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Research Article

Detection sand Gene Expression of Regulatory Gene (*icaR*), and Its Control of the Expression of Biofilm Genes (*icaA* and *icaD*) in *Staphylococcus aureus*

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I N F O

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A B S T R A C T

Introduction: The development of biofilms in *Staphylococcus aureus* (*S. aureus*) is heavily influenced by environmental factors and governed by multiple regulatory mechanisms, posing difficulties in the treatment of infections. *S. aureus* relies on the polysaccharide intercellular adhesin expressed by the *icaADBC* operon. A gene that influences the *ica* operon expression is the *icaR* gene, which is situated next to the *icaADBC* operon. The *icaR* gene encodes a negative regulator of *icaADBC.*

Aim: To investigate the correlation between the regulatory gene *icaR* and biofilm genes in *S. aureus*

Methods: Different clinical samples (n = 250) were collected from patients who attended Al-Husain Medical City and Al Imam Al Hassan Al Mujtaba Teaching Hospital in Karbala City from December 2021 to June 2022 for the isolation of *S. aureus*. The isolates were identified as *S. aureus* using cultural and biochemical assays. The susceptibility of *S. aureus* isolates to antibiotics was determined using the Kirby–Bauer disk diffusion method. The potential of *S. aureus* isolates for biofilm production was quantitatively evaluated using the microtitre plate method. After that, molecular detection of biofilm genes (*icaA* and *icaD*) and the regulatory gene (*icaR*) in bacterial isolates was done using Polymerase Chain Reaction (PCR). Finally, the expression of the biofilm genes and the regulatory gene was assessed using quantitative real-time PCR.

Results: Sixty isolates were identified as *S. aureus* from the 250 clinical samples. The antibiotic susceptibility of *S. aureus* isolates indicated multidrug resistance: 100% of isolates were resistant to oxacillin, 98% to penicillin P, 63% to tetracycline, 51% to azithromycin, 43% to doxycycline, 15% to gentamicin, 13.3% to levofloxacin, 13.3% to ciprofloxacin, 11.6% to trimethoprim, 10% to clindamycin, and 3.33% to chloramphenicol. Regarding the ability of *S. aureus* isolates for biofilm formation, 15 (25%) isolates were strong biofilm producers, 40 (66.6%) were moderate biofilm producers, and 5 (8.3%) were weak

biofilm producers. Furthermore, all *S. aureus* isolates (100%) had biofilm genes (*icaA* and *icaD*) and the regulatory gene *icaR*. The upregulation of regulatory gene *icaR* was found to be associated with the downregulation of biofilm genes *icaA* and *icaD* in the isolates.

Conclusion: There is an inverse relationship between the biofilm *ica* operon and the regulatory gene *icaR* in *S. aureus* isolates.

Keywords: *Staphylococcus aureus,* Biofilm Formation, Biofilm Genes *icaA, icaD*, PIA/*ica*, Regulatory Gene *icaR*

Introduction

Staphylococci, a type of spherical gram-positive cocci, are organised in clusters resembling irregular grape formations. The human skin and mucous membranes are naturally colonised by the commensal bacteria *Staphylococcus aureus* (*S. aureus*).[1,2] It is a notable pathogen, often leading to severe infections that are associated with considerable morbidity, mortality, and substantial healthcare expenses.[3,4] The the anterior nares, is the primary site of pathogen carriage and serves as a reservoir for its transmission.[5,6] Methicillin-resistant *S. aureus* (MRSA) and coagulase-negative staphylococci (MR-CoNS) are frequently responsible for healthcare-associated infections.[7,8]

Biofilms are defined as bacterial populations that attach to surfaces by encapsulating themselves in a self-generated extracellular polymeric matrix. The matrix of staphylococcal biofilms may consist of exopolysaccharides, proteins, and extracellular DNA.[9] The primary exopolysaccharide found in staphylococcal biofilms is called polysaccharide intercellular adhesion (PIA). PIA is a kind of N-acetyl glucosaminoglycan that is linked by beta-1,6 bonds. The genes responsible for producing the enzymes needed for PIA synthesis are located in a region called the intercellular adhesion (*ica*) locus.[10,11]

