Original Article

# Molecular Typing of Community-acquired Methicillin-Resistant *Staphylococcus aureus* Isolated from 2- to 6-year old Children by Staphylococcal Protein A and *Agr* Typing in Isfahan, Iran

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## Abstract

**Background:** Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a considerable public health concern in the entire world due to the rapid spread of this bacterium in human community; also the epidemiology of MRSA has changed, as the isolation of MRSA strains from healthy and non-healthy patients. Therefore, the objective of this study is to determine the genetic diversity and antibiotic resistance profile of community-acquired (CA)-MRSA nasal carriage in the Iranian samples. **Materials and Methods:** A total of 25 CA-MRSA were isolated from the anterior nares of 410 healthy preschool children. All MRSA isolates were characterized by the detection of the toxic shock syndrome toxin-1 (TSST-1) and typed by γ-hemolysin genes, *agr* groups, and staphylococcal protein A (*spa*) typing. Kirby-Buyer antibiotic susceptibility testing was performed and interpreted as per the standard guidelines. **Results:** A total of 25 (6.1%) MRSA isolates were recovered from the anterior nares of 410 preschool children. Sixteen isolates (64%) were positive for the TSST-1 gene. Three *agr* specificity groups were determined, as follows: eight (32%) isolates belonged to *agr* Group I, five (20%) isolates belonged to *agr* Group II, and 12 (48%) isolates belonged to *agr* Group III. The repeated profiles of these *spa* types of 25 isolates were organized into eight different lineages groups. Five of lineages contained a single strain, three of lineages contained two strains, and three of lineages consisted of more than three strains. **Conclusions:** The results of our study show that the rate of MRSA in our region is significantly high. Additionally, *spa* type t037 was the predominant type among CA *S. aureus*.

**Keywords:** *Agr protein, Methicillin-resistant Staphylococcus aureus, staphylococcal protein A, toxic shock syndrome toxin 1*

## Introduction

*Staphylococcus aureus*, especially methicillin-resistant *S. aureus* (MRSA), is one of the most important bacterial pathogens in human and is responsible for the constantly increasing the number of community-acquired (CA) and nosocomial infections, including endocarditis, food poisoning, toxic shock syndrome, septicaemia, skin infections, soft-tissue infections, and bone infections.[1,2] Furthermore, the appearance of CA-MRSA and the potential risks of its introduction into hospitals are the matters of great concern.[3] In the past few years, the isolation of CA-MRSA has been more frequent, especially in the geographical areas with a high prevalence, where these strains have also started to replace HA-MRSA in the hospital settings.[4] Epidemiological data on CA-MRSA carriage and infection and clonal diversity of CA-MRSA in our region are low.[4,5]

*S. aureus* is a heterogeneous species that was recently found to have a clonal population structure and show a high degree of linkage disequilibrium (non-random associations between genetic loci).[6] The distribution of MRSA clonal types (CT) has been not well characterized across different geographic regions and care settings, especially in developing country.[7] Therefore, understanding the spread of specific CT of *S. aureus*, in both the hospitals and community settings, is of great importance for the prevention of this infection.

The use of adequate and accurate epidemiological typing methods is a prerequisite for screening and limiting the

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occurrence and spreading of dangerous clones within and between hospitals and community settings.[7,8] Therefore, typing systems must enable the differentiation between unrelated isolates as well as the recognition of isolates belonging to the same clonal lineage in order to determine whether epidemiologically related isolates are also genetically related.[7]

Recently, similar to other bacteria, *S. aureus* typing has been dominated by the molecular techniques based on the various analysis of specific gene sequences in isolated strain.[7] Different molecular methods were used for typing of *S. aureus* such as staphylococcal cassette chromosome *mec* typing (SCC*mec),* multi-locus sequence typing (MLST), multilocus enzyme electrophoresis (MLEE), pulsed-field gel electrophoresis (PFGE), and staphylococcal protein A (*spa*).[9,10] Among these methods, *spa* typing is a rapid, easy, and relatively inexpensive typing technique, it has shown effectively different genetic variation associated with CA-MRSA strains and they are associated with different geographical areas.[11]

Due to the literature in the current study, we aimed to determine the prevalence of the virulence genes, *spa* typing, and molecular characteristics of nasal carriage CA-MRSA isolates from healthy preschool children in Isfahan, Iran.

