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Molecular typing of methicillin and vancomycin-resistant *Staphylococcus aureus* isolated from clinical specimens by double-locus sequence typing (DLST) method

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Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are the essential cause of infections in communities and hospitals. The present study was conducted to determine the molecular typing of MRSA, isolated from hospitalized patients, using the double-locus sequence typing (DLST). In total, 280 *S. aureus* isolated from clinical specimens by phenotypic (catalase, coagulase, DNase, oxacillin, vancomycin screening agar and antibiotic disk diffusion), and molecular methods (PCR for determining the *mecA, vanA and nuc* genes). The DLST and sequencing was performed for MRSA containing *mecA*. Out of 280 specimens, confirmed as *Staphylococcus aureus* (*S. aureus)*, 123 (43.9%) strains were MRSA. The highest resistance toward the erythromycin (15 µg), followed by ciprofloxacin (5 µg), clindamycin (2 µg), tetracycline (30 µg), gentamicin (10 µg) and rifampicin (5 µg), was 98.3%, 97.5%, 94.3%, 90.2%, 83.7% and 41.4%, respectively. Also, the least resistance (0%) was observed in each of teicoplanin (30 µg), linzolide (30 µg), and vancomycin (30 µg). All (100%) of MRSA strains had the *mecA*, and none of them have had the *vanA*. The results of DLST showed that the most common sequence types were BPH 2003 and 0217. The DLST type 18-32 was a significant cluster of MRSA. By sequencing MRSA and comparing the dominant types via the DLST, it is possible to establish the etiology of the disease in a much shorter time, and prevent the complications of the disease. Therefore, the combination of partial sequences of *clfB* and *spa* can serve as useful genetic markers for MRSA typing. It concluded that the MRSA in our region was relatively high, but no vancomycin resistance was found. The majority of the MRSA DLST type was 18–32.

Introduction

Staphylococcus aureus (*S. aureus*) causes a wide range of diseases, such as infective endocarditis, osteomyelitis, food poisoning, septicemia, skin infections, boils, carbuncles, soft tissue infections, and scalded skin syndrome in humans (Shittu and Lin, 2006; Gordon and Lowy, 2008). Today, resistance to antibiotics is rising due to excessive consumption, causing concerns all around the world. Also, due to the resistance of this bacterium against antimicrobial agents, many antibiotics have been proposed for treatment. *Staphylococci* have recently received much scholarly attention owing to the emergence of penicillin-resistant strains, methicillin and vancomycin-resistant *Staphylococcus aureus* strains (MRSA and VRSA), and the combination of these strains with the epidemic clashes of severe hospital infections (Walsh and Howe, 2002; Carvalho *et al.*, 2009). Penicillin-resistant strains were first isolated in hospitals in 1942, and afterwards in communities. In addition, *S. aureus* strains have also become methicillin and vancomycin-resistant by the acquisition of genes such as the *mecA* and *vanA*. The *MecA* is located on a mobile genetic segment (mobile genome island) that is known as *staphylococcal* cassette chromosome *mec* (SCC*mec*). The drug resistance is caused by the acquisition and replacement of SCC*mec* elements within the chromosomes of susceptible strains. The 2.1 kb *mecA* is located between the locus of the protein A coding genes (*spa*), and the protein responsible for purine biosynthesis. The *mecR* and *mecI* regulatory genes and *mecA* are located in the *mec* operon. This operon is carried on the SCC*mec*. The *mecA*, sensitive to the presence of beta-lactamase, is regulated by the *MecI* repressor protein, and

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the *MecRI* membrane transducer signal protein (Elements IWGotCoSCC, 2009; Turlej *et al*., 2011). The prevalence of MRSA strains varies in cities, countries and even throughout the continents, due to the proliferation of the local clones of MRSA strains (Ko *et al*., 2005; Goering *et al*., 2008). It is important to study the *mecA*, *vanA*, *clfB* and *spa* (encoding PBP2a, ligase enzyme, *ClfB* and protein A, respectively) in MRSA (Kuhn *et al*., 2007; Turlej *et al*., 2011).

