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Spa typing of Methicillin-Resistant *Staphylococcus aureus* isolated from clinical samples of hospitalized patients, a study in the Wasit province of Iraq

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A B S T R A C T

Introduction: Since its discovery in 1961, methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognized as a significant healthcare-associated pathogen (HA-MRSA) and a notorious 'superbug'. Typing is crucial for surveillance, epidemiology analysis, infection control of MRSA and sequencing of the *spa* gene is one of the most common methods used for determining the origin of this bacterium in humans and animals. This research aimed to determine the antibiotic resistance and *spa* type of *S. aureus* strains collected from outpatients in two hospitals in the Wasit province of Iraq.

Material & Methods: The study analyzed 200 outpatient MRSA isolates by collecting nasal and sputum samples from patients. Standard biochemical and molecular methods based on the nuc gene were used to identify *S. aureus* bacteria and amplify the *mecA* and *spa* genes. The Kirby-Bauer disc diffusion method was employed to determine the antibiotic sensitivity of the isolates using penicillin, cefoxitin, vancomycin, gentamicin, erythromycin, tetracycline, imipenem, clindamycin, chloramphenicol and rifampicin.

Results: Thirty-five (17.5%) out of 200 isolates were identified as *S. aureus* by biochemical and molecular methods. The prevalence of MRSA was more common in women than in men. Antibiogram results showed that most of the isolates were resistant to penicillin (94.2%) and sensitive to imipenem (100%), clindamycin (100%), and chloramphenicol (100%). Of these 35 isolates, 30 (87.5%) and 26 strains (74.3%) were positive for the *mecA* and *spa* genes. Typing based on *spa* gene sequencing revealed four different patterns: t386, t3579, t10002 and t10234.

Conclusion: Variations in the *spa* gene among different *S. aureus* isolates may be of clinical importance when treating staphylococcal infections. In this study, *spa* typing revealed four different patterns in Iraq, representing diagnostic and therapeutic implications.

Keywords: *Staphylococcus aureus*, MRSA, PCR, *mecA*, *spa* typing

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Introduction

Staphylococci are Gram-positive cocci, facultative anaerobes, non-sporulating, non-motile, catalase-positive and oxidase-negative bacteria, first identified by Ogston in the 1880s in the purulent fluid from a leg abscess and not long after, formally isolated by Rosenbach (1). *Staphylococcus aureus* is a normal inhabitant of the skin and mucous membranes of healthy animals and humans. However, it can be an opportunistic pathogen and cause several infectious diseases such as skin and soft tissue infections, endocarditis, bacteremia and osteomyelitis infections in humans and animals. This bacterium can spread through air, contaminated surfaces and carrier animals or humans (1).

Antibiotic resistance is the most important challenge in treating bacterial infections (2). Although penicillin provided short-term relief from β-lactamase-positive bacterial infections, considerable resistance was raised in the 1940s. The first semi-synthetic anti-staphylococcal penicillin was developed around 1960. Within a year of clinical use, methicillin-resistant *S. aureus* (MRSA) was reported. Genomic evidence suggests that methicillin resistance existed before the first clinical use of anti-staphylococcal penicillin and it is mediated by the *mecA* gene which can be achieved by bacteria through the horizontal transfer of a mobile genetic element encoded by the staphylococcal cassette chromosome *mec* (SCC*mec*). The *mecA* gene encodes penicillin-binding protein 2a (PBP2a), an enzyme localized in the peptidoglycans of the bacterial cell wall responsible for binding to the antibiotic. Moreover, PBP2a has a low affinity for β-lactams, resulting in resistance to this entire group of antibiotics. MRSA was first observed in clinical samples isolated from hospitalized patients in the 1960s and since then, MRSA infections have spread rapidly in the community. Due to their high morbidity and mortality rates, MRSA infections are considered one of the most significant nosocomial diseases in the world and a major public health problem (3). Also, MRSA is frequently encountered as a source of infections in healthy people without any obvious health risk factors outside of medical facilities (4).

