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Estimation the Expression of Glucose-Dependent Biofilm-Encoding *icaA* and *icaD* Genes in Methicillin Resistant *Staphylococcus aureus* Isolates

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Abstract: *Staphylococcus aureus* is known globally for causing different illnesses, ranging from mild to life-threatening infections such as septicaemia. *S. aureus* can develop a complicated structure of extracellular polymeric biofilm that shelters the cells against hostile conditions, where drugs are becoming increasingly partially or fully inactive against *S. aureus* as these agents fail to penetrate properly biofilm matrix surrounding the bacterial cells properly. Different clinical specimens were collected from different hospitals in Baghdad, Iraq. Identification of *S. aureus* was accomplished by using conventional biochemical reactions then confirmed by detection of *Sa442* gene specific to *S. aureus* by PCR technique. Vitek 2 compact system was employed to identify antimicrobial susceptibility pattern of 39 isolates against 14 antibiotics. The ability of *S. aureus* isolates to form biofilms was assessed using the microtiter plate technique. Furthermore, the presence of *icaA* and *icaD* genes was examined in all isolates using PCR. Moreover, the biofilm thickness as well as the expression of icaA and *icaD* genes was measured at different concentrations of glucose (0, 0.5, 1, 1.5 and 2%) for selected MRSA and MSSA isolates. All isolates identified as *S. aureus* by PCR exhibited the presence of the *Sa422* gene. The highest resistant percentage was identified against Benzylpenicillin (100%) followed by oxacillin and Clindamycin (82% and 62 % respectively). All isolates were capable of producing biofilm layers with 15% being strong biofilm producers and all of the isolates harboured both of *icaA* and *icaD* genes. The biofilm increased with increasing glucose concentration while the expression of *icaA* and *icaD* genes was downregulated. It was concluded that there is a pressing need for enhanced surveillance, improved antibiotic stewardship, and the development of novel anti-biofilm strategies to combat the rising threat of multidrug-resistant *S. aureus* in healthcare settings.

Keywords: Antibiotic resistance, Biofilm, Nosocomial infections, Polymerase chain reaction, *Staphylococcus aureus*.

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Introduction

A biofilm refers to the aggregation of single or multiple microbial species that are adhered to a surface, as opposed to the planktonic cells that reside as separate entities. The establishment of biofilm by bacterial cells not only evades the immune system's detection but also restricts the capability of antibiotics to enter the biofilm matrix(1). A direct relationship exists between antibiotic resistance and biofilm formation in clinical *Staphylococcus aureus* isolates. Bacteria that form biofilms have been observed to exhibit antibiotic resistance levels that are roughly 100–1000 times greater than their planktonic

equivalents(2). The reasons for this phenomenon can be attributed to the following factors: Firstly, antibiotics have a reduced ability to penetrate the biofilm structure. Secondly, biofilm bacterial cells exhibit slower growth and proliferation rates. Thirdly, biofilms have diverse metabolic processes that differ from planktonic bacteria. Lastly, biofilms seem to contribute to the horizontal transfer of antibiotic resistance genes. Furthermore, biofilm bacterial cells exhibit slower growth and proliferation rate (3).

Staphylococcus aureus is a common bacterium that often resides in the human body and is one of the leading causes of illness that may lead to eventual death worldwide, particularly in vulnerable individuals with immunocompromised status (4).

Colonization poses no immediate harm but it increases the likelihood of developing subsequent infections, often caused by the same strain that colonized the body. These infections can differ in severity, ranging from minor soft tissue and skin infections to more serious invasive infections such as pneumonia, osteomyelitis, endocarditis, septic arthritis and bacteraemia or septicaemia(5). Also many studies demonstrated that *S. aureus* may pose negative effects on several sperm parameters and thus may be responsible for some cases of male infertility (6). *S. aureus* infections can be manifested as acute, recurring, or chronic and persistent.

Staphylococcus aureus strains have the ability to create biofilms on the surfaces of many medical devices utilized in hospitals; the treatment options for *S. aureus* biofilm-associated infections are increasingly becoming narrowed due to the decreased sensitivity of the bacteria to antibiotics(7).