## Materials and Methods

### Bacterial isolates

From December 2017 to September 2018 in a cross-sectional study conducted on 410 healthy 2-to 6-year-old preschool children in Isfahan, Iran, 25 CA-MRSA isolates were detected using the phenotypic biochemical test and the polymerase chain reaction (PCR) amplification *mec*A gene PCR.[4] In the present study, all of the 25 CA-MRSA isolates were included. This study was approved by the ethics committee of Isfahan University of Medical Sciences and Social Welfare Organization under which the private and public day-care nurseries (Grant No. 392063). A parent of any child participants provided informed consent on their behalf.

### Ethics statement

This study was approved by the ethics committee of Isfahan University of Medical Sciences and Social Welfare Organization under which the private and public day-care nurseries or kindergartens are organized and operate (Grant No. 392062). A parent or guardian of any child participants provided informed consent on.

### Antimicrobial-susceptibility testing

The recovered *S. aureus* strains were tested for antimicrobial susceptibility pattern to a panel of 16 antibiotic discs with the disk-diffusion technique on Mueller-Hinton agar (Mast, UK), and the results were recorded after incubation for 18 h at 37°C. The interpretive criteria for susceptibility were used by the clinical and laboratory standards institute.[12] The antimicrobial drugs tested included penicillin (PG 10 μg), clindamycin (CD 2 μg), ampicillin (AP 10 μg), vancomycin (VA 30 μg), levofloxacin (LEV 5 μg), teicoplanin (TEC 30 μg), ceftriaxone (CRO 30 μg), gentamicin (GM 10 μg), amikacin (AK 30 μg), tobramycin (TN10 μg), linezolid (LZD 30 μg), erythromycin (E 15 μg), kanamycin (K 30 μg), gatifloxacin (GAT 5 μg), ciprofloxacin (CIP 5 μg), and trimethoprim-sulfamethoxazole (TS 25 μg). Intermediate susceptibility was scored as resistance. Multidrug resistance was defined as resistance to three or more unique antibiotic classes in addition to beta-lactams. *S. aureus* ATCC25923 was used as a quality control strain in each test run.

### Molecular assays

#### *DNA extraction and purification*

Chromosomal DNA of isolates was extracted using a simple boiling method. In summary, several colonies of bacteria were added to 200 ml TES buffer, (50 mM Tris hydrochloride (pH 8.0), 5 mM EDTA, 50 mM NaCl), and the suspension was heated at 95°C for 10 min and centrifuged at 11000 g for 10 min. The supernatant was transferred to another sterile microtube and centrifuged at 20 000 g for 10 min. The supernatant was resuspended in 50 μl Milli-Q water and stored at −20°C.

#### *Toxic shock syndrome toxin and gene detection*

The presence of Toxic Shock Syndrome Toxin-1 (TSST-1) genes in our isolates was determined using the specific PCR amplification strategy developed by Motoshima.[13]

#### *Multiplex polymerase chain reaction for determination of agr group*

The *agr* specific groups of CA-MRSA isolates were determined by 2 duplex PCR according to the methods of Saïd-Salim *et al*.[14]

#### *Staphylococcal protein A typing*

The polymorphic X region of the protein, a gene of all CA-MRSA isolates was determined by using the specific PCR protocol described by Strommenger *et al*.[15] Then, sequencing was performed by the Bioneer Company (South Korea), and the sequence data received were aligned manually with existing sequences of *spa* genes retrieved from the GenBank database. *spa* types were determined by using Ridom Staph-Type software (version 1.4; Ridom GmbH, Wu ̈rzburg, Germany), as described by Harmsen *et al*.[16] Finally, diversity and phylogenetic analyses of *spa* types of isolates were conducted with MEGA 8 software (version 8.1; Penn State University , Pennsylvania , USA http://www.megasoftware.net).

