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Scientific Research

New *Spa* Types among MRSA and MSSA Isolates in North of Iran

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Abstract

Staphylococcus aureus typing using gene encoding protein A (*spa* typing) seems to have a high potential discriminatory power for typing this bacterium. This study was designed based on *spa* typing method to compare the *S. aureus* types among healthy carrier vs patients, and MRSA vs MSSA isolates. Method: This study was carried out on 182 *spa* typeable *S. aureus* isolates, including 52 MRSA. DNA was extracted by phenol chloroform-isoamyl alcohol method and it was amplified by specific primer of polymorphic X region of *spa*. *Spa* types were determined by *Ridom Staph Type* software. The *spa* types distribution among MRSA vs MSSA and healthy carrier vs patients, isolates were statistically compared by X2 method and $P < 0.05$ was considered as significant. Result: The most common types of *spa* in our region were t037 (18.3%) and t937 (13.9%) from 50 *spa* types which were identified in this study. *spa* types in this study were isolated from various age groups but t660 *spa* types were only isolated from children. Distribution of all *spa* types among MRSA and MSSA isolates was 16 and 38 types, respectively which is significant. In this study we found seven new *spa* types (belong to twelve isolates) which are reported for the first time. In 5 out of the above 7 new types the 24 bp repeated sequences in *spa* gene X region were already recognized but their 24 bp arrangement is introduced in our present investigation. In the remaining 2 new types we found new 24 bp nucleotide sequences which are also introduced for the first time in our present study. Conclusion: The distribution of *spa* types in MSSA strains was significantly higher than MRSA isolates, but there are not any specific *spa* types for discrimination between MRSA and MSSA. The novelty of our study is the introducing 7 new *spa* types including 2 new 24 bp repeated sequences in the X region of *spa* gene.

Keywords

***S. aureus*, *Spa* Types, Healthy Carrier, MRSA, New Type**

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1. Introduction

Staphylococcus aureus is a well known agent as commensal organism lived on the human skin and as a leading cause of human disease. This bacterium responsible for a variety of disorders ranging from superficial skin infections up to serious abnormalities such as pneumonia, Bacteremia and Endocarditis and Staphylococcus aureus colonizes the anterior nares of 20% - 80% of the human population [1] [2].

There are many molecular methods that have been employed to type, differentiate and group the *S. aureus* isolates, such as pulsed-field gel electrophoresis (PFGE), which have been used as the gold-standard method [3], Multilocus Sequence Typing (MLST), *coa* typing, phage typing and *SCCmec* typing. Sequence-based methods have been developed to provide fast, unambiguous, and exportable typing data. Among them, the sequence determination of the polymorphic X region of *spa* gene or short sequence repeat (SSR) region of the protein A gene (*spa*) has been proposed as an alternative to current techniques for the typing of *S. aureus* [4] [5].

The X region of *spa* gene as an epidemiological marker initially was employed in 1994 by Frenay and his colleague. The X region was amplified and its size estimated by electrophoresis [6].

In 1996, the same group improved the technique by performing sequence analysis of the X region [7]. Since then many researchers have evaluated the usefulness of this technique for diverse epidemiological purposes and its credibility for S, typing. They found also it is a cost effective and simple method to apply.

Koreen *et al.* stated that the *spa* typing was shown to have a higher potential discriminatory power than other typing methods such as, microarray, multilocus enzyme electrophoresis (MLEE), Pulsed field gel electrophoresis (PFGE) and *coa* typing [8]. Based on these later findings we chose the *spa* typing method to type *S. aureus* in our region.

The aim of this study was to compare the *S. aureus spa* types isolated from healthy carrier and patients, also among MRSA and MSSA isolates.

2. Materials and Methods

2.1. Bacterial Isolates

This study was carried out on 190 *staphylococcus aureus* which was isolated from 81 healthy carrier and 109 patients in Gorgan located in northern Iran during one year period (2009-10). Out of this sample population, 54 (28.4%) were MRSA [9] [10]. The nature of *S. aureus* isolates samples were confirmed using biochemical tests including catalase, cogulase, growth on MSA and DNase.

2.2. DNA Extraction

Genomic DNA for subsequent PCR was isolated from 1 ml of overnight culture, lysed with lysozym-phenol chloroform method and treated with N-lauroyl sarcosine sodium salt 2% (300 µL), proteinase k 100 µg (30 µl), and RNase A (5 µl). DNA was extracted by phenol chloroform—isoamil alcohol, chloroform, and cold ethanol. Extracted DNA were examined by electrophoris on gel agarose 1% and stored in −20˚C until experimental procedures were applied.