For many years prior to the 21st century, vancomycin had been used to treat the MRSA infections. However, in July 2002, the US Center for Disease Control and Prevention (CDC) released its first report on MRSA strain resistance to vancomycin. Reduced sensitivity to vancomycin is reported to be caused by changes in the cell wall due to the presence of *vanA* (Assadullah *et al*., 2003). Epidemiological studies are essential to determine the source of infection, recognize the transmission of cross-pathogens in hospitals and the prevalence of the disease, identify the major pathogenic strains, and to evaluate the effectiveness of control measures. The conventional epidemiological typing methods such as antibiograms, biotyping, serotyping and phage typing have been sometimes useful in describing the epidemiology of infectious diseases, but they reveal little variability. As a result, DNA-based typing methods are deemed necessary for the study of the epidemiology of most microbial pathogens (Van Belkum *et al*., 2007). Given the presence of *S. aureus* in the environment and in the normal flora of hospitalized patients, molecular typing studies are of great importance in controlling infections in communities and in hospitals. The epidemiology of *S. aureus* has been analyzed by a set of different molecular typing methods, such as pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and gene sequencing of *Staphylococcus* Protein A (*Spa*). Among these, PFGE is considered as the golden standard method for its high differentiation power (van Belkum *et al*., 1998; Turlej *et al*., 2011). Recently, it has been shown that an epidemiological study on *S. aureus* is possible by sequencing small regions in only two highly variable loci (for the *clfB* and *spa* loci, respectively) (Kuhn *et al*., 2007; Senn *et al*., 2013). Considering the very limited number of studies in the world, using the double-locus sequence typing (DLST) technique, as only few studies are available in foreign countries (Basset *et al*., 2009; Okon *et al*., 2009) and since no epidemiological study on *S. aureus* has so far been conducted in Iran, using the DLST technique, and the high prevalence of MRSA in different parts of the world, it is necessary to determine the kinds of clones and MRSA types to be able to implement the strain control strategies, both at the hospital and the community levels. Therefore, the aim of this study was to determine the MRSA and VRSA relative prevalence, antibiotic susceptibility and the molecular typing of *S. aureus* strains with methicillin-resistant *mecA*, isolated from the hospitalized patients, admitted to university hospitals in the city of Ahvaz, Khuzestan province, Iran, using the DLST technique.

Materials and Methods

Clinical specimens. During the 6 months (from October to March 2016), all *Staphylococci* isolated at the microbial division of laboratories of university hospitals (Razi, Taleghani, Imam and Golestan) in Khuzestan province, Iran, were included in our study. A total of 280 isolates of *S. aureus*, out of 693 *Staphylococci* specimens from clinical sources, were analyzed. The specimens were obtained from tracheal secretions, blood, wound, urine, and the tracheal tube of patients, collected from different wards of the hospital, including: Women Surgery, Men Surgery, Intensive Care Unit (ICU), Women Internal, Men Internal, Out patient Department (OPD), Orthopedic, Infectious, Dermatology and Adult Nephrology Departments. Samples were transported to the microbiology laboratory of the School of Medicine at Ahvaz Jundishapur University of Medical Sciences (AJUMS).

Phenotypic methods for isolation of S. aureus

After the collection of the isolates as *Staphylococci* bacteria, they were identified and confirmed as *S. aureus*, using standard microbiological tests, including Gram-staining, catalase, coagulase, DNase and mannitol salt agar fermentation (McClure *et al*., 2006).

Antibacterial susceptibility pattern of S. aureus *(Wayne, 2014)*

An antibiotic sensitivity test was performed, using the Kirby Bauer disk diffusion method (all disks were provided by MAST, UK).

The tests for isolation of MRSA strains

1. **Oxacillin disk diffusion test.** The isolation of the MRSA strains, using oxacillin disk (1 µg), and according to the standards of the Clinical and Laboratory Standards Institute (Wayne, 2014).
2. **Identifying methicillin-resistant strains, using cefoxitin.** To identify the MRSA strains based on the cefoxitin disk (30 µg), Mueller-Hinton agar was used according to the CLSI guideline; the resistant strains to cefoxitin (30 µg) were considered as MRSA (Wayne, 2014).
3. **Oxacillin agar screening.** Mueller-Hinton agar (MHA) plates, containing 4% NaCl and 6 µg/mL of oxacillin were prepared. The plates were inoculated with 10 µL of the 0.5 McFarland turbidity standard suspension of the isolate by streaking in one quadrant, and incubated at 35°C for 24 h. Then, the plates were observed carefully by transmitted light, any growth after 24 h was considered as oxacillin resistant (Wayne, 2011).

Vancomycin agar screening

Based on the CLSI guideline, Brain Heart Infusion agar (BHI), containing 4% NaCl and 6 µg/mL of vancomycin powder (Sigma) was prepared. A 10 µl inoculum of 0.5 McFarland suspensions of each specimen spotted into the medium (Wayne, 2011).