The polysaccharide intercellular adhesion (PIA); which is produced by the ica operon, plays a crucial role in the production of biofilms in staphylococci. The intercellular adhesion (*ica*) locus is composed of the *ica*ADBC operon, which comprises four genes responsible for encoding the essential proteins necessary for the production of PIA (8). The genes *icaA* and *icaD* play a crucial role in the synthesis of exopolysaccharides. The *icaA* gene encodes a transmembrane protein that exhibits enzymatic activity as N-acetyl-glucosaminyl transferases, resulting in the formation of the poly-N acetyl glucosamine polymer. Research has shown that the *icaD* gene product is crucial for optimizing the enzymatic activity of the *icaA* gene product (9). Therefore, the present work aim was to highlight the ongoing biofilm-forming capacity of locally isolated *S. aureus* as well as the distribution of *icaA* and *icaD* genes in these isolates.

Materials and methods

Microorganisms

Different clinical specimens were collected for the period of February 2022 to April 2022 from patients attending different hospitals in Baghdad (Baghdad Medical City, Al-Imamain Al-Kadhimain Medical City, and Al-Karama Teaching Hospital). Sputum samples as well as swabs collected from wounds, burns, anterior nares and ears were first inoculated onto Mannitol Salt Agar plates (MSA) and then incubated at 37°C for 24 hours. Afterwards, the colonies were subsequently subjected to

several different biochemical reactions including (Haemolysin Production, Acetoin production, Catalase, Coagulase and Oxidase tests) in order to identify *S. aureus* isolates (10).

Determination of minimal inhibitory concentration

Forty different *S. aureus* isolates were screened for their antimicrobial susceptibility using the AST p580 cards in the Vitek 2 compact system. The test measures the minimal inhibitory concentration for Benzylpenicillin, Oxacillin, Vancomycin, Linezolid, Gentamicin, Moxifloxacin, levofloxacin, Clindamycin, Erythromycin, Tetracycline, Teicoplanin, Rifampicin, Trimethoprim/ Sulfamethoxazole and Nitrofurantoin. Moreover, the test can perform Cefoxitin Screen and detect Inducible clindamycin resistance.

Polymerase Chain Reaction

The genomic DNA of all tested bacterial isolates was extracted using Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan); furthermore, AccuPower® PCR PreMix (Bioneer, USA) was used to carry out all amplification reactions using the gradient master cycler (Eppendorf, Germany) using the primers listed in Table (1).

All *S. aureus* isolates were screened for the presence of the *Sa442* gene to amplify the 108 bp segment of the respective gene. The reaction protocol was carried out as follows: The Initial temperature for denaturation was set at 92°C for 3 min that is followed by 30 cycles of 92°C 1 min, 56°C 1 min, and 72°C 1 min; following that 3 min at 72°C for a final extension.

Furthermore, the isolates were tested for the presence of *icaA* and *icaD* genes to amplify 102 bp and 82 bp segments of the *icaA* and *icaD* genes, respectively. The reaction protocol was as follows: Initial denaturation at 95°C for 10 min followed by 40 cycles of 94°C 1 min, 50°C 1 min, and 72°C 1 min; following that 5 min at 72°C for final extension.

Table (1): List of primers used for PCR study.

Primer name	Sequence 5' ⟶ 3'	Amplicon size (bp)	Reference
SA	F- AATCTTTGTCGGTACACGATATTCTTCACG	108	(11)
	R- CGTAATGAGATTTCAGTAGATAATACAACA		
icaA	F-CAATACTATTTCGGGTGTCTTCACTCT	102	(12)
	R-CAAGAAACTGCAATATCTTCGGTAATCAT		
icaD	F-TCAAGCCCAGACAGAGGGAATA	82	(12)
	R-ACACGATATAGCGATAAGTGCTGTTT		
rpoB	F-CAGCTGACGAAGAAGATAGCTATGT	/	(12)
	R-ACTTCATCATCCATGAAACGACCAT		