2.3. PCR and *Spa* Typing

Standard primers which was introduced by Ridom *spa* typing (http://www.ridom.com/spa-server/) was used for the amplification of the polymorphic X region of the protein A gene (*spa*). The sequences of used primer are as follow:

Spa-1113f (5'- TAA AGA CGA TCC TTC GGT GAG C -3') and *spa*-1514r (5'-CAG CAG TAG TGC CGT TTG CTT -3') and its amplicon size varied between 300 - 500 bp.

The PCR master mix and program was as follow:

1) Add genomic staphylococcal DNA in a PCR mixture to achieve 50 m L of final volume containing 1.25 units of *Taq* polymerase, 1.5 m *M* MgCl 2, 200 m *M* dNTPs, 0.2 m *M* of each primer (*spa*-1113f, *spa*-1514r) and 5 m L of 10× PCR buffer.

2) Cycling conditions consist of an initial denaturation step of 5 min at 80˚C, followed by 35 cycles of 45 s of denaturation at 94˚C, 45 s of annealing at 60˚C, 90 s of extension at 72˚C, and a final extension step of 10 min at 72˚C. The PCR product was assessed by electrophoresis on 1.5% agarose gel.

2.4. Sequencing Xr *Spa*

All PCR products were sequenced by company "Macrogene" in Korea. *Spa* types were assigned by using *S. aureus* Type software (version 1.4; Ridom GmbH, Würzburg, Germany), as described by Harmsen *et al.*

3. Results

3.1. Type Ability

Among the 190 *S. aureus* isolates, all but 8 (4.2%) were typable by *spa* typing.

3.2. Diversity of *Spa* Types

The 182 typable isolates of *S. aureus* belong to 43 known different *spa* types and 7 new *spa* type which is reported for the first time in this present article. The distribution *spa* types among healthy carriers and patients were 32, 29 types respectively, this difference was not statistically significant ($P > 0.05$). On the other hand 38 types were diagnosed in MSSA isolates but only 16 *spa* types were present among MRSA isolates which statistically was significant ($P < 0.05$) (**Table 1**).

3.3. Distribution of *Spa* Type's t037 and t937

Spa type's t037, t937 with 18.3% and 13.9% were the most predominant types (**Table 2**). The distribution of t037, t937 between carriers vs patients and MRSA vs MSSA were not significantly different. The mean age of all subjects in this study was 31.4 ± 19.1 years, but the average age in cases particularly harboring types t37 and t937 were 39.2 ± 21.3 and 34.1 ± 15.5 years respectively, which is higher than mean age of sample population. On the other hand the *spa* type t660 was specifically isolated from children with mean age of 1.75 ± 1.5 years (**Table 1**).

Type t012 and t267 was predominantly isolated from clinical samples and all type t660 (4 cases) were isolated from patients.

Five types including t267, t330, t436, t1149 and t2313, with frequency 9, 5, 6, 5 and 4 isolates, respectively were detected only in methicillin sensitive *S. aureus* (MSSA).

3.4. Detection of New *Spa* Types

We found 12 isolates which have new *spa* types; and classified in 7 *spa* types. The new type 1 was the most prevalent (4 isolates) and all of them were MSSA which were isolated from patients. Apart from 2 cases which belong to new types of 6 and 7, all others cases were isolated from patients (**Table 2**).

In the new types 1, 2, 4, 5 and 6 the 24 repeated sequence in X region *spa* gene, already was recognized but the novelty this 5 types is due to how these 24 bp are arranged in gene.

But in the new type 3 and 7 we found new 24 nucleotide sequences which are introduced for the first time (**Figure 1**).

4. Discussion

Rapid and accurate determination of the different *Staphylococcus aureus* isolated from patients and carriers are a great help in understanding the epidemiology of this bacteria and its infection control [7].

PFGE is still considering the gold standard in molecular typing, owing to its excellent discriminatory ability [11], but the main disadvantages of this technique are related to the technical demands, the costs of the equipment, the time and labor required. Accordingly, there is a need for a rapid, inexpensive, and reliable method in routine epidemiological surveillance. Several PCR-based methods have been applied for molecular typing, for example PCR-RFLP, AP-PCR, MLST, SCC*mec*, *coa* typing and *spa* typing based on sequencing. In this study we used *spa* typing, due to its high degree of polymorphism in X region, for discrimination of different *S. aureus* isolated from patient, healthy carriers and between MRSA and MSSA.

