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ORIGINAL RESEARCH

Characterization of *SCCmec*, *Spa* Types and Multidrug Resistant of Methicillin-Resistant *Staphylococcus aureus* Isolates in Ahvaz, Iran

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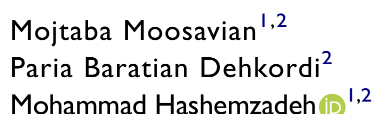

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Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most pathogens associated with health care. Molecular typing methods are vital for outbreak investigations of MRSA. The aim of this study was characterization of *SCCmec*, *spa* types and multidrug resistant of methicillin-resistant *Staphylococcus aureus* isolates in Ahvaz, Iran.

Methods: A total of 50 MRSA isolates were determined by using the phenotypic method and *mecA* gene. Antibiotic resistance profile and *SCCmec* types were screened using disc diffusion method and PCR, respectively. For *spa* typing of MRSA isolates, two molecular typing methods including the PCR-sequencing and high-resolution melting (HRM) analysis were used.

Results: In the present study, the highest sensitivity of MRSA was to vancomycin and linezolid and the lowest to clindamycin. In the MRSA isolates, 22% were XDR and 78% were MDR. *SCCmec* type III was found commonly among MRSA. Based on PCR-sequencing and HRM results, 10 different spa types were identified. The *spa* types t037 and t030 were the most common in this study.

Conclusion: This study emphasizes the *spa* variation among MRSA isolates, which may be considered as an important criterion when treating staphylococcal infections. Accurate and early detection of MDR, XDR, or even PDR MRSA isolates strains must be commenced by all clinical microbiology laboratories to reduce the menace of antimicrobial resistance.

Keywords: high-resolution melting, HRM, *spa*-typing, multidrug resistant *Staphylococcus aureus*, Antibiotic resistance

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is still a major cause of community and healthcare-associated infections worldwide.[1,2] In Iran, the frequency of MRSA infection has increased over the past decade. In a recently published meta-analysis, MRSA infections were reported to be highly prevalent (43.0%) among confirmed *S. aureus* isolates, underscoring the increasing prevalence of MRSA isolates over the recent years.[3] The emergence of MRSA strains is associated with the presence of the *mecA* gene, which encodes an alternative 78 kDa penicillin-binding protein (PBP2′) that has decreased affinity for β-lactam antibiotics.

MRSA has been detected by disk diffusion method, agar dilution method and oxacillin screen agar test as recommended by Clinical Laboratory Standard

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Institution. In a number of clinical microbiology laboratories, the routine procedure for MRSA detection was based on phenotypic assays such as disk diffusion and broth microdilution. Culture requires a long turnaround time of approximately 18–48 hour with low sensitivity and 100% specificity. Whereas detection of *mecA* gene is the "Gold Standard" offering high sensitivity and rapid results but these methods are not always been possible in many facilities constrains laboratories.[4]

The *mecA* gene is located on a mobile genetic element called the staphylococcal cassette chromosome mec (*SCCmec*). Moreover, *SCCmec* is comprised of two main components, namely, *ccr* gene complex ccr and *mec* gene complex. The combination of ccr allotypes with the *mec* gene complex, 11 types (I–XI) *SCCmec* has already been reported. According to previous studies, *SCCmec* types I, II, and III are related to hospital-acquired MRSA.[5]

The molecular typing of MRSA is crucial for outbreak investigation and epidemiological studies.[6] The techniques most widely employed for MRSA typing are pulsed-field gel electrophoresis (PFGE), staphylococcal cassette chromosome mec (SCC *mec*) typing, sequence-based typing methods such as *S. aureus* protein A (*spa*) typing and multi-locus sequence typing (MLST).[7] In the last decade, the *spa* gene is the most widely used for *S. aureus* typing, which is based on repeats of the hypervariable X region in the *spa* gene.[8] The Ridom *spa* server database currently contains 18857 *spa* types with data from 141 countries worldwide (http://www.spaserver.ridom.de, 2019). Although sequencing technology is currently very effective, non-sequence-based genotyping methods can also offer advantages. In particular, the real-time PCR platform supports single step and closed tube genotyping methods and can potentially be carried out simultaneously with diagnosis and interrogate different classes of genetic polymorphisms.

These features provide real advantages for the clinical microbiology laboratory. The high-resolution DNA melting analysis (HRMA) has been recently assessed for *spa* typing in comparison with DNA sequencing of the *spa* gene.[9] This rapid, simple, and cost-effective assay is able to differentiate the PCR products with minimum differences in GC content, length, and compositions, resulting in melting temperature (TM) variation.[10]

In the previous studies performing HRM and *spa* typing, certain *spa* types could not be separated from each other based on their Tm.[9,11]

The present study had three objectives: i) characterization of *SCCmec*, *spa* types, and multidrug resistance of methicillin-resistant *Staphylococcus aureus* isolates in Ahvaz, Iran; ii) application of high-resolution melting curve analysis for a rapid identification of endemic *spa* types in multidrug resistant *staphylococcus aureus*.

Materials and Methods

Ethics Statement

The study was approved by the Research Ethics Committee (REC) of the Ahvaz Jundishapur University of Medical Sciences (No: IR.AJUMS.REC.1395.187), Ahvaz, Iran, and all patients provided written informed consent. The REC has a mission to protect the dignity, rights, safety, and well-being of subjects who participate in biomedical research and to offer public accountability through the publication of their decisions.