The *spa* gene encodes protein A, which contains three distinct functional regions: Fc-binding region, X region and C terminus. The gene sequence corresponding to the X region has a variable number of 24-bp repeats. In this regard, *spa* typing based on the sequencing of highly polymorphic region X offers a reliable method for differential subtyping of *S. aureus*. The *spa* gene encodes a surface protein that enhances *S. aureus* pathogenicity by binding to immunoglobulins, disrupting their opsonizing function and preventing the phagocytosis of bacteria. The *spa* gene carries variable numbers of 21- to 27-bp repeats and *spa* typing of *S. aureus* isolates shows diverse patterns in different geographic locations around the world (5).

The polymorphic region of the *spa* gene, encoding staphylococcal protein A, is particularly useful for differentiating between various *S. aureus* isolates and investigating both the local and global epidemiology of *S. aureus*. Genetic typing methods hold significant importance for investigating the origin and transmission routes of MRSA. It is crucial to use these methods to characterize the genetic profile of clinical isolates and to discern between different bacterial isolates (6, 7).

Various molecular epidemiological methods have been used for MRSA surveillance and one of the most important procedures used for MRSA typing is through sequencing of the *spa* gene, which offers a valuable, simple, cost-effective and standardized nomenclature tool. This process requires the assessment of polymorphic repeats within the X region of the *spa* gene (8).

The rate of MRSA infections has considerably increased in Iraq over time. Therefore, the present study was conducted to determine the prevalence and types of MRSA isolates from outpatients admitted to different wards in two hospitals in the Wasit province.

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Materials and methods

Sample collection

Between March and April 2022, 200 sputum and nasal mucosal samples were collected from 40-45-year-old men and women who were suffering from different illnesses and clinical symptoms such as fever and referred for treatment to the Al-Zahra and Al-Karama hospitals in the Wasit province of Iraq.

S. aureus isolation and identification

The specimens were enriched in brain heart infusion broth (incubation at 37°C for 24 h) and then cultured on mannitol salt agar (MSA) on the following day (incubation at 37°C for 24 h) (9).

After growing the cultures, the colonies showing mannitol fermentation in the mannitol salt agar medium were designated as pathogenic staphylococci. *Staphylococcus* spp. and *S. aureus* were characterized by Gram staining, coagulase test, catalase test and growth in CHROM agar medium (for chromogenic *S. aureus*, 24 h at 37°C). Coagulase-positive, catalase-positive and purple or marshmallow-colored colonies were identified as *S. aureus* (10). *Staphylococcus aureus* isolates were inoculated in trypticase soy broth (TSB) (Merck, Germany) containing 15% glycerol and kept at −20°C until use.

Antibiotic susceptibility test

Antibiotic susceptibility was assessed using 10 antibiotic disks using the Kirby-Bauer disc diffusion method and according to Clinical Laboratory Standards Institute (CLSI) guidelines on Mueller-Hinton agar (11). The disks and concentrations of the antibiotics were as follows:

penicillin (10 units), cefoxitin (30 µg), vancomycin (30 µg), gentamicin (10 µg), erythromycin (15 µg), tetracycline (30 µg), imipenem (5 µg), clindamycin (2 µg), chloramphenicol (30 µg) and rifampicin (15 µg).

Molecular identification

DNA extraction and PCR

The extraction of *S. aureus* genomic DNA was carried out according to the instructions noted in the guidebook of the genomic DNA extraction kit (Geneaid, Germany). DNA samples were stored at −20°C until PCR amplification. The 25 µL PCR reaction mixtures contained approximately 100 ng chromosomal DNA (2 µL), 2 µL oligonucleotide primers (10 pM each), 12.5 µL 2× MasterMix (Amplicqon, Danmark) and 8.5 µL DNAase free double distilled water. A thermal cycler (Labnet, USA) was used for amplification with an initial denaturation step (94 °C, 5 min) followed by 35 cycles of denaturation (95 °C, 45 s), an annealing step (58 °C, 45 s), and an extension step (72 °C, 60 s); and a final extension step at 72 °C for 7 min. Three pairs of primers, targeting nuc, *mecA* and *spa* genes were used as detailed in Table 1.