Biofilm Formation Assay

The biofilm-forming potential of all *S. aureus* isolates was investigated using the Microtiter plate method (13). Briefly, an overnight growth of *S. aureus* isolates were suspended in Tryptic Soy Broth (TSB) with 1% glucose (the density matched the McFarland standard no. 0.5); after that, 200 µl of the suspension were dispensed in 96-well polystyrene microtiter plate, every isolate was tested three times and negative control wells contained only 200 µl of TSB. The plate was incubated

for 24 h at 37°C. Afterthat, wells were washed 3 times with phosphate buffered saline (PBS; pH 7.2), and fixed using methanol for 20 min, left to dry at room temerature, and then stained with 0.1% crystal violet. After rinsing, the dye bound to the adherent cells was dissolved with 200 µl of 33% glacial acetic acid per well. Ultimately, the optical density (OD) of each well was measured at 600 nm using a microplate reader (Biotek, UK). Cut off value (ODc) was calculated as the mean of OD of control wells plus 3 standard deviations. The isolates were then interpreted as Non–producer (OD ≤ ODc), weak producer (ODc < OD ≤ 2*ODc), moderate producer (2*ODc < OD ≤ 4*ODc), or strong producer (4*ODc < OD). Moreover, The biofilm thickness was also measured under different concentrations of glucose (0, 0.5, 1, 1.5 and 2%) for the MRSA isolate S27 and the MSSA isolate S35.

Gene expression

The levels of *icaA* and *icaD* gene expression were assessed for MRSA isolate S27 and the MSSA isolate S35 biofilms formed in a 6-well microtiter plate under 5 different concentrations of glucose (0, 0.5, 1, 1.5 and 2%) using the primers listed in Table 1; *rpoB* gene was used as a housekeeping gene.

RNA was extracted from biofilm cells using Genezol Reagent according to the manufacturer's instructions (Geneaid, Taiwan). The concentration of the extracted RNA was determined using a nanodrop instrument.

The extracted RNA and primers were combined with a qPCR master mix (New England Biolabs, USA) and were vortexed to ensure homogeneous contents, resulting in a qPCR mixture with a final volume of 20 µl. Ten microliters of master mix and 0.8 microliters of each primer were used in the reactants, while the Rt volume was 1µl, moreover, about 50 mg of RNA was added then the volume was completed to 20 µl using nuclease-free water. The following protocol (Table 2) was adopted:

Table (2): RT- qPCR protocol.

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Reverse Transcription	55°C	10 minutes	1
Initial Denaturation	95°C	1 minute	1
Denaturation	95°C	10 seconds	40-45
Extension	60°C	30 seconds	
Melt Curve	60-95°C	variable	1

A melting curve was obtained with temperatures ranging from 95°C to 60°C with a 0.5°C C increment every 15 seconds.

Statistical analysis

Data obtained from the effect of different glucose concentrations on biofilm thickness expirement were statistically analyzed using T test.

Results and discussion

Detection of *Sa442* gene

The result revealed that all of the 40 isolates that were identified as *S. aureus*, based on some biochemical tests, were also identified as *S. aureus* depending on this molecular technique (Figure 1).

