Molecular Detection of *SPA*-Type of Methicillin-Resistant *Staphylococcus aureus* from Urinary Tract Infection Patient in Wasit Province, Iraq

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Abstract

Background: *Staphylococcus aureus,* a commonly occurring pathogen, is associated with severe diseases both in community and hospital settings and has been a significant concern for public health. Methicillin-resistant *S. aureus* (MRSA) infections have become widespread in many regions worldwide. **Objective:** The objective of their study was to employ polymerase chain reaction to identify certain virulence genes and determine the antibiotic susceptibility profiles of *S. aureus* strains. **Materials and Methods:** Initially, the identification of these isolates involved culture, microscopic examination, and biochemical tests. *S. aureus* accounted for 36.4% of the growth observed. **Results:** The findings of antibiotic susceptibility testing for MRSA *S. aureus* indicated the highest resistance rates to cefoxitin and amoxicillin-clavulanic acid, followed by clindamycin and tetracycline. On the other hand, *S. aureus* showed maximum sensitivity to gentamicin and nitrofurantoin. It was suggested that Imipenem and nitrofurantoin exhibited the least antibiotic resistance in urinary tract infection patients. The DNA was extracted from the isolates, and the purity of the nucleic acid samples ranged from 1.8 to 2.0, while the concentration varied from 50 to 360 mg/μL. Molecular analysis of the genes showed that 33.3% of the isolates possessed the *icaD* gene, whereas none of the MRSA strains exhibited the presence of the *icaA* gene. **Conclusion:** The study findings indicate a relatively high prevalence of MRSA strains among *S. aureus* isolates in hospitals located in Wasit Province. Moreover, a significant proportion of these MRSA strains exhibit robust biofilm production capabilities.

Keywords: Antibiotic-resistant, *icaA*, *icaD*, MRSA, *SPA*; *Staphylococcus aureus*

Introduction

Staphylococcus aureus is the primary causative agent of both hospital-acquired and community-acquired infections among Gram-positive bacteria. *S. aureus* infections can vary in severity, ranging from mild skin and soft tissue infections to more serious conditions such as bacteremia, sepsis, endocarditis, and osteomyelitis.[1] A significant challenge in treating these infections is the development of antibiotic resistance. Methicillin-resistant *S. aureus* (MRSA) has been spreading its notorious presence across the globe, occupying approximately 40% of the microbial landscape.[2] The eruption of MRSA strains exerts a profound impact on morbidity, mortality, and healthcare costs, casting a shadow of concern and financial strain in the realm of healthcare.[3,4] In the relentless battle against MRSA, infection control measures act as vigilant sentinels, relentlessly screening patients, healthcare workers, and the environment to thwart its insidious transmission; however, to investigate the origins and transmission routes of MRSA, it is crucial to use genetic typing methods for characterizing the genetic profiles, as they assume a critical role in comprehending the dissemination of the bacteria, these measures hold significant importance.[5]

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Various molecular epidemiological methods are employed for MRSA surveillance, and one of them is the sequencing of the staphylococcal protein A (*SPA*) gene.[6] *SPA* typing is considered a valuable tool due to its simplicity, cost-effectiveness, and standardized nomenclature, as supported by Strommenger *et al.*[7] The process entails evaluating the repetitive region within the X region of the *SPA* gene. This polymorphic X region, responsible for encoding a segment of the *SPA* protein, demonstrates variations in tandem repeats and base sequences within those repeats, as elucidated by Hallin *et al.*[8] The importance of *SPA* types lies in their role in identifying outbreak isolates of *S. aureus* and shaping infection control policies worldwide. Recent investigations have undertaken numerous studies to explore the distribution of *SPA* types and other associated factors in diverse geographic regions.[9,10]

Extensive research and documentation have shed light on the crucial role of biofilm production in the pathogenicity of *S. aureus* and the emergence of multidrug-resistant strains.[11,12] In the realm of adhesion, the polysaccharide intercellular adhesion (PIA), encoded by the ica operon, assumes a pivotal role in the formation of biofilms in staphylococci, the ica locus encompasses the *ica*ADBC operon, comprising four genes that are accountable for the synthesis of PIA. Notably, the genes *icaA* and *icaD* play a crucial role in the production of exopolysaccharides.[13] The *icaA* gene encodes a transmembrane protein with an enzymatic activity called *N*-acetyl-glucosaminyl transferase, which aids in the synthesis of the poly-*N*-acetyl glucosamine polymer.[14] Conversely, the product of the *icaD* gene plays a critical role in optimizing the enzymatic activity of the *icaA* gene product.[15] The formation of biofilms is regulated by genes that oversee the production of biofilm factors or proteins.