Molecular methods

DNA extraction by the kit (Sinaclon, Iran). The colonies, grown after 24 h were taken, and the DNA extraction was performed according to the manufacturer's protocol (Sinaclon Co., Tehran, Iran). Lysis buffer (400 µl) was added to the sample and vortexed at max speed for 20 seconds, then 300 µl precipitation solution was added and vortexed

at max speed for 5 s. Subsequently, the solution was transferred to a spin column with collection tube, and centrifuged for 1 min. Then, wash buffer (400 µl) was added to the spin column, and centrifuged for 1 min. Afterwards, the spin column was washed with 400 µl of wash buffer II by centrifugation for 1 min (twice), and the spin column was placed in the collection tube, centrifuged for 1 min, and carefully the column was transferred to a new 2 ml tube, and 30 µl of 65°C pre-heated elution buffer was placed in the center of the column, and incubated for 3–5 min at 65°C. Then, the DNA was eluted after 1 min centrifugation.

The reconfirmation of *S. aureus* by molecular method. *S. aureus* isolates, identified and confirmed by culture and phenotypic methods, were reconfirmed by amplifying nuclease gene (*nuc*). The primer sequences for the *nuc* have been shown in Tab. 1.

PCR reaction for the *nuc*. The PCR was performed in the final volume of 20 µl, consisting of 2 µl of DNA template, 2 µl of PCR buffer (1x), 1.5 mM of $MgCl_2$ (50 mM), 0.2 µl of dNTPs (10 mM), 0.4 µM of each *nuc1* and *nuc2* primers (10 pmol), 1U of Taq DNA polymerase (5 u/µL) and 13.2 µl of double distilled water. PCR amplifications were done in a thermocycler (Eppendorf, Germany), under the subsequent cycle circumstances: an initial denaturation step at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min, and a final extension step at 72°C for 10 min.

Identification of the *mecA* and *vanA* in MRSA specimens by PCR method. The primer sequences for the *mecA* and *vanA* have been shown in Tab. 1. The composition of the reagents in master mix and the temperature has been indicated under the PCR reaction for *nuc*, annealing temperature for *mecA* and *vanA* were 52°C and 56°C, respectively. The PCR was performed on the thermocycler (Eppendorf, Germany).

Electrophoresis of the PCR Products. The PCR products were electrophoresed on 1.5% agarose gel, and then the gels were stained with 5 µg/mL ethidium bromide. The standard strains ATCC 33591, 25923 and 52199 were used as the positive control for the *mecA*, *nuc* and *vanA*, respectively, and distilled water as the negative control for all PCR reaction (Kalorey *et al.*, 2007; Japoni *et al.*, 2011; Havaei *et al.*, 2012).

The DLST for isolates with *mecA*. Methicillin-resistant strains were examined, using the DLST. After DNA extraction, PCR was performed on two highly variable loci of *clfB* and *spa* at 800–1300 bp and 1000–1500 bp, respectively. The primers have been listed in the following Table. The standard electrophoresis and gel staining with ethidium bromide were utilized (Sinaclon, Iran).

In this study, bioinformatics software programs such as MEGA 06 (with UPGMA method, based on the Dice coefficient), Bioedite and Gene runner were used to analyze the DNA sequences and determine the homology of the sequences. Inconcise, the extracted DNAs of MRSA were used for the PCR amplification, using specific primers. The standard gel electrophoresis was applied, and the gels were stained with ethidium bromide (Sinaclon, Iran). Electrophoresis was carried out on 1.2% agarose gel, at 85 V for 1 h. The length of the PCR product was variable among the isolates. The PCR products were purified and sequenced by the Bioneer Corporation (Bioneer, Daejeon, South Korea). The nucleotide sequences were analyzed by BLAST, and then saved as a FASTA format for sequence alignment and phylogenetic purposes. The *S. aureus* DLST website (http://www.dlst.org/S.aureus/) was used to assign numbers to each distinct allele within a locus, after submitting good quality trace file sequences for each of *clfB* and *spa* loci per each tested isolates.

Results

According to phenotypic methods, our results were indicative of the fact that a total of 693 specimens was collected as *staphylococci*, 280 specimens were confirmed as *S. aureus*, and the rest were coagulase negative *staphylococci*. According to the oxacillin disk (1 µg) diffusion test, 121 strains of *S. aureus* were identified as MRSA. Also, based on the cefoxitin disk (30 µg) diffusion, 123 isolates of *S. aureus* were identified as MRSA. According to the oxacillin agar screening test, 122 strains were MRSA. The number and frequency of MRSA isolates, according to the type of

