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* (SCCmec). The drug resistance is caused by the acquisition and replacement of *SCCmec* 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 *SCCmec*. The *mecA*, sensitive to the presence of betalactamase, 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 the 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 pattern 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 maximal 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 Table 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 MgCl₂ (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 Table 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. 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.

**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

| Gene name | Primer sequence | Size (bp) | Reference |
|-----------|-----------------|-----------|-----------|
| *mecA*    | F:5′-GTAGAAATGACCTGAAACGCTCCGATAA-3′ R:5′-CCCAATTCCCACATTTGTTTCCGCTCAA-3′ | 310 | (McClure et al., 2006) |
| *Nuc*     | F:5′-GGCGATTGATGGTGATACGGTT-3′ R:5′-CGCCAGCCTGACCAAGTAAACGC-3′ | 279 | (Zhang et al., 2004) |
| *vanA*    | F:5′-ATG AAT AGA ATA AAA GTT GC-3′ R:5′-TCA CCC CTT TAA CGC TAA TA-3′ | 1032 | (Saha et al., 2008; Chakraborty et al., 2011) |
| *SA_clfB* | TCGGTGTGAAATAATGAGATACGGTTGCAATCATCGCTCC | 1300 | (Kuhn et al., 2007) |
| *SA_clfB* | TTCGTTGCTTTGCCTCTTATCACCTATCC | 1500 | (Kuhn et al., 2007) |
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 MRSA isolates, 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 MRSA isolates. Based on the antibiotic resistance pattern, the results revealed that erythromycin (98.3%) accounted for the highest resistance of MRSA isolates. 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 cefoxitin 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 SCCmec 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 (SCCmec). So far, 11 major SCCmec types (I-XI types) have been identified. The SCCmec elements are one of the unique genomic islands that contain the ccr (Cassette chromosome recombinase)

| Type of clinical specimens | MRSA isolates (N0) | MRSA isolates (%) |
|----------------------------|--------------------|-------------------|
| 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                      | MRSA isolates (N0) | MRSA isolates (%) |
|----------------------------|--------------------|-------------------|
| 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).
gene complex and the mec gene complex. All MRSA strains contain SCC\textit{mec} elements that are located in a specific locus of \textit{S. aureus} chromosomes called \textit{attBsc} (Bacterial chromosomal attachment site) (Turlej \textit{et al.}, 2011; Hiramatsu \textit{et al.}, 2013).

The present study was shown that the mean prevalence of \textit{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 \textit{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 MRSA 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 \textit{et al.}, 2013;
Ali et al., 2014). In another study, out of 783 \textit{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, SCCmec and MLST) has been described in \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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

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.
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.

| Isolate | Sample/ward | DLST Type | Antibiotic pattern |
|---------|-------------|-----------|--------------------|
| 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 R S 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 R 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 R 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 R S 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 R S 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 R S 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 (5μg), Tec: Teicoplanin (30 μg), Lzd: Linezolide (30 μg), Van: Vancomycin (30 μg). DLST Type: Double-locus sequence type
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 MRSA in Alvaz 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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**References**

Akanbi B, Mbe J (2013). Occurrence of methicillin and vancomycin resistant *Staphylococcus aureus* in University of Abuja Teaching Hospital, Abuja. *Nigeria African Journal of Clinical and Experimental Microbiology* **14**: 10–13.

Ali R, Al-Achkar K, Al-Marriri A, Safi M (2014). Role of polymerase chain reaction (PCR) in the detection of antibiotic-resistant *Staphylococcus aureus*. *Egyptian Journal of Medical Human Genetics* **15**: 293–298. DOI 10.1016/j.ejmhg.2014.05.003.

Assaadallah S, Kakru D, Thoker M, Bat F, Hussain N, Shah A (2003). Emergence of low level vancomycin resistance in MRSA. *Indian Journal of Medical Microbiology* **21**: 196.

Basset P, Blanc D (2014). Fast and simple epidemiological typing of *Pseudomonas aeruginosa* using the double-locus sequence typing (DLST) method. *European Journal of Clinical Microbiology & Infectious Diseases* **33**: 927–932. DOI 10.1007/s10096-013-2028-0.

Basset P, Hammer NB, Kuhn G, Vogel V, Sakwinska O, Blanc DS (2009). *Staphylococcus aureus* clfB and spa alleles of the repeat regions are segregated into major phylogenetic lineages. *Infection, Genetics and Evolution* **9**: 941–947. DOI 10.1016/j.meegid.2009.06.015.

Basset P, Senn L, Prod’hom G, Bille J, Francioli P, Zanetti G, Blanc D (2010). Usefulness of double locus sequence typing (DLST) for regional and international epidemiological surveillance of methicillin-resistant *Staphylococcus aureus*. *Clinical Microbiology and Infection* **16**: 1289–1296. DOI 10.1111/j.1469-0691.2009.03070.x.

Carvalho MJd, Pimenta FC, Hayashida M, Gir E, Silva AMd, Barbosa CP, Canini SRMdS, Santiago S (2009). Prevalence of methicillin-resistant and methicillin-susceptible *S. aureus* in the saliva of health professionals. *Clinics* **64**: 295–302. DOI 10.1590/S1807-59322009000400005.