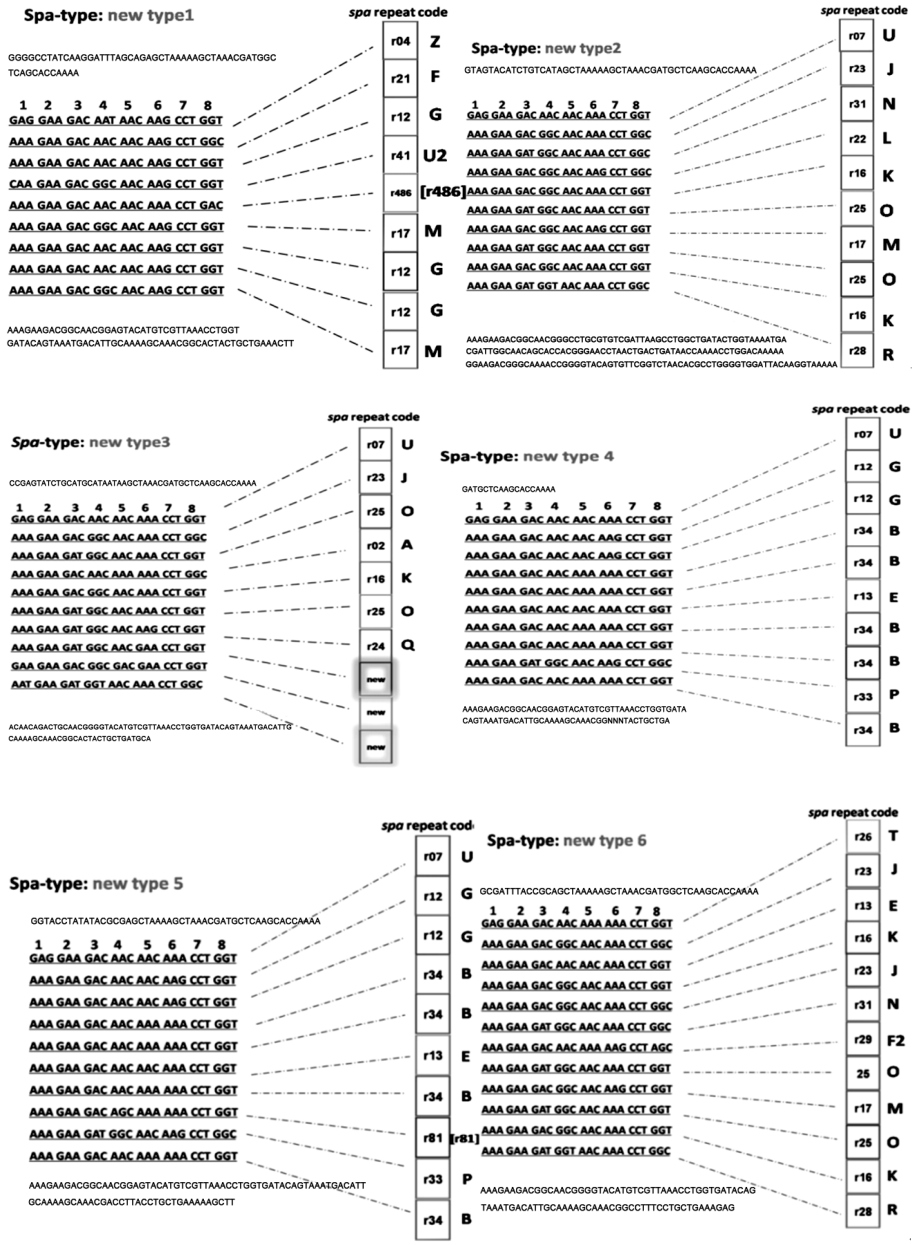
In a study Nuno A. Faria and *et al.* (2008) used different method for typing 116 MRSA isolates and found that there were 32, 34, and 51 types in PFGE, MLST and *spa* methods respectively. This result indicated that discriminatory effect of Xr *spa* typing is more powerful even than PFGE and MLST [12].

Table 1. Distribution of different *spa* types *Staphylococcus aureus* in North of Iran.