Staphylococcus aureus is a persistent human and animal pathogen that causes a wide range of infections with varying clinical manifestations and severity. The pathogenesis of *S. aureus* is a complex process involving multiple virulence determinants, both secreted and surface-associated, which are coordinated at different stages of infection.[16] Additionally, an exemplar of such proteins is *SPA*, which is produced by the *ica* operon in conjunction with PIA encoded by the *ica* ADBC genes. Therefore, the current research aims to determine the virulence and antimicrobial resistance profiles of MRSA isolates using *SPA* typing. Ravaioli *et al.*[9] further emphasized the assessment of biofilm formation by evaluating genes present in the ica locus. This approach allows for the detection of specific virulence genes and antibiotic susceptibility profiles in isolated *S. aureus* strains using the polymerase chain reaction (PCR) technique. The objective of their study was to employ PCR to identify certain virulence genes and determine the antibiotic susceptibility profiles of *S. aureus* strains.

Material and Methods

In this study, a collection of 52 rine samples (male and female) was obtained from patients seeking medical care for urinary tract infections (UTIs) at Al-Karamaa Teaching Hospital, Al-Zahraa Teaching Hospital, and Al-Kut Hospital for Gynecology, Obstetrics, and Pediatrics. To analyze the samples, they were cultured on Mannitol salt agar and blood agar plates and subsequently incubated under aerobic conditions at 37°C for a duration of 24 h. The identification of the isolated bacteria was carried out by assessing their morphological characteristics, conducting biochemical tests, and utilizing the Vitek2 system.

The antibiotic susceptibility testing in this study was conducted using the Kirby-Bauer method on Muller Hinton agar, following the protocol established by Bauer *et al.*[17] The interpretation of the results was conducted in accordance with the guidelines established by the Clinical and Laboratory Standards Institute.[18] Cefoxitin FOX 30 (µg), Ciprofloxacin CIP 5 (µg), Clindamycin CD 2 (µg), *Nitrofurantoin* F 300 (µg), and Gentamicin CN 10 (µg).

For the molecular analysis, the primers were obtained from the manufacturer (Integrated DNA Technologies, USA) in the form of lyophilized powder, supplied in 1.5 mL Eppendorf tubes. The primer usage procedure was implemented as described by Dahwash *et al.*[19] [Table 1], following their guidelines for primer handling and application.

DNA extraction

The DNA of Gram-positive bacteria was extracted and purified using the Presto™ Mini gDNA Bacteria

Table 1: Primer's sequence of *Staphylococcus aureus* virulence genes

Primer name	Primer Sequence (5′-3′)	Size (bp)	References
SPA	F: 5′-TCAAGCACCAAAAGAGGAAGA-3′	300	[20]
	R: 5′-ACGACATGTACTCCGTTGCCG-3′		
icaA	F: 5′-GAGGTAAAGCCAACGCACTC-3′	151	[21]
	R: 5′-CCTGTAACCGCACCAAGTTT-3′		
icaD	F: 5′-ATGGTCAACCCAGACAGAG-3′	198	[22]
	R: 5′-AGTATTTTCAATGTTTAAAGCAA-3′		
16SrRNA	F: 5′-GTGCCAGCAGCCGCGGTAA-3′	886	[23]
	R: 5′-AGACCCGGGAACGTATTCAC-3′		

kit from Geneaid. The purity and concentration of the extracted DNA were confirmed using a Nanodrop spectrophotometer. The nucleic acid purity in the samples was found to be within the range of 1.8–2.0, indicating good quality. The concentration of the nucleic acid ranged from 50 to 360 mg/µL, reflecting variations in the amount of nucleic acid present. The primers used in the study were supplied by Integrated DNA Technologies in the form of lyophilized powder enclosed within 1.5 mL Eppendorf tubes. To prepare the primers for use, the tubes were centrifuged at 8000 rpm for 5 s. This centrifugation step aimed to precipitate the lyophilized primers, ensuring their proper reconstitution before opening the tubes. Then, 1× TE buffer (pH 8.0) was added to each tube to obtain a stock solution of 100 µM, following the manufacturer's instructions. The tubes were incubated at 65ºC for 10 min using a water bath. The primer tubes were stored as stock solutions at -20°C until used. Working solutions of the primers were prepared based on the general dilution equation: C1V1 = C2V2 (refer to Table 2 for details).

Integrated DNA Technologies, USA, as a lyophilized powder in eppendorf tubes (1.5 mL) as procedure by Dahwash *et al.*[19] This was carried on by monoplex PCR.