Chakraborty SP, KarMahapatra S, Bal M, Roy S (2011). Isolation and identification of vancomycin resistant *Staphylococcus aureus* from post operative pus sample. *Al Ameen Journal of Medical Sciences* **4**: 152–168.

Deurenberg RH, Stoberingh EE (2008). The evolution of *Staphylococcus aureus* epidemiology. *Infection, Genetics and Evolution* **8**: 747–763. DOI 10.1016/j.meegid.2008.07.007.

El Kholy A, Baseem H, Hall GS, Procop GW, Longworth DL (2003). Antimicrobial resistance in Cairo, Egypt 1999-2000: a survey of five hospitals. *Journal of Antimicrobial Chemotherapy* **51**: 625–630. DOI 10.1093/jac/dkg101.

Elements JWGottCoSCC (2009). Classification of *staphylococcus* cassette chromosome mec (SCCmec): guidelines for reporting novel SCCmec elements. *Antimicrobial Agents and Chemotherapy* **53**: 4961–4967. DOI 10.1128/AAC.00579-09.

Garcia MS, De la Torre MA, Morales G, Peláez B, Tolón MJ, Domingo S, Candel FJ, Andrade R, Arribi A, García N (2010). Clinical outbreak of linezolid-resistant *Staphylococcus aureus* in an intensive care unit. *JAMA* **303**: 2260–2264. DOI 10.1001/jama.2010.757.

Goering RV, Morrison D, Al-Doori Z, Edwards G, Gemmell C (2008). Usefulness of mec-associated direct repeat unit (dru) typing in the epidemiological analysis of highly clonal methicillin-resistant *Staphylococcus aureus* in Scotland. *Clinical Microbiology and Infection* **14**: 964–969. DOI 10.1111/j.1469-0691.2008.02073.x.

Gordon RJ, Lowey FD (2008). Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clinical Infectious Diseases* **46**: S350–S359. DOI 10.1086/533591.

Havaei SA, Azimian A, Fazeli H, Naderi M, Ghazvini K, Samiee SM, Masoumi Z, Akbari M (2012). Genetic characterization of methicillin resistant and sensitive, vancomycin intermediate
Staphylococcus aureus strains isolated from different Iranian Hospitals. ISRN Microbiology 2012: 1–6. DOI 10.5402/2012/215275.

Hiramatsu K, Cui L, Kuroda M, Ito T (2001). The emergence and evolution of methicillin-resistant Staphylococcus aureus. Trends in Microbiology 9: 486–493. DOI 10.1016/S0966-842X(01)02175-8.

Hiramatsu K, Ito T, Tsukabishita S, Sasaki T, Takeuchi F, Morimoto Y, Katayama Y, Matsuo M, Kuwahara-Arai K, HisInuma T (2013). Genomic basis for methicillin resistance in Staphylococcus aureus. Infection & Chemotherapy 45: 117–136. DOI 10.3947/ic.2013.45.2.117.

Huang CC, Ho CM, Chen HC, Li CY, Tien N, Fan HM, Ge MC, Lu JJ (2017). Evaluation of double locus (clfB and spa) sequence typing for studying molecular epidemiology of methicillin-resistant Staphylococcus aureus in Taiwan. Journal of Microbiology, Immunology and Infection 50: 604–612. DOI 10.1016/j.jmii.2015.10.002.

Japoni A, Jamalidoust M, Farshad S, Ziyaeyan M, Alborzi A, Japoni S, RafaeiPour N (2011). Characterization of SCCmec types and antibacterial susceptibility patterns of methicillin-resistant Staphylococcus aureus in Southern Iran. Japanese Journal of Infectious Diseases 64: 28–33.

Kalorey DR, Shanmugam Y, Kurkure NV, Chousalkar KK, Barbuddhe SB (2007). PCR-based detection of genes encoding virulence determinants in Staphylococcus aureus from bovine subclinical mastitis cases. Journal of Veterinary Science 8: 151–154. DOI 10.4142/jvs.2007.8.2.151.

Ko KS, Lee JY, Suh JY, Oh WS, Peck KR, Lee NY, Song JH (2005). Distribution of major genotypes among methicillin-resistant Staphylococcus aureus clones in Asian countries. Journal of Clinical Microbiology 43: 421–426. DOI 10.1128/JCM.43.1.421-426.2005.

Kuhn G, Francioli P, Blanc D (2007). Double-locus sequence typing using clfB and spa, a fast and simple method for epidemiological typing of methicillin-resistant Staphylococcus aureus. Journal of Clinical Microbiology 45: 54–62. DOI 10.1128/JCM.01457-06.

McClure JA, Conly JM, Lau V, Elsayed S, Louie T, Hutchins W, Zhang K (2006). Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methicillin-susceptible from-resistant staphylococci. Journal of Clinical Microbiology 44: 1141–1144. DOI 10.1128/JCM.44.3.1141-1144.2006.

Narukawa M, Yasuoka A, Note R, Funada H (2009). Sequence-based spa typing as a rapid screening method for the areal and nosocomial outbreaks of MRSA. Tohoku Journal of Experimental Medicine 218: 207–213. DOI 10.1620/tjem.218.207.