Sample Collection and Bacterial Isolates

A total of 146 non-duplicated *S. aureus* isolates collected from patients admitted to Golestan, Taleghani and Razi teaching hospitals, were recorded from January to December 2018. We have described the aim of the study and its methods to the individuals, and if they consented to participate in the study, the data information of each person was entered into a previously prepared form. The participants have included the personnel of Surgery, Emergency, ICU, Women, pediatrics, pediatrics ICU, nephrology and orthopedic wards of the hospital due to the higher prevalence of infection in these wards. The *S. aureus* was isolated from various clinical samples including blood, urine, wound, synovial fluid, and abscesses. The collected samples were immediately transferred to the Department of Microbiology of Ahvaz Jundishapur University of Medical Sciences. First, the isolates were subcultured on Blood Agar (Merck, Germany), and the single colony was inoculated on Mannitol Salt Agar (Merck, Germany) at 37°C for 24 h. The suspicious colonies were subjected to biochemical tests including Gram staining, catalase, tube-Coagulase and Dnase.[12] The *S. aureus* ATCC 29213 strain was used as the reference strain. Finally, *S. aureus* isolates were inoculated in Trypticase soy broth (TSB) (Merck, Germany) with 20% glycerol and were kept at −80°C until use.

Investigation of Susceptibility to Antimicrobial Agents

Antibiotic susceptibility testing was performed for 11 drugs covering all the 11 antimicrobial categories comprising aminoglycosides, ansamycins, fluoroquinolones, folate pathway

inhibitors, tetracyclines, glycopeptides, oxazolidinones, phenicols, macrolides, lincosamides and penicillinase-stable penicillins were determined using the disc diffusion susceptibility test according to clinical and laboratory standards institute (CLSI) guidelines. Commercial antibiotic discs of rifampin (5 µg), linezolid (30 µg), ciprofloxacin (5 µg), tetracycline (10 µg), gentamycin (10 µg), erythromycin (15 µg), clindamycin (2 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), cefoxitin (30 µg) and chloramphenicol (30 µg) (MAST Diagnostics, Merseyside UK) were used in disc diffusion test. Then, MDR/XDR/PDR phenotype of these isolates was established according to the results obtained from the disc diffusion test.[13]

Criteria for Defining MDR, XDR and PDR in *S. aureus*

As per standardized international terminology created by European Centre for Disease Control (ECDC) and Centre for Disease Control & Prevention (CDC), the multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) bacteria have been well defined. Multidrug-resistant (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. Extensively drug-resistant (XDR) was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two antimicrobial categories). Pandrug resistance (PDR) was defined as non-susceptibility to all agents in all antimicrobial categories. According to reference, MRSA isolates should be considered MDR.[14]

Screening for Methicillin Resistance by Cefoxitin Disc Diffusion Test

All of *S. aureus* isolates were tested for susceptibility to methicillin using a cefoxitin (30 µg) disc. Results were interpreted according to the criteria established by the CLSI. *S. aureus* ATCC 29213 strain were used as a positive control and distilled water as a negative control.[13]

Screening for Vancomycin Resistance

Resistance to vancomycin (MAST Diagnostics) was prepared by Mueller–Hinton agar (Merck, Germany) containing vancomycin 6 µg/mL and 4% NaCl. The plates were incubated at 37°C for 24 hrs according to CLSI guidelines. Any visible growth after 24 h was considered vancomycin-resistant.[13]

Molecular Identification

DNA Extraction

Genomic DNA was extracted from pure colonies of MRSA strains using the high pure PCR template preparation kit (QIAGEN, Germany) according to the manufacturer's procedure. The concentration of extracted DNA was measured at 260 nm, using a Nanodrop instrument (Thermo Scientific, USA) and gel electrophoresis. The samples were stored at −20°C until PCR amplification.

Detection of *mecA* Gene

The presence of the *mecA* gene was evaluated using the PCR amplification. An approximately 359 bp fragment of *mecA* gene was amplified by two specific primers *mecA* F (5′- AGA AGA TGG TAT GTG GAA GTT AG-3′) and *mecA* R (5′- ATG TAT GTG CGA TTG TAT TGC -3′) as previously described.[15] *S. aureus* ATCC 33591 strain was used as a positive control and *S. aureus* ATCC 25923 strain as the negative control.

Screening for of *SCCmec* Elements

The presence of *SCCmec* genes in *S. aureus* isolates was checked using PCR amplification, as previously explained by Moosavian et al.[16] Five MRSA strains, NCTC10442 (*SCCmec* I), NCTC N315 (*SCCmec* II), NCTC 85/2082 (*SCCmec* III), NCTC CA05 (*SCCmec* IVa), and JCSC3624 (*SCCmec* V) were used as a positive control and uninoculated medium as negative control.

Conventional *Spa* Typing (Sequence-Based Method)

All MRSA isolates were subjected to *spa* typing according to the method of Oliveira et al[17] The polymorphic X regions of the *spa* gene were amplified and sequenced using the primers *spa* 1113F (5′- TAA AGA CGA TCC TTC GGT GAG C -3′) and *spa*-1514R (5′CAG CAG TAG TGC CGT TTG CTT -3′). The sequences of the products were determined using the ABI PRISM} 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA) following the standard protocol of the supplier. The Ridom *Spa* Server database (http://www.spaserver.ridom.de) (Ridom, Wurzburg, Germany) was used to assign the edited sequences to particular *spa* types.

