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Identification of virulence genes from clinical isolates of Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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Abstract. *Mohammad SQ, Awayid HS, Zarrouk-Mahjoub S. 2024. Identification of virulence genes from clinical isolates of Methicillin-Resistant* Staphylococcus aureus *(MRSA). Biodiversitas 25: 5083-5093.* Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of *Staphylococcus aureus* that has developed resistance to beta-lactam antibiotics, including penicillin, methicillin, amoxicillin, and oxacillin. This resistance complicates treatment compared to non-resistant strains. While previous studies have defined MRSA phylogeny and transmission in well-resourced settings, this study specifically highlights the effectiveness of five targeted loci analyses (*erm*A, *erm*B, *erm*C, *hla*-α, and *tsst*-1) for understanding MRSA evolution in diverse clinical contexts. This study analyzed the phylogenetic relationships among 20 MRSA isolates, which were amplified from the targeted loci. The research focused on the *erm*A locus in three isolates (A1-A3), the *erm*B locus in nine isolates (B1 to B9), the *erm*C locus in six isolates (C1 to C6), the *hla*-α gene in one isolate (D1), and the *tsst-1* gene in one isolate (E1). Genetic diversity was assessed based on the variants identified within these loci. Direct sequencing of the amplified fragments was conducted to detect genetic polymorphisms, and coding variants were translated to evaluate their potential effects on protein function. A phylogenetic tree was constructed to evaluate the associations and distributions of the experimental variables. Sequencing results confirmed that the isolates were *S. aureus*. The alignment revealed no variants in samples A1-A3, C1-C6, and D1. One variant (176G>A) was found in group B1-B9, resulting in a missense mutation (p.100Ser>Asn). Sample E1 contained a single nucleotide deletion (56C-del). Phylogenetic analysis revealed distinct clades of isolates that corresponded with various clinical and non-clinical sources. The *erm*A locus proved to be more effective in tracking the evolutionary trajectories of the studied *S. aureus* isolates compared to the other loci. These findings suggest that *erm*A markers could be valuable for broader applications in monitoring the evolutionary distributions of bacterial strains across diverse contexts. Further investigation may enhance our understanding of bacterial evolution in various environments.

Keywords: Diversity, *erm*A, *erm*B, *erm*C, *hla*-α , *tsst*-1

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first identified by scientists in the UK in 1961. It is a major bacterial pathogen that is responsible for a wide range of illnesses and poses a significant global health risk due to its high mortality and morbidity rates. The pathogenicity of MRSA is attributed to extracellular toxins and biofilm production, which facilitate its rapid spread and prevalence worldwide, including in the USA, Europe, North Africa, and the Middle East (Sami Awayid and Qassim Mohammad 2022). Biofilms are primarily composed of water and organic components, including polysaccharides, DNA, and proteins. Biofilm formation occurs in multiple stages: adhesion, maturation, and dispersion. Initial bacterial adhesion can take place in the biotic (blood vessels, bones, or joints) or abiotic environment (medical device) (Kaushik et al. 2024). MRSA is categorized into three distinct types based on transmission patterns and environmental context: hospital-acquired (MRSA-HA), community-acquired (MRSA-CA), and livestock-associated (MRSA-LA). MRSA-HA predominantly affects immunocompromised patients undergoing extended hospital stays. MRSA-CA is commonly found among individuals who frequently visit healthcare facilities or undergo medical intervention. MRSA-LA, first identified in 1972 in cases of bovine mastitis in Belgium, has since been detected in various regions, affecting farm animals such as cattle and swine (Krishnamoorthy 2019; Crespo-Piazuelo and Lawlor 2021; Nikolic and Mudgil 2023). *S. aureus* is part of the natural microbial flora and can be found on the skin, nails, or nasal passages. However, it can also spread to other areas of the human body. More than 25% of individuals infected with MRSA are asymptomatic, facilitating its transmission across various environments. This pathogen primarily resides in burns, wounds, and urinary tract infections and ranks second to *Escherichia coli* (Saeed et al. 2021; Yurnaliza et al. 2024). The World Health Organization recognizes MRSA as a significant public health concern due to its antibiotic resistance (Shehab et al. 2023).

The prevalence of MRSA has increased in clinical samples from various countries in recent years. Three primary factors contribute to the dissemination of MRSA: social behavior, antibiotic usage, and medical procedures.

High rates of MRSA infection have been reported in several countries, including Sri Lanka (86.5%), Korea (77.6%), Vietnam (74.1%), Taiwan (65.0%), Thailand (57.0%), Hong Kong (56.8%), and Iran (43.5%) (Yan et al. 2016). In India and the Philippines, it reached 38.1% and 22.6%, respectively (Mehta et al. 2020). The prevalence of MRSA isolates in Iraq varied across different regions. A study conducted in Dhi-Qar Governorate in southern Iraq found a prevalence of 89%, while Basra reported a prevalence of 94% (Alkhafaji 2020). In central Iraq, a study conducted in the capital, Baghdad, reported a prevalence of 78%. Additionally, a study in Diyala and Baghdad indicated an MRSA rate of 80.7% (Hami and Ibrahim 2023).

