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Detection of Novel *spa* Types in Clinical Isolates of Methicillin-Resistant *Staphylococcus aureus* in Iraq

Hussam Sami Awayid*

Department of Medical Laboratory Techniques, Suwaira-Technical Institutes, Middle Technical University, Wassit, Iraq

Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a gram-positive bacterium that is an opportunistic pathogen, causing infections in hospital settings and communities. MRSA has become a significant and increasing problem in Iraq. The aim of this study was to evaluate the genetic mutations of MRSA strains, especially in the *spa* gene, from patients in Wassit, Iraq.

Methods and Results: Biochemical tests were conducted to identify *S. aureus* isolates and then on MRSA. The MRSA was identified by the Chrome Agar method and confirmed by PCR with genotyping of the *mecA* gene. The disk diffusion method was used to detect antibiotic resistance to three different common antibiotics used at Wassit hospitals. The Vitek-2 compact system was utilized for the detection of the minimum inhibitory concentration (MIC) of vancomycin. All MRSA strains in this study were tested to screen the *mecA* gene, with 21 strains subjected to the molecular typing method for the *spa* gene. Out of 166 samples, 132(79.5%) contained *S. aureus* and 34(20.4%) were identified as MRSA. Genotyping showed that out of 34 MRSA, 31(91.2%) isolates were *mecA*-positive. The *spa* gene was detected in 21(61.8%) isolates out of 34 MRSA samples. The *spa* typing of 21 MRSA samples revealed four different *spa* types, as follows: t386 (3/14.3%), t3576 (1/4.8%), t10002 (1/4.8%), and t10234 (1/4.8%). High polymorphism rates were shown in isolates of *spa* type t386.

Conclusion: Our data represent the first report to detect novel mutations in the *spa* gene in the MRSA clinical isolates from Wassit hospitals, Iraq.**(International Journal of Biomedicine. 2024;14(1):127-133.)**

Keywords: methicillin-resistant *Staphylococcus aureus* • *mecA* • *spa* type

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Abbreviations

MIC, minimum inhibitory concentration; **MRSA**, methicillin-resistant *Staphylococcus aureus*

Introduction

Staphylococcus aureus is the main pathogen that infects humans.[1] Hospital-acquired methicillin-resistant *S. aureus* (MRSA) is accountable for the worldwide spread of MRSA.[2] This pathogen has several virulence factors, such as staphylococcal protein A (spa), with multidrug resistance ability.[3]

However, it should be noted that the detection of resistance via phenotypic procedures remains prevalent, and the amount of available information about the molecular characterization of clinical MRSA strains is relatively limited.[4] MRSA strains resistant to multiple antibiotics are considered a significant challenge to the efficacy of relevant drug therapy. It is imperative to investigate the prevalence of these types of MRSA that are resistant to treatment.[5] Genotypic techniques can quickly and reliably classify the interrelatedness of clinical isolates. These techniques have a promising role in detecting eruptions and monitoring isolates spreading through specific regions.[6]

The mutation in the *spa* gene of this bacteria results from the diversity of the site in chromosome called the x region in the bacteria, and the typing method relies on varying genetic elements at area x in the *spa* gene.[7] The *spa* gene that determines virulence is intimately linked to the mechanism

***Correspondence:** Hussam Sami Awayid, husam.sami@mtu.edu.iq

and potency of infection. This marker exhibits a high degree of variability and offers valuable insight into strain.[8]

Risks associated with MRSA colonization and infections are no longer restricted to hospitals; therefore, sequence typing and *S. aureus* protein A (spa) detection, which can help track outbreaks, are useful for identifying MRSA strains and colonization sources, and for differentiating between infections in the community and in the hospital. The development of community-associated MRSA has altered MRSA epidemiology. Genetically, hospital infection differs from community infection in that the latter is more sensitive to non-lactam antibiotics.[9] The molecular features of MRSA can exhibit variability across hospitals located in the same nation.[10] A big advantage of molecular methods such as sequencing DNA is that they give simple, fast results for huge amounts of data that can be easily utilized to understand the evolution of these strains.[11] The molecular *spa* typing method is a powerful tool for investigating and revealing genetic diversity, especially in multidrug-resistant MRSA.