Table 1. List of oligonucleotide primers used in this study.

Primer name	Sequence (5´-3´)	Target gene	PCR amplicon (bp)	Reference
nuc1	GCGATTGATGGTGATACGGTT	*nuc*	297	12
nuc2	AGCCAAGCCTTGACGAACTAAAGC			
***mec*F**	ACGAGTAGATGCTCAATATAA	*mecA*	293	13
***mec*R**	CTTAGTTCTTTAGCGATTGC			
***Spa*1**	ATCTGGTGGCGTAACACCTG	*spa*	Variable	14
***Spa*2**	CGCTGCACCTAACGCTAATG			

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Sequence and spa type analyses

To determine the sequences of the *spa* gene, the PCR product of 33 representative selections of the isolates (Table 3) was purified using an available PCR product purification kit (Yekta Tajhiz Azma, Tehran, Iran) following the manufacturer's instruction. The purified PCR products were sequenced commercially (Macrogen, Seol, Korea), using both primers used in the PCR. The obtained sequences were assembled using the SeqMan module in the Lasergene suite (DNAstar, Inc.) and analysed using the Ridom StaphType™ software, (Ridom GmbH, Würzburg, Germany).

Results

S. aureus identification

The results of bacterial culturing and biochemical analysis of the 200 clinical samples isolated from outpatients showed that 35 (18%) isolates were identified as *S. aureus*.

The identification of these isolates was confirmed by 24 hours of culturing on mannitol salt agar (yellow discoloration due to the fermentation of mannitol) and chromogenic agar (pink to mauve colonies) (Figure. 1 A, B).

The results showed that more women (57.1%) were infected with *S. aureus* compared to men (42.8%) (Figure 2).

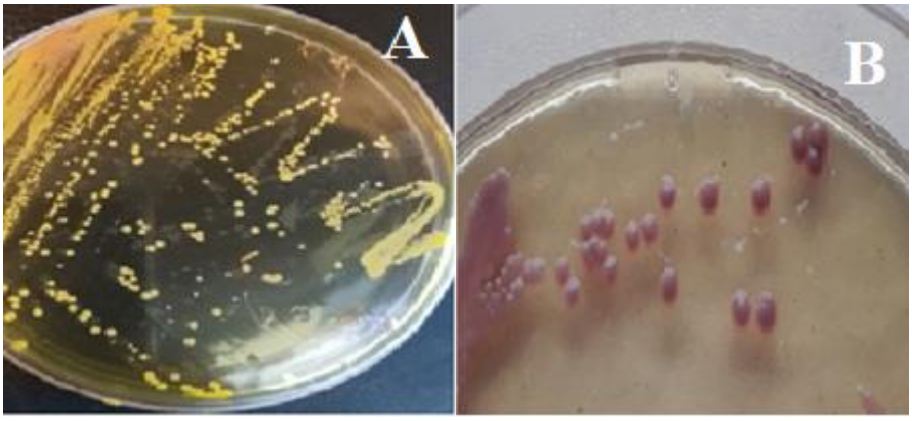


Figure 1. A) *S. aureus* colonies on MSA, B) *S. aureus* colonies on chromogenic agar.