High-Resolution Melting Curve Analysis (HRMA)

The polymorphic X region of the *spa* gene was amplified on the Rotor-gene Q instrument (Qiagen, Hilden, Germany) using the same DNA extract and primer pair (Spa1113F

and Spa1514R). In a final volume of 10 µL, the HRM PCR reaction mix contained 5 ng of sample gDNA, 0.3 pmol of each primer, and the Type-it HRM PCR Kit (QIAGEN, Germany) using EvaGreen as the fluorescent dye. The settings for HRM were used as recommended by the manufacturer. Briefly, on the Rotor-Gene Q, PCR conditions were 10 min activation at 94°C, 42 cycles 94°C for 7 s, 60°C for 30s and a final step at 95°C for 1 min and cooling to 40°C for 1min. HRM was performed from 61°C to 87°C with a ramp rate of 0.01°C/s and 100 reads/s. Reactions were performed in duplicate. The reproducibility of the assay was tested by three different persons and all operators gained similar results. For each separate run, 4 control samples with known *spa* types (t037, t030, t034 and t304) were included as melting curve standards. The curves obtained from each type were considered to determine a standard curve for the rest of the procedure. The melting curves of all samples were generated automatically by the Rotor Gene Q Series Software 2.1.0 and were normalized by using the default setting. HRM difference plot was also generated by the software, which could give a better comparison of melting temperatures. In the experiment, HRM analysis was performed 3 times on the 50 isolates, and the results proved to be completely reproducible concerning calling the *spa* types.

Statistical Analysis

Differences of MRSA isolates frequencies among hospitals and specimens and relationship between antibiotic resistance and *spa* typing, we performed the chi-square test and Fisher's exact test using the SPSS version 22 was used to analyze data (SPSS, Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

Results

We confirmed a total of 146 clinical *S. aureus* isolates from various clinical specimens based on culture and standard biochemical criteria. Out of the 146 clinical *S. aureus* isolates (*n*= 50, 34%) were resistant to methicillin by cefoxitin disc diffusion test. As indicated by PCR, all 50 isolates were positive for *mecA*, leading to MRSA and MSSA (methicillin-susceptible *S. aureus*) prevalence rates of (*n* = 50, 34%) and (*n*= 96, 66%), respectively. In the study group, (*n*= 29/50, 58%) of the patients were women and (*n*= 21/50, 42%) were men. The mean age of the patients included in the study was 45.06 years and mean ± standard deviation (SD) was 11.59 (Table 1). Our results revealed that Razi Hospital had a higher prevalence of MRSA (*n*=29,58%) while Taleghani had the lowest prevalence (*n*=3,6%). Table 2 shows the total distribution of MRSA in various types of clinical specimens in three major hospitals. The prevalence rate of MRSA in different specimens was (*n*=13/50, 26%) blood, (*n*=14/50, 28%) wound, (*n*=15/50, 30%) urine, (*n* =4/50, 8%) synovial fluid and (*n*= 4/50, 6%) abscesses in clinical samples. MRSA isolates were subjected to disk diffusion to assess the antibiotic resistance pattern.

Tables 1 and 3 present the phenotypic pattern of antibiotic resistance in MRSA isolates. A high rate of resistance was detected against erythromycin (*n*=38/50,76%), chloramphenicol (*n*=18/50,36%), gentamicin (*n*=35/69%), clindamycin (*n*=43/50,86%), ciprofloxacin (*n*=34/50,68%), and tetracycline (*n*=32/50,64%) antibiotic agents. MRSA isolates had the lowest prevalence of resistance against rifampin (*n*=29/50,58%), trimethoprim-sulfamethoxazole (*n*= 19/50,38%), and antibiotic agents.

All MRSA isolates were susceptible to vancomycin and linezolid. In the MRSA isolates, (*n*=11/50,22%) were XDR and (*n*=39/50,78%) were MDR. PDR strain was not observed. Based on our results, Razi Hospital had a higher prevalence of MDR (*n*=22/50,44%) while Taleghani had the lowest prevalence (*n* =3/50,6%). Statistically significant differences were found among different hospitals regarding the prevalence of MDR (χ^2=14.1, P=0.001). Our findings showed that Razi Hospital had a higher prevalence of XDR (*n*=7/50,14%) while Golestan had the lowest prevalence (*n* =4/50,8%). Statistically non-significant differences were detected among different hospitals regarding the prevalence of XDR (χ^2=0.82, P=0.366). The prevalence rate of MDR in different specimens was (*n* =10/50, 26%) in blood, (*n* =12/50, 28%) in wound, (*n*=11/50, 30%) in urine, (*n* =3/50, 8%) in synovial fluid, and (*n*= 3/50, 6%) in the abscesses of clinical samples. Statistically significant differences were seen as far as the prevalence of MDR and type of specimens (χ^2=10.1, P=0.039) are concerned. The prevalence rate of XDR in different specimens was (*n* =3/50, 26%) in blood, (*n* =2/50, 28%) in wound, (*n*=4/50, 30%) in urine, (*n* =1/50, 8%) in synovial fluid, and (*n*=1/50, 6%) in the abscesses of the clinical samples. Statistically non-significant differences existed regarding the prevalence of XDR and type of specimens (P=0.664). The predominant simultaneous resistance patterns were (*n*=11/50, 22%) for nine antibiotics, (*n*=2/50, 4%) for eight antibiotics, (*n*=6/50,12%) for seven antibiotics, (*n*=7/50,14%) for six antibiotics, (*n*=11/50,22) for five antibiotics, (*n*= 7/50,14%) for four antibiotics, and (*n*=5/50,10%) for three antibiotics (Table 4). Of the 50 MRSA