Detection of virulence genes

The detection of *SPA, icaA*, and *icaD* sequences was performed using monoplex PCR. For each PCR reaction, 25 µL of the PCR Mastermix was used, and all primers were used at a concentration of 30 pmol/µL (the PCR cycle program settings in this experiment were shown in Table 3).

For the analysis of the PCR products and DNA ladder, a 1.5% agarose gel was prepared, which included Ethidium Bromide. Subsequently, the samples were loaded into the wells of the gel. Electrophoresis was performed at a voltage of 70 V, allowing the DNA fragments to migrate through the gel based on their size. After the electrophoresis run, the agarose gel was carefully removed from the gel tank. To visualize the DNA bands, the gel was subjected to UV light illumination, as Ethidium Bromide intercalates with the DNA and emits fluorescence under UV light. The DNA bands were then observed and recorded. To determine the size of the DNA bands, a DNA ladder was used as a reference, following the protocols described in Sambrook and Russell.[24]

Statistical analysis of data was performed using statistical analysis system (SAS) (Users Guide for Personal Computer, Release 9.1. SAS Institute, Inc., Cary, NC, USA). The percentages were compared using the Chi-square test. $P < 0.05$ is considered statistically significant.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before a sample was taken. The study protocol and subject information, and consent form were reviewed and approved by a local ethics committee according to document number 1644 (including the number and the date on July 31, 2022) to get this approval.

Results

This study involved the analysis of 52 urine samples obtained from patients with UTIs. All the specimens mentioned were subjected to inoculation on both blood agar and mannitol salt agar. Subsequently, they were incubated at a temperature of 37°C for a period of 18–24 h. This incubation period allowed for the growth and development of microorganisms present in the specimens on the respective agar plates. Among these 52 clinical specimens, 19 (36.5%) showed no growth, while 33 (63.5%) yielded positive growth. Out of the positive growth isolates, twelve were identified as *S. aureus*, accounting for 36.4% of the total growth. The remaining 63.6% consisted of gram-negative bacteria such as *E. coli, Klebsiella,* and *Proteus*. To identify the bacterial isolates and confirm the diagnosis, the VITEK 2 system was utilized. Additionally, the isolates were cultured on two different media to assess colony shape, size, and characteristics. *S. aureus* colonies displayed beta-hemolytic properties on blood agar and exhibited yellow zones on mannitol salt agar. Biochemical tests conducted on *S. aureus* bacteria demonstrated positive results for catalase, coagulase, Simmon citrate, methyl red, mannitol fermentation, urease hydrolysis, and hemolysis tests showing β-hemolysis. However, the bacteria tested negative for the KOH test, oxidase, motility, and swarming tests.

The antibiotic susceptibility results for MRSA *S. aureus* showed a maximum resistance rate of 100% for cefoxitin and ciprofloxacin with 91.6 %; also, the results also indicated moderate resistance levels to clindamycin (66.6%). On the other hand, *S. aureus* showed high sensitivity to gentamicin (100%) and nitrofurantoin (91.9%).

Detection of 16 *SrRNA* gene

The *16SrRNA* gene is a highly sensitive and specialized assay used for the detection and differentiation of bacterial genera. It serves as a diagnostic tool for

Table 2: Amplification conditions of genes were used by PCR reactions final volume 25 µl

Master mix	DNA template	Forward primers	Reverse primers	Deionized water
12.5 µl	2 µl	1 µl	1 µl	8.5 µl

identifying bacterial species. In this study, the diagnostic test targeting the *16SrRNA* gene confirmed the presence of *S. aureus* in all 12 samples (100% detection rate). The PCR products were analyzed and visualized using agarose gel electrophoresis. The results of this electrophoresis are depicted in Figure 1, which presents the outcomes of the gel electrophoresis procedure.

Molecular detection of *icaA* and *icaD* genes

In the molecular study of 12 MRSA strains, it was found that four isolates (33.3%) possessed the *icaD* gene, while none of the MRSA strains showed the presence of the *icaA* gene. Furthermore, eight isolates did not exhibit either of these genes. In this study, most *S. aureus* strains were found to have the *icaD* gene, indicating their potential for biofilm formation [Figure 2].

Molecular detection of *SPA* gene

In this study, a total of 10 out of 12 isolates showed positive results in *SPA* typing, indicating a positivity rate of 83.3% among the MRSA isolates [Figure 3].

A study revealed that 10% of healthy carriers exhibited mutations in the *SPA* gene. The variation in tandem repeats within the *SPA* gene, which is responsible for encoding protein A, plays a critical role in understanding host-parasite interactions and can help bacteria evade the host immune system.