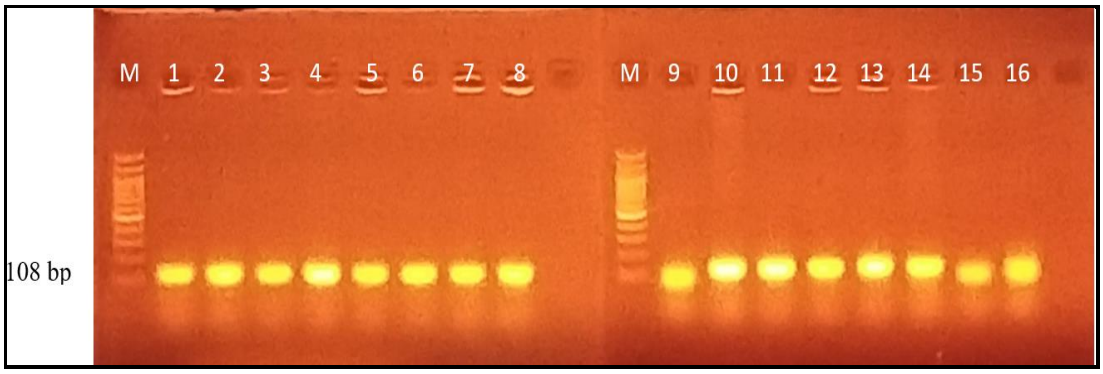

Figure (1): Agarose gel electrophoresis of amplified PCR product of *Staphylococcus aureus Sa442* gene. LaneM: 100 bp DNA ladder;Lanes 1-16: amplicons of *S.aureus* isolates (1-16). (Expected amplicon size: 108, agarose (1.5%), TBE buffer (1x), 70-volt for 1 hr. stained with Ethedium bromide stain.

The 442-bp gene which is a chromosomal fragment that is only located in *S. aureus* has been frequently used for the purpose of achieving identification of *S. aureus* in a multiplex polymerase chain reaction (PCR) manner (14). The *Sa442* DNA fragment, which is a popularly employed DNA target for the identification of *S. aureus* by PCR methods, originally was described by Martineau, Picard (11).

The advancement of molecular technologies has provided investigators with the ability to conduct more thorough and precise examinations of microorganisms compared to traditional culture testing methods (15) These technologies primarily involve the detection and sequencing of specific bacterial DNA, allowing for the identification and classification of various groups of bacteria, through the amplification of a specific target region using PCR. Another method involves the amplification of particular genes associated with specific species, focusing on distinct characteristics such as virulence factors or genes responsible for antibiotic resistance (16).

Molecular approaches offer a high level of sensitivity, speed, and precision in identifying specific infections, eliminating the need for time-consuming older methods. Furthermore, the advancement of cost-effective molecular techniques enables their extensive integration into regular clinical diagnostic methods. Molecular diagnostic approaches are advantageous in their ability to concurrently detect several bacterial infections that were difficult to identify with commercial biochemical procedures (17).

Detection of *icaA* and *icaD* genes

The results revealed that all (100%) of the isolates of *S. aureus* harboured both of *icaA* and *icaD* genes (Figures 2 and 3).

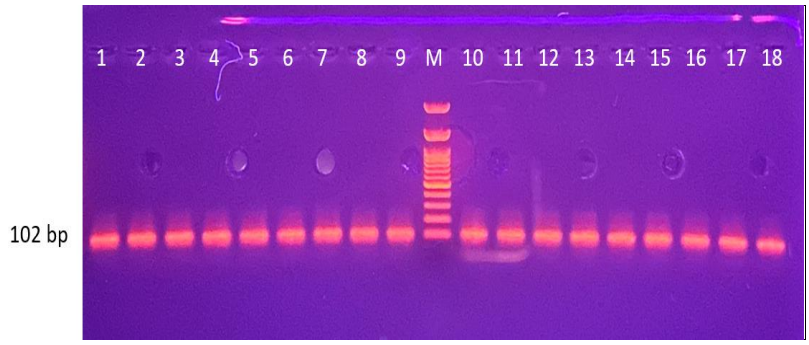

Figure (2): Agarose gel electrophoresis of amplified PCR product of *icaA* gene *in S. aureus* isolates. LaneM: 100 bp DNA ladder;Lanes 1-18: amplicons of *S.aureus* isolates (1-18). (Expected amplicon size: 102, agarose (1.5%), TBE buffer (1x), 70-volt for 1 hr. stained with Ethedium bromide stain.