Types	Frequency (%)	MecA		Isolated from		X Repeated Numbers	Repeats in Ridom *Spa* Serve
		MSSA	MRSA	CARRIER	PATIENT		
t008	1 (0.5%)	1	-	-	1	10	YHGFMBQBLO
t012	7 (3.4%)	3	4	1	6	10	WGKAKAOMQQ
t021	3 (1.4%)	3	-	1	2	9	WGKAKAOMQ
t030	1 (0.5%)	-	1	-	1	6	WGKAQQ
t037	**38 (18.3%)**	15	23	12	26	7	WGKAOMQ
t065	2 (1.0%)	1	1	-	2	6	XKAKBB
t084	2 (1.0%)	2	-	1	1	11	UJGBBGGJAGJ
t127	1 (0.5%)	1	-	-	1	7	UJFKBPE
t160	2 (1.0 %)	2	-	-	2	7	UJFQPLM
t162	1 (0.5%)	1	-	-	1	8	I2Z2GMMJH2M
t258	1 (0.5%)	1	-	1	-	10	ZFGU2DMGGGM
t267	9 (4.3%)	9	-	2	7	10	UJGFMBBBPB
t279	5 (2.4%)	3	2	2	3	12	UJGBBBGGJAGJ
t284	1 (0.5%)	1	-	1	-	8	I2Z2EMMJH2M
t325	2 (1.0%)	2	-	1	-	10	UGFMBEBBPB
t330	5 (2.4%)	5	-	1	1	9	A2AKBBMBKB
t346	1 (0.5%)	1	-	1	-	10	UJGBGGJAGJ
t359	2 (1.0%)	2	-	1	1	9	UJGFMBBPB
t409	1 (0.5%)	1	-	1	-	6	S2W3BLBM
t436	6 (2.9%)	6	-	3	3	6	ZFGU2DM
t608	1 (0.5%)	-	1	1	-	10	TJNF2MOMOKR
t660	4 (1.9%)	2	2	-	4	8	YGU2DMGGM
t701	8 (3.8%)	7	1	5	3	10	YC2FMBQBLOO
t779	2 (1%)	2	-	2	-	1	X1
t790	2 (1.0%)	1	1	1	1	12	TJEJNF2MOMOKR
t932	1 (0.5%)	-	1	-	1	6	GKAOMQ
t937	**29 (13.9%)**	26	3	16	13	8	XKBQBBMM
t1077	2 (1.0%)	2	-	-	2	7	I2Z2GMJH2M
t1149	5 (2.4%)	5	-	3	2	10	ZMOKJBT2T2T2M
t1358	6 (2.9%)	3	3	2	4	11	TJEJNF2MOMOR
t1497	1 (0.5%)	1	-	1	-	8	UJFLKBPE
t1810	1 (0.5%)	1	-	-	1	5	ZFGU2D
t2120	1 (0.5%)	1	-	1	-	9	XAKBBMBKB
t2147	2 (1.0%)	1	1	1	1	8	GKAKAOMQ
t2313	4 (2.9%)	6	-	6	-	7	XKBQBBM
t3543	2 (1.0%)	1	1	1	1	11	I2Z2GMMJH2MJH2M
t3572	1 (0.5%)	1	-	1	-	6	I2Z2GMT2M
t3992	1 (0.5%)	1	-	1	-	7	WFFKAOM
t4478	1 (0.5%)	-	1	-	1	11	TJEJNF2OMOKR
t6352	1 (0.5%)	-	1	1	-	9	TJMBMAMMK
t8027	1 (0.5%)	1	-	1	-	11	UJGBBGGGAGJ
t9017	2 (1.0%)	2	-	1	1	8	[r468]KAKAOMQ
t9830	1 (0.5%)	1	-	-	1	8	ZMBME2JQQ

Table 2. Distribution of new *spa* types *Staphylococcus aureus* in North of Iran.