MRSA acquires resistance to antibiotics through several mechanisms, including altering the antibiotic's target by modifying the protein, which prevents methicillin from inhibiting cell wall synthesis. It also acquires resistance genes such as *mec*A, which codes for penicillin-binding protein 2a (PBP2a), and develops an antibiotic-impermeable cell membrane. Additionally, MRSA can pump antibiotics out of the cell using efflux pumps. These mechanisms render MRSA unresponsive to conventional antibiotic therapy (Nadeem et al. 2020; Yaseen et al. 2023). Key virulence factors of MRSA include the erythromycin resistance gene (*erm*), the alpha toxin gene (*hla*), and the toxic shock syndrome toxin-1 gene (*test*-1) (Afnani et al. 2022; Zhu et al. 2023; Anggraini and Yunita 2024). In this study, the genetic profiles of the bacterial isolates were determined through a comprehensive sequence analysis of the target sites. Subsequent protein translation studies evaluated the potential effects of the identified genetic variants on the encoded proteins. Finally, a detailed phylogenetic tree was constructed to accurately represent the distribution and classification of these variants with their evolutionary context. The study aimed to sequence MRSA genes and Investigate the spread of MRSA from different clinical sites.

The novelty of the findings in this study lies in the comprehensive genetic profiling of MRSA isolates through targeted sequence analysis of key loci (*erm*A, *erm*B, *erm*C, *hla*-α, and *tsst*-1). While existing literature has established the prevalence and resistance mechanisms of MRSA, this research provides a detailed examination of the genetic diversity and phylogenetic relationships among isolates from various clinical settings.

MATERIALS AND METHODS

Collection of samples

Three hundred human samples were taken from patients at four hospitals in the Diyala Governorate, Iraq: Baquba Teaching Hospital, Muqdadiyah General Hospital, Al-Zahraa Hospital, and Al-Batoul Hospital, as well as from outpatients. The samples included wound swabs (n=65), burn swabs (n=60), sputum swabs (n=75), and urine cultures (n=100), all collected under optimal conditions for laboratory examination.

Isolation and identification of MRSA

All samples were directly cultured on nutrient agar, blood agar, and mannitol salt agar, then incubated for 24 h at 37°C. Following incubation, the samples were streaked on mannitol salt agar, a differential and selective medium for diagnosing *S. aureus* due to its high salt content that inhibits the growth of other microorganisms. Bacterial colonies were prepared on glass slides and Gram stain was performed. The shape, color, and arrangement of the bacterial cells were observed using a microscope. Microscopic examination revealed cocci arranged in pairs or clusters, resembling grape-like formations with a blue colony color. Subsequently, biochemical tests - including catalase, oxidase, coagulase, gelatinase, and urease were conducted to confirm the diagnosis (William et al. 2009).

MRSA isolates were initially identified using HiCrome MeReSa Agar Base (HIMEDIA, India). This selective medium facilitates the isolation and identification of methicillin-resistant strains based on their color. The MeReSa Agar Base media were inoculated with pure bacterial colonies and incubated at 37°C for 24 h, resulting in colonies exhibiting a bluish-green color. Some MRSA strains may demonstrate poor growth, necessitating further incubation for up to 48 hr before result interpretation.

Molecular identification of MRSA

S. aureus isolates were used to extract genomic DNA, with PCR primers synthesized by Macrogen Laboratories according to the sequences available in the National Centre for Biotechnology Information (NCBI) (Table 1). The PCR program involved an initial denaturation at 95°C for 5 min, 30 cycles of denaturation, annealing (50°C to 60°C), and extension steps with a final extension at 72°C for 7 min (Kmiha et al. 2023).

PCR program

To pellet the resulting solution at the bottom of the tube, the response solution was placed in a microcentrifuge tube and centrifuged at 15000 rpm for ten seconds. The tubes were carefully inserted into the thermocycler to complete the amplification according to the specific program for each primer, as shown in Table 2.

DNA loading and sequencing

After loading the PCR products onto the agarose gel, electrophoresis was performed for 60 min at 100V. A gel imaging device was then used to visualize bands stained with ethidium bromide. According to the recommended guidelines provided by the sequencing laboratory (Macrogen Company, South Korea), the specific PCR amplicon products were submitted for sequencing from their forward termini. The retrieved sequencing chromatograms were manually analyzed using SnapGene Viewer, which was employed to annotate the nucleic acid peaks accurately.

Table 1. A list of PCR primer sequences employed in this study

Genes	Primer	Sequence 5`-3	Annealing temperature (°C)	Product size (bp)	Reference
*erm*A	F	AAGCGGTAAACCCCTCTGA	55	190	(Krishnamoorthy et al. 2019)
	R	TTCGCAAATCCCTTCTCAAC			
*erm*B	F	CATTTAACGACGAAACTGGC	60	425	
	R	GGAACATCTGTGGTATGGCG			
*erm*C	F	AATCGTCAATTCCTGCATGT	55	299	
	R	TAATCGTGGAATACGGGTTTG			
*mec*A	F	GTG AAG ATA TAC CAA GTG ATT	50	146	
	R	ATG CGC TAT AGA TTG AAA GGA			
hla-α	F	CGG TAC TAC AGA TAT TGG AAGC	60	744	(Kshetry et al. 2016)
	R	TGG TAA TCA TCA CGA ACT CG			
tsst-1	F	TTA TCG TAA GCC CTT TGT TG	60	398	
	R	TAA AGG TAG TTC TAT TGG AGT AGG			

Table 2. PCR program used in the studied genes

Steps	Temperature (°C)	Time (min: sec)	Number of cycles
Initial denaturation	95.0	05:00	1
Denaturation	95.0	00:30	30
Annealing		00:30	
ermA	55		
ermB	60		
ermC	55		
hla-α	60		
tsst-1	60		
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Data analysis and phylogenetic tree construction

Bio Edit Genome Alignment Designer software was utilized to align and evaluate the sequencing results, and the genes were submitted to NCBI to obtain unique accession codes. The neighbor-joining approach was used to construct a phylogenetic tree, which was subsequently displayed as a rectangular cladogram using iTOL software.