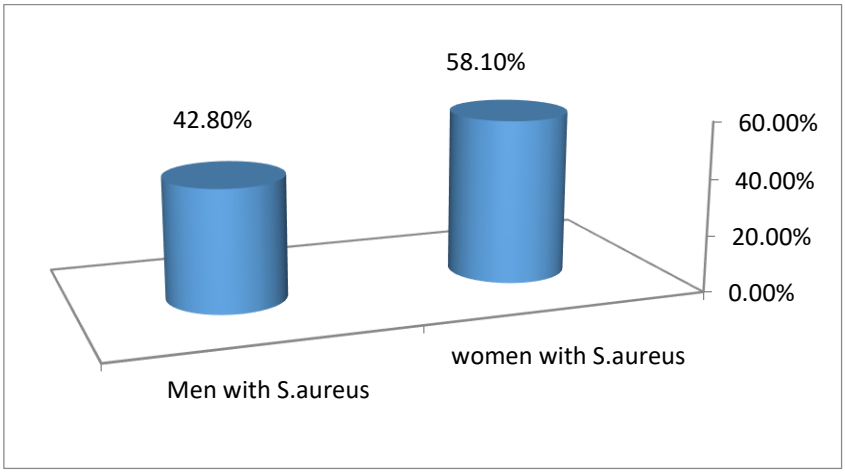


Figure 2. The ratios of women and men infected with *S. aureus*

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Antibacterial susceptibility

Antibacterial susceptibility results showed that all the isolates were sensitive to chloramphenicol and clindamycin and most of the isolates were resistant to tetracycline, penicillin and cefoxitin (Table 2).

Table 2. Antimicrobial susceptibility of *S. aureus* isolates.

Antibiotics	Abbreviations	Results (Resistant isolates%)
Cefoxitin	FOX	68.6%
Chloramphenicol	CHL	0.0%
Imipenem	IPM	0.0%
Rifampicin	RIF	48.5%
Clindamycin	CLA	0.0%
Penicillin G	PCG	94.2%
Tetracycline	TE	54.2%
Erythromycin	ERY	42.8%

Distribution of the mecA and spa genes

Out of 35 isolates identified as *S. aureus*, 30 isolates were positive for the *mecA* gene (293-bp product, 85.7%) and 23 isolates were positive for the *spa* gene (variable product size of 1150-1500 bp, 65.7%) (Figures 3 and 4).

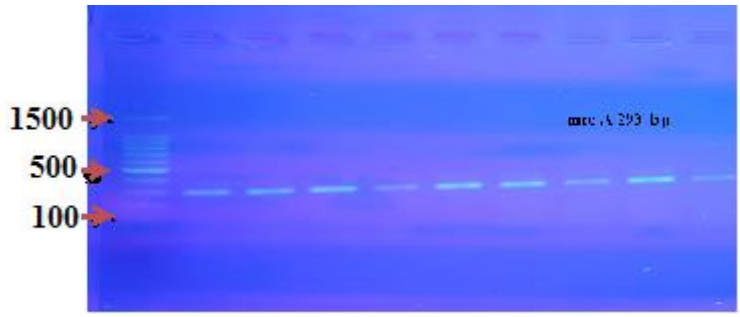


Figure 3. Gel electrophoresis of the PCR product of *mecA* amplicon. L: Ladder (100 bp), Lanes 2-9: *Staphylococcus aureus* isolates

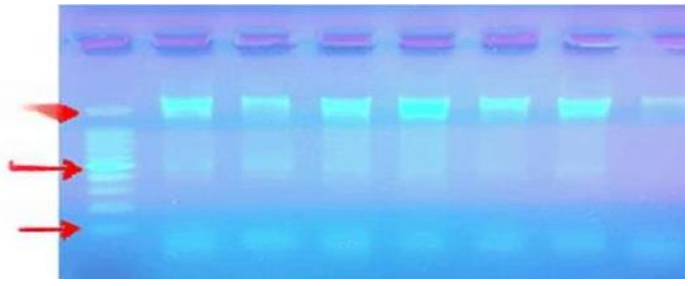


Figure 4. Gel electrophoresis of the PCR product of the *spa* gene amplicon. L: Ladder (100 bp), Lanes 2-8: *Staphylococcus aureus* isolates

spa gene sequence analysis

A detailed overview of all detected *spa* types is given in Table 3. Out of 26 amplicons subjected to sequencing, only 6 isolates could be sequenced

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successfully. Four different *spa* types were identified: t386, which was identified in isolates no 5, 6, 8 and t3579, t10002 and t10234 which were recognized in isolates no. 12, 14 and 27 respectively.

The most frequent *spa* types were t386, t3579, t10002 and t10234, representing the frequencies of 13%, 4.3%, 4.3% and 4.3% respectively.