The staphylococcal *ica* locus comprises two transcription units that are oriented in opposite directions. One of these units contains the *icaADBC* operon, which encodes the enzymes needed for PIA production. The other unit contains *icaR*, a TetR family transcription factor. A strong regulator of the *icaADBC* operon transcription, IcaR (whose crystal structure has been solved), binds to a region upstream of *icaA*.[12,13] The regulation of the *ica* locus is complex, with several environmental signals known to affect PIA production. Many of these cues have a direct or indirect impact on the transcription of *icaR*.[14,15] The expression of *icaR* is also regulated at the post-transcriptional level by RNA-mediated processes that impact the stability and translation of the *icaR* mRNA. These mechanisms have direct effects on the synthesis of PIA and the development of biofilms. The presence of PIA/*ica* was initially observed in *Staphylococcus epidermidis* and subsequently identified in *S. aureus* and other staphylococcal species.[16] The *ica* locus is typically located inside the chromosomal DNA of bacteria in all staphylococcal species.

The *icaR* gene encodes a protein that is a member of the TetR family of transcriptional regulators. This protein acts as a repressor and inhibits the transcription of the *icaADBC* gene. It does so by binding to a specific area located just before the start codon of the *icaA* gene.[17] Furthermore, it has been documented that environmental variables such as glucose, ethanol, elevated temperatures, and increased osmolarity might impact the formation of biofilms.[18] Ethanol enhances the expression of *icaA* by inhibiting the transcription of *icaR*.[19]

Materials and Methods

This study was designed as a laboratory-based cross-sectional study. Non-duplicate clinical isolates of Staphylococcus aureus were included, while duplicate isolates and samples with incomplete data were excluded. Data were analyzed using SPSS software version 24. Descriptive statistics were applied, and the Chi-square test was used where appropriate, with a p-value < 0.05 considered statistically significant. Ethical approval for the study was obtained from the Scientific and Ethical Committee of the College of Science, University of Baghdad. Informed consent was obtained from participants prior to sample collection.

Bacterial Culture Conditions

Clinical samples (n = 250) were collected from outpatients who attended Al-Husain Medical City and Al Imam Al Hassan Al Mujtaba Teaching Hospital in Karbala City in Iraq, from December 2021 to June 2022. The samples included nares swabs (n = 55), abscesses (n = 60), ulcers (n = 5), blood (n = 30), midstream urine (n = 40), and wound swabs (n = 60). Mannitol salt agar[20] was used to identify *S. aureus* isolates from these samples and the growing colonies were submitted for biochemical assays (production of catalase and coagulase). Furthermore, VITEK 2 Compact (bioMérieux, America) was employed for conclusive verification.[21]

Antibiotic Susceptibility Test

As recommended by the Clinical and Laboratory Standards Institute (CLSI), the Kirby−Bauer disk diffusion method was used to examine the susceptibility of *S. aureus* isolates to antibiotics.[22,23] The antibiotics used in this study included oxacillin (30 µg), penicillin P (10 µg), tetracycline (30 µg), azithromycin (15 µg), doxycycline (30 µg), gentamicin (10 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), trimethoprim (5 µg), clindamycin (2 µg), and chloramphenicol (30 µg).

Detection of Quantifiable Biofilm Production

The production of biofilms by bacterial isolates was evaluated using the microtitre-plate technique, utilising

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sterile 96-well U-shaped bottom polystyrene microplates.[24,25] The well was filled with 200 µL of Tryptic Soy Broth (TSB) solution with 0.75% glucose (for a total of 1% glucose in TSB), and the bacterial concentration was adjusted to match the 0.5 McFarland standard. The plates were then covered and subjected to aerobic incubation at 37 °C for 24 hours. The experiment involved conducting three replicates for each isolate. The control wells were prepared by introducing sterile TSB without bacteria. The wells were subjected to aspiration and subsequently washed three times using 200 µL of sterile Phosphate Buffered Saline (PBS). The bacteria that remained attached to the wells were then fixed by treating them with 200 µL of methanol for 15 minutes. Following the process of air drying, the wells were subjected to staining with a 200 µL solution of 0.1% crystal violet. This staining process was carried out for 15 minutes, maintaining the ambient room temperature. The plates were placed under running water to remove any remaining discolouration and were then dried. After 15 minutes, 200 µL of 33% glacial acetic acid was used to resolubilise the adhering cells. Finally, an enzyme-linked immunosorbent assay (ELISA) reader (Biotek, UK) was used to measure the amount of biofilm in each well at 600 nm.[26] The optical density cut-off value (ODc) was determined by calculating the mean optical density (OD) of the control wells plus three standard deviations. The OD value of the negative control in this study was determined to be 0.092. The standard deviation of the OD value was determined to be 0.00173. Consequently, the cut-off value was computed to be 0.0971. The biofilm gradation in clinical isolates was then assessed based on the criteria outlined in Table 1.[27,28]

Extraction of Bacterial Genomic DNA

The genomic DNA from *S. aureus* isolates was extracted using a gram-positive DNA extraction kit (Norgen, Canada), following the manufacturer's instructions. The extracted DNA was analysed for both quality and quantity using 2% agarose gel electrophoresis and a Qubit™ dsDNA HS (High Sensitivity) assay kit (Thermo Fisher®, USA). The DNA samples were ultimately placed in a 1 µL microtube and stored at -20 °C for subsequent investigations.

