Archives of Razi Institute, Vol. 77, No. 6 (2022) 2423-2430

DOI: 10.22092/ARI.2022.358867.2320

Original Article

The Relative of *Spa* Gene Types, Prevalence and Antibiotic Resistance in Methicillin-Resistant *Staphylococcus aureus*

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Received 17 May 2022; Accepted 7 June 2022
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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the primary cause of nosocomial and animal, community-acquired infections. *S. aureus* is a common inhabitant of the upper respiratory tract. Molecular typing methods are vital for investigations of MRSA. The MRSA has significantly increased in Iraq. This study aimed to determine the prevalence of this bacteria and know the distribution of *Spa* type among antibiotics-resistant local isolates from different sources**.** A total of 150 samples were collected from three different sources: humans, animals and the environment. Among all these samples, 55 MRSA isolates were determined using the phenotypic method and *the mecA* gene. Antibiotic resistance profiles were screened using the disc diffusion method. Whereas *Spa* types were identified by using PCR technique and nucleotide sequencing analysis. The MRSA presence rates were 67.5%, 80 %, and 31.3% in bovine, human, and environmental sources, respectively. The highest sensitivity of MRSA was to vancomycin, and the lowest was to penicillin. Multi-drug resistance was found to be in all isolates. Molecular investigation showed that 100% of the tested MRSA isolates harboured a *Spa* gene; *Spa* gene typing assay reveals that the most repetitive *spa* type was t304, t8986, and t14870, which were reported in humans and animals, followed by type t 304 t14870 in environmental isolates. This study's findings could help identify the genetic variants responsible for the emergence and spread of these bacteria in the region.

Keywords: *Spa* Gene Types, Prevalence, Antibiotic Resistance

1. Introduction

Staphylococcus aureus is a major pathogen of increasing importance due to the rise in antibiotic resistance; in recent decades, with the evolution of bacteria and the abuse of antibiotics, the infection rate of MRSA has increased worldwide (1). Recently, methicillin-resistant *S. aureus* was disseminated in Iraq intensively (2, 3). *S. aureus* has been studied in Basrahprovince by several researchers (4-7). Hence, studying these bacteria is important to control their spread in the community and hospital and determine their source. In 2005, Livestock-Associated MRSA (LA-MRSA) CC398 was detected for the first time in pigs and pig farmers. The pig population was a reservoir for LAMRSA, but LA-MRSA was also found in a wide range of animals such as chickens, horses, sheep, goats, calves, and dairy cattle (8).

Several laboratory techniques have been followed to detect and identify these bacteria. The most popular technique was related to the molecular characterization of bacterial genes. The molecular genetic identification of *S. aureus* strain types can be achieved by applying the *Spa* gene nucleotide sequence genotyping technique. The *Spa* genotyping is based on the polymorphism of the *Spa* gene, which encodes protein A. Protein A consider one of the essential virulence factors of *Staphylococcus aureus.* This protein consists of five IgG binding sites (A, B, C, D, E) and a cell wall

attachment portion (C-terminal) (9). The *Spa* gene, approximately 1350bp in length, is composed of 3 distinct regions. Their names in sequence order are Fc protein section, x region, and c terminal section. *Spa* molecular genotyping is related to the nucleotide sequencing of protein A's polymorphism x section. The x section comprises a flexible number of 24bp nucleotide repeats that might differ due to spontaneous mutation, deletion, or duplication. It can be used as a good tool to describe the natural bacterial population of *S. aureus* strains and investigate its outbreaks (10). The *spa* gene is variable in length among various strains of this species due to the diversity of the Xr region, which arises from deletion, duplication of the repetitive units, or point mutation. *Spa* typing has been proposed as a rapid sequence-based approach to characterize MRSA. It is a DNA nucleotide sequence-based technique that has become a gradually popular method in many laboratories worldwide (11).

Several studies established many *spa* gene types among MRSA isolates from animal and human patients worldwide. *Spa* typing depending on PCR and DNA sequencing, has some advantages like the speed of analysis, the convenience of setting up huge databases, and the easy use along with the high data clarity. Additionally, for exploring the initial origin region and the evolutionary history of the *S. aureus* strains. That is based on DNA sequencing, which has proven exceptional type ability and repeatability.

This study aimed to determine the prevalence of this bacteria and know the distribution of *Spa* type among antibiotics-resistant local isolates from different sources.