Table 1 Characteristics of MRSA isolates from Patients Seen at the University Hospitals of Ahvaz, Iran

MSRA	Age	Gender	Hospital	Source	Ward	HRM-Based spa-Typing	PCR-Based spa-Typing	ARPs	MDR	XDR	*SCCmec* Typing
1	29	F	Razi	Blood	ICU	t037	t037	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	III
2	33	M	Golestan	Urine	Nephrology	t037	t037	CD,E,T,FOX	+	–	II
3	39	F	Golestan	Blood	Surgery	t030	t030	CIP,CD,E,GN,RP,T,FOX	+	–	II
4	54	F	Razi	Urine	ICU	t037	t037	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	IV
5	63	F	Golestan	Urine	Pediatric	t030	t030	CIP,CD,RP,T,FOX	+	–	III
6	51	F	Golestan	Blood	Pediatrics ICU	t030	t030	CIP,CD,E,GN,RP,T,FOX	+	–	II
7	45	M	Razi	Blood	Women	t037	t037	CD,E,RP,T,FOX	+	–	NT
8	64	F	Razi	Abscesses	Surgery	t037	t037	SXT,CD,RP,T,FOX	+	–	II
9	54	F	Razi	Urine	Emergency	t363	t363	CIP,CD,T,FOX	+	–	II
10	42	M	Taleghani	Urine	Emergency	t037	t037	CIP,CD,E,RP,T,FOX	+	–	I
11	47	F	Golestan	Abscesses	Pediatrics ICU	t030	t030	CIP,CD,E,RP,T,FOX	+	–	NT
12	43	F	Razi	Urine	Women	t037	t037	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	III
13	52	F	Razi	Urine	Emergency	t037	t037	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	III
14	28	M	Razi	Blood	ICU	t037	t037	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	III
15	32	F	Golestan	Abscesses	ICU	t030	t030	CIP,CD,E,T,FOX	+	–	I
16	36	F	Taleghani	Wound	ICU	t030	t030	CIP,CD,T,FOX	+	–	II
17	21	F	Razi	Blood	ICU	t037	t037	CIP,CD,GN,RP,T,FOX	+	–	II
18	51	F	Golestan	Synovial fluid	Surgery	t030	t030	CIP,CD,GN,RP,FOX	+	–	II
19	53	M	Taleghani	Blood	ICU	t037	t037	SXT,C,CD,GN,RP,T,FOX	+	–	IV
20	36	F	Razi	Wound	ICU	t037	t037	CIP,CD,GN,RP,T,FOX	+	–	IV
21	37	F	Golestan	Blood	ICU	t030	t030	SXT,C,RP,FOX	+	–	II
22	51	F	Razi	Wound	Women	t030	t030	CD,E,GN,RP,FOX	+	–	I
23	62	M	Razi	Blood	Surgery	t275	t275	CD,E,FOX	+	–	II
24	41	M	Golestan	Wound	Pediatrics ICU	t037	t037	C,CD,FOX	+	–	III
25	48	M	Razi	Blood	ICU	t030	t030	SXT,C,CD,E,GN,RP,T,FOX	+	–	III
26	58	M	Razi	Wound	Surgery	t034	t034	CD,E,FOX	+	–	III
27	54	F	Razi	Wound	ICU	t037	t037	CD,E,GN,FOX	+	–	III
28	27	M	Golestan	Blood	Pediatric	t037	t037	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	III
29	41	M	Razi	Blood	ICU	t030	t030	CIP,CD,E,GN,FOX	+	–	III
30	52	F	Golestan	Blood	Surgery	t037	t037	C,E,GN,FOX	+	–	II
31	36	M	Razi	Synovial fluid	Orthopedic	t030	t030	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	III
32	63	F	Razi	Wound	ICU	t459	t459	CIP,GN,FOX	+	–	III
33	30	M	Golestan	Wound	Pediatric	t037	t037	SXT,C,CD,E,GN,RP,T,FOX	+	–	III
34	63	M	Razi	Urine	Surgery	t030	t030	CIP,E,GN,FOX	+	–	III
35	61	F	Golestan	Wound	ICU	t037	t037	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	II

(*Continued*)

Table 1 (Continued).