## Results

In this study, total of 25 (6.1%) MRSA isolates were recovered from the anterior nares of 410 preschool children. Among the 25 CA-MRSA isolates, 16 isolates (64%) were positive for the TSST-1 gene. The antimicrobial susceptibility pattern was observed among our isolates given in Table 1.

## *Agr* groups

In the current study, three *agr* specificity groups were determined as follows: 8 (32%) isolates belonged to *agr* Group I, 5 (20%) isolates belonged to *agr* Group II, and 12 (48%) isolates belonged to *agr* group III [Table 1].

### Reproducibility

To control the intra-laboratory reproducibility of the sequence-based typing method, every 25 isolates in this study was typed repeatedly. All previous typing results could be confirmed, thus leading to an intra-laboratory reproducibility of 100%.

### Diversity of staphylococcal protein A types

As described in the literature,[16] a *spa* type is composed of various repeats, each of which represents eight codons (24 nucleotides). In this study, a total of 25 distinct *spa* types were observed from 25 CA-MRSA strains. The repeat profiles of these *spa* types of 25 isolates were organized into eight different lineage groups [Figure 1]. Five of lineages contained a single strain, 3 of lineages contained two strains, and 3 of lineages consisted of more than three strains.

**Table 1: Antimicrobial susceptibility pattern and distribution of staphylococcal protein A types *agr* group and tst in 24 multidrug resistance isolates from healthy children in Isfahan**

| *Spa* types | *agr* | tst | *hlȧ* | Resistance pattern |
|---|---|---|---|---|
| 30 | 3 | + | + | PG, AP, E, AK, CD, CIP, GM |
| 2 | 3 | + | | PG, AP, AK, CD, TN, GAT, LEV |
| 3 | 3 | | | PG, AP, AK, CD, TN, GAT, LEV |
| 6 | 1 | + | | PG, AP, E, CIP, CD, GM, K, TN |
| 7 | 3 | + | | PG, AP, E, AK, CD, CIP, GM |
| 10 | 1 | | + | PG, AP, E, CIP, AK, GM, K, TN, TS, GAT |
| 14 | 2 | + | | PG, AP, E, CIP, CD, GM, K, TN |
| 15 | 2 | + | | PG, AP, AK, CD, TN, GAT, LEV |
| 16 | 1 | | | PG, AP, E, CD, GAT, TS, TN, LEV, CRO |
| 17 | 3 | + | | PG, AP, CD, K, GAT, TS, CRO |
| 18 | 3 | + | | PG, AP, CD, K, GAT, TS, CRO |
| 20 | 3 | | | PG, AP, E, CIP, CD, GM, K, TN |
| 22 | 1 | | | PG, AP, AK, CD, TN, GAT, LEV |
| 25 | 1 | + | | PG, AP, E, AK, CD, CIP, GM |
| 26 | 3 | + | | PG, AP, AK, CD, TN, GAT, LEV |
| 27 | 2 | + | | PG, AP, E, AK, CD, CIP, GM |
| 28 | 3 | | + | PG, AP, E, CIP, AK, GM, K, TN, TS, GAT |
| 29 | 3 | + | | PG, AP, AK, CD, TN, GAT, LEV |
| 31 | 1 | + | | PG, AP, E, CD, GAT, TS, TN, LEV, CRO |
| 32 | 3 | + | | PG, AP, AK, CD, TN, GAT, LEV |
| 34 | 1 | | | |
| 35 | 1 | + | | PG, AP, E, CIP, CD, GM, K, TN |
| 37 | 3 | | | |
| 38 | 3 | + | + | PG, AP, E, CIP, CD, GM, K, TN |

