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*Research Article*

# Staphylococcal variable number tandem repeat (VNTR)-spa genotyping and their role in phylogenic study

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**Abstract**
*Staphylococcus aureus* protein A is considered a vital virulence element determinant of its pathogenicity. Its sequence diversity aids in staphylococcal typing and phylogeny. The present study aimed to study the genotyping method for *S. aureus* isolates by applying Spa typing (variable number tandem repeat) and their role in the phylogenic study. Twenty *S. aureus* isolates were achieved from various clinical isolates and subjected for complete identification and diagnosis. Later on, these isolates were subjected to DNA extraction and PCR for amplification and sequencing of SPA gene. Spa genotyping showed that out of 20 isolates and their amplified *spa* gene, 8 different types among the 18/20 isolates were detected and 2/20 isolates could not be typed, as the most commonly observed Spa were t304 (35%), t491 (15%) followed by t078 and t059 (10%). Finally, depending on the bacterial phylogenetic relationships, *S. aureus* isolates were categorized into 2 clades. The first one contained 18 isolates and the second one contained 2 isolates. Most Spa types were included in clade A (18 isolates) whereas only 2 isolates were involved in clade B. The isolates in clade A were grouped into 3 different groups established on the dissimilarity in tandem repeats of the Spa gene. Cluster 1 contained t304, t078, t044 Spa types, cluster 2 contained t059, t4870 and t386 Spa types and cluster 3 contained t491 and t091 Spa types. Clade B contained 2 Spa types (unknown). The utility of the present work is the application of repetitive tandem repeats within the spa gene for phylogenetic analysis of Staph aureus clinical isolates.

**Keywords:** *spa* gene, SPA genotyping, *Staphylococcus aureus*, Xr region

## INTRODUCTION

*Staphylococcus aureus* is a highly virulent pathogen that settles different sites of the body, including skin and mucous membranes of the nasopharynx, Gastrointestinal tract GIT and perineum and the genitourinary tracts (den *et al*.,2013). It can be a cause of range of minor to critical infections in nearly most body tissues, chiefly in immune-deficient people like pneumonia, bloodstream infections and septic shock, skin and soft-tissue infections, burns and surgical-site infections in addition to endocarditis and many numerous infections (Lu & DeLeo, 2015). It can cause extensive series of human and animals' infectious diseases that carry a weighty reverse influence on public health.

*S. aureus* is a medically important pathogen that is associated with serious diseases and pathogenesis, as it has the ability to spread from an initial entry site, such as from venous and indwelling urinary catheters to whole organs, including the bones, lungs, and cardiac valves; where it expresses various virulence factors involved in adhesion, invasion, immune evasion, and toxin production to facilitate its establishment on these vital sites, in addition to its ability to acquire and transfer resistance to many types of commonly used antibiotics (Gnanamani *et al*., 2017).

These virulence factors can work together in order to aid the pathogenesis process and disease production and aid in its ability to cause different infections, varying from minor to life-threatening infectious diseases (Rasheed and Hussein,2021). Different staphylococcal

surface proteins can act as adhesins, and tissue invasion or are involved in the evasion of immune system mechanisms. One of these pathogenicity factors involved in immune evasion is the staphylococcal protein A (SPA), produced by all staphylococcal isolates. It binds to immunoglobulins molecules, inhibits opsonization and phagocytosis mechanisms, and can act as a superantigen (Balachandran *et al*.,2018).

Genotyping of *S. aureus* is an important tool for investigating and detecting healthcare-associated infections and helps to control and prevent MRSA that causes hospital-acquired or community-acquired infections (Rezai *et al*.,2020). This typing can be done depending on variable molecular typing procedures. Many techniques have been developed and applied for the documentation and comparison of staphylococcal isolates in different epidemiological works, but many drawbacks are associated with these conventional typing methods, as typing methods using pulsed-field gel electrophoresis and phage typing can be accomplished only in expert laboratories and time-consuming (Javid *et al*.,2018). Thus, the sequencing of DNA repeated short sequences of the polymorphic X region of the staphylococcal protein A gene, *spa*, that comprising of a variable number sequences of 21 to 27-bp (24 bp average) length, is an unconventional technique for *S. aureus* genotyping (Park *et al*.,2017).