RESULTS AND DISCUSSION

Isolation and identification of MRSA

A total of 300 medical specimens, including sputum, urine, burns, and wounds, were collected from various clinical sources. These samples were cultivated for 24 h at 37°C on agar with mannitol salts and blood agar. Among the 170 isolates (57%), 125 (41%) exhibited positive growth and were identified as *S. aureus*. Using HiCrome MeReSa Agar Base, 25 isolates (20%) of them were identified as MRSA. On this selective medium, the MRSA colonies showed bluish-green coloration, validating their ability to resist profile (Figure 1). This selected growth pattern validates the efficacy of HiCrome MeReSa Agar in distinguishing resistant varieties of *S. aureus* it is consistent with known protocols for identifying MRSA strains (Singh et al. 2017).

Distribution of MRSA isolates by source

The MRSA isolates were categorized based on their infection origins. The data indicated 12 isolates (48%) were derived from urinary tract infections, 6 isolates (24%) from burn infections, 4 isolates (16%) from post-surgical wound infections, and 3 isolates (12%) from throat infections, as shown in Figure 2.

Figure 1. Colonies *S. aureus* bluish-green color on HiCrome MeReSa agar

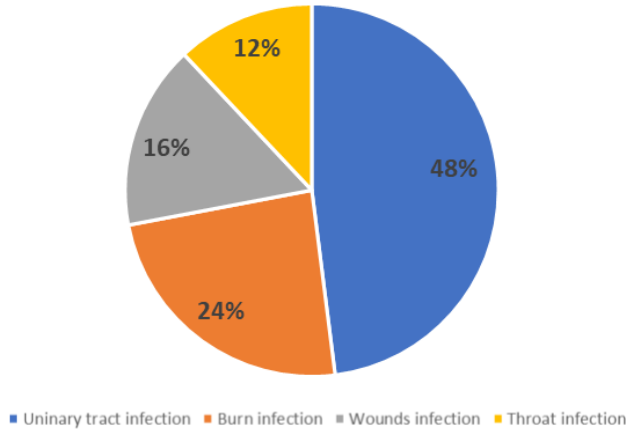

Figure 2. Distribution of the identified bacterial isolates according to the source of isolation

Table 3. The details of the identified variants in the investigated sequences that partially covered the *erm*A, *erm*B, *erm*C, *hla*-α, and *tsst*-1 sequences within the investigated isolates of *S. aureus*, respectively

No.	Sample	Position in PCR amplicon	Variation	SNP	Position in protein	Consequences
A	*ermA*					
	No variation detected	-	-	-	-	-
B	*ermB*					
	B5, B6, B7, B8, B9	176	G>A	176G>A	Ser100	Missense (p.100Ser>Asn)
C	*ermC*					
	No variation detected	-	-	-	-	-
D	*hla-α*					
	No variation detected	-	-	-	-	-
E	*tsst-1*					
	E1	56	C-del	56C-del	Pro28	Frameshift (56C-del)

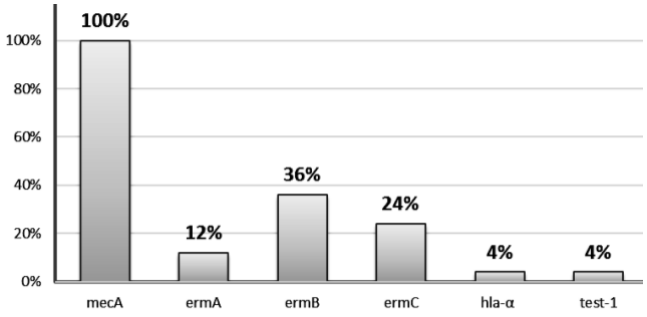

Figure 3. Distribution of various genes of MRSA isolates

Genetic detection of resistance and virulence genes

The *erm*B gene was present in 36% of the isolates, while *erm*C had an occurrence rate of 24%, and the *erm*A gene was found in only 12% of the isolates. Among the virulence genes present, *hla-α* and *tsst*-1 were detected at a frequency of 4% each. All MRSA isolates were found to carry the *mecA* gene, which is a definitive indicator of methicillin resistance, as shown in Figure 3.

Sequencing and genetic variations

To establish the variances in this study, the sequencing chromatograms for each sample must be analyzed and documented with additional comments. Furthermore, the chromatograms illustrating the sequence locations within the PCR amplicons for *S. aureus* were also presented. All original chromatograms can help rule out some technical errors from variations observed. e DNA sequencing results were confirmed in their chromatograms by showing that there were no nucleotide changes between any of the A1-A3 specimens and the *S. aureus* standard genomes (GenBank acc. number NG_047791.1). Further sequencing of 20 clinical samples identified specific genetic mutations in the MRSA isolates. A missense mutation (176G>A) in the *erm*B gene was detected, resulting in a serine-to-asparagine substitution at position 100, which could potentially affect the protein's function and confer antibiotic resistance. Additionally, a frameshift mutation characterized by a cytosine deletion at the 56th position was also observed in the *tsst*-1 gene.

Total genetic variations in the amplified genes of *S. aureus* in this study

Research data on the genetic variants discovered in *S. aureus* amplified genes are presented in Table 3. The location of each nucleotide alteration in the protein is indicated by its position in the protein's structure column. For example, "Ser100" designates the 10th amino acid existing in the protein. The influence of each mutation on the gene functions is described accordingly.

Phylogenetic analysis

In order to investigate the genetic variation among MRSA isolates, phylogenetic analysis was conducted. The findings from the sequenced *erm*A, *erm*B, *erm*C, *hla-α,* and *tsst*-1 genes are illustrated in various phylogenetic trees, which indicate distinct clades representing significant genetic variability.