Discussion

They identified the bacterial isolates and confirmed the diagnosis are consistent with previous studies conducted in Iraq,[25,26] as well as studies by Raheema and Abed.[27] *S. aureus* bacteria have the ability to grow in high-salt environments and ferment mannitol, leading to a change in the phenol red pH indicator color from red to yellow, which aligns with findings from Ayeni *et al.*[28]Biochemical tests conducted on *S. aureus* bacteria in accordance with Thaker *et al.*[29]

The antibiotic susceptibility results for MRSA *S. aureus* are consistent with the findings of Al-Dahbi and Al-Mathkhury[30] and Idbeis,[31] who also reported

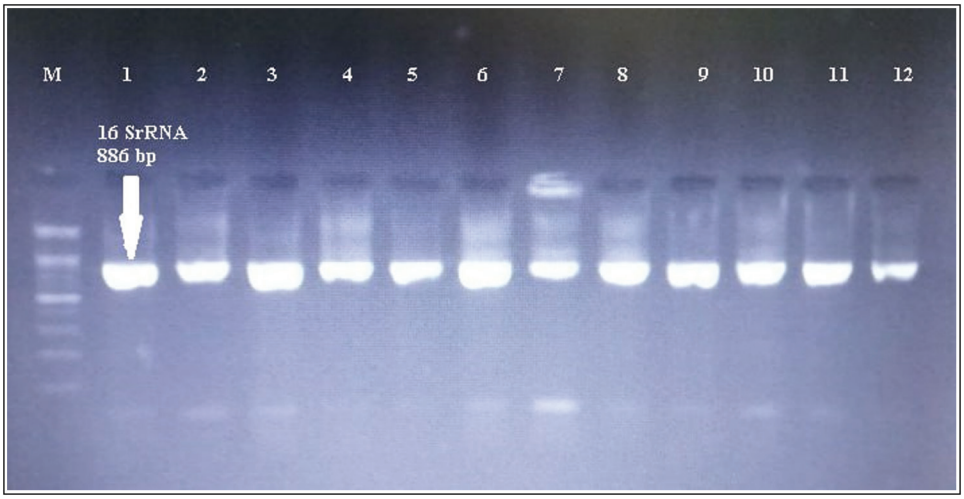

Figure 1: The 16SrRNA gene from *S. aureus* was amplified using conventional PCR and analyzed by electrophoresis on a 1.3% agarose gel. A 2000 bp DNA ladder was used as a size marker in lane (M), while the 886 bp 16SrRNA PCR products were loaded in lanes 1 through 12

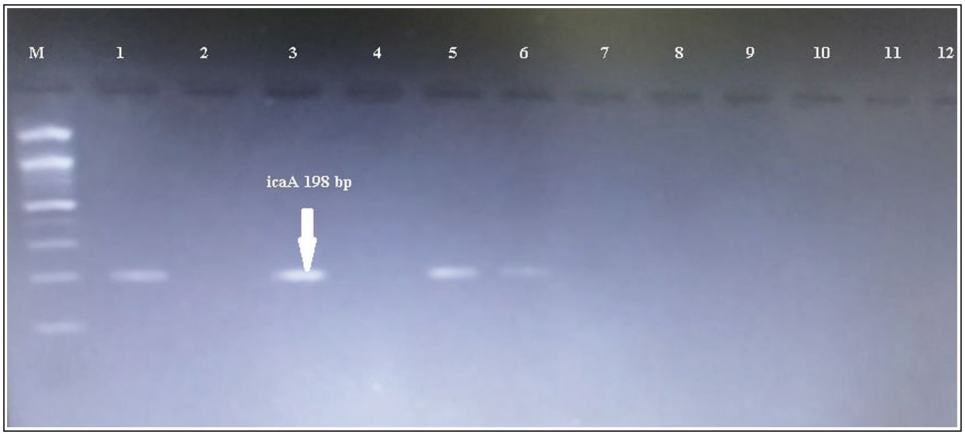

Figure 2: PCR amplicon of *S. aureus icaA* gene. Product size 198 bp. Lane (L), DNA marker (2000 bp ladder), lanes (1–12)