TABLE 1

Primers used in molecular identification and the DLST typing of *S. aureus*

Gene name	Primer sequence	Size (bp)	Reference
mecA	F:5'-GTAGAAATGACTGAACGTCCGATAA-3	310 bp	(McClure *et al.*, 2006)
	R:5'-CCAATTCCACATTGTTTCGGTCTAA-3'		
Nuc	F:5'-GCGATTGATGGTGATACGGTT-3	279 bp	(Zhang *et al.*, 2004)
	R:5'-AGCCAAGCCTTGACGAACTAAAGC-3		
vanA	F:5′-ATG AAT AGA ATA AAA GTT GC-3'	1032 bp	(Saha *et al.*, 2008; Chakraborty *et al.*, 2011)
	R:5'-TCA CCC CTT TAA CGC TAA TA-3'		
SA_*clfB*_1586.forward (5'-3')	TCGGTTGGAATAATGAGAATGTTG	1300 bp	(Kuhn *et al.*, 2007)
SA_*clfB*_2658.reverse (5'-3')	TTTGTGTTTTCGCTCTTATCTCCT		
SA_*spa*_729.for (5'-3')	ACTAGGTGTAGGTATTGCATCTGT	1500 bp	(Kuhn *et al.*, 2007)
SA_*spa*_1984.reverse (5'-3')	TCCAGCTAATAACGCTGCACCTAA		

hospitals, were as follows: Imam 24 (19.8%), Razi 18 (14.9%), Taleghani 51 (41.5%) and Golestan 30 (24.8%). Based on the frequency of distribution of MRSAs, the isolates were compared according to the admitted wards. Clinical specimens and associated hospitals have been presented in Tab. 2.

The highest number of isolated MRSA 51 (41.5%) was observed at Taleghani Hospital; also, ICU wards accounted for the majority of isolated MRSAs. Based on the antibiotic resistance pattern, the results revealed that erythromycin (98.3%) accounted for the highest resistance of MRSAs isolated. The minor resistances were belonged to teicoplanin (0%), linzolide (0%) and vancomycin (0%). Based on the vancomycin agar screening test, all of MRSA strains were sensitive to vancomycin. In this study, the 280 specimens identified as *S. aureus*, using the phenotypic method were reconfirmed as *S. aureus* by *nuc* gene as mentioned below (Fig. 1). Furthermore, 100% of all 123 (43.9%) MRSA strains, identified by the cefoxcitin disk diffusion method showed the presence of *mecA* by the molecular examination (Fig. 2). The sensitivity for oxacillin disk diffusion and oxacillin screening agar was 98% and 99%, respectively. However, the sensitivity and specificity of cefoxitin and PCR were 100%; it was also demonstrated that the specificity of all four methods was 100%. In addition, 30 MRSA strains (24.4%) were selected randomly for DLST. The sequences were submitted to the DLST website for allele assignment. The allele profiles were compared and clustered, using an online data analysis service. The results of the DLST showed a high similarity with the results obtained from the MLST method. The most common sequence types were BPH 2003 and 0217. The type 18–32 was a significant cluster of MRSA isolates, using DLST. The DLST showed a great typeability and discriminatory power.

The evaluation of the electrophoresis induced by the amplification of *clfB* and *Spa*, illustrated the presence of bands with a length of 1200 bp and 1100 bp, respectively (Figs. 3 and 4). The isolate, sample/ward, DLST types and antibiotic pattern of MRSA isolates have been presented in Tab. 3. Also, the phylogenetic relationships of MRSA isolates have been indicated in Fig. 5.

Discussion

S. aureus is one of the most important human pathogen, causing a wide range of infections. Furthermore, the prevalence of MRSA strains, particularly in hospital infections, is a global clinical challenge. Many epidemiological studies revealed that more than 50% of *S. aureus* infections are caused by MRSA strains (Stefani and Varaldo, 2003; Schito, 2006). The appearance of methicillin-resistant isolates in MRSA, carrying SCC*mec* with multiple drug-resistant genes, is problematic in the treatment of *S. aureus* infections.

Methicillin resistance in MRSA strains is caused *by mecA* acquisition (Deurenberg and Stobberingh, 2008). The *mecA* is located on a mobile genomic island called the *staphylococcal* cassette chromosome *mec* (SCC*mec)*. So far, 11 major SCC*mec* types (I-XI types) have been identified. The SCC*mec* elements are one of the unique genomic islands that contain the *ccr* (Cassette chromosome recombinase)

TABLE 2

The frequency of MRSA isolates according to the type of clinical specimens and admitted wards

Origin	MRSA isolates	MRSA isolates (N0)	MRSA isolates (%)
Type of clinical specimens	Wound	74	60.2
	Blood	25	20.3
	Tracheal tube	14	11.4
	Urine	8	6.5
	Others	2	1.6
	Total	123	100
Wards	ICU	41	33.3
	MS	10	8.1
	WS	4	3.3
	WIW	7	5.7
	OPD	11	8.9
	OW	7	5.7
	IW	27	22
	DW	6	4.9
	ANW	7	5.7
	MIW	3	2.4
	Total	123	100

Note: Intensive care unit (ICU), Men Surgery (MS), Women Surgery (WS), Women Internal ward (WIW), Out patient department (OPD), Orthopedic ward (OW), Infectious ward (IW), Dermatology ward (DW), Adult Nephrology ward (ANW), Men Internal ward (MIW).