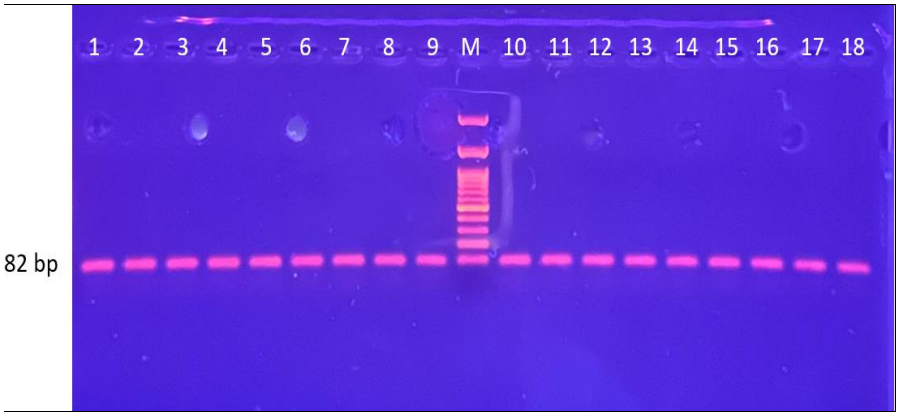

Figure (3): Agarose gel electrophoresis of amplified PCR product of *icaD* gene *in S. aureus* isolates. LaneM: 100 bp DNA ladder;Lanes 1-18: amplicons of *S.aureus* isolates (1-18). (Expected amplicon size: 82, agarose (1.5%), TBE buffer (1x), 70-volt for 1 hr. stained with Ethedium bromide stain.

The findings are consistent with the study by Ahmed, Abdullah (18) who reported that a substantial majority (99.2%) of the *S. aureus* isolates harboured both the *icaA* and *icaD* genes.

It also agrees with Mohamad (19) who also found that the *icaD* gene was found in all *S. aureus* isolates; however, the findings disagree with the study concerning the *icaA* gene as it was missing in all of the isolates that were included in the study.

Infections caused by *S. aureus*, especially those strains that produce an extracellular slime and contribute to the formation of a biofilm, pose significant challenges in clinical treatment. Polysaccharide intercellular adhesion, generated by enzymes encoded by the intercellular adhesion cluster (*ica*), is necessary for the process of biofilm development. The occurrence of adhesion molecules is very crucial for the initiation of an infection. The existence of *ica* adhesion genes may reveal the function of different adhesion mechanisms in the development of infections in hospital environments (8).

It can be inferred that infections generated by *S. aureus* strains containing the *ica* locus can result in clinically challenging conditions to cure. Detecting the presence of the *ica* locus in clinical *S. aureus* isolates can enhance clinical decision-making on

treatment and prevention choices. It can also aid in the development of techniques to counteract the ability of the bacteria to colonize and penetrate medical devices thus, PCR identification of the *ica* operon can serve as a reliable technique for distinguishing between pathogenic and non-pathogenic strains.

Antibiogram

The results summarized in Figure 4 demonstrate that the highest percentage of antibiotic resistance was towards Benzylpenicillin (100%) followed by Oxacillin and Clindamycin (82% and 62 % respectively). On the other hand, the isolates showed complete susceptibility (100%) towards Linezolid, Teicoplanin, Vancomycin and Nitrofurantoin.