Detection of Biofilm Genes and Regulator Gene in *S. aureus* Isolates

The biofilm *ica* genes encoding *icaA* and *icaD*, and the regulator gene *icaR*, were detected in the bacterial isolates using Polymerase Chain Reaction (PCR). The primers were designed according to the *S. aureus* genome available in the National Center for Biotechnology Information (NCBI) database and supplied in a lyophilised state by the Macrogen Company (Korea). The size of the genes under investigation and their corresponding genetic sequences were specifically determined for the purpose of this study (Table 2). PCR technique was employed using primers specific to the target gene to validate the identification of *S. aureus* isolates.[29] The PCR reaction mixture, with a final volume of 20 µL, consisted of various components that included 2 µL of a 10X PCR buffer, 1.5 µL of a 25 mM $MgCl_2$ solution, 2 µL of a 10 mM dNTPs solution, 1 µL of each primer, 2.5 µL of the DNA sample, and 1.25U of Taq DNA polymerase from New England Biolabs® (England). The final volume was then adjusted to 20 µL using 10.5 µL of nuclease-free water. The amplifications were conducted using a DNA Thermal Cycler SimpliAmp (Thermo Fisher, Germany). The PCR product (4 µL) was subjected to analysis using 1.5% agarose gel electrophoresis in Tris-acetate-EDTA (TAE) 1X buffer, stained with ethidium bromide, visualised under ultraviolet transillumination, and documented using Gel Doc imaging.

Molecular Detection of Reference Gene *16S rRNA*

This step was carried out by adding 12.5 µL of OneTaq (New England Biolabs®) Master Mix, 3 µL of DNA sample, 1 µL of each primer solution (10 pmol/µL), and 7.5 µL of nuclease-free water in a PCR tube. The reaction was carried out under the optimal PCR conditions for the gene, as shown in Table 3.

Molecular Detection of Biofilm Genes *(icaA and icaD)* and Regulatory Gene *icaR*

This step was the same as that mentioned for *16S rRNA.* The PCR conditions are mentioned in Tables 4–6.".

Table 1.Criteria of Classification of Biofilm Generated by *Staphylococcus aureus* Strains[27]

Optical Density Values (OD)	Criteria Outlined
OD ≤ 0.0971	Non-biofilm formation
0.0971 < OD ≤ 0.194	Weak biofilm formation
0.194 < OD ≤ 0.388	Moderate biofilm formation
0.388 < OD	Strong biofilm formation

Table 2.Sequence and Product Size of Primers for the Studied Genes

Gene	Function	Nucleotide Sequences 5'–3'	Amplicon Size (bp)	Annealing Temperature (°C)
icaA	Intracellular adhesion A	F: CGACGTTGGCTACTGGATAC R: ACACATGGAAGCGGTTCATA	106	56

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icaD	Intracellular adhesion D	F: TCGCTATATCGTGTGTCTTTTGGA R: CGCGAAAATGCCCATAGTTTCA	164	53
icaR	Intracellular adhesion R	F: TCCACTGCTCCAAATTTTTGCGA R: ACGCCTGAGGAATTTTCTGGAAAT	194	54
16S rRNA	Housekeeping	F: CTCAACCGTGGAGGGTCATTG R: CCTGTTTGACCCCACGCTTT	169	57

Table 3.PCR Conditions for *16S rRNA* Gene

Cycle No.	Stage	Temperature (°C)	Time
1	Initial denaturation	94	5 min
38x	Denaturation Annealing Extension	94 57 72	30 sec 45 sec 45 sec
1	Final extension	72	7 min

Table 4.PCR Conditions for *icaA* Gene

Cycle No.	Stage	Temperature (°C)	Time
1	Initial denaturation	94	5 min
35x	Denaturation Annealing Extension	94 54 72	30 sec 45 sec 45 sec
1	Final extension	72	7 min