The phylogenetic tree based on the *erm*A gene revealed various related clades, suggesting separate origins or different evolutionary paths over time. Twenty-nine nucleic acid sequences were aligned across the entire phylogenetic tree in relation to the *erm*A sequences. Thirteen Japanese bacterial strains were identified when clade-1 was combined with the A1-A3 samples that were investigated (GenBank AP019543). Clade 2, which had two clinical varieties of *S. aureus*, was positioned adjacent to clade 1. Due to its proximity to the tree's roots, clade 1 appears to be related to the progenitors from whence the other clades originated (Figure 4; Table 4).

Concerning the *erm*B sequences, a total of twenty-four nucleic acid sequences were aligned within this comprehensive tree. Notably, the investigated B1-B9 samples were incorporated into two different clades within the same tree. Due to the evolutionary effect of the identified variant of 176G>A, it has been found that samples having this variant located in the Cla de 2 beside four American strains from the same clinical source (CP092548). On the other hand, clade 3 originated from clades 1 and 2, respectively. Given the close positioning of clade 1 toward the roots of the tree, it is expected that this clade is the ancestor from which clades 2 and 3 originated (Figure 5; Table 5).

Concerning the *erm*C sequences, the total number of aligned nucleic acid sequences in this comprehensive tree was twenty. For this reason, the strains of clade 1, in which the investigated C1-C6 samples were positioned, may represent the main ancestor from which clade 2 and 3 respectively originated (Figure 6; Table 6).

Concerning the hla-α sequences, a total of thirty-one nucleic acid sequences were aligned within this comprehensive tree. As observed with the other loci, the only bacterial species represented among the incorporated sequences was *S. aureus*. This finding underscores the high specificity of this genetic fragment for identifying *S. aureus* without confusion with related bacterial organisms. What has characterized this fragment over the other fragments is the higher range of diversity among the *S. aureus* strains, attributed to several genetic variations of the investigated *hla*-α, which are represented by five different phylogenetic clades (Figure 7; Table 7).

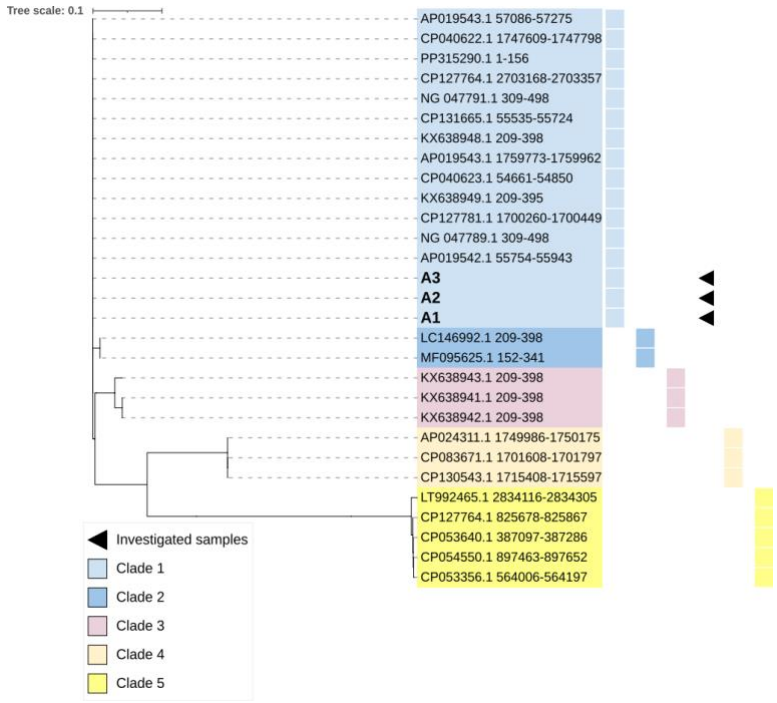

Figure 4. Phylogenetic tree illustrating the evolutionary relationships of *erm*A gene fragment in three *S. aureus* samples

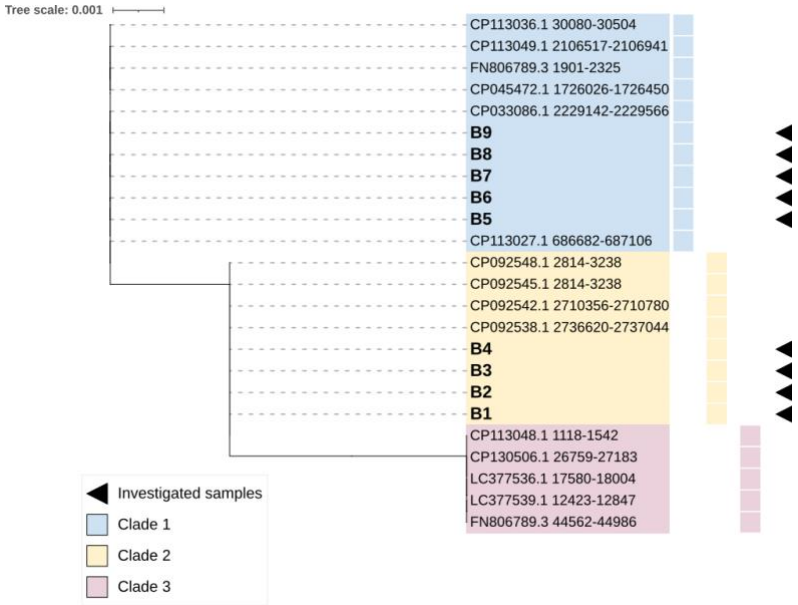

Figure 5. Phylogenetic tree illustrating the evolutionary relationships of the *erm*B gene fragment in nine *S. aureus* samples