2. Materials and Methods

2.1. Samples Collection

One hundred fifty samples were collected from three different groups, including animals, humans and the environment. Fifty samples from each group were selected as nasal swabs from animals, humans (veterinarians and animal owners), and swabs from the environment in Basra Governoratefrom September 2020 to January 2021. Sterile cotton swabs were used for nasal swabs samples by inserting them into the bottom of the nostril and then rotating the swab. Sterile cotton swabs were rotated on the surfaces, instruments and hands for the environmental samples. The swabs were then inoculated in a brain–heart infusion (BHI) medium and transported immediately to the microbiological laboratory.

2.2. Bacterial Isolation

After samples had been transported to the laboratory, they were inoculated on a selective medium (mannitol salt agar) and incubated for 24 h. Morphology and colony characteristics were observed. Gram stain was used to identify the colonies. Furthermore, the isolates were differentiated by performing biochemical tests such as catalase and tube coagulase. After that, Pure growth was stored in Soyabean Casein Digest Medium containing 40% glycerol as stock at -20 ºC.

2.3. Antibiotic Resistance Profile and MRSA Detection

Several antibiotics, such as ampicillin, oxacillin, cefoxitin, tetracycline, vancomycin and erythromycin, were used to identify the bacterial antibiotic resistance profile. MRSA strains were primarily detected by identifying the oxacillin resistance ability. For this purpose, Mueller-Hinton agar plates were inoculated with bacterial suspension equivalent to 0.5 McFarland. Then a commercial disk containing oxacillin was put on the medium surface. The resulting inhibition zone around the disk was interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

2.4. Genomic DNA Extraction

Genomic DNA was extracted from all bacterial isolates using a commercially available kit (Promega, USA). Each isolate grew a single pure colony on 5 ml nutrient broth overnight at 37 ºC. The producing bacterial biomass was collected by centrifugation of the overnight incubated tube at 4000 rpm for 4 mins. The pellets were washed twice with sterile distal water. Then all the extraction steps were done as recommended by the manufacturing protocol. The concentration and purity of extracted DNA were

estimated by the Nanodropspectrophotometry technique (Quawell, USA).

2.5. Polymerase Chain Reactions (PCR)

The PCR technique detected three genes of interest during the current study. A specific pair of primers was used to amplify each target gene. *Staphlococcus aureus* genus-specific forward and reverse primers were used to confirm the bacterial genus. Furthermore, a pair of primers was used to amplify the *spa* gene's X region, and the last pair was applied to detect the mecA gene. All these primers were provided by the Macrogen company, Soul/ South Korea (Table 1). Every individual PCR reaction tube contains 12.5 µl master mix (*Taq* Green Master Mix 2x, Promega, USA), 1µl of 10 pmol forward primer (F), 1µl of 10 pmol reverse primers (R) and approximately 3µl of gDNA (regards its concentration). The final volume of each reaction mixture was made up to 25µl with Nuclease–free water.

As mentioned in table 2, the thermocycling conditions were accomplished by (BioNeer, Korea) thermocycler. Then, agarose gel (1%) containing Safe Dye was used to electrophorese the PCR products, and the gels were visualized under UV light to detect the expected band sizes.

Table 1. PCR primers used during this study

Primers name	Primer sequence 5' ⟶ 3'	Product size	Reference
16srRNA	F: AACTCTGTTATTAGGGAAGAACA R: CCACCTTCCTCCGGTTTGTCACC	756 bp	(12)
Meca gene	F: AAAATCGATGGTAAAGGTTGGC R: AGTTCTGGAGTACCGGATTTGC	533bp	(13)
Spa gene	F: AGACGATCCTTCGGTGAGC R: GCTTTTGCAATGTCATTTACTG	330bp	(14)

Table 2. Polymerase Chain Reaction amplification programmes used in this study

Step	Temp. C° TC	Time T	OP	Cycles	Reference
16SrRNA					
I De	95	5 min		1	
De	95	30 sec			
An	55	30 sec		35	(12)
Ex	72	1 min			
F Ex	72	5 min		1	
mecA					
I De	95	3 min		1	
De	94	*30 sec	1 min		
An	53	30 sec		30	(13)
Ex	72	1 min			
F Ex	72	5 min		1	
Spa					
I De	94	3 min		1	
De	94	30 sec			
An	50	30 sec		35	(14)
Ex	72	1 min			
F Ex	72	5 min		1	

* Modified parameters; I De: initial denaturation; De: denaturation; OP: original program An: annealing; Ex: extension; F Ex: final extension

2.6. DNA Sequencing and Analysis

The DNA nucleotide sequences of the amplified X region of the *spa* gene were performed by Macrogene company, South Korea. The amplicons were extracted from the agarose gel after being analyzed and electrophoresed. The same PCR primers were used for sequences. Primer concentrations were sent for sequencing following Macrogene's instructions.