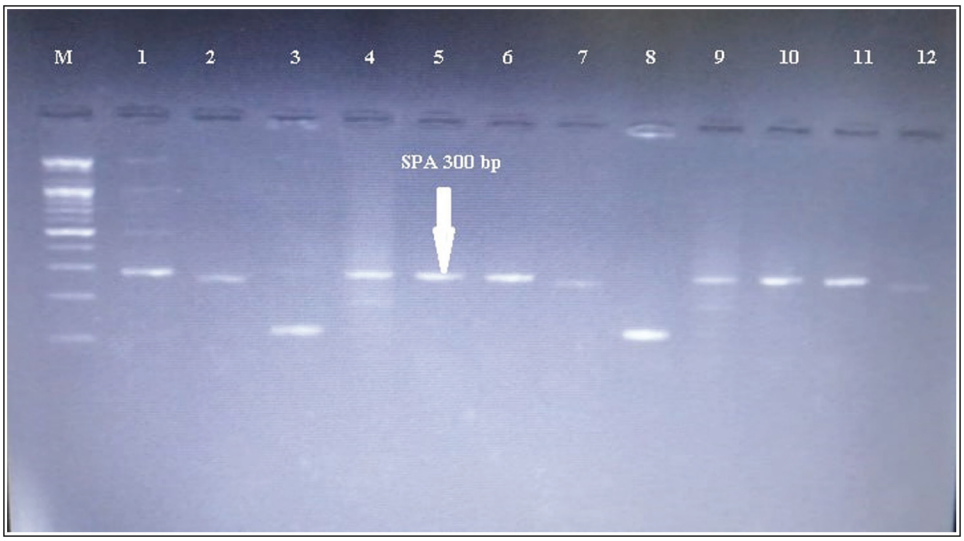

Figure 3: PCR amplicon of *S. aureus SPA* gene. Product size 300 bp. Lane (L), DNA marker (2000 bp ladder), lanes (1–12)

Table 3: Thermal cycling program for monoplex *SPA*, *icaA,* and *icaD* genes

Steps	Temperatures	Time	No. of cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 s	30
Annealing	48, 60, 59°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	7 min	1

complete resistance (100%) to oxacillin and cefoxitin. Also aligns with the results of Raheema *et al.*[25] and Khudher and Jabur[32] but differs from the findings of Saber *et al*.[33] A *similar finding* was *reported* by Ibrahim *et al.*[34] and Mohammed *et al.*,[35] the use of antibiotics should be kept under the supervision and advice of doctors and should be given in appropriate doses for an appropriate period of time.

Bacteria have developed complex resistance mechanisms to ensure their survival, and the declining interest in antibiotic development by the pharmaceutical industry emphasizes the importance of preserving our current arsenal of antibiotics through responsible antibiotic stewardship. Furthermore, the exploration and development of novel compounds such as teixobactin have shown promise in addressing antibiotic resistance; results contradict the findings of Munive Nuñez *et al.*,[36] who reported a higher prevalence of the *icaD* gene (94.7%) and the *icaA* gene (66.7%). In this study, most *S. aureus* strains were found to have the *icaA* and *icaD* genes, indicating their potential for biofilm formation, consistent with Andrade *et al*.[37] Other results suggested that adhesion and biofilm formation in *S. aureus* may primarily rely on the *icaA* operon-dependent mechanism.

In this study, the rate of *SPA* typing is higher compared to another study conducted in Iraq by Kareem *et al*.,[20] which reported a positivity rate of 67.2% for *SPA* typing among MRSA isolates. The selected region of the *SPA* gene typically contains short sequence repeats that exhibit sufficient polymorphism, allowing for effective typing of bacterial isolates, as noted by O'Hara *et al.*[38] *SPA* typing is highly valued for its efficiency and effectiveness, making it an excellent tool for both national and international surveillance and short-term local epidemiology, as emphasized by Shakeri *et al.*[39] The growing incidence of MRSA in our society has led to more concern and a strict antimicrobial policy being awarded for using without medical guidance.[40]

Conclusion

The study findings indicate a relatively high prevalence of MRSA strains among *S. aureus* isolates in hospitals located in Wasit Province. Moreover, a significant proportion of these MRSA strains exhibit robust biofilm production capabilities. In addition to the *ica* genes, recent advancements have highlighted the role of surface proteins such as *icaA* and *icaD* in facilitating intercellular accumulation during biofilm development, even in ica-deficient *S. aureus* strains. These findings suggest the possibility that other surface proteins of *S. aureus* may also contribute to biofilm formation. Additionally, the analysis of the SPA gene demonstrated significant diversity among

MRSA strains isolated from different hospitals. However, to strengthen this observation, more extensive studies incorporating sequencing and precise typing methods such as MLST (multilocus sequence typing) would be required.

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Conflicts of interest
There are no conflicts of interest.

Author contributions
Each author contributed equally to the creation of this manuscript.

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