PG: Penicillin, VA: Vancomycin, TEC: Teicoplanin, TN: Tobramycin, LZD: Linezolid, TS: Trimethoprim-sulfamethoxazole, AK: Amikacin, AP: Ampicillin, CD: Clindamycin, CIP: Ciprofloxacin, CRO: Ceftriaxone, E: Erythromycin, GAT: Gatifloxacin, GM: Gentamicin, K: Kanamycin, LEV: Levofloxacin, *Spa*: Staphylococcal protein A

## Discussion

Due to the advances in bacterial genomics, DNA sequencing, and bioinformatics have enriched the molecular tools of population geneticists, evolutionary biologists, and infection control facility to track and control the suspected outbreaks. MLEE, MLVA, PFGE, and MLST have been the most common methods for the application in population geneticists and those studying global epidemiology because these techniques accumulate genetic variation slowly.[17]

Different genotyping techniques are available for classifying *S. aureus* strains for epidemiological investigation, including "band-based" as well as "sequence-based" methods. Thereby, sequence-based typing methods, such as *spa* typing and MLST, have several obvious advantages, for instance they are easy to use, transportable, reproducible, and they can provide comparable results, compared to band-based methods, such as small macro restriction analysis.[11,17] Due to the issue, the aim of the current study was the evaluation of the *spa* typing use as an appropriate tool for the routine typing of staphylococcal isolates in the clinical laboratory reference center for staphylococcus isolates, in accordance with previously proposed guidelines.

The repeated profiles of these *spa* types of 25 isolates were organized into eight different lineage groups. Lineages

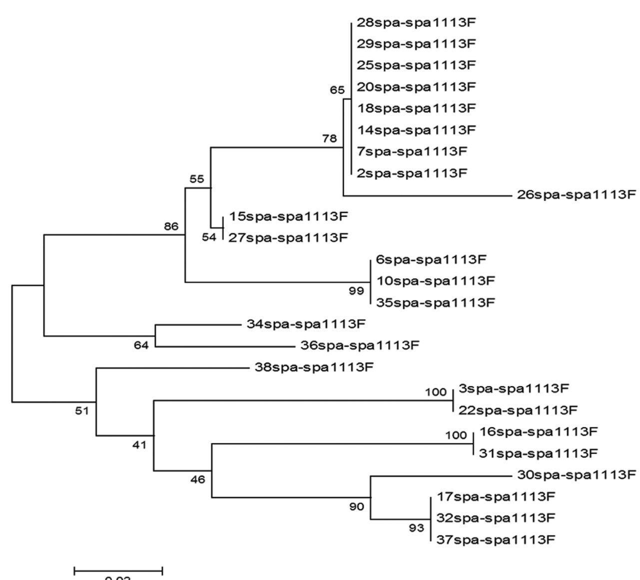


**Figure 1: Staphylococcal protein A type sequence-based phylogenetic tree for the methicillin-resistant *S. aureus* isolates, produced using the neighbor-joining method by staph Type software. The number at each node represent the bootstrapping values**

were formed by grouping strains together with similar *spa* repeated profiles, which presents the accumulation of identical point mutations, suggesting a genetic relatedness and descent from a common ancestor [Figure 1].[18] Same *spa* profile groupings were obtained by utilizing a multiple-sequence alignment program (MEGA 8). As an example, although *spa* type 46 (YMBQBLO) differed in the number of repeats from *spa* type 7 (YHGCMBQBLO), these two were grouped together in *spa* lineage 1 because they contained many identical repeats representing exact nucleotide polymorphisms shared by both.

In the current study, we found that 25 different *spa* types such as t037, t030, t7685, t7689, t030, t3096, that correspond with the results of other studies in Iran such as studies conducted by Mirzaii *et al*. and Japoni-Nejad *et al*. that were isolated and *spa* typed MRSA from personnel, patients, air, and intensive care unit in Iran in 2014.[19,20] These result showed that the *spa* types t037, t030, t7685, and t7689 were the most common *spa* type our region.