The aim of this study was to evaluate the genetic mutations of MRSA bacteria, especially in the *spa* gene responsible for evading the immune system, which makes it difficult to treat patients in Wassit.

Materials and Methods

This study was carried out in Al-Aziziyah Hospital and Al-Suwaira Hospital (Wassit Governorate) in the period between August 2022 and May 2023.

A total of 207 non-duplicated strains of *S. aureus* were isolated from outpatients and patients upon admission to hospitals. Clinical specimens included urine, wounds, blood, abscesses, and sputum. When included in the study, demographic characteristics, diagnosis, and smoking habits were considered.

Collection of the different clinical samples

Samples were instantly transported to the Laboratory of Microbiology, Department of Medical Laboratory Techniques, and Middle Technical University. According to Bailey and Scott's Diagnostic Microbiology, blood agar plates were inoculated with collected samples and incubated for 24 hours at 37°C. A single colony was picked up from blood agar plates and inoculated on Mannitol Salt Agar for 18–24 hours at 37°C. Morphological and biochemical features were then conducted to identify the isolates. The biochemical tests used in this study were catalase and coagulase. Ultimately, *S. aureus* isolates were inoculated with Trypticase soy broth (Himedia, India) with 20% glycerol and kept at -80°C in the freezer until used for the following test.[12]

Diagnosis of MRSA by Chromogenic Modified Agar

To ensure the diagnosis of MRSA bacteria was cultured, the suspected colonies were inoculated on the chromogenic modified agar base (CAMB), including cefoxitin (30 µg) (CandaLab, Spain) as a supplement, and were aerobically incubated for one day at 35±2°C.[13]

Antibiotics Susceptibility Testing

All isolates were tested against vancomycin (30 µg), methicillin (10 µg), and oxacillin (1 µg) (Bioanalysis, Turkey) through the disk diffusion method, according to the CLSI 2010 recommendations.[14] The Vitek-2 system was used to detect the MIC for vancomycin and quality control for *S. aureus* ATCC 29213.

DNA extraction

The DNA was extracted from colonies of MRSA strains by a Wizard® genomic DNA Extraction kit based on the manufacturer's instructions. The purity of extracted DNA was measured by the nanodrop spectrophotometry technique at 260 nm, and the DNA samples were then stored at -18°C to keep samples when needed for use.

Detection of the mecA gene

For genetic identification, a specific primer to detect the *mecA* gene encoding for methicillin resistance (F: 5'ACGAGTAGATGCTCAATATAA3' and R: 5'-CTTAGTTCTTTAGCGATTGC-3') was used to amplify *mecA* loci in MRSA DNA. The amplicons were then run on agarose gel using electrophoresis and visualized by UV light using an ultra-violet transilluminator. The amplification of the *spa* gene for all MRSA isolates from different clinical samples according to the previous study. [15]

Detection of the spa gene

All isolates were subjected to the *spa* gene. Specific primers (*spa* 1 5'-ATCTGGTGGCGTAACACCTG-3' and s*pa* 2 5'-CGCTGCACCTAACGCTAATG-3') (Alpha, Canada) were for a variable region, amplifying a PCR product size of 1500 bp.

PCR reaction and conditions

The final volume is 25 µl, containing 12.5 µl of master mix (Taq-DNA polymerase, dNTPs, $MgCl_2$, and reaction buffers), 5 µl of DNA, 1µ (10 pmol) forward and reverse primer, and 5.5µl of nuclease-free water (Promega, USA). run under the following conditions: initial denaturation at 95°C for 3 minutes for 35 cycles, followed by denaturation at 95°C for 30 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 1 minute for 32 cycles, followed by a final extension at 72°C for 1 minute. A total of 32 cycles, and then used the next step at 72°C for five minutes, while Spa used 55°C for 30 seconds in the annealing step. Next run-on agarose 1-1.5% (Sigma-Aldrich, USA) and the band of products visualized by a trans-illuminator.