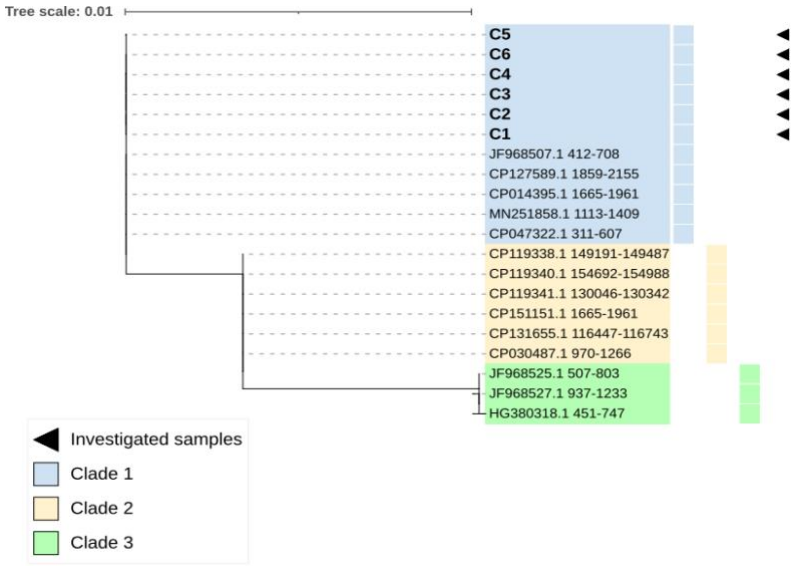

Figure 6. Phylogenetic tree illustrating the evolutionary relationships of the *erm*C gene fragment in six *S. aureus* samples

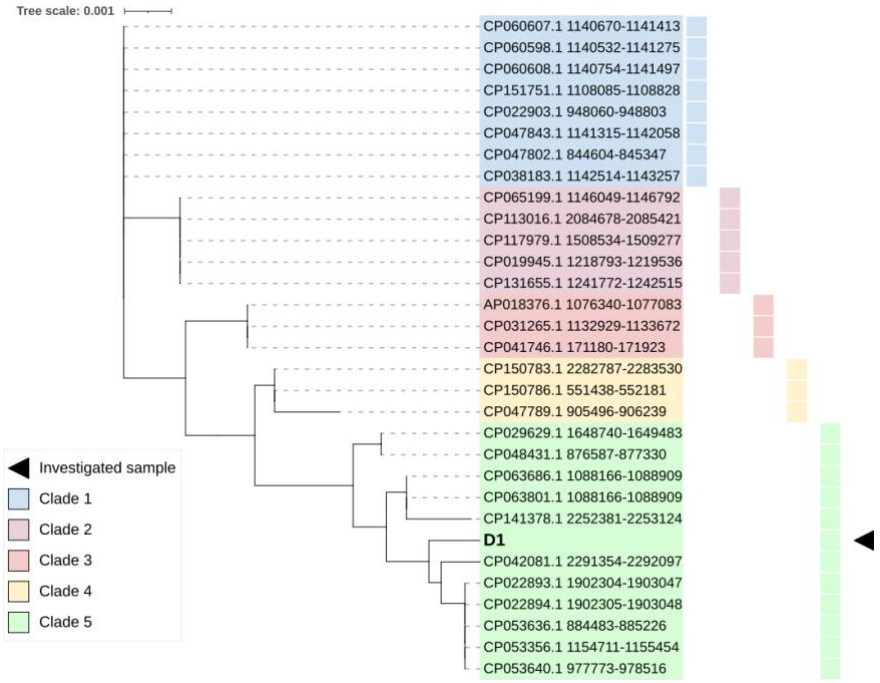

Figure 7. Phylogenetic tree illustrating the evolutionary relationships of *hla*-α gene fragment in six *S. aureus* samples.

The tree based on the *tsst*-1 gene indicated a more recent divergence among certain isolates, which may imply the acquisition of distinct genetic traits for adaptation and virulence-specific environments. A total of twenty-six nucleic acid sequences were aligned within this comprehensive tree. Notably, the only species identified among the integrated bacterial sequences was *S. aureus*. This finding, consistent with the results from previous loci, highlights the remarkable specificity of this genetic fragment in accurately identifying this particular type of bacterial sequence, without ambiguity with other closely related bacterial organisms. In the constructed cladogram, the incorporated samples were clustered into three phylogenetic clades within *S. aureus* (Figure 8, Table 8).

Discussion

This study involves a comprehensive investigation into the prevalence, virulence, and antibiotic resistance of MRSA in Diyala Governorate of Eastern Iraq. Samples were collected via swabs from human patients across various clinical sources including several hospitals in the region. The detailed methodology includes several microbiological techniques specifically designed to identify MRSA. Initial culturing was performed on mannitol salt agar swabs, which is both selective and differential for *S. aureus*. The high salt concentration inhibits the growth of most other

bacteria, while the presence of mannitol allows for differentiation based on mannitol fermentation. *S. aureus* typically ferments mannitol, producing in the production of yellow colonies (Musa et al. 2023).

MRSA isolates were diagnosed phenotypically using a color guide after culturing on a selective media, HiCrome MeReSa Agar Base, where the colonies appeared green after incubation for 24 to 48 hours (Singh et al. 2017). The study results were consistent with those obtained by Ghasemian et al. (2016), which reported an MRSA isolation rate of 19%. However, these findings differ from the study conducted by Al Fayyad (2021) in Diyala Governorate, which reported a higher isolation rate of MRSA of 38% from the same isolation sources. Additionally, our study revealed a significantly higher MRSA isolation rate compared to the research by Dağı et al. (2015), where the rate of MRSA isolation from the nasal samples was only 3%.