Table 3. *Spa* types and repeat successions of *S. aureus* isolated from outpatient samples.

Isolate No.	*Spa*-type	Repeat Succession
5	t386	07-23-13
6	t386	07-23-13
8	t386	07-23-13
12	t3579	26-23-17-34-17-20-17-20-17-12-16
14	t10002	11-10-21-17-34-24
27	t10234	11-10-21-17-34-24-34-22

Discussion

This study showed that out of 200 samples collected, 35 isolates (18%) were identified as *S. aureus*, all of which were coagulase-positive and able to ferment mannitol in the mannitol salt agar medium and its yellow discoloration. In clinical settings, the use of CHROM-agar *S. aureus* is very useful compared to routine media for the rapid detection of *S. aureus*. Our results confirmed the identity of *S. aureus* isolates, which formed pink to purple colonies on chromogenic agar media. According to our results and those of previous studies, the CHROM-agar *S. aureus* medium can be used for the final identification of *S. aureus* along with the coagulase reaction.

The prevalence of infection with *S. aureus* was 57.1% in women and 42.8% in men. Few studies are comparing *S. aureus* infections between women and men. Researchers have isolated MRSA from different samples in patients with infectious diseases referring to medical clinics. In the study of Al-Miyahi and Sirhan, out of 68.2% of lactating women with breast abscesses, 29.13% were detected as *S. aureus* isolates (15). In another study by Saleh et al., out of 30 *S. aureus* strains isolated from nasal mucosa, 8 isolates fermented mannitol and were coagulase positive. Prior studies have noted an increase in the incidence of *S. aureus* infections in patients with poor financial status, highlighting risk factors such as overcrowding, limited access to medical services, and poor hygiene (16). Al-Charakh et al., in a study in Iraq, showed that 13 (54.16%) out of 24 *S. aureus* isolates were determined as MRSA, indicating the widespread propagation of MRSA in the community (17). Another study in Iraq by Hallabjaiy et al. also showed a high rate of disease (out of 348 samples from hospitalized patients, 228 isolates (65.5%) were identified as *S. aureus*) (18). The results of these studies were consistent with our findings in the present study.

In the present study, most *S. aureus* isolates were sensitive to chloramphenicol, imipenem, and clindamycin, regardless of their source. Despite the ban on chloramphenicol administration in Europe in 1997, it is commonly used as a broad-spectrum antibiotic in antimicrobial susceptibility tests of staphylococci (19-20).

Of note, most *S. aureus* strains isolated from outpatient samples were resistant to penicillin. Isolates no 24 and 30 that were susceptible to penicillin were also sensitive to cefoxitin and were *mecA* negative. This finding was consistent with many previous global reports (21-22).

Cefoxitin can be a suitable replacement for methicillin. Methicillin has an admirable function in blocking the functionality of *mecA*. Cefoxitin is suggested to be used instead of methicillin to identify

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MRSA isolates because it is easier to implement than the methicillin plate test. Methicillin is not specific for the diagnosis of MRSA, and methicillin resistance is not always investigated (23). The occurrence of MRSA in the present study was 85.7%. Many investigators have also used oxacillin instead of methicillin, but trials on cefoxitin have shown more reproducible and accurate results than studies on oxacillin. Cefoxitin is a potent *mecA* inducer that appears to be less affected than oxacillin by penicillinase-overproducing isolates, rendering more reliable results (24). Despite, the preference for cefoxitin over oxacillin, a discrepancy between the cefoxitin and *mecA* tests was observed in the current study. *Staphylococcus aureus* strains that are positive for the *mecA* gene by PCR but phenotypically susceptible to oxacillin and cefoxitin by disk diffusion or minimum inhibitory concentration (MIC) testing collectively known as oxacillin-susceptible MRSA (OS-MRSA), have been recognized for over a decade and pose a challenge for diagnostic laboratories (25-28). It has been demonstrated that oxacillin susceptibility is associated with mutations in regions of nucleotide repeats within *mecA* while PCR amplification of this gene remains positive (25, 29). In addition, phenotypic tests for detecting resistance are affected by various factors such as temperature, breakpoints for inhibition zone diameter, incubation period, inoculum density, and salt concentration in the culture media (30). This observation emphasizes the use of *mecA* gene amplification for accurate identification of MRSA.