MSRA	Age	Gender	Hospital	Source	Ward	HRM-Based spa-Typing	PCR-Based spa-Typing	ARPs	MDR	XDR	SCCmec Typing
36	39	M	Razi	Wound	Surgery	t363	t363	CIP,CD,E,GN,FOX	+	–	IV
37	47	F	Golestan	Wound	ICU	t037	t037	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	II
38	46	M	Razi	Wound	Surgery	t304	t304	CIP,FOX	+	–	II
39	52	F	Razi	Wound	Women	t030	t030	CIP,E,GN,T,FOX	+	–	III
40	35	M	Razi	Wound	ICU	t030	t030	E,GN,T,CIP,FOX	+	–	III
41	42	M	Razi	Synovial fluid	Orthopedic	t030	t030	SXT,C,CD,E,GN,T,FOX	+	–	III
42	61	F	Golestan	Urine	Nephrology	t037	t037	CIP,CD,E,GN,RP,T,FOX	+	–	III
43	37	M	Razi	Synovial fluid	ICU	t459	t459	CD,E,FOX	+	–	II
44	33	M	Razi	Urine	Emergency	t189	t189	CD,E,GN,RP,T,FOX	+	–	IV
45	46	F	Razi	Urine	Emergency	t030	t030	SXT,CIP,CD,E,GN,FOX	+	–	III
46	28	M	Razi	Abscesses	Surgery	t044	t044	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	III
47	53	F	Golestan	Urine	ICU	t037	t037	CIP,CD,E,GN,FOX	+	–	II
48	27	F	Golestan	Urine	ICU	t037	t037	SXT,CIP,CD,E,GN,RP,FOX	+	–	II
49	48	F	Razi	Urine	Emergency	t030	t030	CIP,CD,E,GN,FOX	+	–	IV
50	62	F	Golestan	Urine	Emergency	t037	t037	SXT,C,CIP,CD,E,GN,RP,T,FOX	–	+	III

isolates, *SCCmec* type I (*n*=3/50,6%), type II (*n*=17/50, 34%), type III (*n*=22/50,44%), and type IV (*n*=6/50,12%) were the most prevalent. None of the tested isolates were type V and two isolates were untypable by the routine PCR assays utilized (Table 1). The frequency of MDR and XDR among MRSA isolates carrying the *SCCmec* types II and III was significantly higher than other types, although the differences were statistically significant, respectively ($X2$=19.84, P=0.001). Using PCR-sequencing as a reference method, a total of 10 different *spa* types were detected among the 50 MRSA isolates. The 10 *spa* types were: t037 (*n*=22,46%), t030 (*n*=18,36%), t363 (*n*=2,0.04%), t459 (*n*=2,0.04%), t304 (*n*=1, 0.02%), t034 (*n*=1,0.02%), t275 (*n*=1,0.02%), t325 (*n*=1,0.02%), t044 (*n*=1, 0.02%), and t189 (*n*=1, 0.02%). *Spa* type t037 was the major *spa* type circulating in the study region. Razi Hospital isolates were fully diverse, with six *spa*-types detected among the 29 MRSA isolates. Statistically non-significant differences were detected regarding the type of hospital and *spa* types (P=0.902). Table 1 shows the distribution of the *spa* types isolatesfrom the clinical sources. Figure1 compares the occurrence of antimicrobial resistance among the investigated MRSA isolates concerning different *spa* types. There were statistically significant differences regarding the prevalence of MDR and *spa* types (P<0.0001). Statistically significant differences also existed in the prevalence of XDR and *spa* types (P=0.004). Table 5 presents the distribution of *SCCmec* types in *spa* types. Statistically non-significant differences were further detected as regards the distribution of *SCCmec* types in *spa* types (P=0.841). Following sequencing comparison, the 50 clinical MRSA isolates were subjected to HRM analysis. HRM analysis revealed 10 unique *spa* types out of 10 with melting temperatures ranging between 81.38°C and 82.70°C (Table 5 and Figure 2). Four distinct melting curves and HRM difference plots were obtained by the Rotor-gene Q real-time instrument. All the four control *spa* types showed a minimum of 0.2°C temperature difference (t037: 82:50°C, t030: 82:30°C, t0190: 81:80 and t304:82:00 °C). These empirically obtained criteria were employed to name the melting curves "the same" or "different" using difference graphs. The melting curves were called "the same" as the defined control which the difference graph within ±0.2 relative to the x-axis and did not display reproducible differences such as double peaks or crossing the x-axis more than twice in both replicates.

Table 2 Total Distribution of MRSA Among Different Clinical Specimens of Three Major Hospitals

Hospital						
Clinical Specimens	**Razi Hospital**		**Golestan Hospital**		**Taleghani Hospital**	
	MRSA (29)	**MSSA(49)**	**MRSA (18)**	**MSSA (20)**	**MRSA (3)**	**MSSA (27)**
Blood	7	18	5	10	1	14
Wound	9	12	4	5	1	9
Synovial fluid	3	3	1	1	–	1
Urine	8	11	6	4	1	3
Abscesses	2	5	2	–	–	–

Table 3 Results of Antimicrobial Resistance Tests by Disk Diffusion Method for MRSA

Antimicrobial Category	**Antimicrobial Agent**	**Negative**	**Positive**	**Pvalue for χ^2**
Oxazolidinones	Linezolid	50(100%)	–	
Folat pathway inhibitors	Trimethoprim-sulphamethoxazole	31(62%)	19(38%)	0.090
Phenicols	Chloramphenicol	32(64%)	18(36%)	0.048*
Fluoroquinolones	Ciprofloxacin	16(32%)	34(68%)	0.011*
Lincosamides	Clindamycin	7(14%)	43(86%)	<0.0001*
Macrolides	Erythromycin	12(24%)	38(76%)	<0.0001*
Aminoglycosides	Gentamycin	15(31%)	35(69%)	0.005*
Ansamycins	Rifampin	21(42%)	29(58%)	0.258
Tetracyclines	Tetracycline	18(36%)	32(64%)	0.048*
Glycopeptides	Vancomycin	50(100%)	–	
Penicillinase-stable penicillins	Cefoxitin	–	50(100%)	

Note: *Denotes statistically significant difference between resistance and susceptible MRSA isolated.