Table 5.PCR Conditions for *icaD* Gene

Cycle No.	Stage	Temperature (°C)	Time
1	Initial denaturation	94	5 min
35x	Denaturation Annealing Extension	94 53 72	30 sec 45 sec 45 sec
1	Final extension	72	7 min

Table 6.PCR Conditions for icaR Gene

Cycle No.	Stage	Temperature (°C)	Time
1	Initial denaturation	94	5 min
35x	Denaturation Annealing Extension	94 54 72	30 sec 45 sec 45 sec
1	Final extension	72	7 min

Extraction of RNA from *S. aureus* Isolates

RNA from *S. aureus* isolates was extracted using TRIzol™ (ThermoFisher®, USA), following the manufacturer's protocol. The isolates were cultured in TSB containing 1% glucose for 18 hours before being transferred to microtitre plates to produce biofilm cells. The plates were carefully rinsed with distilled water to eradicate any remnants of methanol, hence eliminating any non-adherent cells from the wells. Once the biofilm on the glass surface became invisible, the biofilm cells were collected by suspending them in cold, sterile normal saline using a pipette. Throughout the whole procedure, the cells were placed on ice to prevent the breakdown of RNA. Subsequently, the bacterial cells were transferred to microcentrifuge tubes

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with a capacity of 1.5 mL. The tubes were then subjected to centrifugation with a force of 14000 g for two minutes. Following centrifugation, the supernatant was entirely removed. The extracted RNA concentration was quantified using a Quantus fluorometer (Thermo Fisher®, USA) to assess the suitability of samples for future use. This assay is highly selective for RNA, and it is accurate for initial sample concentrations from 10 pg/µL to 100 ng/µL. The assay is performed at room temperature, and the signal is stable for 3 hours. Common contaminants such as salts, free nucleotides, solvents, detergents, or proteins are well tolerated in the assay.[30]

Reverse Transcription Synthesis

A ProtoScript cDNA Synthesis Kit (New England Biolabs®, UK) was used to reverse transcribe mRNA from total RNA. and a primer for the resistance genes and *16S rRNA* transcripts was designed in this study which is shown above in Table 2. The experiment was carried out following the manufacturer's (New England Biolabs®) protocol in a reaction volume of 20 µL. Then, cDNA was stored at -80 °C until use.

Real-Time Quantitative PCR Assay

The QUBIT® Real-time PCR System (Thermo Fisher®, USA) and qPCRsoft software were used to perform real-time quantitative PCR (RT-qPCR).[31] The programme for RT-qPCR was set up with the thermocycling protocol as shown in Table 7.

Determination of the Expression of Biofilm Genes (icaA and icaD) and Regulatory Gene icaR Using RT-qPCR

Data was gathered using the FAM/ SYBR channel, exported to an Excel spreadsheet and analysed using the LinRegPCR programme version 2015. The initial concentration of each sample was standardised relative to the concentration of the housekeeping gene *16S rRNA* to determine the expression value. The cDNA template of the *S. aureus* isolate with low biofilm development was used as a control, and each experiment was replicated at least three times.[32] The $2^{-\Delta\Delta CT}$ method was used in this study. It is commonly employed as a relative quantification tool for analysing RT-qPCR data and provides an easy approach to determine the relative levels of gene expression among various samples. It directly utilises the threshold cycles (CTs) provided by the RT-qPCR equipment for computation.[33] The CT of the interset gene was adjusted to be equal to that of the internal control gene. The results were evaluated using CT and Livac formulas as follows:

ΔCT = CT(a target gene)−CT(a reference gene)

ΔCT for the target sample = CT(a target gene A) – CT(a reference gene A)

ΔCT for the reference sample = CT(a target gene B) – CT(a reference gene B)

$\Delta\Delta CT$ = ΔCT(a target sample) − ΔCT(a reference sample)

Normalised CT expression formula = $2^{-(\Delta\Delta CT)}$

Results and Discussion

Isolation and Identification of *S. aureus* Isolates

Two hundred and fifty different clinical samples (wound, nasal cavities, blood, urine, abscesses, and ulcer) were collected from patients who attended two hospitals in Karbala City in Iraq. Sixty isolates were identified as *S. aureus* according to their morphological and biochemical properties.[34,35] The colonies appeared round, smooth, raised, mucoid, and glistening. The microscopic examination illustrated that all these isolates were gram-positive cocci. Furthermore, all these isolates revealed a negative result for oxidase, a positive result for catalase and coagulase, and were able to ferment mannitol. In addition, *S. aureus* colonies on blood agar appeared yellow in colour with a clear zone surrounding the colonies, showing haemolysis.