Table 4. A list of the isolation sources, countries, and clades of *S. aureus* strains included in the *ermA*-based phylogenetic tree

GenBank	Isolation source	Country	Clade
AP019543	Clinical (blood)	Japan	1
CP040622	Clinical	Australia	1
PP315290	Clinical (blood)	Saudi Arabia	1
CP127764	Clinical (sinus swab)	Australia	1
NG_047791	Clinical	New Zealand	1
CP131665	Clinical	Taiwan	1
KX638948	Clinical (human excrement)	China	1
AP019543	Clinical (blood)	Japan	1
CP040623	Clinical	USA	1
KX638949	Clinical (human excrement)	China	1
CP127781	Clinical (sinus swab)	Australia	1
NG_047789	Clinical	USA	1
AP019542	Clinical (blood)	Japan	1
LC146992	Clinical (thorat Swab)	India	2
MF095625	Clinical	India	2
KX638943	Clinical (human excrement)	China	3
KX638941	Clinical (human excrement)	China	3
KX638942	Clinical (human excrement)	China	3
AP024311	Clinical	Japan	4
CP083671	Clinical (blood)	South Korea	4
CP130543	Clinical (secretion)	China	4
LT992465	Clinical	Germany	5
CP127764	Clinical (sinus swab)	Australia	5
CP053640	Clinical	USA	5
CP054550	Clinical	USA	5
CP053356	Clinical	USA	5

Table 5. A list of the isolation sources, countries, and clades of *S. aureus* strains included in the *ermB*-based phylogenetic tree

GenBank	Isolation source	Country	Clade
CP113036	Environmental	Taiwan	1
CP113049	Environmental	Taiwan	1
CP045472	Clinical (blood)	China	1
CP033086	Clinical (sputum)	China	1
CP113027	Environmental	Taiwan	1
FN806789	Cattle	Germany	1
CP092548	Clinical (urine)	USA	2
CP092545	Clinical (urine)	USA	2
CP092542	Clinical (urine)	USA	2
CP092538	Clinical (urine)	USA	2
CP113048	Environmental	Taiwan	3
CP130506	Clinical (secretion)	China	3
LC377536	Clinical	Taiwan	3
LC377539	Clinical	Taiwan	3
FN806789	Cattle	Germany	3

Table 6. A list of the isolation sources, countries, and clades of *S. aureus* strains included in the *erm*C-based phylogenetic tree

GenBank	Isolation source	Country	Clade
CP113036	Environmental	Taiwan	1
CP113049	Environmental	Taiwan	1
CP045472	Clinical (blood)	China	1
CP033086	Clinical (sputum)	China	1
CP113027	Environmental	Taiwan	1
FN806789	Cattle	Germany	1
CP092548	Clinical (urine)	USA	2
CP092545	Clinical (urine)	USA	2
CP092542	Clinical (urine)	USA	2
CP092538	Clinical (urine)	USA	2
CP113048	Environmental	Taiwan	3
CP130506	Clinical (secretion)	China	3
LC377536	Clinical	Taiwan	3
LC377539	Clinical	Taiwan	3
FN806789	Cattle	Germany	3

Table 7. A list of the isolation sources, countries, and clades of *S. aureus* strains included in the *hla-α*-based phylogenetic tree

GenBank	Isolation source	Country	Clade
CP060607	Clinical (nasal cavity)	Germany	1
CP060598	Clinical (nasal cavity)	Germany	1
CP060608	Clinical (nasal cavity)	Germany	1
CP151751	Clinical (blood)	USA	1
CP022903	Clinical (blood)	Germany	1
CP047843	Clinical	Germany	1
CP047802	Clinical	Germany	1
CP038183	Clinical (sperm)	China	1
CP065199	Non-clinical (pig)	Germany	2
CP113016	Clinical (environmental)	Taiwan	2
CP117979	Clinical	USA	2
CP019945	Non-clinical (bovine milk)	China	2
CP131655	Clinical	Taiwan	2
AP018376	Clinical	Japan	3
CP031265	Non-clinical (buffalo milk)	Brazil	3
CP041746	Clinical	South Korea	3
CP150783	Clinical	Japan	4
CP150786	Clinical	Japan	4
CP047789	Clinical	Germany	4
CP029629	Non-clinical (bovine milk)	Ireland	5
CP048431	Non-clinical (bovine)	USA	5
CP063686	Clinical (wound)	India	5
CP063801	Clinical (hand)	India	5
CP141378	Clinical	Kenya	5
CP042081	Non-clinical (retail pork)	USA	5
CP022893	Clinical (blood)	Germany	5
CP022894	Clinical (blood)	Germany	5
CP053636	Clinical	USA	5
CP053356	Clinical	USA	5
CP053640	Clinical	USA	5

