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ORIGINAL PAPER

Spa typing of *Staphylococcus aureus* Isolated from Clinical Specimens from Outpatients in Iraq

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is notorious as a hospital superbug and a problematic pathogen among communities. The incidence of MRSA has substantially increased over time in Iraq. The aim of this study was to determine the prevalence and *spa* types of MRSA isolates from outpatients or patients upon admission into hospitals. Various biochemical tests identified *S. aureus* isolates, and then this identification was confirmed by PCR using species-specific 16S rRNA primer pairs. Antibiotic susceptibility was determined against methicillin, oxacillin, and vancomycin using the disk diffusion method. Vancomycin MIC was detected by VITEK 2 compact system. All the identified isolates were screened for the presence of *mecA* and lukS-PV-lukF-PV genes; 36 of them were subjected to *spa* typing-based PCR. Out of 290 clinical samples, 65 (22.4%) were *S. aureus*, of which 62 (95.4%) strains were resistant to oxacillin and methicillin. Except for two isolates, all MRSA isolates were *mecA* positive. One of the three MSSA isolates was *mecA* positive. Five strains were resistant to vancomycin. Fourteen (21.5%) isolates were positive for the presence of lukS-PV-lukF-PV genes. *Spa* typing of 36 *S. aureus* isolates revealed eleven different *spa* types, t304 (30.3%), t307 (19.4%), t346 (8.3%), t044 (8.3%), t15595 (8.3%), t386 (5.5%), t5475 (5.5%), t17928 (2.8%), t14870 (2.8%), t021 (2.8%), and t024 (2.8%). These findings could be useful for assessing the genetic relatedness of strains in the region for epidemiological and monitoring purposes, which would be essential to limiting the spread of MRSA.

Keywords: Staphylococcus aureus, methicillin resistance, *spa* typing, PCR

Introduction

Staphylococcus aureus is the most common cause of nosocomial and community-acquired infections worldwide (Lakhundi and Zhang 2018; Kourtis et al. 2019). Since the late 1970s, the importance of *S. aureus* increased due to the emergence and spread of their resistance to methicillin and vancomycin (Parker and Hewitt 1970; Bendary et al. 2016; Abd El-Aziz et al. 2018).

Recent studies have shown marked dissemination of methicillin-resistant *S. aureus* in Iraq (Kareem et al. 2015; Kareem et al. 2020). Hence, epidemiological studies of these bacteria are significantly important to determine their source and to control their spread in the community and hospital settings. Different methods such as bacteriophage typing, antibiotyping, genotyping, *spa typing*-based PCR, and DNA sequencing have been used to investigate the hospital and community-onset *S. aureus* infections (Locatcher-Khorazo and Gutierrez 1960; Frénay et al. 1996; van Leeuwen et al. 1999; Yadav et al. 2018). *Spa* typing is based on the polymorphism of the gene encoding protein A (*spa*). Protein A is an essential virulence factor of *S. aureus* consisting of five IgG binding sites (A, B, C, D, E) and C-terminal cell wall attachment portion. The gene encoding this protein (*spa*) consists of two regions, one encodes the Fc-binding domain, and the other encodes X region (Harmsen et al. 2003). The X region includes the Xr region and the Xc region, which encodes the cell wall attachment sequence. The Xr region consists of a variable number of 24 bp repeats and is located immediately upstream of the region encoding the C-terminal cell wall attachment sequence (Guss et al 1984; Uhlén et al. 1984; Schneewind et al. 1992). The Xr region's diversity may arise from deletion, duplication of the repetitive units, or point mutation (Brígido et al. 1991).

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Therefore, the *spa* gene is variable in length in various strains of this species because of its X region diversity. *Spa* typing method is based on the number of tandem repeats and the sequence variation in region X of the protein A gene. Numerous studies demonstrated different *spa* gene patterns among MRSA strains isolated from patients in different geographic locations in the world (Furuya et al. 2010). The main advantages of *spa* typing-based PCR and DNA sequencing is the speed, the simplicity of large database establishment and the ease of use as well as the clarity of data. Furthermore, DNA sequencing has demonstrated outstanding type ability and reproducibility for studying the origin and evolution of *S. aureus* strains (Stefani et al. 2012).