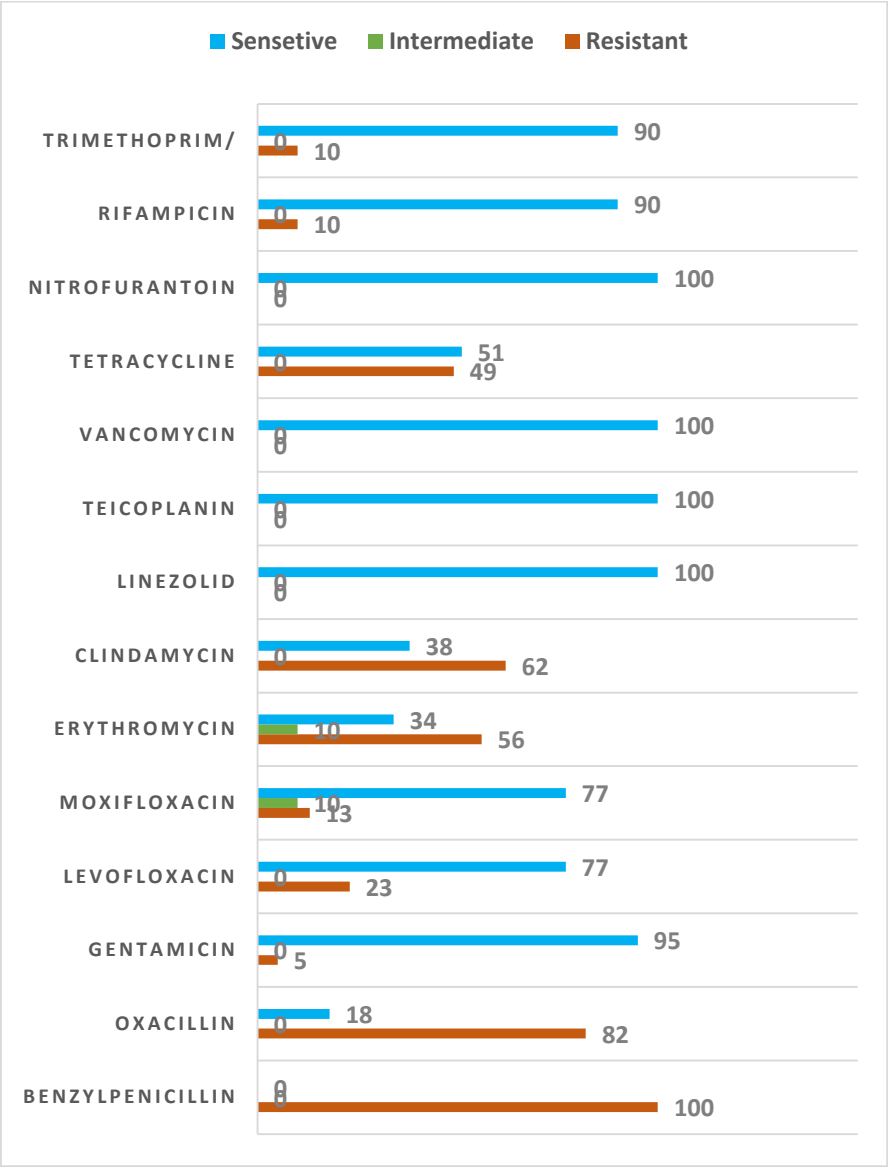

Figure (4): Antibiotic susceptibility of *Staphylococcus aureus*.

It should be noted that although three *S. aureus* isolates were susceptible to Oxacillin (MIC: 012 µg/µl), they were interpreted as resistant since they showed positive for cefoxitin screening test where 82% of the isolates were positive for this test and accordingly identified as Methicillin resistant.

It is also noteworthy to mention that the majority of resistance among the tested strains towards Clindamycin (70%) was inducible and not constitutive with an MIC value of less than 0.25 µg/µl but was positive for the inducible clindamycin resistance test.

Accordingly, the findings highlight the importance of this test as it is vital and should always be included in routine antibiotic sensitivity testing as the inducible resistance phenotype can inhibit the action of clindamycin and affect the effectiveness of treatment.

The result of the current study relatively agrees with Jassim and Kandala (20) who found that about 62.8 % of *S. aureus* isolates were resistant to Oxacillin and 65% were resistant to Erythromycin, while it disagrees with their study concerning Gentamicin and Vancomycin as they found a higher resistance percentage of 34.8% and 74.4% respectively. The results agreed with Mahmood and Flayyih (21) who also found low resistance to Vancomycin (10.8)

The findings of this study completely agree with Al-Saadi and Abd Al-Mayahi (22) and Sami Awayid and Qassim Mohammad (23) who also found that 100% of *S. aureus* were resistant to Penicillin. Moreover, it also agrees with Jafar Alwash and Abed Aburesha (24) that all of the isolates were susceptible towards Vancomycin.

The results also agree with Ibraheem and Al-Mathkhury (25) who found that the majority of *S. aureus* (80%) were Methicillin-resistant.