DNA sequencing

According to the Macrogen Company requirement (Seoul, South Korea), 20 µl of spa gene PCR products of selected 34 MRSA isolates were sent for DNA sequencing for both strands. The primers used for DNA sequencing of the X region of the spa gene were as follows: spa-1113f 5'-TAAAGACGATCCTTCGGTGAGC-3', and spa-1514r 5'-CAGCAGTAGTGCCGTTTGCTT-3' (Oliveira et al. 2001).[16] The ABI 3730xl DNA Sequencer was used.

DNA sequence analysis

The sequences obtained were analyzed and aligned using the Ridom Staph Type program (Ridom, Würzburg, Germany). The *spa* typing and evaluation of *spa* types of *S. aureus* strains were performed using the *spa* database http://www.spaserver.ridom.de and http://spatyper.fortinbras.us/. The *spa* type phylogenetic tree was drawn using the Geneious11.1 Prime software (Auckland, New Zealand). There were evolutionary

relationships between genomic sequences of five local isolates, and they were matched with 12 global isolates of *S. aureus* in NCBI from clinical samples.

Documentation in NCBI

All molecular results of local selective *S. aureus* were sent to the NCBI on May 18, 2023, under accession numbers LC768797, LC768793, LC768796, LC768794, and LC768795 for five isolates of *S. aureus* named Amena, Dimashams, KarBane, Mayar, and Mohammed, respectively. They were then documented as five new allelic variations in the GenBank database (Maryland, USA) and certified on May 23, 2023

Results

Out of 207 participants, only 166 responded to our questions, making an answer rate of 80.2%. The participants were between 20 and 50 years old, with a median of 42. The age group of <40 years was the largest. Among 166 participants, women made up 60.8% and men 39.2%. Analysis of clinical conditions showed that pneumonia and urinary catheters were the most common: 27.1% and 24.1%, respectively (Table 1).

Table 1.

Demographic and clinical characteristics of the study participants

Variable	n	Percentage
Age, years		
< 40	98	59.0%
> 40	68	41.0%
Gender		
Males	65	39.2%
Females	101	60.8%
Smoker		
Yes	28	16.9%
No	138	83.1%
Clinical Characteristics		
Bacteremia	28	16.9%
Pneumonia	45	27.1%
Surgical wound infection	30	18.1%
Urinary catheter	40	24.1%
Meningitis	23	13.8%

Out of 166 samples, 132(79.5%) contained *S. aureus* and 34(20.4%) MRSA. Of the 34(20.4%) MRSA isolates, the maximum number were obtained from sputum samples (15/44.1%), followed by urine samples (9/26.5%), wound samples (7/20.6%), pus from abscesses (2/5.9%), and blood samples (1/2.9%) (Table 2). Also confirmed by the used subculture, isolates on the CMAB included cefoxitin. MRSA was maintained without contamination with other genera of bacteria. For more accuracy in the following procedures of phenotypic and genotypic assays, and based on the manufacturer's instructions, all MRSA isolates showed color rose to mauve of the colony after incubation for 24 hours (Figure 1).

Table 2.

Distribution of MRSA isolates between clinical samples.

Samples	n	*S. aureus*	MRSA
Urine	40	31 (23.5%)	9 (26.5%)
Blood	28	27 (20.4%)	1 (2.9%)
Wound	30	23 (17.4%)	7 (20.6%)
Pus from abscesses	23	21 (15.9%)	2 (5.9%)
Sputum	45	30 (22.7%)	15 (44.1%)
Total	166	132 (79.5%)	34 (20.5%)

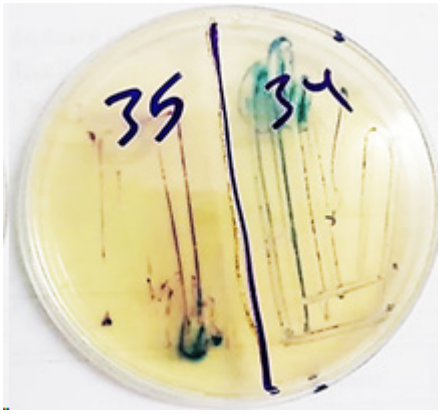

Fig. 1. *Left side: The mauve color shows the growth of MRSA isolates on CMAB media. Right side: The green color shows the growth of Staphylococcus. spp. on CMAB media.*

A phenotypic assay of antimicrobial resistance test for 34 isolates showed high resistance to methicillin and oxacillin in 32 strains of MRSA (94.1%). Genotyping showed that out of 34 MRSA, 31(91.2%) isolates were *mecA*-positive (Table 3, Fig.2).