In the light of the above, typing of methicillin-resistant *S. aureus* isolates is a useful tool for studying the genetic diversity of the pathogens, clonal relatedness and tracking the spread of MRSA infections. Currently, there is not adequate information about typing of community-associated methicillin-resistant *S. aureus* in Iraq. This study aimed to determine the *spa* types among clinical isolates of *S. aureus* obtained from outpatients in the South of Iraq.

Experimental

Materials and Methods

Bacterial strains and susceptibility test. A total of 65 *S. aureus* strains isolated from 290 clinical specimens were obtained from outpatients or patients upon admission into hospitals in Basrah and Thi-Qar city, South of Iraq. The following patients were excluded: recent admission to hospitals, patients on hemodialysis, recent surgical operation, or an intravenous cannula at the time of swab taking. *S. aureus* isolates were collected from urine samples, tonsil swabs, nasal swabs, wound swabs, burn swabs, blood samples, and sputum (Table I). All strains were identified as *S. aureus* according to the standard microbiological techniques (Merlino et al. 2000).

Antibiotic susceptibility testing to methicillin (10 μg), oxacillin (1 μg), and vancomycin (30 μg) were carried out on Mueller-Hinton agar (Oxoid Limited, Hampshire, England) using the Kirby-Bauer disk diffusion method, according to the recommendations by the Clinical and Laboratory Standard Institute (CLSI 2019). Vancomycin MIC was detected by VITEK 2 compact system (bioMérieux, Inc., Durham, NC, software version 8.01 and AST-GP580).

DNA isolation and PCR conditions. DNA extraction was carried out with a mercantile DNA isolation kit (Promega, USA) according to the manufacturer's instructions.

Molecular identification of *S. aureus*. PCR was used to amplify 228 bp region of 16S rRNA gene fragment of *S. aureus,* which is highly conserved at a species level, using specific primers F 5'-GTAGGTGG-CAAGCGTTATCC-3' and R 5'-CGCACATCAGCGT-CAG-3' (Monday and Bohach 1999).

Detection of *mecA* gene. All identified *S. aureus* isolates were tested for the presence of the 310 base pair bp PCR product of the *mecA* gene using the following primers: F 5'-GTAGAAATGACTGAACGTC-CGATAA-3' and (R 5'-CCAATTCCACATTGTTTCG-GTCTAA-3' (Geha et al. 1994).

Detection of *lukS-PV-lukF-PV*. All isolates were tested for the presence of the *lukS-PV-lukF-PV* genes, which encode for Panton-Valentine leucocidin (PVL); in PCR assays using previously described primers and protocols (Lina et al. 1999).

***Spa* typing.** Primers *spa* 1 (F 5'-ATCTGGTG-GCGTAACACCTG-3') and *spa* 2 (R 5'-CGCTGCAC-CTAACGCTAATG-3') were used to amplify a portion of the *spa* gene (products Variable: 1,150–1,500 bp) of the 65 isolates (Wichelhaus et al. 2001).

PCR mix reaction. The PCR reaction mix had a final volume of 25 μl consisting of 2 μl (50–100 ng)

Table I
Prevalence and characterization of *S. aureus* isolates in different clinical samples.

	Sample	Strain No	Vancomycin test		Methicillin and oxacillin test		The *mecA* gene		*The spa* gene		The *pvl* gene
			VSSA	VRSA	MRSA	MSSA	*mecA+*	*mecA-*	*spa+*	*spa–*	
1	Urine	34	32	2	32	2	31	3	23	11	5
2	Nasal swab	7	4	3	7	0	7	0	7	0	2
3	Wound	9	9	0	9	0	9	0	9	0	3
4	Burn	5	5	0	5	0	5	0	5	0	2
5	Tonsil	6	6	0	6	0	5	1	6	0	2
6	Blood	2	2	0	1	1	2	0	2	0	
7	Sputum/pleural	2	2	0	2	0	2	0	2	0	
	Total	65	60	5	62	3	61	4	54	11	14

DNA, 1 μl (20 pmol) of each primer, 12.5 μl of master mix (Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers; Promega) and 8.5 μl of nuclease-free water, under the following conditions: initial denaturation at 94°C for 4 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 5 min. The *mecA* and 16S rRNA genes were amplified under similar conditions except that the extension was 1 min. PCR amplification products were separated on 1–2% agarose gels and visualized by staining with ethidium bromide using a UV light transilluminator.