Antimicrobial resistance poses a significant risk to the health and well-being of both humans and animals, as a considerable number of conventional antimicrobial drugs are becoming increasingly less effective, either partially or completely; based on the 2019 report by the World Health Organization (WHO), the widespread bacterial resistance is responsible for an estimated 700,000 deaths annually. In addition to being a significant contributor to illness and death in both humans and animals, antimicrobial resistance is also resulting in substantial economic losses for the world economy(26).

Antibacterial drugs suffer from decreased permeability when crossing the biofilm matrix, enabling *S. aureus* cells to sustain in the presence of these antibiotics at lowered concentrations. Bacterial biofilms play a significant role in drug resistance. However, there are additional variables that also contribute to the development and expansion of antimicrobial resistance. These factors include the ability to evade the innate host immune system, the ability to modify and adapt through gene mutation and the interchange of genetic material via horizontal transfer (27).

Biofilm

The results depicted in Figure 5 indicated that about 6 isolates (15%) were strong biofilm producers whereas 65% (26 isolates) and 8 isolates (20%) were moderate and weak biofilm producers, respectively.

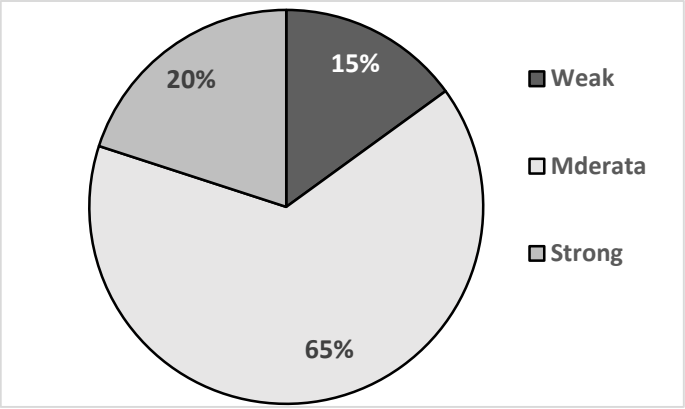

Figure (5): Biofilm forming capacity by *Staphylococcus aureus* isolates.

The findings of the current study come in partial agreement with Alkhafajy and Al-Mathkhury (28) who also found that all of the tested isolates were biofilm producers where 37.93%, 48.28% and 13.79% were strong, moderate and weak biofilm producers respectively. However, the results indicated a disagreement with the findings of Abdrabaa and Abd Aburesha (29) who found a higher percentage (42%) of *S. aureus* isolates that were capable of producing strong biofilm on abiotic surfaces and about 20% and 38% of the isolates were moderate and weak biofilm producers respectively.

Infections caused by *S. aureus* are challenging to manage because of the development of biofilms by these bacteria. *S. aureus* is capable of establishing biofilms within the tissues of the infected host. These infections pose a greater challenge because of antibiotic resistance, and their treatment typically requires the surgical removal of affected tissues (30). The antimicrobial resistance associated with biofilms is partially attributed to the existence of dormant *S. aureus* cells, often referred to as persister cells, which are surrounded by the biofilm structure. These cells remain dormant throughout antimicrobial treatment and become active once the treatment is stopped, leading to a persistent recurring infection (31).

Novel therapeutic techniques will be necessary to treat the aforementioned infections associated with *S. aureus* biofilms. Therefore, it is very crucial to properly investigate *S. aureus* biofilm and gain a comprehensive understanding of it through the use of appropriate microbiological along with other techniques (32).

Effect of glucose concentration on biofilm thickness

Regarding MRSA isolate, the results summarized in Table 3 illustrate a notable significant increase ($p < 0.05$) in biofilm thickness at 1.5% glucose concentration, suggesting enhanced biofilm formation with rising glucose levels. However, At 2% glucose concentration, the optical density significantly ($p < 0.05$) drops, indicating a reduction in biofilm thickness. This may be due to osmotic stress or metabolic inhibition at high sugar levels, which could disrupt cellular processes and biofilm stability. On the other hand, MSSA biofilm shows a steady significant increase ($p < 0.05$) in thickness across all glucose concentrations from 0% up to 2%. This trend suggests that MSSA continuously

benefits from increased sugar availability, enhancing biofilm formation without the inhibitory effects observed in MRSA at higher concentrations.