An automatic computer software-based assignment method was followed to translate the obtained nucleotide sequences into *spa* types. The resulting DNA sequences were initially analyzed using Snapgene software. The X region FASTA type sequence located between the conserved SL and SR sequences was submitted to the *spa* typer website (http://spatyper.fortinbras.us/). This region is assessed as consensus nucleotide sequence repeats consisting of 24 bp, called the repeated ID (r) region. There are more than 830 identified repeats. Regarding the r types and numbers forming the X region, the result would be either an existing *spa* type or a newly detected type (for example, see figure 1).

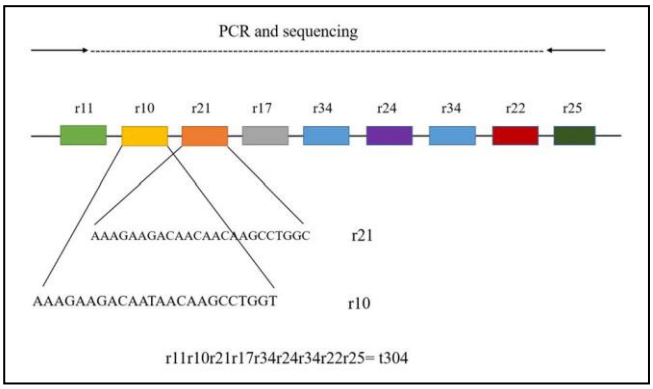

Figure 1. The *spa* types nomenclature system

3. Results

Only 86 isolates of the tested bacteria were identified as *S. aureus* regarding their phenotypic characterization. These results were confirmed by the presence of the *spa* gene in all *S aureus* isolates. Whereas MRSA isolates were confirmed for 55 isolates of the total isolated *aureus*.

Among the 86 test isolates, the highest resistance percentage was documented with ampicillin (97%), followed by cefoxitin (86%), penicillin G (83.72%), oxacillin, erythromycin (63.95%), while resistance to vancomycin was found to be very low at 1% (Table 3).

All MRSA isolates were assessed for the presence of *the mecA* gene using a specific pair of primers in the PCR technique (Table 1). The results revealed that 58.2% (31/55) of the tested isolates harboured the *mecA* gene (Figure 2).

The Staphylococcus aureus protein A (*spa*) gene detection method relied on the partial amplification of the *spa* gene by using specific primers in the PCR technique. Interestingly, the *spa* gene was detected in all the tested *S. aureus* isolates. The band of the amplified gene characterized by approximately 330 bp was determined using a standard molecular DNA ladder of 1500 bp. Positive and negative amplified samples were shown with a right-size band and no band on the agarose gel, respectively (Figure 3).

Spa gene type patterns were identified by sequence analysis of the amplified part of the *spa* gene. Each *spa* typically consists of multi-repetitive DNA sequences (r regions). They are specific categorized DNA sequences made up of 24 nucleotides. Regarding the r types, located between the LS and RS conserved *spa* nucleotide sequence and their repeats, Several *spa* types were determined in this study by using *the spa* typer server (Table 4).

A range of *spa* gene types was obtained regarding the bacterial sources (human, animal and environment). Moreover, each source of the isolated bacteria showed diversity in *spa* gene types. The most repetitive type was t304 and t8986, reported in humans and animals, followed by type t14870 in environment isolates. Interestingly, some isolates from all three sources or the same source shared the same *spa* type, such as t304 (Table 4). Whereas approximately half of them harboured different types. Furthermore, some *spa* types were reported only once, such as t1543 in animal isolates.

Table 3. The effectiveness of the antibiotics used in this study against the isolated *Staphylococcus aureus*

Antibiotics	Resistance %	Intermediate %	Susceptible %
Ampicillin	97.68	1.16	1.16
Cefoxitin	86	9.3	4.7
Oxacillin	63.95	0	36.05
Tetracycline	6.98	90.7	2.32
Vancomycin	1	0	99
Penicillin G	83.72	0	16.28
Erythromycin	63.95	23.25	12.8

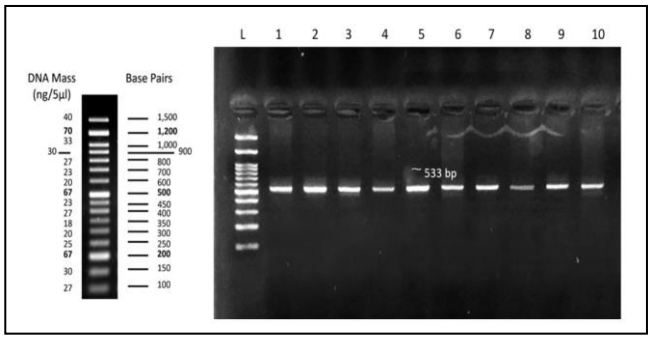

Figure 2. An agarose gel electrophoresis image displayed the amplified *mecA* gene. The image shows the presence of *the mecA* gene by amplifying 533 bp of the target gene. Lane L: 1500 bp DNA ladder, lanes 1-10: positive results