Therefore, this work aimed to study the genotyping method for *S. aureus* isolates by applying Spa typing (variable number tandem repeat) and their role in the phylogenic study.

## MATERIALS AND METHODS

### Collection of samples

A total of (110) clinical samples that obtained from diverse infection sites (burn, wound and urine) at Al-Hilla General Teaching Hospital, Babylon city, Iraq, looking for *Staphylococcus aureus* isolates. Its identification and the definitive diagnosis were accomplished according to Forbes *et al*. (2007).

### DNA extraction

The whole genomic DNA was purified by applying a Kit and used according to the manufacturing company's instructions (Genaid, UK).

### D-Primer sequences and PCR

Two primers for amplification of *spa* gene and detection of single locus-variable number tandem repeat among the Xr region of this gene; these primers are *spa*-1113 F: (5′-TAAAGACGATCCTTCGGTGAGC-3′) and *spa*-1514 R: (5′-CAGCAG TAGTGCCGTTTGCTT-3′), a PCR was performed in a full volume of 25 µl (Primers 1.5 x2, DNA 3 µl, Master mix of 12.5 µl, nuclease free water 6.5 µl), then DNA amplification was carried out with the thermal cycler by the application of primers with conditions of amplification as (Initial denaturation at 94ºC for 5 min., followed by 35 cycles of the three main steps of denaturation at 94ºC for 45 sec, Annealing at 60ºC for 45 sec and extension at 72ºC for 90 sec, finally 10 min of extension to ensure adequate extension) with a product of about 200-500bp. After completion, products were visualized by applying gel electrophoresis, stained with ethidium bromide and capturing photos with digital camera.

### DNA sequencing and *Spa* analysis

According to the manufacturer's instructions, all sequencing reactions were performed at Macrogen Company in Korea using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit and an ABI 3100 Avant Genetic Analyzer (Applied Biosystems). StaphType software (version 1.4; Ridom GmbH, Würzburg, Germany) was used to assign spa types (Harmsen *et al*.,2003). Sequence annotations (repeat score) of studied isolates were done according to Kreiswirth Method (Shopsin *et al*.,1999; Koreen *et al*.,2004).

## RESULTS AND DISCUSSION

Out of 20 isolates, 100% showed the PCR products of *spa* Gene with a size range from 200-500 bp with a single PCR band showing the number of 24bp repeat units; within *Spa* Genes, as revealed in Fig. 1.

Out of 20 strains identified, 8 different types among the 18/20 isolates were detected, and 2/20 isolates could not be typed, as the most commonly observed Spa were t304 (35%), t491 (15%) followed by t078 and t059 (10%) as shown in Table (1).

This method was found in sequencing the polymorphic Xr region's VNTR. The highly conserved areas around the Xr region allowed for the annealing of the primers needed for amplification and sequencing, as well as the analysis of sequence data and the identification of iso-

**Table 1.** Number and percentages of *Spa* types among the studied *S. aureus* isolates

| Spa types | Number | Percentage |
|---|---|---|
| t304 | 7 | 35% |
| t491 | 3 | 15% |
| t386 | 1 | 5% |
| t078 | 2 | 10% |
| t059 | 2 | 10% |
| t044 | 1 | 5% |
| t14870 | 1 | 5% |
| t091 | 1 | 5% |
| Unknown* | 2 | 10% |
| Total | 20 | 100% |