DNA sequencing. According to the Macrogen Company requirement (Seoul, South Korea), 20 μl of spa gene PCR products of selected 36 *S. aureus* 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') (Kahl et al. 2005).

DNA sequence analysis. The sequences obtained were analyzed and aligned using the Bio Edit program (Hall 1999). The *spa* typing and evaluation of *spa* types of *S. aureus* strains were performed using the *spa* database http://spatyper.fortinbras.us/) and (http://www.ridom.de/spaserver). The *spa* types' phylogenetic tree was drawn using the Molecular Evolutionary Genetics Analysis (MEGA 7.0) (Kumar et al. 2016).

GenBank accession numbers. The DNA sequences of the partial *spa* gene from the representative isolates have been deposited in the GenBank database under accession numbers LC577038-LC577073 for isolates KAZ1-KAZ37, respectively, and LC586070.1 for KAZ7.1 isolate.

Results

A total of 65 (22.4%) *S. aureus* strains were isolated from 290 clinical specimens. *S. aureus* isolates were identified according to the standard microbiological techniques. All 65 *S. aureus* (100%) samples exhibited positive results for the 16S rRNA gene.

Differentiation was based on sensitivity testing using oxacillin, methicillin, and vancomycin discs, confirmed by detecting the amplified 310 bp *mecA* gene using PCR in MRSA strains.

Among the 65 *S. aureus* isolates, the highest number of isolates were from urine samples (n = 34, 52.3%) followed by wound swabs (n = 9, 13.8%) nasal swabs (n = 7, 10.8%), tonsils (n = 6, 9.2%), burns (n = 5, 7.7%), blood (n = 2, 3.1%) and sputum and tracheal aspirates 2 (3.1%) as shown in Table I.

Antimicrobial susceptibility testing of MRSA isolates. Out of 65 isolates, 62 (95.4%) strains were resistant to oxacillin and methicillin. The *mecA* gene was detected in 60 (96.8%) MRSA strains, whereas 2 (3.2%) MRSA strains lacked the *mecA* gene (Table I). Out of the four *mecA*-negative *S. aureus*, two strains were resistant to oxacillin and methicillin. On the other hand, a single strain was *mecA*-positive but sensitive to oxacillin and methicillin. Five (7.7%) strains were resistant to vancomycin in addition to resistance to oxacillin and methicillin, as shown in Table I.

The *lukS-PV-lukF-PV* genes detection. All 65 *S. aureus* isolates were screened for the presence of the *lukS-PV-lukF-PV* genes, which encode for Panton-Valentine leucocidin (PVL). Fourteen (21.5%) isolates were positive for the presence of the *lukS-PV-lukF-PV* genes, these isolates were isolated from burns, tonsil swabs, and urine samples (Table I).

***Spa* typing.** Out of 65 strains, 54 (83.1%) showed the *spa* gene PCR products with different sizes, reflecting the number of 24 bp repeat units within the *spa* gene (Fig. 1, Table I). These PCR products generated two different *spa* types, 52 strains (96.3%) showed a single PCR band, and only two strains (3.7%) showed two PCR bands. The *spa* gene PCR products were not detected in eleven strains, which were all isolated from urine samples (Table I). The absence of the *spa* gene could be due to mutations in the primer-binding region or true deficiency of the *spa* gene in these isolates; however, this finding needs further study to be confirmed. PCR products of the *spa* gene of representative 36 isolates were sequenced and typed.

Spa typing of 36 *S. aureus* isolates revealed eleven different *spa* types, t304 (12 isolates, 30.3%), t307 (7 isolates, 19.4%), t346 (3 isolates, 8.3%), t044 (3 isolates, 8.3%), t15595 (3 isolates, 8.3%), t386 (2 isolates, 5.5%), t5475 (2 isolates, 5.5%), t17928 (1 isolate, 2.8%), t14870 (1 isolate, 2.8%), t021 (1 isolate, 2.8%), and t024 (1 isolate, 2.8%).