Table (3): Impact of glucose concentration on biofilm.

Glucose concentration %	Mean OD	
	MRSA	MSSA
0	0.089	0.094
0.5	1.489	0.310
1	1.666	0.335
1.5	1.758	0.402
2	0.952	0.505

The findings align with the results of Singh, Prakash (33) who found that *S. aureus* biofilm increased significantly by supplementation with glucose.

Gene expression

To study the effect of different glucose concentrations on the expression of *icaA* and *icaD* genes, the RNA was extracted from the established biofilm under different concentrations of glucose to measure the expression of the genes using qRT-PCR.

Melting curve analysis revealed a single distinct peak representing a pure single discrete amplicon (34).

The results summarized in Table 4 demonstrate that in MRSA isolate both *icaA* and *icaD* genes showed a reduction in expression levels with all concentrations of glucose when compared with control (0% glucose). This suggests that higher glucose levels may not favour the expression of these biofilm-associated genes in MRSA. On the other hand, there was a decrease in gene expression levels with decreasing glucose concentrations. Similarly, MSSA isolate shows a decrease in the expression of both *icaA* and *icaD* genes with increasing glucose concentrations. This consistent reduction indicates that the MSSA isolate also exhibited a downregulation in these genes in response to higher glucose levels.

The observed increase in biofilm thickness with higher glucose concentrations contrasts with the downregulation of *icaA* and *icaD* gene expression. This phenomenon suggests that while glucose promotes biofilm formation, it may do so through mechanisms that do not rely heavily on the upregulation of *ica* genes. Previous studies have shown that glucose can modulate biofilm formation through various mechanisms, including metabolic changes that affect the intracellular environment (35).

Table (4): Impact of glucose concentration on gene expression.

Fold change				
Glucose %	MRSA		MSSA	
	icaA	*icaD*	*icaA*	*icaD*
0.5	0.2973	0.3439	0.2832	0.2872
1	0.2793	0.409	0.1743	0.2316
1.5	0.4353	0.683	0.183	0.3439
2	0.5322	0.5	0.1416	0.2333

Another possible explanation involves the global stress response regulator sigmaB (σB) and other regulatory proteins such as SarA and Agr are involved in the regulation of biofilm formation and can be influenced by changes in glucose levels. These regulators may modulate biofilm formation independently of *ica* expression (36).

Kot, Sytykiewicz (37) conducted a study on the expression of various biofilm-associated genes, including *icaA* and *icaD*, in *S. aureus* over different periods. Their findings indicated that the expression levels of both *icaA* and *icaD* genes peaked at 12 h. and subsequently decreased over time. These observations suggest that the decrease in gene expression observed in the current experiment with increasing glucose concentrations may also be influenced by time-dependent factors.

Conclusion

Methicillin-resistant *S. aureus* strains are widespread among patients attending hospitals in Baghdad, many of these strains possess the ability to establish a biofilm when tested *in vitro*, which further complicates the situation and causes many treatment regimens to become more or less obsolete. Moreover, both MRSA and MSSA strains exhibited increased biofilm thickness with higher glucose concentrations accompanied by downregulation of *icaA* and *icaD* gene expression. This suggests that biofilm formation under these conditions is not heavily dependent on the upregulation of these specific genes.

Conflicts of Interest

The authors declare no conflict of interest.

Funding

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Ethics Statements

This work has been approved by the College of Science Research Ethics Committee (ref. CSEC/0422/0159). All of the participants were allowed to provide the researchers with the specimens. Informed consent according to the Declaration of Helsinki was obtained from all participants.

Author Contribution

Both of the authors, Ali A. Mussa and Harith Jabbar Fahad Al-Mathkhury, have equal contributions in all aspects of this study including study conception and design, data collection, analysis and interpretation of results, draft manuscript preparation ending with revisions to reach the final version of the manuscript.

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