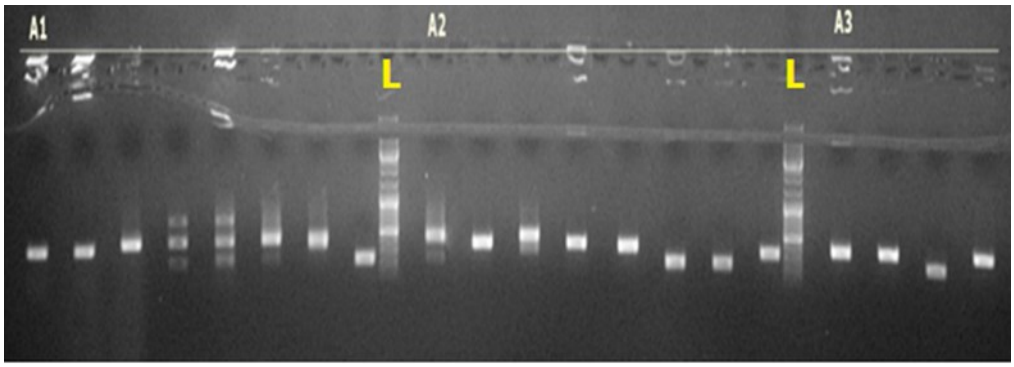


**Fig. 1.** *Showing the agarose gel electrophoresis of PCR products obtained by using Spa-specific primers. Lanes A1-D3 represent the identified spa gene products with variable sizes (200-<500bp). L: Ladder, GeneRuler DNA Ladder was used as 100bp DNA ladder*

lates' Spa types, which was done using the ridom Staph types software, as shown in Table 2.

The results in this study agree with Mohammed *et al*. (2021), who found that the tested isolates belonged to t304 (30.3%) but disagreed with this study which found the *Spa* type t037 detected in 19.4%, but in the present study, this type was not detectable.

The difference in the *Spa* types described in the neighboring countries or local regions may be due to cross-border patients' motility or migIration from one country to another over the years (Mohammed *et al*.,2021). Another study detected (52) various *Spa* types amongst (616) MRSA strains. The most common type included t003, t586, t014 and t002, which were not typable in the present study. It is significant to remember that the dominancy of just one Spa type (or few) in a certain region does not mean that the other types of Spa do not exist in these parts of the area (Pomorska *et al*., 2021).

Spa gene typing involves sequencing a single locus with a size of 200–600 kb. The Polymorphic Xr in the Spa gene is comparatively stable and has MSLT and PFGE-level discrimination power (Khademi *et al*., 2016). In present study, tandem repeats of Spa were created and calculated using bioinformatics analysis, and the results revealed partial identity. They were detected using the coordinate of the repeat in the sequence alignment, the quantity and length of repeat units, and the length of the entire variable number tandem repeat. As a result, Spa typing represents an excellent standard for both long-term local epidemiology and international and national surveillance.

Additionally, Mohammed *et al*. (2021) found that *Spa* typing of (36) *Staph. aureus* isolates displayed (11) different *Spa* types; t304 detect in (30%), t044 (8%), t386 (5%) and t14870 in (2.8%), which agree with the

**Table 2.** Spa typing of *S. aureus* isolates

| Strain Name | start pos[1] | repeat units[2] | len in bp[3] | Spa type4 |
|---|---|---|---|---|
| 1_spa | 45 | 9 | 216 | t304 |
| 2_spa | 45 | 9 | 216 | t304 |
| 3_spa | 51 | 11 | 264 | t491 |
| 4_spa | 51 | 11 | 264 | t491 |
| 5_spa | 51 | 11 | 264 | t491 |
| 6s_spa | 45 | 9 | 216 | t304 |
| 7G_spa | 45 | 9 | 216 | t304 |
| 8S_spa | 46 | 3 | 72 | t386 |
| 9S_spa | 292 | 3 | 72 | * |
| 10S_sp | 49 | 9 | 216 | t078 |
| 11S_spa | 150 | 9 | 216 | * |
| 12S_spa | 47 | 9 | 216 | t304 |
| 13S_spa | 47 | 9 | 216 | t304 |
| 14S_spa | 50 | 3 | 72 | t059 |
| 15S_spa | 45 | 3 | 72 | t059 |
| 16S_spa | 43 | 7 | 168 | t044 |
| 17S_spa-1113f | 46 | 9 | 216 | t078 |
| 18S_spa | 44 | 9 | 216 | t304 |
| 19S_spa | 45 | 4 | 96 | t14870 |
| 8G_spa | 49 | 10 | 240 | t091 |