Based on phylogenetic relationships, *S. aureus* strains were classified into two main clades (Fig. 2). Except for t14870 and t386, all other *spa* types were included in

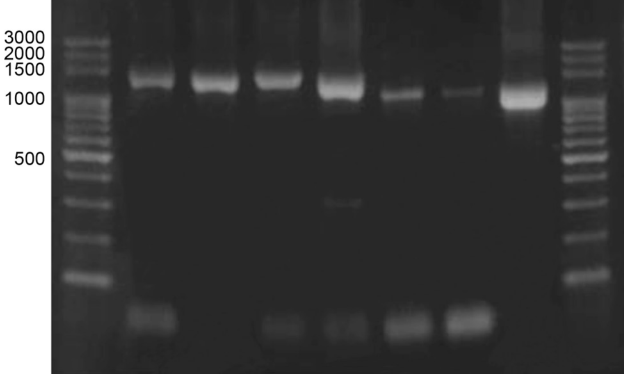

Fig. 1. The variable PCR product of the *spa* gene; lanes 2–8. Lanes 1 and 9, 100-bp DNA ladder.

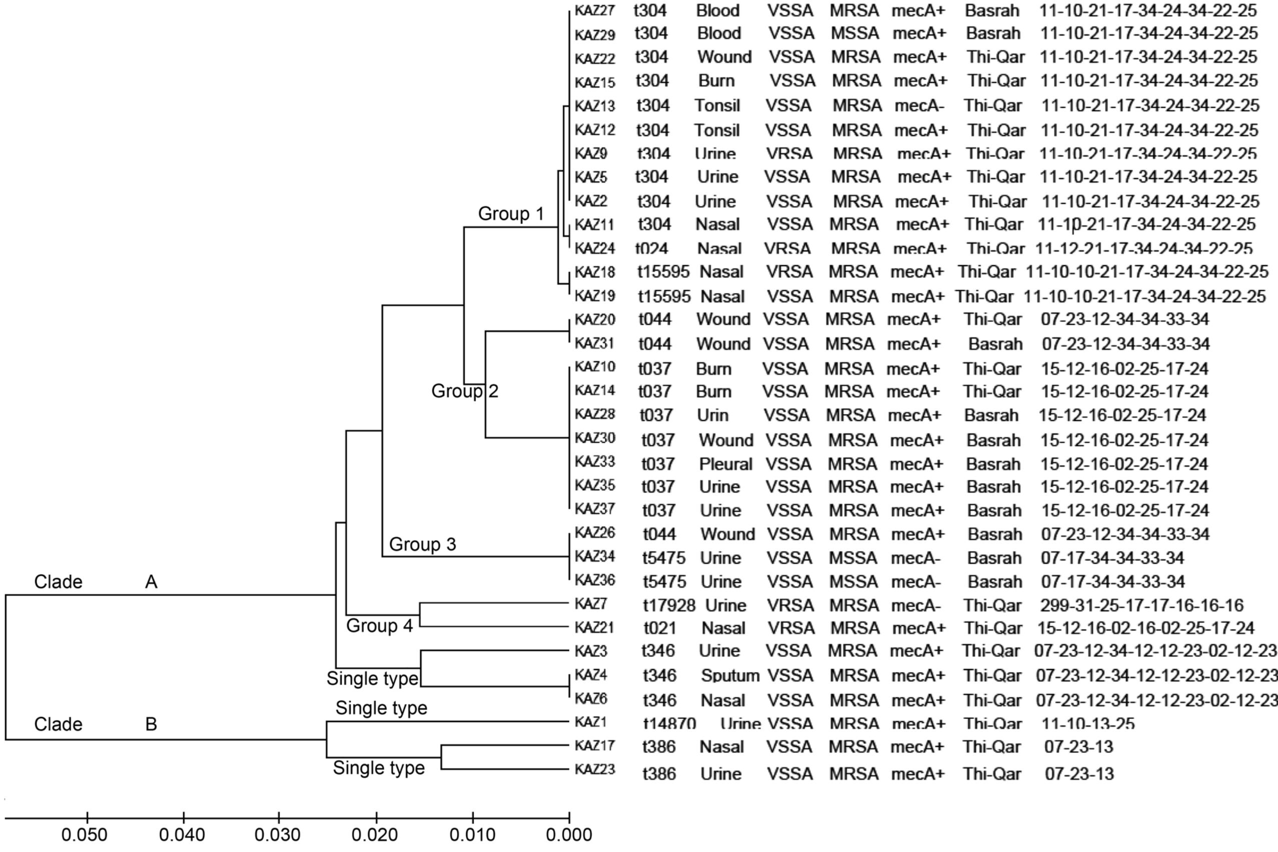

Fig. 2. Phylogenetic tree based on specimens, strain types, *spa* types, vancomycin and methicillin resistance, the *mecA* gene, geographical location, and *spa* repeats.