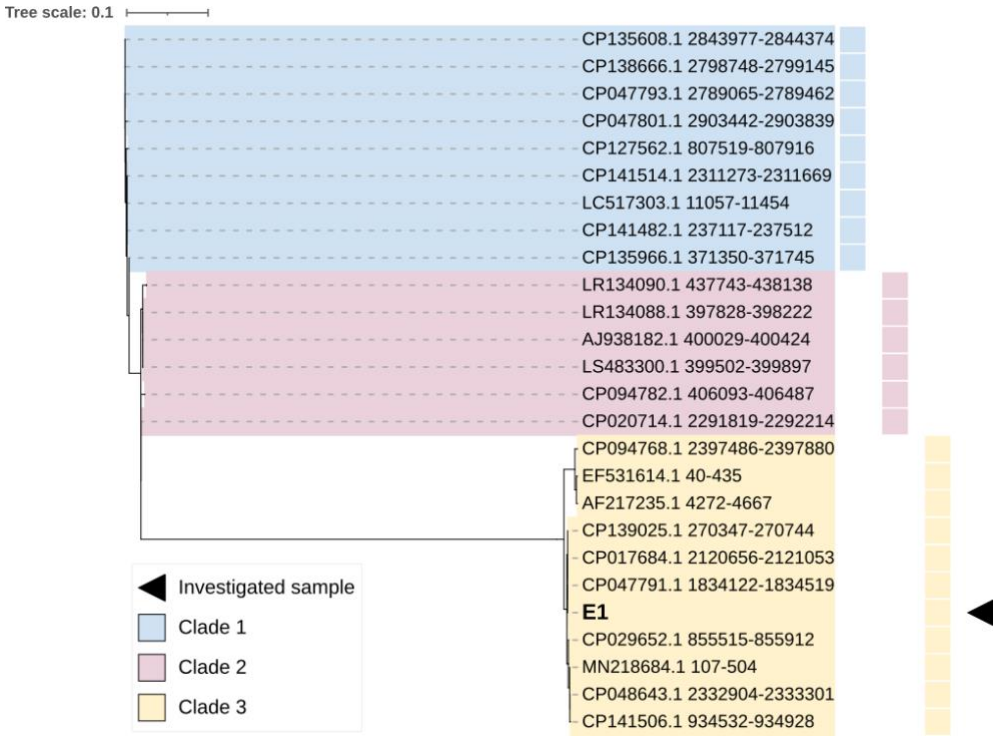

Figure 8. Phylogenetic tree illustrating the evolutionary relationships of the *tsst-1* gene fragment in six *S. aureus* samples

Table 8. A list of the isolation sources, countries, and clades of *S. aureus* strains included in the *tsst*-1-based phylogenetic tree

GenBank	Isolation source	Country	Clade
CP135608	Clinical (wound)	USA	1
CP138666	Clinical	Spain	1
CP047793	Clinical	Germany	1
CP047801	Clinical	Germany	1
CP127562	Clinical (sinus swap)	Australia	1
CP141514	Clinical	Kenya	1
LC517303	Clinical (synovial fluid)	Japan	1
CP141482	Clinical	Kenya	1
CP135966	Non-clinical (bovine)	Tanzania	1
LR134090	Unknown	UK	2
LR134088	Unknown	UK	2
AJ938182	Non-clinical (bovine)	Ireland	2
LS483300	Unknown	UK	2
CP094782	Non-clinical (bovine milk)	Kenya	2
CP020714	Non-clinical (buffalo milk)	India	2
CP094768	Non-clinical (bovine milk)	Kenya	3
EF531614	Non-clinical (bovine)	Switzerland	3
AF217235	Non-clinical (bovine)	Ireland	3
CP139025	Clinical (wound)	Sweden	3
CP017684	Non-clinical (environment)	USA	3
CP047791	Clinical	Germany	3
CP029652	Clinical	USA	3
MN218684	Clinical (sputum)	Japan	3
CP048643	Clinical (blood)	China	3
CP141506	Clinical	Kenya	3

Merdaw and Abdul-Kareem (2018) indicated in their study, conducted from January to June 2018 across 14 Iraqi governorates, that *S. aureus* was the third most common bacterial contaminant in operating rooms. This suggests a significant risk of infection and underscores the need for stringent infection control measures in surgical settings. In 2015, the Kurdistan Region of Northern Iraq reported a high prevalence of MRSA, reaching 53%, indicating a substantial burden of MRSA in the region (Hussein et al. 2015).

According to multiple studies conducted in the Middle East, the prevalence of MRSA was approximately 78% between 1999 and 2020. These studies indicate that MRSA strains have demonstrated high resistance to the antibiotics penicillin and ciprofloxacin (Nikmanesh et al. 2022). This elevated prevalence presents significant challenges in treating MRSA infections in the region, as treatment options become increasingly limited due to the resistance of these strains to common antibiotics. Consequently, there is a growing need to develop effective strategies to control infections and reduce the spread of these resistant bacteria.

Globally, numerous studies have investigated the prevalence of MRSA. A study conducted in Nepal indicated that the isolation rate of MRSA from various clinical cases reached 37%, highlighting the widespread prevalence of this microbe in the country (Kshetry et al. 2016). Another study in Iran demonstrated that *S. aureus* is a common pathogen in burn wound infections, ranking second in *Pseudomonas aeruginosa*, with an infection rate of 20%. This underscores the significance of *S. aureus* as a pathogenic factor in burn infections (Ekrami and Kalantar 2007). Additionally, a separate Iranian study conducted in Tehran found that the isolation rate of MRSA from various clinical cases reached 66%.

This distribution indicates that MRSA is more common in urinary tract infections than any other type of infection. These findings align with previous studies which have reported similar patterns; suggesting that MRSA is more likely to colonize and infect urothelial tissues due to specific microflora and host factors associated with these infections (Ghasemian et al. 2016).

The establishment of multiple resistance genes indicates that MRSA strains have evolved various means by which they evade antibiotic treatment. We employed PCR to detect several resistance and virulence genes, allowing for a more detailed characterization of MRSA isolates. The identification of virulence factors suggests that these MRSA strains may exacerbate infection severity. These findings are consistent with earlier studies on the distribution patterns of resistance and virulence genes in MRSA isolates, reflecting their broad phenotypic adaptability and survival strategies in the hospital environment (Hussein et al. 2019; Li et al. 2019).