The frequency of *S. aureus* isolates in the samples was different: wounds (n = 15, 35%), nasal cavities (n = 14, 23.33%), blood (n = 5, 8.33%), urine (n = 10, 16.66%), abscesses (n = 9, 15%), and ulcer (n = 1, 66%)(Figure1).

It has been observed that bacteria in chronic wounds develop biofilms that contribute to a delay in healing. In a mature biofilm, bacteria grow slowly due to a deficiency of nutrients that results in the resistance of bacteria to antibiotics.[36,37] *S. aureus*, a prominent bacterium found in both hospital and community settings, has a crucial role in causing wound infections. The onset and development of *S. aureus* infection can be influenced by the virulence of the bacterium and the immunological components of the host. Pathogen-related factors encompass the intricate bacterial structure and functional traits that confer metabolic and adhesion qualities, enabling them to evade the host immune response. The virulence markers and toxins of *S. aureus* exhibit significant similarities across several wound models. The presence of distinct clonal lineages and virulence factors, such as TSST-1, leukocidins, enterotoxins, and exfoliatins, greatly influences the outcomes of wounds.[38]

Antibiotic Susceptibility Test

The antibiotic susceptibility results showed that all isolates were resistant to oxacillin. The highest resistance rate of the isolates was to penicillin P (98%). Resistance rate to other antibiotics was observed as follows: 63% of the isolates were resistant to tetracycline, 51% to azithromycin, 43% to doxycycline, 15% to gentamicin, 13.3% to levofloxacin, 13.3% to ciprofloxacin, 11.6% to trimethoprim, 10% to clindamycin, and 3.33% to chloramphenicol. According to

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the findings of this investigation, *S. aureus* has a significant level of resistance to antibiotics (Figure 2).[39]

S. aureus is a highly adaptable bacterium capable of causing infections in several settings, including hospitals and communities, through contaminated food and animals. Clinical infections caused by *S. aureus* result in higher rates of illness and death, as well as increased treatment expenses, which are further compounded by the advent of drug-resistant strains. It is crucial to gather and integrate information about its pathogenesis, which encompasses the regulation of virulence, the development of resistance to antimicrobial agents, and the formation of biofilms, in order to effectively address this complex pathogen. This will enable the development of diverse treatment approaches.[40,41]

MRSA is a highly significant pathogen that is resistant to multiple drugs and is prevalent worldwide. MRSA develops when methicillin-susceptible *S. aureus* (MSSA) obtains a gene called *mecA,* which confers resistance to methicillin. This gene is carried by a mobile genetic element called staphylococcal cassette chromosome mec (SCCmec), which is believed to be capable of spreading among the different species of staphylococci.[42]

Biofilm Formation by *S. aureus* Isolates

S. aureus isolates were distributed into three groups depending on their ability to form biofilm: 15 (25.0%) isolates were strong biofilm producers, 40 (66.6%) were moderate biofilm producers, and 5 (8.3%) were weak biofilm producers (Figure 3).

Quantitatively, the data revealed that biofilm production was greater in multidrug-resistant (MDR) strains. The production of biofilms and the emergence of MDR strains have been identified as significant factors contributing to the pathogenicity of chronic infections caused by *S. aureus*, posing a potential hazard to public health.[43,44] Furthermore, it should be noted that host tissues, as well as abiotic surfaces, such as implant devices and catheters, have the potential to facilitate the initial adhesion of bacterial cells and subsequent biofilm development. The presence of persister cells inside the biofilm matrix provides a mechanism for evading the host immune system and resisting the effects of antimicrobial agents.[45,46]

Biofilm Genes (*icaA* and *icaD*) in *S. aureus*

All isolates exhibited the presence of biofilm genes (*icaA* and *icaD*) (Figure 4) at the genotypic level, as was determined using the particular primers (Table 2). The observed prevalence rates of the genes in question were as follows: *icaA* (100%) and *icaD* (100%). The pathogenicity of *S. aureus* relies on its capacity to generate a diverse array of virulence factors that facilitate bacterial invasion. The production of biofilms has been documented to augmnt the pathogenicity of *S. aureus*, and strains that exhibit biofilm formation display heightened resistance to antibiotics, disinfectants, and other environmental variables.[47] The *ica* genes have a significant impact on the generation of slime in *S. aureus*. Strains that possess the *icaADBC* cluster are recognised as potential producers of biofilm.[48,49]

