Islamic Azad University approved the study protocol.

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Tables 1 And 2

Table 1 Oligonucleotide primer sequences used in this study

Gene type	Encoded protein	Target Gene	Primer sequences (5′→3′)	Amplicon size (bp)	References
Virulence association genes	Collagen-Binding Protein	*can*	F=5′-GTCAAGCAGTTATTAACACCAGAC-3′ R=5′-AATCAGTAATTGCACTTTGTCCACTG-3′	423	[16]
	Clumping Factors A/B	*clfA*	F=5′- ATTGGCGTGGCTTCAGTGCT-3′ R=5′- CGTTTCTTCCGTAGTTGCATTTG-3′	292	[17]
		clfB	F=5′- ACATCAGTAATAGTAGGGGGCAAC-3′ R=5′- TTCGCACTGTTTGTGTTTGCAC-3′	205	
	Fibronectin–Binding Proteins A/B	*fnbA*	F=5′-GTGAAGTTTTAGAAGGTGGAAAGATTAG-3′ R=5′- GCTCTTGTAAGACCATTTTTCTTCAC-3′	643	
		fnbB	F=5′-GTAACAGCTAATGGTCGAATTGATACT-3′ R=5′- CAAGTTCGATAGGAGTACTATGTTC-3′	524	
	Fibrinogen-Binding Protein	*fib*	F=5′-CTACAACTACAATTGCCGTCAACAG-3′ R=5′-GCTCTTGTAAGACCATTTTCTTCAC-3′	404	[16]
	Laminin-Binding Protein	*eno*	F=5′- ACGTGCAGCAGCTGACT-3′ R=5′-CAACAGCATYCTTCAGTACCTTC-3′	302	
	Hemolysin-encoding genes	*hla*	F=5′-CTGATTACTATCCAAGAAATTCGATTG-3′ R=5′-CTTTCCAGCCTACTTTTTTATCAGT-3′	209	[18]
		hlb	F=5′- GTGCACTTACTGACAATAGTGC-3′ R=5′- GTTGATGAGTAGCTACCTTCAGT-3′	309	[16]
		hld	F=5′-AAGAATTTTTATCTTAATTAAGGAAGGAGTG-3′ R=5′- TTAGTGAATTTGTTCACTGTGTCGA-3′	111	[18]
		hlg	F=5′- GCCAATCCGTTATTAGAAAATGC-3′ R=5′-CCATAGACGTAGCAACGGAT-3′	937	[16]
	Toxic Shock Syndrome Toxin-1	*tsst-1*	F=5′- ATGGCAGCATCAGCTTGATATTT-3′ R=5′- CCAATAACCACCCGTTT-3′	350	[18]
	Panton-Valentine Leukocidin	*pvl*	F=5′-ATCATTAGGTAAAATGTCTGGACATGATCCA-3′ R=5′-GCATCAASTGTATTGGATAGCAAAAGC-3′	433	[16]
	Staphylococcal Enterotoxin A/B/C/D	*sea*	F=5′- TTGCGAAAAAAGTCTGAATTGC-3′ R=5′- ATTAACCGAAGGTTCTGTAGAAGTA-3′	552	[20]
		seb	F=5′- GTATGGTGGTGTAACTGAGC-3′ R=5′- CCAAATAGTGACGAGTTAGG -3′	164	[21]
		sec	F=5′- GACATAAAAGCTAGGAATTT-3′ R=5′- AAATCGGATTAACATTATCC-3′	257	[21]
		sed	F=5′- CTAGTTTGGTAATATCTCCT-3′ R=5′- TAATGCTATATCTTATAGGG-3′	317	
	Exfoliative Toxin A/B	*eta*	F=5′- GCAGGTGTTGATTTAGCATT-3′ R=5′- AGATGTCCCTATTTTTGCTG-3′	93	[22]
		etb	F=5′- ACAAGCAAAAGAATACAGCG-3′ R=5′- GTTTTTGGCTGCTTCTCTTG-3′	226	
Resistance encoding genes	Resistance to Methicillin	*mecA*	F=5′-AAAATCGATGGTAAAGGTTGGC-3′ R=5′-AGTTCTGGAGTACCGGATTTGC-3′	533	[23]
	Resistance to Mupirocin	*ileS-2 (mupA)*	F= 5′-TATATTATGCGATGGAAGGTTGG-3′ R= 5′- AATAAAATCAGCTGGAAAGTGTTG-3′	456	