Nia MK, Hasani A, Hasani A, Sharifi Y, Ahmadi SM, Deganhi L (2013). Investigation and identification of the nuc, fem B meca and aac (6’)/aph (2’)-Ia genes in the staphylococcus aureus isolated from northwest Iran by multiplex PCR method. Medical Journal of Tabriz University of Medical Sciences and Health Services 35: 68–73.

Okon KO, Bassett P, Uba A, Lin J, Oyawyoe B, Shittu AO, Blanc DS (2009). Cooccurrence of predominant Panton-Valentine leukocidin-positive sequence type (ST) 152 and multidrug-resistant ST 241 Staphylococcus aureus clones in Nigerian hospitals. Journal of Clinical Microbiology 47: 3000–3003. DOI 10.1128/JCM.01119-09.

Robinson D, Enright M (2004). Multilocus sequence typing and the evolution of methicillin-resistant Staphylococcus aureus. Clinical Microbiology and Infection 10: 92–97. DOI 10.1111/j.1469-0691.2004.00768.x.

Saha B, Singh AK, Ghosh A, Bal M (2008). Identification and characterization of a vancomycin-resistant Staphylococcus aureus isolated from Kolkata (South Asia). Journal of Medical Microbiology 57: 72–79. DOI 10.1099/jmm.0.47144-0.

Schito G (2006). The importance of the development of antibiotic resistance in Staphylococcus aureus. Clinical Microbiology and Infection 12: 3–8. DOI 10.1111/j.1469-0691.2006.01353.x.

Senn L, Bassett P, Greub G, Prod’hom G, Frei R, Zhbendin R, Gaia V, Balmelli C, Pfpyffer G, Mühlemann K (2013). Molecular epidemiology of methicillin-resistant Staphylococcus aureus in Switzerland: sampling only invasive isolates does not allow a representative description of the local diversity of clones. Clinical Microbiology and Infection 19: E288–E290. DOI 10.1111/j.1469-0691.12185.

Shittu AO, Lin J (2006). Antimicrobial susceptibility patterns and characterization of clinical isolates of Staphylococcus aureus in KwaZulu-Natal province, South Africa. BMC Infectious Diseases 6: 125. DOI 10.1186/1471-2334-6-125.

Stefani S, Varaldo P (2003). Epidemiology of methicillin-resistant staphylococci in Europe. Clinical Microbiology and Infection 9: 1179–1186. DOI 10.1111/j.1469-0691.2003.00698.x.

Tiwari HK, Sen MR (2006). Emergence of vancomycin-resistant Staphylococcus aureus (VRSA) from a tertiary care hospital from northern part of India. BMC Infectious Diseases 6: 156. DOI 10.1186/1471-2334-6-156.

Turlej A, Hryniewicz W, Empel J (2011). Staphylococcal cassette chromosome mec (SCCmec) classification and typing methods: an overview. Polish Journal of Microbiology 60: 95–103. DOI 10.33073/pjm-2011-013.

Van Bekum A, Tassios P, Dijkshoorn L, Haeggman S, Cookson B, Fry Vogel V, Falquet L, Calderon-Copete SP, Basset P, Blanc DS (2012). Fan Belkum A, Tassios P, Dijkshoorn L, Haeggman S, Cookson B, Fry Vogel V, Falquet L, Calderon-Copete SP, Basset P, Blanc DS (2012). Performance st andards for antimicrobial susceptibility testing. 21st informational supplement. Clinical Microbiology and Infection 13: 1–46. DOI 10.1111/j.1469-0691.2007.01786.x.

Van Bekum A, Van Leeuwen W, Kaufmann M, Cookson B, Forey F, Etienne J, Goering R, Tenover F, Steward C, Grubb W (1998). Assessment of resolution and intercenter reproducibility of results of genotyping Staphylococcus aureus by pulsed-field gel electrophoresis of Smal macrorestriction fragments: a multicenter study. Journal of Clinical Microbiology 36: 1653–1659.

Vogel V, Falquet L, Calderon-Copete SP, Basset P, Blanc DS (2012). Short term evolution of a highly transmissible methicillin-resistant Staphylococcus aureus clone (ST228) in a tertiary care hospital. PLoS One 7: e38969.

Walsh TR, Howe RA (2002). The prevalence and mechanisms of vancomycin resistance in Staphylococcus aureus. Annual Reviews of Microbiology 56: 657–675. DOI 10.1146/annurev.micro.56.012302.160806.

Wayne PA (2014). Performance standards for antimicrobial disk susceptibility tests. Approved standard, 11th ed. CLSI document M02-A11. Clinical and Laboratory Standards Institute.

Wayne PA (2011). Performance standards for antimicrobial susceptibility testing. 21st informational supplement. CLSI document M100-S21. Clinical and Laboratory Standards Institute.

Zhang K, Sparling J, Chow BL, Elsayed S, Hussain Z, Church DL, Wayne PA (2011). Performance standards for antimicrobial susceptibility testing. 21st informational supplement. Clinical and Laboratory Standards Institute.