Regulator Gene (*icaR*) in *S. aureus*

S. aureus isolates were analysed to identify the presence of a regulatory gene (*icaR*). The electrophoretic pattern of the *icaR gene* is presented in (Figure 5); *icaR* was detected in all isolates under investigation (100%). Poly-N-acetylglucosamine (PNAG) represents a major constituent of *S. aureus* biofilm. The synthesis of PNAG is facilitated by the products of four genes, namely *icaADBC*, which are transcribed in the *ica* operon. The primary regulator of this operon is IcaR, which is encoded just upstream i*caADBC*.[50]

Table 7.Thermocycling Protocol for Real-Time Quantitative Polymerase Chain Reaction

Cycle No.	Stage	Temperature (°C)	Time
1	Initial denaturation	95	60 sec
40–45	Denaturation Extension	95 60	15 sec 30 sec
1	Melt curve	60–95	40 min

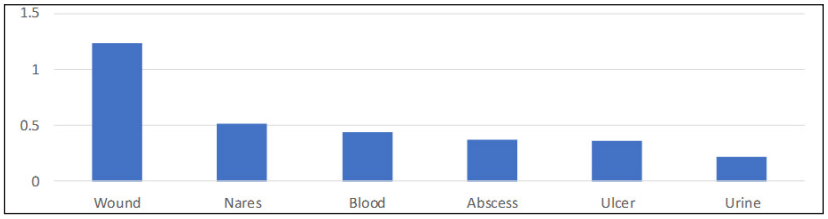

Figure 1.Frequency of Staphylococcus aureus isolates recovered from different sites of infection(The y-axis represents the sites of infection

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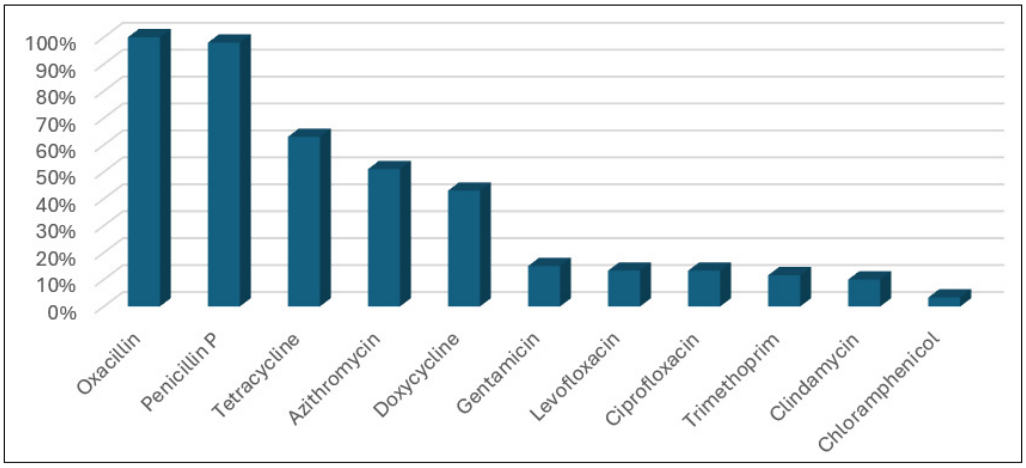