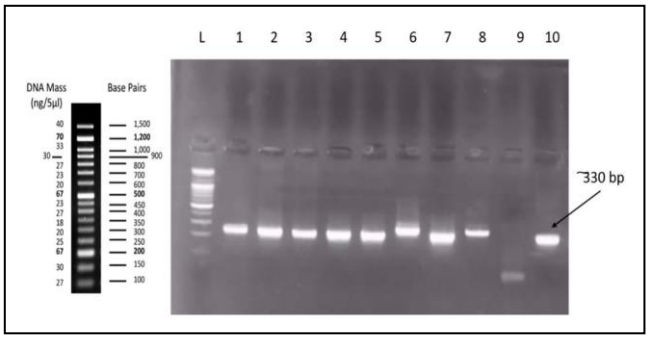

Figure 3. An agarose gel electrophoresis image shows a partially amplified *spa* gene pattern. The detected band corresponds to approximately 330 bp. Lane L: 1500 bp DNA ladder (Promega, USA); Lanes 1,2,3,4,5,6,7,8: *spa* gene bands of bacterial isolates; lane 9: negative control and lane 10: positive control

Table 4. Repetitive of the identified *spa* gene types among the isolated MRSA, regarding their sources

Spa type Source	t304	t8986	t14870	t9121	t1534	t3576	t18052	t14061	Total
Human	8	8	6	-	-	-	1	1	24
Animal	14	7	-	1	1	2	-	-	25
environment	3	-	3	-	-	-	-	-	6
Total	25	13	8	1	1	2	1	1	55

4. Discussion

S. aureus is a frequent microbe inhabitant of the upper part of the respiratory tract. These bacteria, from the nasal cavities, were observed to be related to hospital and community-associated infections. In the last decade, numerous antimicrobial agents have been documented to be less efficient in *S. aureus* infections therapy, which led to drug treatment failure. Methicillin-resistant *S. aureus* is an important microbial pathogen that is presently receiving considerable attention in animal and public health.

The present study reveals that *S. aureus* was widely distributed in different host types. It showed that 60% (30/50) of human samples contained *S. aureus* isolates. These results notice increases in *S. aureus* presence when compared to a previous study, which identified *S. aureus* bacteria from humans and animals; the studied bacteria (*S. aureus*) were detected in 55% of human samples (15). These findings agree with another researcher, Mezban, Khudor (6), who found that 59.83% of human nasal samples were carried *S. aureus*

isolates. Meanwhile, animal nasal samples (bovine nasal swabs) were 63.33%. The current study showed that *S. aureus* was detected in 74% (37/50) of animal nasals, which is a bit higher than the *S. aureus* presence percentage (65%) in a previous Iraqi study reports 65% of isolates from cow nasal swabs (6). Generally, most similar studies showed that animal samples contained more *S. aureus* than human samples. These differences might be related to personal human care and body hygiene.

In contrast, the rate of *Staphylococcus aureus* isolated from the different environmental samples (during this study) was 38% (19/50), which was higher than what Khudaier, Abbas (15) found in 2013, who reported that *S. aureus* presence in 12.5% of the environmental samples. This highness may be explained by the fact that the environment, including hospital tools from which the bacteria were isolated, was not subject to similar strict sterilization in different organizations. On the other hand, the environmental samples were curried much less *S. aureus* isolates. The presence of *S. aureus* in the environment is low compared to other sources that the bacteria isolated from; these results are attributed to a lack of nutrients and appropriate and viable conditions for proliferation and growth. The expected reasons for this high distribution of *S. aureus* bacteria in several hosts (particularly the live host) could be related to owning several virulence elements that help these microbes to invade, colonize and infect diverse hosts (15). Moreover, it might be attributed to exposure to antibiotics and gaining the genes for resistance are much lower in the environment.

The highest MRSA presence rate was recorded in Human samples at 80% of the isolated *S. aureus*. This result is higher than what Khudaier, Abbas (15), and his colleagues found in 2013 in the same city and Alkhafaji in 2020 reported MRSA isolates rate as 65.28% and 53%, respectively. In contrast, the lowest MRSA presence percentage was recorded in the environmental samples at 31.58% (6/19), followed by the bovine nasal samples at 67.57% (25/37). Generally, the incidence of MRSA has substantially increased over time in Iraq.