**Fig. 2.** *Sequence Annotations (repeat score) of studied isolates according to Kreiswirth Method*

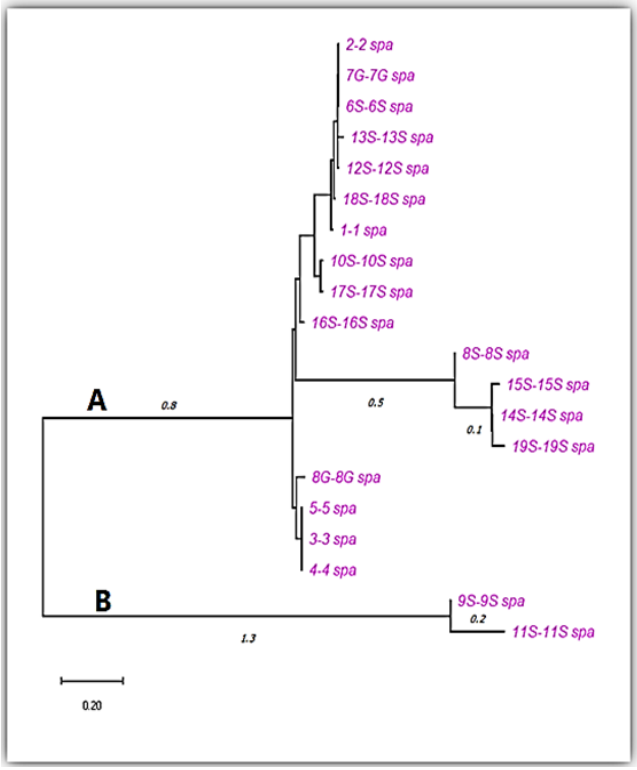


**Fig. 3.** *Phylogram of S. aureus isolates using spa sequences. In this analysis, the Maximum Likelihood method and Tamura-Nei model (1,2) were used to build a tree. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches) ; S*= the number of *S. aureus* isolates

results detected in the present study.

In the present work, *S. aureus* strains, depending on phylogenetic relationships, were classified into two clades. The first one contained 18 isolates and the second one contained 2 isolates, as shown in (Fig. 3). The most *Spa* types were included in clade A (18 isolates). In contrast, only 2 isolates were involved in clade B. The isolates in clade A were clustered into 3 various groups based on the discrepancy in tandem repeats of the Spa gene, cluster 1 contained the t304, t078, t044 Spa types, cluster 2 contained t059, t4870 and t386 Spa types and cluster 3 contained t491 and t091 Spa types and clade B contain 2 Spa types (unknown).

Khademi *et al*. ( 2016) demonstrated that any two Spa genotypes that shared the majority of the same repeats or differed in a single deletion or insertion of the nucleotide sequence fit into the same clump. The high Spa gene diversity found among different strains' sources was compared to the consensus and control obtained from National Center for Biotechnology Information NCBI. The results publicized that some chains took place in nucleotide sequence compared to the control Spa gene and most isolates displayed different genetic variations.

The different genetic cluster groups were shown dendrogram and phylogenetic tree, found that the different genetic clusters may exhibit the same type of Spa tandem repeats. This may be due to the similarity between more than one type of Spa repeats, The discrimination between *S. aureus* isolates is possible by determining the repeat sequence number within the x-region of spa gene (Kareem *et al.*, 2020).

Since every Spa sequence contained a unique pattern, the enormous variety in the Spa genopattern could be observed. As a result, there was no genetic relationship regarding the sources of infection.

## Conclusion

spa genotyping is a good tool for rapid diagnosis, typing and epidemiological studies of *S. aureus,* especially during epidemics and multidrug-resistant nosocomial staphylococcal infections. Spa genotyping indicated that out of 20 isolates and their amplified *spa* gene, 8 different types among the 18/20 isolates were detected and 2/20 isolates could not be typed, as the most commonly observed Spa were t304 (35%), t491 (15%) followed by t078 and t059 (10%). Based on phylogenetic relationships, *S. aureus* strains were classified into two clades, the first one contained 18 isolates and the second one contained 2 isolates. The novelty of this work were application of repetitive tandem repeats within the spa gene for phylogenetic analysis of Staph aureus clinical isolates.

### Ethical approval

The study was conducted in accordance with the moral guidelines found in the Declaration of Helsinki. Before collecting any samples, the patients' verbal and written consents were obtained. The agreement formula, personal data and study protocol were studied and accepted by a local ethics committee according to Certificate number 112 (including the number and the dated 11/08/2021) .

## Conflict of interest

The authors declare that they have no conflict of interest.

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