Table 3.

Phenotypic and genotypic resistance testing of MRSA isolates.

Samples	n	Van	Oxa	Meth	*mecA*
Urine	10	9	10	10	5
Blood	3	3	4	3	3
Wound	5	2	1	1	7
Abscesses	2	4	3	4	4
Sputum	14	13	14	14	12
Total	34	33	32	32	31

Van = vancomycin, Oxa = oxacillin, Meth = methicillin

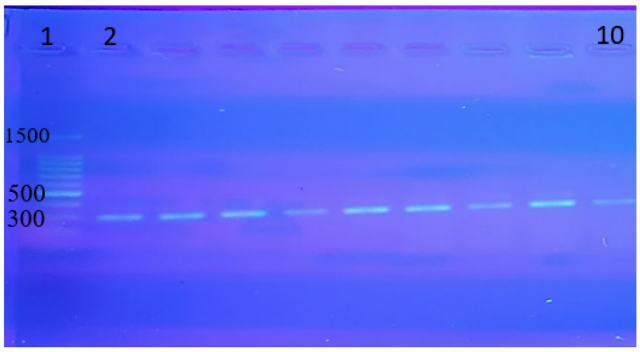

Fig. 2. *PCR products of mecA gene (300 bp) for MRSA isolates (Lanes 2-10); Lane 1 - DNA ladder (1500 bp).*

As for the vancomycin MIC value <0.063-16 µg/ml, at breakpoints ≤2/4–8/≥16 µg/ml, 33(97.1%) strains showed resistance to vancomycin.

The *spa* gene was detected in 21(61.8%) isolates out of 34 MRSA samples (Figure 3). The *spa* typing of 21 MRSA samples revealed four different *spa* types, as follows: t386 (3/14.3%), t3576 (1/4.8%), t10002 (1/4.8%), and t10234 (1/4.8%). High polymorphism rates were shown in isolates of *spa* type t386 (Table 4).

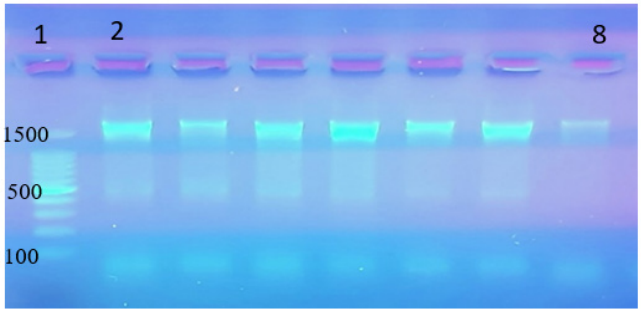

Fig. 3. *PCR products of the spa gene for MRSA isolates (Lanes 2-8); Lane 1 - DNA ladder (1500 bp).*

Table 4.

The spa typing of 21 MRSA samples.

Isolate number of MRSA	*spa*-type (Ridom)	Tandem Repeats
7,10,16	t386	07-23-13
5	t10002	11-10-21-17-34-24
19	t10234	11-10-21-17-34-24-34-22
35	t3576	26-23-17-34-17-20-17-20-17-12-16

The sequences of the *spa* gene for the five MRSA local isolates were analyzed by BLAST with Geneious software. The results showed an alignment feature as described for genomic sequences for linear DNA lengths (1,066 bp, Accession number: LC768794 *S. aureus* Mayar), (728 bp, Accession number: LC768797 *S. aureus* Amena), (961 bp, Accession number: LC768793 *S. aureus* Dimashams), and (932 bp, Accession number: LC768796 *S. aureus* KarBane) with a database of NCBI, except for one in length. Accession number LC768795 *S. aureus* Mohammed had 99% genetic identity with duplicates of the genetic bases for query nucleotide sequence from 839 to 899 bp, and two nitrogen bases (TT) were not found in the query sequence, which indicated deletion mutation at locus 241 bp. In addition, two nitrogen bases (GA) were found in the query sequence but not found in the database, meaning an insertion mutation at locus 298 bp, according to the report of the BLAST result. There are clear reasons for the different identities of the last sequence compared to the first four sequences.