Types	Frequency (%)	*meca*		Isolated from		Samples	Repeats in Ridom *Spa* Serve
		MSSA	MRSA	CARRIER	Patient		
New Type 1	4 (1.9%)	4	-	-	4	3 urine-1 sputum	ZFGU2 [r486] MGGM
New Type 2	2 (1.0 %)	1	1	-	2	1 wound-1 sputum	UJNLKOMOKR
New Type 3	1 (0.5%)	1	-	-	1	Urine	UJOAKOQ-**new-new-new**
New Type 4	1 (0.5%)	-	1	-	1	Wound	UGGBBEBBPB
New Type 5	1 (0.5%)	1	-	-	1	Blood	UGGBBEB [r81] PB
New Type 6	2 (1.0 %)	-	2	1	1	Nose	TJEKJNF2OMOKR
New Type 7	1 (0.5%)	1	-	1	-	Nose	**new-new** MBQBLOO



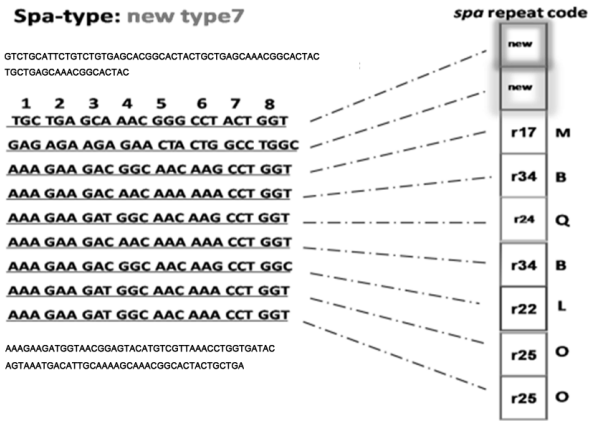


Figure 1. The sequence of 7 new *spa* types of *S. aureus* were isolated from north of Iran. *The letters A, B, G, K, M, O, Q, X and W in known *spa* types means a specific 24 nucleotide sequences of X region *spa* gene which equal to one of numbered r(r01, r08,..). In new *spa* types (except 3, 7) the r types was previously determined but there arrangement is new but in new types 3, 7 we find 5 new specific 24 nucleotide sequences which marked as new.

In our pervious study PCR-RFLP method on *spa* gene indicated that only 8 types out of 190 isolates are *S. aureus* [13]. In another survey conducted in India by this method only 5 *spa* types were detected [14], but in this present study *spa* typing was carried out based on its Xr region sequencing and found about 50 *spa* types. This means PCR-RFLP *spa* gene had lower discriminative effect than Xr *spa* sequencing method, therefore Xr *spa* sequence typing preferred to PCR-RFLP method of *spa* gene.

We found that the *spa* types distribution in MSSA (44 types) was significantly more than MRSA (21 types) isolates. Nuno A. Faria *et al.* showed *spa* types among 116 MRSA isolates and 82 MSSA isolates was 51 and 55, respectively (Faria *et al*. 2008) and Strommenger *et al.* (2006) in a study on 283 MSSA and 1176 MRSA isolates found 128 and 121 types, respectively [3]. These latter findings are in accordance with our data which mean that the distribution of *spa* types in MSSA is much higher than MRSA types.

We were not able to find any *spa* types which we confidently label it as MRSA, although t037 was the most common type among MRSA isolates in our region but it was also found among 15 MSSA isolates. In contrast, most isolates belonging to type t937 were MSSA and a few of them belong to MRSA, and this difference was statistically meaningful. The common *spa* types in MRSA isolates in different region was not similar, in study conducted by Strommenger in Germany, common types were t032 and t003 in MRSA and t008 in MSSA isolates [15] and in Larry Koreen study *the* common type was t033 [8]. Based on these latter findings the prominent *spa* type of *S. aureus* are varied in different parts of the world.

We detected that some *spa* types, t021, t330, t267, t436, t1149, t2313, are conclusively found among MSSA isolates. Based on our findings the question is whether this observation is due to any relation between these types and sensitivity to methicillin or it is an accidental phenomenon? Answering to this question requires a larger sample population.

The distribution of *spa* types among *S. aureus* which were isolated from healthy carrier, and patients, was similar. The most common types among healthy carriers and patients were t937 and t037 respectively. Although none of *spa* types was restricted to either of patients or healthy carriers, but some *spa* types t012, t267 and t660 predominantly detected in patients and some other *spa* types including t701, t779 and t2313 were more common in healthy carriers. *Spa* type t660 only was detected in children less than 3 years old, can this type be considered as a marker of infection on children? To answer these question extensive studies on this *spa* type are suggested.

We found seven new *spa* types in our region where out of them five types exclusively were isolated from patients, and further studies are required to address the key role played by these 5 *spa* types in pathogenicity, virulence and toxicity of *S. aureus*.

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