The *mecA* gene was molecularly detected in the isolated MRSA using the PCR technique. Interestingly, it was detected in 31 of 55 isolates only. Even though all the tested isolates showed a clear inhibition zone as positive results in the antibiotic resistance phenotypic method (Oxacillin and Cefoxitin disc diffusion), these results were in line with those of, who reported only 51.7% of the MRSA isolates were carrying the *mecA* gene. This finding might be related to the presence of a *mecA* homolog gene such as the *mecC*. Several researchers from different countries suggested that *mecC* mediated resistance to the penicillinase-stable penicillin (oxacillin, nafcillin, cloxacillin, dicloxacillin, and flucloxacillin) Furthermore, the *mecB* gene (which is a plasmid curried gene) was detected in *S. aureus* as a responsible gene for methicillin resistance ability. In addition to the possibility of the *mecD* gene being detected in some bacteria as a methicillin resistance encoding gene from canine and bovine sources.

Staphylococcus aureus protein A gene (*spa*) is a nucleotide sequence encoding for an S protein A formation, which is mainly located in all *S. aureus* isolates. Protein A is a cell wall component that prevents neutrophils from phagocytosing. It has a distinguished region called the X-segment. The XR-region, which is a small part of the X-segment of the *spa* gene sequence, frequently undergoes repetition (approximately 24 nucleotide repeats) which is variable among different strains with no effect on the gene expression (16). Regarding the Xr-region nucleotide sequence variation among *S. aureus* strains, the *spa* gene typing analysis has been applied worldwide as a valuable tool for the molecular genetic characterization of *S. aureus* isolates (17). Last few years, the molecular *spa* gene analysis was the most utilized method for the *S. aureus* genotyping technique, which is a hypervariable repeat-based method of the X region in the *spa* nucleotides sequence (18). The present study showed that all the identified MRSA isolates (100%)

harboured *spa* genes. A direct method (Xr-region-specific primers and PCR amplification) was used to scan the presence of the gene of interest. Meanwhile, each of these *spa* genes was recognized and classified in a previously identified category (typable) based on the Ridom® *spa* server website database, which currently contains more than 20300 novel *spa* types supplied from approximately 70 countries around the world (http://www.spaserver.ridom.de, 2022). Even though several studies reported non-typeable-*spa S. aureus* strains (14, 19, 20), we have not detected anyone during this study.

Eight *spa* types were reported among the three bacterial sources (Table 3). The dominant *spa* type, in this work, was t304. Which has the highest prevalence among humans (33.33 %) and animals (56 %) in addition to 50% of isolated environmental MRSA. Our results are in line with several previous researchers (21, 22), who reported that *spa* type t304 was the dominant type among MRSA isolated from human and animal samples from different countries such as Norway, Denmark, France, Sweden and the UK. On the other hand, many studies conducted in Europe, Asia, America, Africa and Australia (including France and the UK) stated that *spa* type t304 was rare among the studied MRSA (23). These results suggest that the sample type and collection time are important factors for identifying style distribution for *spa*-type MRSA. Furthermore, they could be used to study the presence and epidemiology of bacterial infection.

Interestingly, the environmental MRSA isolates were identified as t304 (50%) and t14870 (50%) only. Both these *spa* types were detected in the human samples, and only t14870 was shared with animal samples. That would suggest the ability of a human to contaminate the surrounding environment more than animals. This might be related to the limited movement and access of animals to specific places compared to humans and human control over the number and existence of domestic animals such as cows. Another reason could be due to the origin of environmental samples used in this study (door handles, chairs and surgical instruments).

No clear relationship between *spa* type and antibiotic resistance was detected. Even though, the majority of *spa* type t304 was found to be multidrug-resistant including cefoxitin and penicillin. Whereas erythromycin divided the t304 strains into three groups; antibiotic sensitive, resistant and intermediate within 16, 6 and 3isolates respectively.

Authors' Contribution

Study concept and design: N. S. A. and M. Y. A.
Acquisition of data: N. S. A.
Analysis and interpretation of data: M. Y. A.
Drafting of the manuscript: M. Y. A.
Critical revision of the manuscript for important intellectual content: N. S. A. and M. Y. A.
Statistical analysis: N. S. A.
Administrative, technical, and material support: N. S. A. and M. Y. A.

Ethics

All ethical procedures were approved by the ethics committee of the University of Basrah, Basrah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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