The colored accession number of local MRSA isolates for the spa gene showed variable diversity among bases, compared to control obtained from NCBI in clinical samples. The results data has been split into three monophyletic branches; the first branch contains the control strains NCBI (LT992466, CP077922, CP013616, CP060597, AP019713, CP038270, and CP006630). Most isolates showed diversity in three groups (Figure 4).

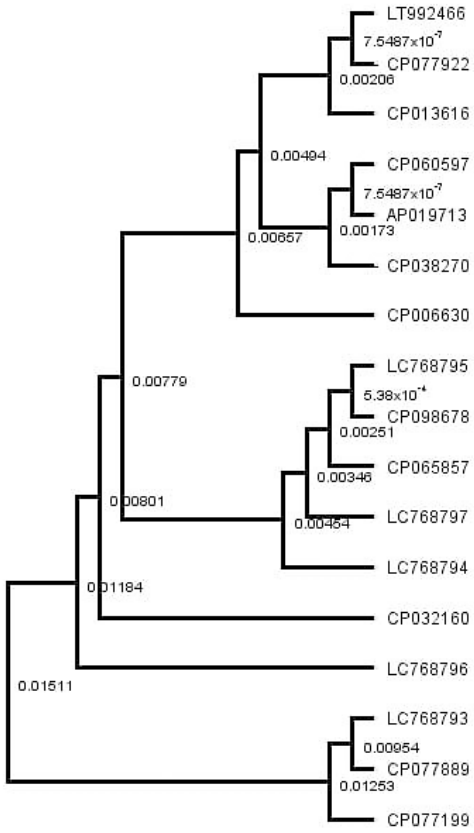

Fig. 4. *Phylogenetic tree to the sequence of the spa gene related to MRSA local isolates from clinical samples.*

Group 1 contains the NCBI control strains (CP098678 *S. aureus* strain TCD12 and the CP065857 *S. aureus* strain CC1153-MRSA). The result revealed that the local isolate

(LC768795 *S. aureus* Mohammed) was similar to the NCBI control strain (CP098678 *S. aureus* TCD12). In contrast, the other local isolates (LC768797 *S. aureus* Amena and LC768794 *S. aureus* Mayar) had distant genetic values of 0.00346 and 0.00454, respectively, with the control CP065857 *S. aureus* strain CC1153-MRSA.

In Group 2, a local isolate (LC768796 *S. aureus* KarBane) had a genetic distance with CP032160 *S. aureus* strain SA G5 at a value of 0.001511.

In Group 3, a local isolate (LC768793 *S. aureus* Dimashams) showed a genetic distance with NCBI control (CP077889 *S. aureus* strain 333) at a value of 0.00954 (Figure 4).

Discussion

Identifying bacterial species typically relies on culture-dependent phenotypic tests. Nevertheless, the reliability of these methods can be limited due to the variable expression of phenotypic characteristics. Additionally, the databases used in these tests are often restricted to a subset of bacterial species, further limiting their overall efficacy.[17] The distinction between *Staphylococcus* species and *Micrococcus* bacteria was made using the catalase and oxidase tests, respectively, with positive outcomes. The bacterial colonies from swabs cultured on an MSA medium were shown as yellow, and under a microscope, they were cocci in form, clustered, and had positive stains based on cultural and morphological criteria. The isolates were reported as positive for coagulase, as revealed by the biochemical analysis. This was in agreement with the study achieved in Jordan.[18]

Detecting accurate species is of immense value to the diagnosis and helps distinguish rare drug-resistant features. A desirable way must possess the potential to distinguish between closely related species with high discriminatory power. It should also be cost-effective, rapid, and reproducible. In this regard, genetic techniques that rely on PCR or sequencing are promising alternatives for identification.[19]

As per our data, the notable spreading of the *mecA* gene in local *S. aureus* isolates of MRSA was at a percentage of 20.4%, which was in agreement with the study achieved in the USA at a percentage of 20% from different clinical samples.[20]