This frameshift mutation likely disrupts the normal function of the gene, contributing to increased virulence. Such mutations are significant because they can alter the efficacy of common antibiotics and the effect of the treatment of MRSA infections (Kmiha et al. 2023). The term "missense SNP" denotes an alteration in the order of amino acids (100Ser>Asn denotes the substitution of Asparagine for Serine at position 100), which might impact protein function. The intricate genetic profile of MRSA is reflected in the diverse forms it exhibits on phylogenetic trees, which contribute to its resistance against environmental pressures such as antibiotic treatment and the host's immune system. Analyzing these genetic relationships is essential for developing more effective infection control measures tailored to combat specific MRSA strains, thereby facilitating appropriate treatment.

The sequencing processes have been validated by analyzing established phylogenetic trees, clarifying the actual neighbor-joining placement of studied sequences. Additionally, the identification of the bacterial species was accurately performed using the *erm*A, *erm*B, and *erm*C genomes in this study as well as *hla-α* and *tsst*-1. Results of our study indicate that *erm*A, *erm*B, *erm*C, *hla-α,* and *tsst*-1 are valuable for genome sequencing of *S. aureus* strains and for classifying their phylogenic relationships. Understanding the genetic differences among isolated bacteria is crucial for elucidating the evolutionary relationships among different groups of *S. aureus* and for recognizing how each isolate fits within its true biological diversity.

Concerning the *hla-α* sequences, clade 1 serves as the main clade from which the other clades likely derived due to its proximity to the roots of the phylogenetic tree. Within the clade 1, eight strains were included, sources from various clinical contexts. These stains were isolated from several German (GenBank CP060607.1, CP060598.1, CP060608.1, CP022903.1, CP047843.1, and CP047802.1), American (GenBank CP151751.1), and Asian (GenBank CP038183.1) sources. Adjacent to clade 1 is clade 2, composed of twelve clinical and non-clinical strains isolated from diverse sources. Similarly, clade 3 consists of three strains from Asian and South American origins, some of which were not collected from clinical settings. In contrast, all strains in clade 4 were clinical strains sourced from Asia and Europe, as shown in Table 7. Interestingly, clade 5 exhibits the highest level of diversity and includes the investigated D1 sample. Within this clade, the D1 sample was suited near various bacterial strains isolated from American (GenBank CP048431.1, CP042081.1, CP053636.1, CP053356.1, and CP053640.1), European (GenBank CP029629.1, CP022893.1, and CP022894.1), African (GenBank CP141378.1), and Asian (GenBank CP063686.1 and CP063801.1) sources. Notably, this clade contains several non-clinical strains positioned alongside clinical strains within the same phylogenetic context. Due to this positioning, the *hla*-α fragment may not be as specific as the *erm*A fragment in terms of accurate clinical identification and characterization. Clade 5 represents the most recent descendent clade from the others because of its distant position from the roots of the tree. Consequently, it is reasonable to speculate that the investigated D1 sample has undergone more recent evolutionary changes compared to the other samples.

Concerning the *tsst*-1 sequences, clade 1 included nine strains of *S. aureus*, which were isolated from diverse sources: American (GenBank CP135608.1), European (GenBank CP138666.1 and CP047793.1, CP047801.1), Australian (GenBank CP127562.1), Asian (GenBank LC517303.1), and African (GenBank CP141514.1, CP141482.1, and CP135966.1). Notably, not all the incorporated samples have been isolated from clinical sources; one non-clinical isolate was identified (GenBank CP135966.1), as shown in Table 8. A similar observation was noted in the other two *tsst*-1-based clades. The presence of non-clinical strains alongside clinical strains may indicate a reduced specificity of the *tsst*-1 fragment in identifying the clinical sources of these strains. Given its position near the roots of the tree, clade 1 is likely the ancestor from which the other two clades descended, a relationship evident in clade 2, which is derived from clade 1. Both clades 1 and 2 share a high level of nucleic acid similarity. In contrast, clade 3 occupies a more distant phylogenetic position relative to clades 1 and 2, suggesting its separate origin from these clades. The investigated E1 sample is grouped within clade 3 along various clinical and non-clinical strains isolated from several sources: African (GenBank CP094768.1 and CP141506.1), European (GenBank EF531614.1, AF217235.1, CP139025.1, CP047791.1), and Asian (GenBank MN218684.1 and CP048643.1). Thus, it is reasonable to speculate that the E1 sample may have originated from these multinational regions. As with the *hla*-α-based phylogenetic tree, it is possible to infer a recent evolutionary positioning of the investigated *tsst*-1 sample compared to the other samples.

In conclusion, this study underscores the significant prevalence of MRSA in clinical specimens and highlights the genetic diversity of these isolates. The identification of multiple resistance and virulence genes, along with observed genetic variations and phylogenetic diversity, indicates ongoing evolution and adaptation of MRSA, presenting substantial challenges for infection control and treatment. The highest occurrence of *S. aureus* was found

in urine samples from patients with urinary tract infections. The analysis of genetic diversity across multiple loci demonstrated high specificity in identifying *S. aureus*, reflecting the accuracy of sequencing and amplification techniques used. The phylogenetic trees categorized strains into distinct clades, providing insights into their evolutionary relationships. The *ermA* gene exhibited superior accuracy in tracing the clinical sources of infections, suggesting its potential for enhancing diagnostic strategies. Future research should focus on developing targeted interventions and monitoring the emergence of new MRSA strains to improve clinical outcomes and public health responses.

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