clade A. Thirty-three (91.7%) isolates were included in clade A, whereas only 3 (8.3%) isolates were included in clade B. Within clade A, the isolates were further clustered into four different groups and three single types based on the variation in tandem repeats of the *spa* gene, methicillin resistance, specimen source, and geographical location (Fig. 2). Cluster 1 consists of 13 out of 36 (36.1%) isolates, including *spa* type 304, t024, and t11595. Cluster 2 consists of nine out of 36 (25%) isolates, including type t044 and t037. Cluster 3 consists of three out of 36 (8.3%) isolates, including t5475 and t044. Cluster 4 consists of two out of 36 (5.5%) isolates, including t17928 and t021. The *spa* types t346, t14870, and t386 showed a single type.

Discussion

The high prevalence of MRSA is becoming a great public health concern. The resistance of *S. aureus* to multiple drugs restricts therapeutic options and causes severe morbidity and mortality in hospitalized patients and among communities (Gajdács 2019). The rate of methicillin resistance in our study was 95.4%, which is comparable with the findings of previous local studies (Al-kadmy 2013; Ibed and Hamim 2014; Kareem et al. 2015; Kareem et al. 2020) and higher than other studies in different geographic regions of the world (Wang et al. 2012; Cirkovic et al. 2015; Akanbi et al. 2017; Gitau et al. 2018). Many factors could contribute to the variation in the rate of resistance, such as the population studied, type of isolates, and prescription of certain antibiotics in different geographic areas. Two MRSA isolates were negative for the *mecA* gene; this could indicate that these MRSA isolates have a different mechanism for methicillin resistance than through the *mecA* gene (Ba et al. 2014). On the other hand, a single MSSA isolate was *mecA*-positive. It could be due to an effective mutation leading to an inactivate *mecA* gene (Kuwahara-Arai et al. 1996). Our findings show that the presence or absence of the *mecA* gene may not be sufficient for the confirmed characterization of MRSA and MSSA isolates. However, further study is needed to understand the mechanism behind such a phenomenon of these isolates (MRSA *mecA*-negative and MSSA *mecA*-positive).

In Iraq, as indicated in this study and other recent studies (Kareem et al. 2015; Kareem et al. 2020), the prevalence of MRSA has significantly increased. Therefore, rapid and efficient typing of MRSA isolates is essential for epidemiological survey and infection control.

In the present study, *spa* typing based PCR and DNA sequencing were used to determine MRSA types

isolated from clinical samples. A selected number of clinical isolates (33 MRSA and three MSSA) as representative isolates collected from two regions in the south of Iraq were subjected to *spa typing*. Since it has several advantages such as simplicity, high discriminatory power, ease of interpretation, and reproducibility.

Our results revealed 11 different *spa* types, which were clustered into different groups (Fig. 2). This highlights the relationship between *spa* types and the potential application of *spa* typing in assessing phylogenetic and clonal relationships among clinical isolates. For epidemiological purposes, the clinical isolates were classified into two clades (Fig. 2). Out of 36 isolate, 33 strains belonged to t304, t037, t024, t044, t346, t021, t15595, and t5475, t17928 were clustered in clade A, indicating that 91.7% of the tested isolates were clonally related in the south of Iraq. Only three out of 36 strains (8.3%) belonging to two *spa* types (t386, t14700) were included in clade B, which could be explained by patient mobility from a different region in Iraq.

The *spa* types obtained in the present study varied in length between 10 (t346, t15595) and three (t386) repeats. The majority of the tested isolates belonged to t304 (30.3%) followed by t037 (19.4%). To our knowledge, this is the first time that these *spa* types have been found in Iraq. The phylogenetic tree (Fig. 2) showed that t037 is the predominant *spa* type in Basrah province, and all isolates included in this type are methicillin resistance. *Spa* type t037 was widely reported as the second common *spa* type in many Asian countries (Korea, Taiwan, Malaysia, Iran) and African countries (Kim et al. 2011; Asadollahi et al. 2018; Zarizal et al. 2018; Lee et al. 2019). The situation was quite different in Turkey (Güven Gökmen et al. 2018), where t030 was the predominant type, and t037 was detected as a low percentage (8%). Shakeri and Ghaemi (2014) found that the *spa* type t037 was the most predominant and the most common type among MRSA isolates in Iran. The regional clusters of *spa* type t037 found in both countries (Iran and Iraq) could be explained by cross-border patient mobility between Iraq and Iran. Accordingly, the cross-border transfer of patients may have a vital influence on the spreading and prevalence of MRSA in a certain clone (t037).