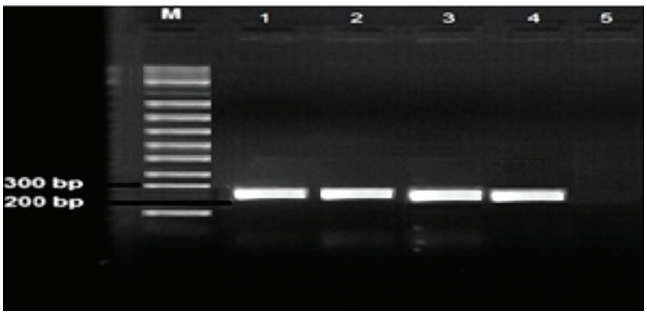

FIGURE 1. Electrophoresis results from *nuc* gene (279 bp) amplification. Electrophoresis was carry out using 1.2% agarose gel at 85 V for 1 h.
Lane M 100 bp DNA ladder, lane 1 to 3 *nuc* gene, lane 4 Positive control, and lane 5 negative control.

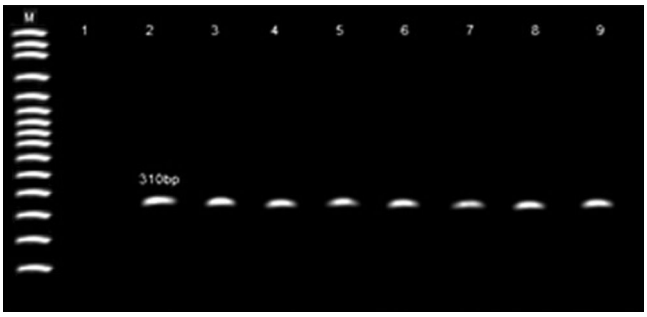

FIGURE 2. Electrophoresis results from *mecA* (310 bp) gene amplification. Electrophoresis was carry out using 1.2% agarose gel at 85 V for 1 h.
Lane M molecular size marker (100 bp), lane 1 Negative control, lane 2 Positive control, lane 3 to 9 *mecA* gene.

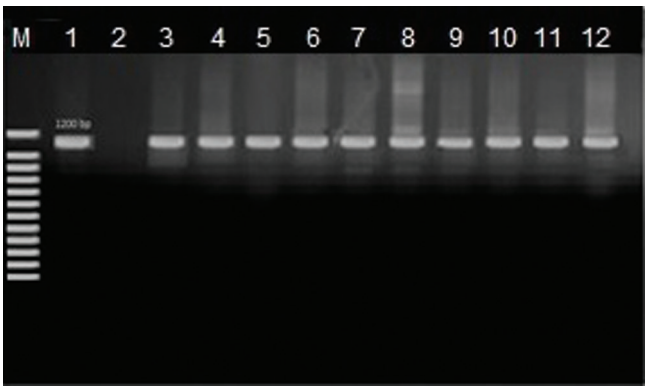

FIGURE 3. Electrophoresis results from *clfB* gene amplification (Fig. 3). Electrophoresis was carry out using 1.2% agarose gel at 85 V for 1 h. Lane M 100 bp DNA ladder, lane 1 Positive control, lane 2 Negative control, lane 3 to 12 *clfB* gene.

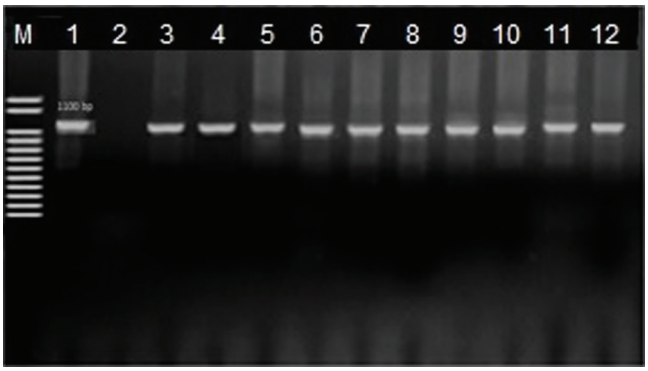

FIGURE 4. Electrophoresis results from *Spa* gene amplification (Fig. 4). Electrophoresis was carry out using 1.2% agarose gel at 85 V for 1 h. Lane M100 bp DNA ladder, lane 1 Positive control, lane 2 Negative control, lane 3 to 12 *Spa* gene.

gene complex and the *mec* gene complex. All MRSA strains contain SCC*mec* elements that are located in a specific locus of *S. aureus* chromosomes called *attBscc* (Bacterial chromosomal attachment site) (Turlej *et al.*, 2011; Hiramatsu *et al.*, 2013).