Figure 2.Percentage Resistance of S. aureus Isolates to Antibiotics

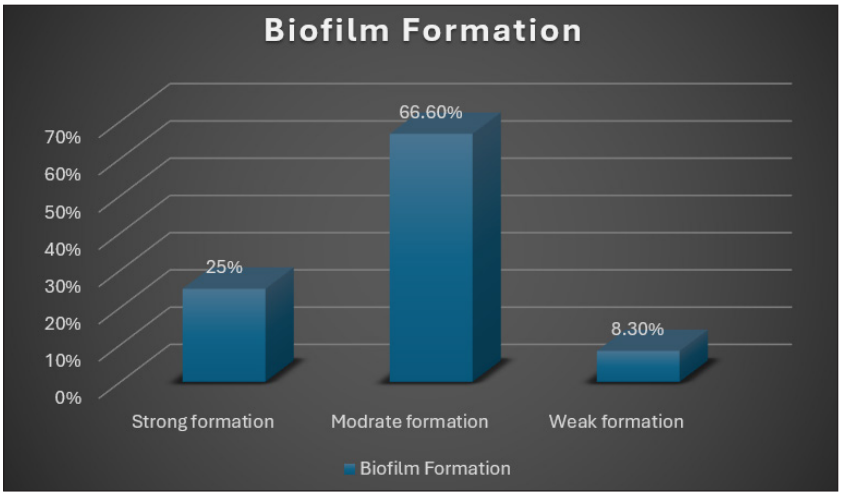

Figure 3.Biofilm Formation by *S. aureus* Isolates

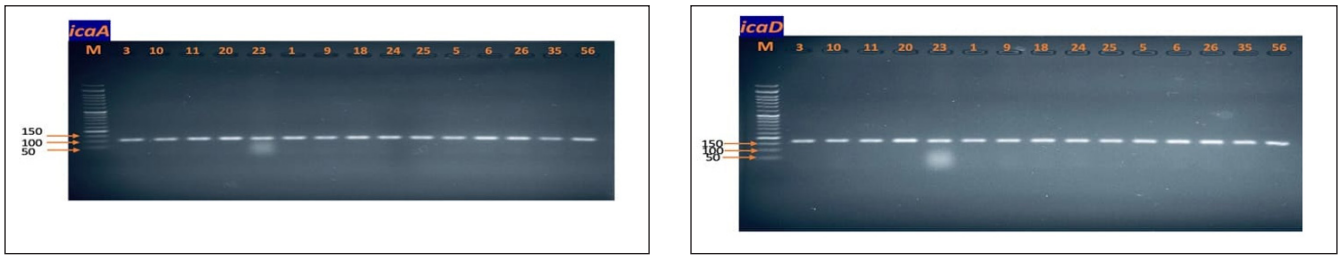

Figure 4.Gel Electrophoresis Indicating the Presence of Biofilm Genes, *icaA* (106 bp) and *icaD* (164 bp), in *S. aureus* Isolates. M: Molecular Size Marker

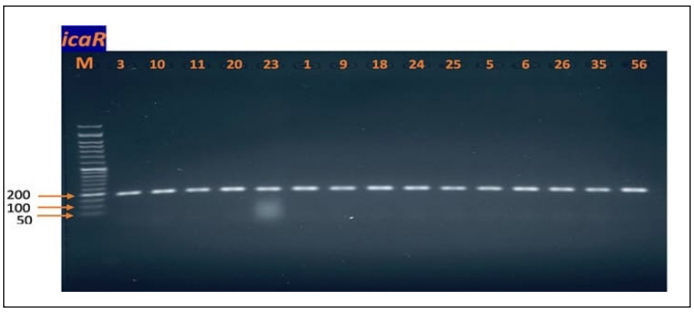

Figure 5.Gel Electrophoresis Indicating the Presence of Biofilm Genes, *icaA* (106 bp) and *icaD* (164 bp), in *S. aureus* Isolates. M: Molecular Size Marker

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Expression of *icaA*, *icaD* and *icaR* Genes in *S. aureus* Isolates

The expression of biofilm production genes (*icaA* and *icaD*) and regulatory gene (*icaR*) in 15 biofilm-producing *S. aureus* isolates was measured using RT-qPCR. The average of weak biofilm values was used as a control to compare the values of strong, moderate, and weak biofilm production. The results indicated that the expression of the biofilm genes *icaA* and *icaD* was upregulated in the *S. aureus* isolates, which exhibited strong and moderate biofilm production. In contrast, the expression of *icaA* and *icaD* was downregulated in the isolates that had weak biofilm production (Tables 8 and 9). Table 10 reflects the inverse relationship between biofilm formation genes (*icaA* and *icaD*) and the regulatory gene *icaR*. This implies that when the expression of *icaA* and *icaD* was upregulated, the expression of regulatory gene *icaR* was downregulated (and vice versa).

Indeed, the regulation of biofilm-associated operon *icaADBC* is complex; several regulatory elements are involved in controlling its activity. Some of these elements are well-recognised as global transcriptional regulatory factors. The regulatory gene (*icaR)*, which is positioned next to the *ica* operon, belongs to the TetR family of transcriptional regulators.[17–19] The regulatory gene *icaR* was previously documented to inhibit the transcription of *icaADBC* by attaching to the 42-base pair segment inside the *ica* promoter.[51,52] It has been previously reported that *icaR* is a repressor of *ica* transcription in *S. epidermidis* and *S. aureus*.[53,54] concluded that *icaR* is a repressor of *ica* transcription in *S. aureus*. The deletion of this regulatory gene will result in a substantial increase in PNAG, which is considered a major carbohydrate component in bacterial biofilm. In addition, glucose impacts *ica* transcription and PNAG production by counteracting the negative regulatory effect of *icaR*.[12,55]