Discussion

In 2018, the Center for Disease Control and Prevention (CDC) estimated that more than 70,000 invasive infections and more than 9000 deaths were caused by MRSA.[18] In Iran, the total number of MRSA infection cases has increased, thereby introducing a serious problem in the form of nosocomial infections.[16]

A previous study on MRSA in Khuzestan province revealed a dramatic annual increase in the proportion of isolates resistant to methicillin during the recent years, rising from 60% in 2017 and 2018 to a peak of 77% in 2019.[19–22] Such differences in the prevalence of MRSA infections in our region might reflect the fact that in these areas, different policies are considered for infection control and other factors.[23]

There is a continuous increase in the global prevalence of MRSA in European countries. In Belgium and Spain, 19% and 29% of the isolates *S. aureus* strains are methicillin-resistant, respectively.[24,25] Similar antibiotic resistance patterns of MRSA strains have also been reported against aminoglycosides, glycopeptides, penicillins, macrolides, tetracyclines, fluoroquinolones, lincosamides, folate inhibitors, and ansamycins groups of antibiotics.[26–28] In the present study, MRSA had the highest sensitivity to vancomycin and linezolid (100%) and the lowest to clindamycin (86%). This limits the use of this drug and often warrants prolonged treatment. Results obtained by Huang et al showed that MRSA had no resistance to vancomycin and 91% resistance to clindamycin.[29]

The present study highlighted that a large number of MRSA were MDR, being in line with the previous findings reporting a high rate of multidrug resistance in MRSA.[30] The observed MDR and XDR rates in hospitals indicate that antibiotics resistance is increasing at an alarming rate and pathogenic bacteria circulating in hospitals are becoming more resistant to all available antibiotics.

The first reason might be associated with the lack of an antibiotics resistance surveillance and stewardship program in Iran. There is sufficient evidence indicating that an antibiotics resistance surveillance and stewardship program contributes to understanding the pattern of resistance and improving the utilization of antibiotics to prevent

Table 4 Multidrug Resistance Patterns Among MRSA Isolates

Number of Resistant Antibiotics	Antibiotics	Number of Species
9	SXT,C,CIP,CD,E,GN,RP,T,FOX	11
8	SXT,C,CD,E,GN,RP,T,FOX	2
7	CIP,CD,E,GN,RP,T,FOX	3
	SXT,C,CD,GN,RP,T,FOX	1
	SXT,C,CD,E,GN,T,FOX	1
	SXT,CIP,CD,E,GN,RP,FOX	1
6	CIP,CD,RP,T,CIP,FOX	1
	CIP,CD,E,RP,T,FOX	2
	CIP,CD,GN,RP,T,FOX	2
	CD,E,GN,RP,T,FOX	1
	SXT,CIP,CD,E,GN,FOX	1
5	CD,E,RP,T,FOX	1
	SXT,CD,RP,T,FOX	1
	CIP,CD,E,T,FOX	1
	CIP,CD,GN,RP,FOX	1
	CD,E,GN,RP,FOX	1
	CIP,CD,E,GN,FOX	4
	CIP,E,GN,T,FOX	2
4	CD,E,T,FOX	2
	CIP,CD,T,FOX	2
	SXT,C,RP,FOX	1
	C,E,GN,FOX	1
	CIP,E,GN,FOX	1
3	CD,E,FOX	3
	C,CD,FOX	1
	CIP,GN,FOX	1
2	CIP,FOX	1

antibiotic resistance. The second reason might be the lack of comprehensive national antibiotic policies and problems associated with their implementation. In Iran, it is a common practice to buy any antibiotic from private drug vendors and pharmacies without any prescription.[31] Studies in other countries have reported a lower prevalence. For instance, Ullah et al found that the prevalence of MDR and XDR was 15.84% and 6.93%, respectively.[32] A major concern for establishing a reliable infection control system in hospitals is the time to perform molecular typing tests. Monitoring the spread of MRSA strains in a hospital setting requires the use of accurate and rapid typing methods. The region X of the *spa* gene as a genetic marker allows for the global investigation outbreak by analyzing a single locus.[33] The conventional PCR-sequencing typing method has been extensively used worldwide. Based on the results of PCR-sequencing method, the t037*spa* type was the most prevalent among Iranian patients, accounting for 44% of the isolates and their transfer from the community to hospitals.