The present study was shown that the mean prevalence of *S. aureus* and MRSA were 280 (40.94%) and 123 (43.9%), respectively. The prevalence rates of MRSA were 71% in Egypt and 26.9% in South Africa (El Kholy *et al.*, 2003; Shittu and Lin, 2006). Therefore, the average outbreak is lower than that of hospitals in Egypt and higher, compared with South African hospitals. The highest prevalence was observed at Taleghani Hospital 51(41.5%) and the highest number of isolated MRSAs was seen in ICU wards (33.3%). It is worth mentioning that the frequency of MRSA in the Scandinavian countries is less than 2% and more than 40% in the Mediterranean countries (Stefani and Varaldo, 2003). The results of the current study indicated that no resistance of MRSAs to teicoplanin (0%), linzolide (0%), and vancomycin (0%) was seen. The resistance to vancomycin was reported 12% in the north-west of Iran, 3.7% and 15% in other countries (Akanbi and Mbe, 2013; Nia *et al.*, 2013;

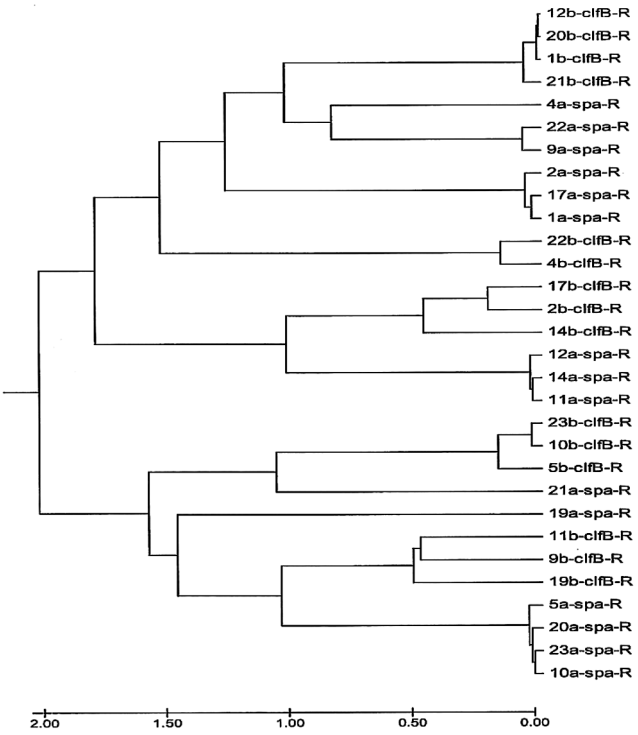

FIGURE 5. Phylogenetic relationships of MRSA isolated in microbial division of laboratories of university hospitals (Razi, Taleghani, Imam, Golestan) in Khuzestan Province, Iran.
MEGA 06 software program, UPGMA method, based on the Dice coefficient.

Ali *et al*., 2014). In another study, out of 783 *S. aureus* strains, two *strains* were found to be vancomycin and teicoplanin resistant (Tiwari and Sen, 2006). In another report, 12 patients with resistance to linzolide (LRSA) were identified (García *et al*., 2010). Hospitalization and prolonged use of antibiotics can cause colonization and infection with the MRSA strains. The infection can be transmitted from patient to patient, the staff to patient, or from the environment to patient (Hiramatsu *et al*., 2001). Recognition, classification and evaluation of the phylogenetic correlation among MRSA strains are essential for epidemiological researches. The DLST design was effectively used for genotyping; and, the local epidemiology of the MRSA isolates in the Khuzestan province were explored. Hence, this study revealed an important role in disease control and identification as well. Recently, several patterns based on the variable number tandem repeat typing (e.g., MLVA, SCC*mec* and MLST) has been described in *S. aureus*, but all of these methods are costly and time-consuming. PFGE is also considered as the golden standard method for epidemiological studies, due to its high differentiation power. It is one of the most important molecular methods for epidemiological studies and typing of *S. aureus* (van Belkum *et al*., 1998).