A comprehensive knowledge of the regulatory mechanism of PNAG elaboration in *S. aureus* is essential for comprehending biofilm formation and might potentially facilitate the development of techniques to suppress the production of this significant virulence factor. However, it is becoming more evident that the control of gene transcription for PNAG synthesis, namely the *ica* genes, is highly complex. In the present study, we identified a novel IcaR, which is a repressor of biofilm elaboration. Furthermore, the icaR gene is located within the icaR–icaA intergenic region and acts as a repressor of the icaADBC operon.[12] *icaR* is a direct repressor of *icaADBC* in both *S. aureus* and *S. epidermidis*; however, the function of TcaR/ icaR is less understood.[56]Yu et al. suggest that PIA is the primary component of biofilm in *S. aureus*. The findings suggest that the activation of *icaR* represses the transcription of *icaADBC*. Taken together, the data demonstrate that induction of icaR negatively regulates icaADBC transcription, ultimately leading to reduced biofilm formation.[57] In this study, we have shown that the transcription of the *icaADBC* genes was affected by downregulating *icaR* transcription and thereby upregulating transcription of *icaADBC.*

Table 8.Expression of Biofilm *Gene icaA* in *S. aureus* Isolates with Different Biofilm Activity

Isolates	Biofilm Production	Fold of Expression	Induction	Reduction
S3	Strong	0.223	-	4.484
S10	Strong	2.549	1.549	-
S11	Strong	4.184	3.184	-
S20	Strong	2.027	1.027	-
S23	Strong	2.337	1.337	-
S1	Moderate	3.930	2.930	-
S9	Moderate	1.610	0.610	-
S18	Moderate	1.630	0.630	-
S24	Moderate	1.427	0.427	-
S25	Moderate	0.278	-	3.597
S5	Weak	0.007	-	142.850
S6	Weak	0.381	-	2.624
S26	Weak	0.005	-	200.000
S35	Weak	0.404	-	0.227
S56	Weak	1.000	-	-

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Table 9.Expression of Biofilm Gene *icaD* in *S. aureus* Isolates with Different Biofilm Strengths

Isolates	Biofilm Production	Fold of Expression	Induction	Reduction
S3	Strong	0.083	-	12.048
S10	Strong	1.840	0.840	-
S11	Strong	3.105	2.105	-
S20	Strong	1.717	0.717	-
S23	Strong	3.398	2.398	-
S1	Moderate	1.582	0.582	-
S9	Moderate	1.019	0.019	-
S18	Moderate	0.372	-	2.688
S24	Moderate	0.294	-	3.401
S25	Moderate	0.215	-	4.651
S5	Weak	0.002	-	500.000
S6	Weak	0.309	-	3.236
S26	Weak	0.004	-	250.000
S35	Weak	0.174	-	5.747
S56	Weak	1.000	-	-

Table 10.Expression of Regulatory Gene (*icaR*) in *S. aureus* Isolates with Different Biofilm Strengths

Isolates	Biofilm Production	Fold of Expression	Induction	Reduction
S3	Strong	0.100	-	10.000
S10	Strong	0.020	-	50.000
S11	Strong	0.537	-	1.862
S20	Strong	0.393	-	2.544
S23	Strong	0.005	-	200.000
S1	Moderate	0.350	-	2.857
S9	Moderate	0.020	-	50.000
S18	Moderate	0.537	-	1.862
S24	Moderate	0.369	-	2.710
S25	Moderate	0.296	-	3.378
S5	Weak	1.647	0.647	-
S6	Weak	0.300	-	3.333
S26	Weak	2.703	1.703	-
S35	Weak	3.823	2.823	-
S56	Weak	1.000	-	-

Conclusion

This study demonstrates that the upregulation of *icaA* and *icaD* was found in bacterial isolates with strong biofilm production, while downregulation of biofilm genes was recorded in weak biofilm isolates. In contrast, the expression of regulatory gene *icaR* was upregulated in isolates with weak biofilm formation, while downregulation of *icaR* was determined in the isolates with strong biofilm formation. An inverse correlation exists between the biofilm *ica* operon (*icaA* and *icaD)* and the regulatory gene (*icaR)* in *S. aureus* isolates.

Authors' Contribution

All authors contributed equally to the conception and design of the study. Data collection, analysis, and interpretation

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were performed collaboratively. All authors participated in drafting and revising the manuscript and approved the final version for publication.

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