	Aminoglycoside-enzymes modifying	*APH(3')-I*	F= 5′-ATGTGCCATATTCAACGGGAAACG-3′ R= 5′-TCAGAAAAACTCATCGAGCATCAA-3′	816	[24]
		APH(3')-IIIa	F= 5′-CTTGATCGAAAAATACCGCTGC-3′ R= 5′-TCATACTCTTCCGAGCAAA-3′	296	
		aac(6′)/aph(2′′)	F= 5′-GAAGTACGCAGAAGAGA-3′ R= 5′-ACATGGCAAGCTCTAGGA-3′	491	[25]
		ANT(4')-Ia	F= 5′-AATCGGTAGAAGCCCAA-3′ R= 5′-GCACCTGCCATTGCTA-3′	135	[26]
	Resistance to Vancomycin	*vanA*	F= 5′-GGCAAGTCAGGTGAAGATG-3′ R= 5′-ATCAAGCGGTCAATCAGTTC-3′	713	
	Inducible Resistance	*ermA*	F=5′-TATCTTATCGTTGAGAAGGGATT-3′ R=5′-CTACACTTGGCTTAGGATGAAA-3′	139	[14]
		ermB	F=5′-CCGTTTACGAAATTGGAACAGGTAAAGGGC-3′ R=5′-GAATCGAGACTTGAGTGTGC-3′	359	
		ermC	F=5′- ATCTTTGAAATCGGCTCAGG -3′ R=5′- CAAACCCGTATTCCACGATT -3′	295	
		ereA	F=5′- AACACCCTGAACCCAAGGGACG-3′ R=5′-CTTCACATCCGGATTCGCTCGA-3′	420	
biofilm-genes	Biofilm-encoding genes	*icaA*	F=5′-GATTATGTAATGTGCTTGGA-3′ R=5′- ACTACTGCTGCGTTAATAAT-3′	770	[27]
		icaB	F=5′- AGAATCGTGAAGTATAGAAAATT -3′ R=5′- TCTAATCTTTTTCATGGAATCCGT -3′	900	[28]
		icaC	F=5′-CATGAAAATATGGAGGGTGG-3′ R=5′-TCAAACTGATTTCGCCCACCG-3′	1000	[28]
		icaD	F=5′-AAACGTAAGAGAGGTGG-3′ R=5′-GGCAATATGATCAAGATAC-3′	381	[16]
SCC*mec* typing	Type II &IV	*ccrA2-B*	F=5′-ATTGCCTTGATAATAGCCYTCT-3′ R=5′-TAAAGGCATCAATGCACAAACACT-3′	937	[29]
	Type III & V	*ccr*	F=5′-CGTCTATTACAAGATGTTAAGGATAAT-3′ R=5′-CATTTATAGACTGGATTATTCAAAATAT-3′	518	
	Type I & IV	*IS1272*	F=5′-GCCACTCATAACATATGGAA-3′ R=5′-CATCCGAGTGAAACCCAAA-3′	415	
	Type V	*mecA-IS431*	F=5′-TATACCAAACCCGACAACTAC-3′ R=5′-CGGCTACAGTGATAACATCC-3′	359	

Table 2 M-PCRs conditions and cycles in the present study

Reaction set	Amplified genes	Reaction compounds	M-PCR program	Cycles of amplification
S1	*Cna/clfA/ fnbA/ fnbB/tsst-1*	1.0 µL of template DNA, 12.1 µL of CinnaGen PCR Master Mix, 0.8 µL of each primer, and 10.3 µL of ddH2O.	initial denaturation at 94°C for 5 min, denaturation at 95°C for 30 s, annealing at 56°C for 60 s, extension at 72°C for 60 s and a final extension at 72°C for 5 min.	30
S2	*clfB/ fib/eno/mecA*	0.9 µL of template DNA, 10.6 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 11.5 µL of ddH2O	initial denaturation at 95°C for 5 min, denaturation at 94°C for 45 s, annealing at 55°C for 50 s, extension at 72°C for 60 s and a final extension at 72°C for 6 min.	
S3	*hla/hlb/hld/hlg/ pvl*		initial denaturation at 96°C for 6 min, denaturation at 95°C for 50 s, annealing at 57°C for 45 s, extension at 72°C for 60 s and a final extension at 72°C for 5 min.	32
S4	*Sea/seb/sec/sed/ ileS-2*	0.8 µL of template DNA, 11.6 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 10.6 µL of ddH2O.	initial denaturation at 95°C for 5 min, denaturation at 95°C for 55 s, annealing at 56°C for 45 s, extension at 72°C for 60 s and a final extension at 72°C for 5 min.	35
S5	*eta/etb*		initial denaturation at 95°C for 7 min, denaturation at 94°C for 45 s, annealing at 55°C for 40 s, extension at 72°C for 50 s and a final extension at 72°C for 7 min.	31
S6	*APH(3')-I/APH(3')-IIIa/aac(6′)/aph(2′′)/ANT(4')-Ia/vanA*	1.0 µL of template DNA, 12.5 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 9.5 µL of ddH2O.	initial denaturation at 95°C for 6 min, denaturation at 95°C for 50 s, annealing at 53°C for 45 s, extension at 72°C for 50 s and a final extension at 72°C for 10 min.	30
S7	*ermA/ermB/ermC/ereA*	1.0 µL of template DNA, 12.3 µL of CinnaGen PCR Master Mix, 0.9 µL of each primer, and 9.9 µL of ddH2O.	initial denaturation at 95°C for 6 min, denaturation at 94°C for 55 s, annealing at 57°C for 50 s, extension at 72°C for 60 s and a final extension at 72°C for 5 min.	35
S8	*IcaA/icaB/icaC/icaD*	0.8 µL of template DNA, 11.7 µL of CinnaGen PCR Master Mix, 0.9 µL of each primer, and 10.7 µL of ddH2O.		33
S9	*ccrA2-B/ccr/IS1272/mecA-IS431*	0.9 µL of template DNA, 12.2 µL of CinnaGen PCR Master Mix, 0.8 µL of each primer, and 10.3 µL of ddH2O.	initial denaturation at 94°C for 5 min, denaturation at 95°C for 45 s, annealing at 55°C for 60 s, extension at 72°C for 60 s and a final extension at 72°C for 10 min.	32

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