The main problem with this method and all other methods based on comparing patterns of DNA fragments, is the difficulty to compare the results obtained in the laboratory (Narukawa *et al*., 2009). In contrast, sequence-based methods, which are common have definitive results and provide a good repeatability. Also, several typographic designs have become available, which are based on the analysis of regions, having a variable repetitive sequence and the sequences of nucleic acids, including MLST and *Spa* sequencing. Such designs are used for epidemiological studies, as well as comparisons of *S. aureus* strains in a geographical region with strains of other parts of the world. Each isolate is characterized by a number of repetitive sequences, observed in many loci. Having multiple and repetitive sequences, complicates the typing. In addition, the MLST is less suitable for the study of the nuances of strains. Although *Spa* classification better reveals such small genomic differences, it is less likely to be implemented due to genomic recombination events. The MLST uses the nucleotide data of some seven housekeeping genes. Despite the fact that this method is useful for understanding the structure of the overall population of *S. aureus*, it is both costly and time-consuming; that is why it is not very practical. Furthermore, it has a low differentiation power for epidemiological investigations, and is used for longitudinal studies as well as phylogeny studies and the evolutionary process of species (Robinson and Enright, 2004; Kuhn *et al*., 2007).

Therefore, the DLST method was performed based on small regions of the 3' sequence. The DLST for *S. aureus* involves multiplication and sequencing, and then analyzing two highly variable gene loci, *clfB* and *spa* (Kuhn *et al*., 2007; Senn *et al*., 2013). In 2014, this technique was first introduced, using three variable loci for *Pseudomonas aeruginosa*. In 2015, this technique was designed, using two highly variable loci (Basset and Blanc, 2014). In recent reviews, the comparison of the DLST with MLST and PFGE has shown that the differentiation power indexes of DLST with regard to *S. aureus* are almost identical to those of MLST, and both techniques are capable of detecting high-risk epidemic clones.

The results of the DLST showed great typeability and discriminatory power (ID = 0.91), the results of this study are consistent with those reported in other countries (Kuhn *et al*., 2007; Basset *et al*., 2009). The most common sequence types were BPH 2003 and 0217. The DLST type, 18–32 was a significant cluster (10/30; 33%) of MRSA isolates. In this

TABLE 3

Isolate, sample/ward, DLST types and antibiotic pattern of MRSA isolates

Isolate	Sample/ward	DLST Type	Antibiotic pattern								
			Ery	Cip	Cli	Tet	Gen	Rif	Tec	Lzd	Van
SA-1	Wound/ICU	18–32	R	R	R	R	R	R	S	S	S
SA-2	Wound/ICU	18–32	R	R	R	R	R	R	S	S	S
SA-3	Urine/ICU	36–30	S	R	R	R	R	R	S	S	S
SA-4	Wound/ICU	18–32	R	R	R	R	R	R	S	S	S
SA-5	Wound/ICU	38–39	R	R	R	R	R	R	S	S	S
SA-6	Wound/ICU	49–47	S	S	R	R	S	R	S	S	S
SA-7	Blood/ICU	30–30	R	R	R	R	R	R	S	S	S
SA-8	Urine/Urology	28–32	S	R	S	R	S	S	S	S	S
SA-9	Wound/Infectious	18–32	R	R	R	R	R	R	S	S	S
SA-10	Wound/ICU	36–30	S	R	R	S	R	R	S	S	S
SA-11	Trachea/Women	29–30	S	R	R	R	R	R	S	S	S
SA-12	Wound/Infectious	18–32	R	R	R	R	R	R	S	S	S
SA-13	Wound/ICU	36–30	S	S	R	R	S	R	S	S	S
SA-14	Wound/Infectious	38–39	R	R	R	R	R	R	S	S	S
SA-15	Wound/ICU	18–32	R	R	R	R	R	R	S	S	S
SA-16	Wound/ICU	49–47	R	R	R	R	R	R	S	S	S
SA-17	Wound/ICU	18–32	R	R	R	R	R	R	S	S	S
SA-18	Urine/ICU	36–30	S	R	R	R	R	R	S	S	S
SA-19	Wound/ICU	18–32	R	R	R	R	R	R	S	S	S
SA-20	Wound/ICU	38–39	R	R	R	R	R	R	S	S	S
SA-21	Wound/ICU	29–30	S	S	R	R	S	R	S	S	S
SA-22	Blood/ICU	30–30	R	R	R	R	R	R	S	S	S
SA-23	Urine/Urology	28–32	S	R	S	R	S	S	S	S	S
SA-24	Wound/Infectious	49–47	R	R	R	R	R	R	S	S	S
SA-25	Wound/ICU	50–30	S	R	R	S	R	R	S	S	S
SA-26	Trachea/Women	29–30	S	R	R	R	R	R	S	S	S
SA-27	Wound/Infectious	30–30	R	R	R	R	R	R	S	S	S
SA-28	Wound/ICU	18–32	S	S	R	R	S	R	S	S	S
SA-29	Wound/Infectious	18–32	R	R	R	R	R	R	S	S	S
SA-30	Wound/ICU	28–32	R	R	R	R	R	R	S	S	S