In correlation with the results of previous studies, we found that *spa* typing has a high degree of typeability and reproducibility in our laboratory.[21] The unambiguous nomenclature also facilitates the submission of comparable typing information to international networks, which is in clear contrast to other methods based on fragment patterns, such as PFGE, that have the lack of interlaboratory reproducibility. Thus, *spa* typing can be an excellent tool for the international multicenter surveillance of MRSA strains.[21,22]

The typing data resulting from those studies can also be used to enlarge our knowledge of *spa*-MLST mapping, which is extremely useful for the daily routine typing of MRSA, in which the Based Upon Repeat Pattern (BURP) algorithm together with "reference *spa* types" enable the classification of isolates into particular clonal lineages. This work, as well as previous studies, have shown that, in general, the typing concordance between *spa* typing-BURP analysis and alternative methods is high; however, the occurrence of "group violations"[16] associated with particular BURP groups and clonal lineages were also demonstrated. Some of these misclassifications (in BURP groups A, E, and G) are due to the related *spa* repeat successions in isolates of different clonal lineages, possibly caused by the recombination events in the *spa*.

The successful application of *spa* typing for the detection of transmission events or clusters of infections was reported in different studies.[11,23] The results of our study are able to monitor clusters of infections over an even longer period of time and also have a correlation with the *in vivo* stability of the *spa* locus as a molecular marker in the epidemiological research.[24] The endemic spread of these highly successful types finally leads to a lack of discrimination in local site epidemiology. To overcome this limitation, recent studies suggest the use of a combination of different typing techniques to increase the ability to discriminate isolates.[25] We have previously described the use of a combination of two techniques (SCC mec and MLST analysis) for the successful subtyping of isolates.[4]

In our study, the majority of MRSA strains recovered from specimens were *agr* group I, which is consistent with previous reports. For example, Shopsin *et al*. 12 showed that *agr* specific group I (42%) was prevalent between children and their guardians, and in the van Leeuwen *et al*. 13 collection of 192 MRSA isolates, 71% of strains belonged to *agr* group I. Other study indicated a high prevalence of *agr* Group II in the nosocomial infection group.[26,27] This is likely due to ecological and geographical structuring.

Considering the possible relation between the *agr* group and the antibiotic resistance in *S. aureus* isolates, we made disk-diffusion agar tests. Resistance pattern to oxacilin between the *agr* groups was relatively even. *Agr*-specific Group II isolates except oxacilin were susceptible to all antibiotic disks tested as opposed to the others, and *agr*-specific group IV isolates were more resistant strains.

MRSA can produce many exotoxins such as TSST-1 and α-hemolysin which were associated with a severe illness that includes shock and multiple organ failure and is called toxic shock syndrome.[28,29] Approximately 20% and 5% of MRSA isolates possess the genes encoding TSST-1, and α-hemolysin, respectively. However, the result of the current study showed that 16 isolates (64%) and 4 isolates (16.6%) were positive for the TSST-1 and α-hemolysin gene, respectively. This result showed that our CA-MRSA have high pathogenicity in compared to CA-MRSA of other regions.

## Conclusion

In conclusion, the authors demonstrated the value of *spa* typing in combination with *agr* groups of the isolates as a suitable and reproducible tool for routine epidemiological typing based on a random sampling of isolates. The results of our study show that this approach yields highly interchangeable and reproducible information that may be used both for local epidemiology and for national as well as international surveillance of MRSA isolates. However, to overcome the limitations of a single locus-based molecular typing method, the use of additional markers is indispensable. To reduce time and cost, those markers should be selected on the basis of the clonal lineage inferred by *spa* typing and also on the basis of the question to be addressed. Additional targets can be SCCmec, *agr* groups, resistance genes or lineage-specific virulence gene, or alternative polymorphic regions of the *S. aureus* chromosome.

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### Conflicts of interest

There are no conflicts of interest.

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