The present study showed that *spa* type t304 was the predominant type in the Thi-Qar province, and most of the strains of this type were methicillin-resistant. Few strains of this type were isolated from patients in Basrah province. *Spa* type t304 has been reported previously in Oman as the predominant type (Udo et al. 2014) but were not detected in Saudi Arabia (Monecke et al. 2012), Qatar (El-Mahdy et al. 2014), and Kuwait (Udo and Al-Sweih 2013). Generally, *spa* type t304 is rare globally and found only in five countries with a small number (Asadollahi et al. 2018). However, our results showed a high frequency of t304 (30.6%) as the dominant type.

Other *spa* types identified with lower frequency in this study corresponded to t346, t044, t15595, t386, t5475, t17928, t14870, t021, and t024. All these *spa* types have been recorded for the first time in Iraq. Two strains belonged to t5475, and were isolated from patients in Basra. Both of them are methicillin-sensitive. This type (t5475) is very rare in the world; there are only six isolates with records of *spa* type t5475 in the Ridom SpaServer database (http://www3.ridom.de/spa-server/). The first MSSA isolates with *spa* type t5475 was reported from Denmark, but MRSA isolates with the same *spa* type have been reported from Sweden (http://www3.ridom.de/spa-server/).

Additionally, the present study showed that only one MRSA strain belonged to *spa* type t17928; noticeably, there is only one MSSA strain with records of this *spa* type that was reported from Sweden (http://www3.ridom.de/spa-server/). Furthermore, our results showed three MRSA strains belonged to t044, and one MRSA strain belonged to each of t021 and t024. All of these *spa* types are frequently reported in Europe (Asadollahi et al. 2018).

The present study explored distinct *spa* types recorded for the first time in Iraq and many of these *spa* types were not reported in the local region or neighboring countries. This could be explained by cross-border patient mobility or migrations from or to Iraq during and after the 2003 Iraq invasion. Although these findings provide vital information on the types of MRSA in Iraq, there were some limitations. The sources and the number of clinical samples were not enough to generalize the entire country's conclusions. Further studies are required to investigate more clinical samples, determine the *spa* types of the MRSA isolates, and trace the origin of these isolates.

This study also, revealed that 21.5% of isolates carried genes code for PVL. It was lower than the 44.3% and 54.2% *lukS-PV-lukF-PV*-positive isolates reported in Oman (Udo et al. 2014) and Saudi Arabia (Monecke et al. 2012), respectively but higher than the 14.6% positive rate obtained in Kuwait (AlFouzan et al. 2013) and the 12.7% positive rate obtained in Turkey (Akoğlu et al. 2010). Our results also showed a lower rate of the *lukS-PV-lukF-PV*-positivity than that obtained in Egypt (Abd El-Hamid et al. 2019). On the other hand, the present results are comparable with positive rates obtained in Iran (Fard-Mousavi et al. 2015), indicating the MRSA diversity harbored *lukS-PV-lukF-PV* genes in the region.

The rate of the prevalence of the *lukS-PV-lukF-PV* genes in MRSA isolated from burn specimens and other sites, in this study, suggests that PVL protein is an effective virulence factor of MRSA infections in our

community. Furthermore, no significant association was observed between *lukS-PV-lukF-PV*-positivity and a particular *spa* type, as the presence of the *lukS-lukF-PV* genes were observed among diverse *spa*-types.

In conclusion, our study reported a significant increase in the prevalence of CA-MRSA in Iraq. Based on *spa* typing, eleven different MRSA *spa* types were identified, with *spa* t037 and t304 being the predominant types. The CA-MRSA data could be useful in characterizing MRSA in Iraq and establishing a proper preventive and curative program. Therefore, further studies should focus on identifying MRSA and the incidence of different *S. aureus spa* types.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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