Note: Resistance rate of MRSA isolates to antibiotics: Ery: Erythromycin (15 μg), Cip: Ciprofloxacin (5 μg), Cli: Clindamycin (2 μg), Tet: Tetracycline (30 μg), Gen: Gentamicin (10 μg), Rif: Rifampicin (5 μg), Tec: Teicoplanin (30 μg), Lzd: Linezolide (30 μg), Van: Vancomycin (30 μg). DLST Type: Double-locus sequence type

study, the DLST results showed that these strains were originated from the hospital. Drug-resistant isolates confirmed that patients were in relatively high antibiotic resistance risk. The *S. aureus* strains, which have been secluded from diverse geographical and infection origins, have illustrated a variety in their DLST types. It can be due to their variety, adaptive capability to diverse geographical niches and illness causes.

In 2010, Basset *et al.* conducted the DLST technique based on the sequencing of two genes, *clfB* and *spa* on 1242 MRSA isolates, isolated from Western Switzerland. Then, they compared them with a collection of isolates typed by PFGE. The results showed 88% of congruence between DLST and PFGE clones. Therefore, the DLST can be included in a routine monitoring plan. In this study, the alleles were classified as DLST-types with *clfB* and *spa* alleles. All isolates derived from each DLST cluster belonged to two clonal complexes (cc). Thus, these two genes are good markers for epidemiological studies. The MRSA isolates were studied by Kuhn *et al.*, (2017) and it was shown that the sequencing of small areas, around 500 bp of only two highly variable *clfB* and *spa* loci, allowed studying this pathogen epidemiologically. It has also been shown that unlike the PFGE method, this technique has a high stability, and yields the similar results over time (over 3 years). Also, (Vogel *et al.*, 2012) reported a gradual transition of ST228 clone to MRSA with a high transmission capability in the hospital care units in Switzerland. They determined the

genetic variation of the southern German clones (ST228) in Switzerland monitored hospitals; for this purpose 8 isolates were compared and the results showed the close interdependence between them. Moreover, the movement of mobile genetic elements (MGE), caused modifications of the family of ST228, leading to its spread in the hospitals. Finally, it was determined that with the development of new sequencing technologies, qualitative and quantitative information is obtained from a single strain, but more data are needed for their interpretation and important investments are required in technology and routine research. Therefore, using this new method proved to be a standard unambiguous typing of *S. aureus* strains. Moreover, this technique has a high power in determining new types. The results of this study are consistent with those reported in other countries (Basset *et al*., 2009; Huang *et al*., 2017). This method is easy to accomplish and helps to reduce costs and save time. Hence, this method can be used as a scientific and practical approach to be easily applied in monitoring programs, including the typology of hundreds of isolates. In addition, this method proves high typeability, differentiation and repeatability. Unambiguous identification of types makes this method useful and effective in epidemiological studies. Also, this method yielded results with a high degree of similarity with the results of the MLST method, while being more practical and requiring much lower cost and time. Therefore, the DLST method was proposed as a valuable typing tool in epidemiological surveillance (Kuhn *et al*., 2007; Basset *et al*., 2010). Infection-inducing clones are limited in number and belonged to a number of pandemic strains. Therefore, in order to prevent the spread of infection with multiple strains, appropriate measures regarding the consumption of antibiotics and the infection controls should be taken. This study can be a step forward in identifying and implementing a useful, practical, and scientific molecular typing method for epidemiological studies in Iran, for *S. aureus*. The results of this study illustrated that the prevalence of MRSAs in Ahvaz hospitals was relatively high, but these strains were totally sensitive to vancomycin, teicoplanin and linzolide antibiotics. The results of the DLST showed that the most common sequence types were BPH 2003 and 0217. The DLST type 18–32 was the significant cluster of MRSA. Therefore, the combination of partial sequences of *clfB* and *spa* can serve as useful genetic markers for the MRSA typing. By sequencing MRSA and comparing the dominant types via the DLST, it is possible to establish the etiology of the disease in a much shorter time and prevent the complications of the disease.

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