Our results are similar to those of the previous studies conducted in Iran and other parts of the world.[9,34] All the 50 MRSA isolates were then subjected to HRMA. Among the 10 different *spa* types belonging to the 50 clinical MRSA isolates, 10 specific *spa* types could be assigned by being matched with the control curves. Contrary to our study, Mayerhofer et al found that certain *spa* types could not be separated from each other based on their Tm.[8,9,11] However, the major advantage of this method is that it is single-step and closed-tube, performed on a moderately priced and generic piece of laboratory equipment. This technique offsets the occasional inability to differentiate between *spa* alleles because *spa* interrogation could be simply combined with the real-time PCR interrogation of clonal-complex specific single-nucleotide polymorphisms and toxin encoding genes for instance. This approach is much less expensive than full *spa* sequencing; each sequencing reaction in our institution costs $20 while each HRM runs costs$0.50 for reagents.[35] In our study, initial HRM results showed some similar melting temperatures and curve shapes; however, optimizing several methods and materials such as DNA extraction method and HRM Master mix, a real-time PCR-based HRM assay was developed to differentiate between *S. aureus* isolates, particularly major MRSA *spa* types (t037 and t030), present in Iran. With the standardization and improvements in the HRM method in the present study, predominant *spa* types in Iran such as t030 and t037 could be differentiated from each other.

Myriad factors (band size, G + C content, real-time thermal cycler, HRM software, DNA and primer concentrations, and DNA quality) can affect HRM results; therefore, this technique can be used as a screening approach, along with other genotyping methods, to increase the discrimination power in molecular typing, reduce the expense, and minimize the risk of sample contamination. This study showed that the presence of (*SCCmec*III-t030) and (*SCCmec*III-t037) was largely disseminated in our region. Interestingly, *spa* types t037and t030, identified in 41 MRSA isolates, were MDR and XDR, including isolates causing severe human infections. Similar to our results, the most common MRSA in Turkey was found to be (91.4%), followed by *SCCmec* type III (91.4%), and

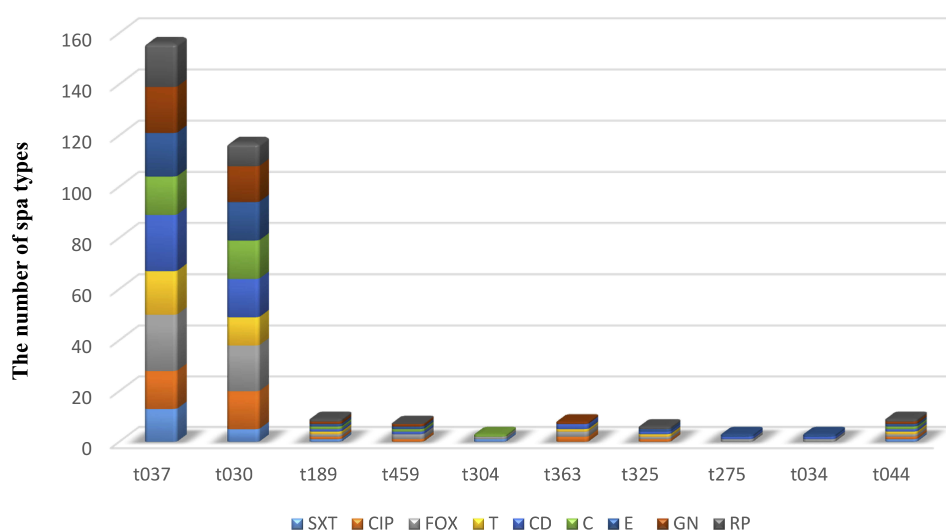

Figure 1 Distribution of antibiotic resistance in MRSA with *spa* types.

t030 (85.1%).[36] Use of genomic methods such as MLST analysis was a limitation of our study; in this regard, clonal complex for correlation with *spa* typing should be designed. In regard to our small sample size and the large number of *spa* types registered in the *Spa*Server database, the use of HRM curve profiling replacing sequence-based *spa* typing is not promising, hence the necessity of future studies.

Conclusion

We found a regionally divergent distribution of MRSA in the largest population of Iranian patients with distinct occurrences of single *spa* types. We hereby conclude that early detection and close monitoring of MDR, XDR, or even PDR MRSA isolates strains must be commenced by all clinical microbiology laboratories to reduce the menace of antimicrobial resistance as a global issue. This study emphasizes the *spa* variation among different *S. aureus* isolates, which might be considered as an important criterion when treating staphylococcal infections. Additionally, two common types of *spa* had an alarming rate of antibiotic resistance, which should be taken into consideration by hospital settings. Furthermore, the HRM-based *spa* typing is extremely rapid, easy to perform, and cost-effective but has to be standardized for different regions, *spa* types, and real-time machinery. In contrast to more expensive and labor-intensive techniques, this method could be used for active screening and rapid detection of common endemic *spa* types. Moreover, the large number of *spa* types registered in the SpaServer database indicates that HRM curve profiling cannot replace sequence-based *spa* typing. However, HRM can be used as a superior screening method for the detection of

Table 5 HRM and *Spa* Sequence Types of the 50 MRSA Isolates, and the Frequencies in the Hospital of Origin

HRM	Tm	Spa-Type	Repeat of Spa Type	Size, bp	CG%	Number of Isolates
1	82.30	T030	15-12-16-02-24-24	144	45	18
2	82.50	T037	15-12-16-02-25-17-24	168	45.2	22
3	82.55	T363	15-16-02-25-17-24	144	45.8	2
4	82.15	T459	15-12-16-02-24	120	44	2
5	82.23	T189	07-23-12-12-21-17-34	168	43	1
6	82.00	T304	11-10-21-17-34-24-34-22-25	216	43.5	1
7	81.80	T034	11-17-34-24-34-22-25	168	44	1
8	82.75	T275	15-12-16-02-25-17-24-24	192	44	1
9	81.38	T325	7-12-21-17-34-13-34-34-33-34	240	40.4	1
10	81.60	T044	7-23-12-34-34-33-34	168	41.7	1

Abbreviations: HRM, high-resolution melting; *Tm*, melting temperature.