Another study in Eritrea showed a high proportion of MRSA isolates—72%.[21] MRSA prevalence has increased due to various risk factors, including MRSA carriage by healthcare workers and patients, improper use and overuse of antimicrobials, inadequate adherence to the hand-hygiene protocol, extended hospital stays, and a lack of comprehensive bundle approaches.[22]

In Iraq, the augmented frequency of incidence and hospitalization has led to a significant concern regarding MRSA. Given the gravity of the situation, prompt and precise classification of MRSA isolates has become an essential prerequisite for effective screening, epidemiology, surveillance, and infection control. In this context, accurate typing of MRSA isolates is crucial in averting the spread of this perilous pathogen. Chrome MRSA agar employs a chromogenic substrate to differentiate *S. aureus*, especially MRSA, from other pathogens and selectively cultivate MRSA in the presence of antibiotics. Chromogenic agars have been instrumental in reducing the time and cost associated with confirmatory testing, which can be time-consuming as well as costly. CHROMagar Staph aureus has been evaluated in several studies, and a high sensitivity for S. aureus has been reported.[23]

Anand et al.[24] evaluated the efficacy of the cefoxitin disc diffusion test to characterize MRSA and compare it with oxacillin agar screening and detection of the *mecA* gene by PCR. The results of the cefoxitin disc diffusion test were in concordance with the PCR for the *mecA* gene. In our study, 91.2% of MRSA isolates showed positive results for the *mecA* gene, which was almost similar to the outcomes reported in a study conducted by Dhungel et al.,[25] who found 94.1% positivity for the *mecA* gene among the MRSA isolates. The absence of the *mecA* gene in our three MRSA isolates suggests the presence of an alternate pathway for methicillin resistance rather than the conventional *mecA* gene-mediated mechanism.[26]

Vancomycin-resistant S. aureus (VRSA) in the current study was 97.1% from different clinical samples, which agrees with the result of a study by Rehman et al. performed in Pakistan (92.6%).[27] The resistance rate for oxacillin among MRSA isolates was 94.1%, while the rate was higher at 100% in the previous study.[28]

Bacterial resistance to a particular antibiotic may be ascribed to random genetic mutations.[29] This leads to the bacterial microbe acquiring an innate resistance to a greater dosage of an antibiotic due to its frequent usage.[30] Bacteria also can acquire antimicrobial resistance through horizontal gene transfer.[31]

In our study, the *spa* typing of 21 MRSA samples revealed four different *spa* types (t386, t3576, t10002, and t10234) with a higher prevalence of t386 (14.3%) versus other types with a prevalence of 4.8% for each. In contrast, in a study by Mohammed et al.[32] the prevalence of t386 was 5.5%. In a study by Hadyeh et al.[33] performed in Palestine, the *spa* type t386 (CC1) was at a percentage of 12.5%.

The first report from Wassit, which was documented in NCBI, revealed three novel types of staphylococcal protein A. These isolates showed methicillin and vancomycin resistance in samples of sputum and wounds from patients in the Aziziyah and Suwaira Hospitals. The current study analyzed unique *spa* types not previously documented in NCBI. Some *spa* types were also absent in neighboring countries or local regions. This discovery could potentially be attributed to the mobility of patients across borders or migration to and from Iraq, both during and following the Iraq War. These findings contribute significantly to the knowledge of these bacteria in central Iraq.

This study has limitations due to the limited number of MRSA samples studied; therefore, additional research is required with a more significant number of samples in different regions of the country.

In conclusion, the investigation revealed a noteworthy escalation in the frequency of MRSA infections in Wassit. Using *spa* typing, this analysis identified four distinct MRSA *spa* types, with *spa* t386 being the most prevalent. The data

on hospital-acquired MRSA infections may be advantageous in the comprehensive features of these bacteria at Wassit Governorate and in devising an appropriate preventive and therapeutic strategy. It is also imperative that future research endeavors to focus on identifying a variety of MRSA types spreading in Iraqi healthcare facilities. This information will be crucial for developing efficient interventions to restrict the spread of this antibiotic-resistant pathogen in the country. The outcomes of the current study also emphasize the need for enhanced surveillance and control measures to minimize the burden of MRSA infections in Iraq.

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Competing Interests

The author declares that there is no conflict of interest.

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