Al-Hasnawi et al. reported that out of 44 isolates resistant to beta-lactams, 13 isolates (29.5%) were resistant to cefoxitin and oxacillin and were designated as MRSA (31). In the recent study, all samples were identified as MRSA, which may be due to the increase of beta-lactam antimicrobial stress in the human host. *mecA*-mediated resistance through beta-lactamases and PBPs is a global problem that needs to be effectively addressed. Resistant organisms and microorganisms are spreading worldwide and causing more fatal infections due to their continuous mutations. MRSA strains are impervious to beta-lactamase-resistant penicillin due to changes in their penicillin-binding proteins in the cell membrane (31) and mutations in the gene encoding the penicillin-binding protein, which is responsible for the development of MRSA. This protein is called PBP2A and has a low affinity for binding to beta-lactam antibiotics, disrupting the ability of the antibiotic to disintegrate the cell wall, rendering it functionally ineffective (32).

In the present study, the presence of the *mecA* gene was assessed in 35 *S. aureus* isolates, 85.7% of which were positive for this gene. The study also found that 74.3% of *S. aureus* isolates had the *spa* gene, indicating that these isolates are highly pathogenic. The *spa* gene allows *S. aureus* to evade the host's immune system by producing protein A, which prevents opsonization and phagocytosis (33). This observation is in agreement with other researchers who reported 62.5% (34) and 86.1% (35) of their isolates harboring the *spa* gene.

Although the PCR amplification of these genes was efficient, *spa*-typing by sequencing was only effective for six groups, identifying four types of *spa*s. The *spa* type t386 comprised three groups of isolates (i.e., half of the groups), while *spa* types t3579, t10002 and t10234 were each identified in a separate group. In addition, about 17% of all *spa* types rendered the same results. In this study, 17% of the isolates showed disconcerting methicillin results, which was consistent with the results of other studies (36-38). The most common *spa* type observed here among MRSA isolates was t386, and the same results were obtained in a study conducted in Palestine (39). In addition, the t386 *spa* variant has been identified in MRSA isolates from Lebanon and Jordan (40).

Overall, the *spa* types identified in the present study may not be consistent with the results of several previous studies conducted worldwide. For example, Harastani et al. reported that the endemic types of t304 and t9129 were predominant in a major clinic in

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Lebanon (41). Also, the results of the present study did not match the results of a study conducted in Tehran, Iran, where the dominant type of *spa* was reported as t7685 (42). In a study conducted in Kuwait, the presence of *S. aureus* t688 was reported in samples isolated from the skin and soft tissue, as the most common type of *spa* in emergency clinics in Kuwait. These different types of *spa*s in Iraq may be justified by the excessive and wrong use of antimicrobial agents in the country, unlike other countries (43). In a study by Saleh et al. in Mosul, Iraq, *spa* typing revealed five distinct patterns (t975, t840, t991, t304 and t386) (16). In a study in Iran, Hashemizadeh et al. reported considerable diversity concerning *spa* types in MRSA isolates, where *spa* type t386 was reported for the first time and *spa* type t030 was found to be the most frequent (44).

Conclusion

This study highlighted the importance of *spa* typing and corresponding variations in *S. aureus* clinical isolates when treating staphylococcal infections. Also, the results showed that the prevalence of MRSA was higher in women compared to men. The most active antibiotics against *S. aureus* were imipenem, clindamycin and chloramphenicol.

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Conflict of interest

The authors declare that no conflict of interest exists.

Authors' contributions

K.A designed and implemented the current training program and participated in data collection. M.N was a promotor and has prepared of the manuscript draft and its final edition. FP was co-promotor and H.S.A was advisor in this dissertation. All authors contributed to the preparation of the manuscript draft and its final edition.

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