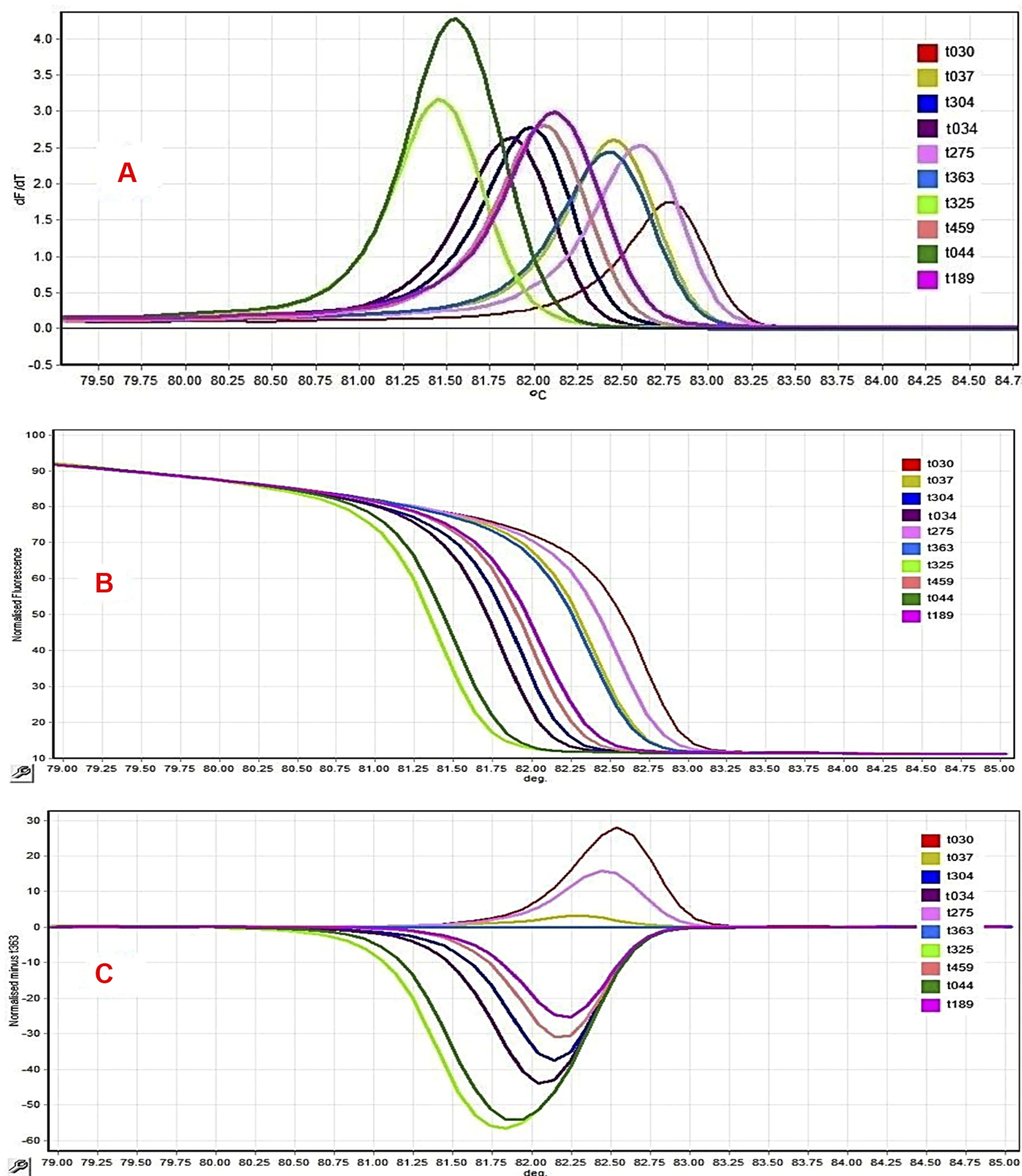

Figure 2 Comparison of different spa polymorphic region X HRM curves obtained from MRSA isolates. (**A**) Negative derivative of fluorescence over temperature (df/dt) plots displaying 10 HRM profiles. (**B**) Normalization data curve shows the decreasing fluorescence vs increasing temperature. (**C**) Difference graph demonstrating the accurate reproduction of eight spa HRM profiles in a run experiment. Isolates with difference plots that fall within the ±0.2 relative fluorescence unit (RFU) cutoffs were considered as the "same" type, while the isolates that lie outside of the ±0.2 RFU cutoffs were denoted as "different".

endemic strains and surveillance of non-endemic strains through detecting new curve profiles. Accordingly, to definitively specify rare types or non-endemic *spa* types, it is inevitable to use a reference method such as DNA sequencing.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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