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Original article

Intracellular adhesion gene expression studies in biofilm-forming of *Staphylococcus aureus* isolated from diffeent clinical sources in Bagdad, Iraq.

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ABSTRACT

Background: *Staphylococcus aureus (S. aureus)*, a bacterial infection typically found in the population is noteworthy for its pathogenicity due to its ability to produce biofilms with high virulence. The aim of this study was to identify the gene icaA, which is responsible for biofilm formation in clinical isolates from wounds and urine samples. **Material and method:** The gene is involved in the construction of slime layers. The present study collected a total of 425 clinical samples, including urine and wound swabs. We used four different methods to identify S. aureus isolates: the biochemical test methodology, the Analytical Profile Index (API) Staph system, the Vitek-2 compact systems, and the polymerase chain reaction (PCR) targeting the 16SrRNA. In the end, we confirmed only 388 isolates to be S. aureus. **Results:** We assessed the ability of *S. aureus* isolates to produce biofilm using 96-well microtiter plates. The examination of 388 isolates revealed that 146 isolates (37.6%) formed robust biofilms, 160 isolates (41.3%) formed moderate biofilms, and 82 isolates (21.1%) formed weak biofilms. Additionally, the expression level of the strong biofilm-producing isolate (6.508) was considerably lower ($P \geq 0.01$) compared to the weak and moderate isolates. The results indicated that the moderate isolate's expression level was higher than that of the weak isolate. **Conclusion:** A quantitative PCR analysis was conducted on three isolates with different biofilm forming capacity (EPS) to evaluate the transcript levels of the icaA gene, which is responsible for exopolysaccharide production.

Introduction

Hospital infections have significantly increased according to epidemiological surveys conducted in clinical settings. This increase is attributable to the therapeutic use of immunosuppressive drugs [1, 2].

Among the most common bacteria, Staphylococcus is traditionally implicated [3]. Today, different species of Staphylococcus cause serious and life-threatening infections with an impact on morbidity as well as mortality [4]. One of the most common is *S. aureus;* usually it naturally comes back on the skin of humans and inside their nostrils. They are ubiquitous in a range of diseases, representing 1-5% of infections in the community and up to 30% among hospital-acquired infection [5, 6].

This organism represents a major concern for general public health, as it is responsible for large numbers of infections ranging from milder

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skin boils to severe and life-threatening conditions like endocarditis, osteomyelitis or sepsis [7]. Numerous researches had been done in order to detect the pathogenic pathways and structures [8-10].

Staphylococcus pathogenesis is a multifactorial process in which different factors are involved, among them the adhesion and also biofilm production [11]. The generation of biofilms is central to infection by shielding from multiple host defenses and protecting bacteria from antimicrobials [12, 13]. Consequently, the ability of S. aureus to adhere onto host tissues is a key mechanism in infection establishment [14], and therefore elucidating those microbial adhesion mechanisms has become an important research field.

Biofilm development basically is dependent upon the generation of an extracellular matrix, which cements individual cells together like polysaccharide intercellular attachment or poly-N-acetyl glucosamine that exert what we believe are very important influences on biofilms accretion and progress [15]. Surface proteins, polysaccharide intercellular adhesion (PIA), and extracellular DNA proteins construct the extracellular matrix; these exo-polymeric compounds frequently envelop, and safeguard bacteria embedded within them [16]. Hence, biofilm bacteria frequently exhibit heightened resistance to diverse antimicrobials because of their protective coating [17].

The ica operon (intercellular adhesion) governs the regulation of polysaccharide intercellular adhesion production in Staphylococcus species. The operon is composed of the ica ADBC open reading frames encoding for IcaA, IcaD, IcaC and Icb respectively [18]. A poly-N-acetyl glucosamine polymer is production depends on the icaA gene. The icaD gene is necessary for full activity [19]. When co-expressed with icaC, both shares participate activity in making N-acetyl-glucosamine oligomers up to 20 residues [18-21]. A further function of the *icaC* product is the export of PIA precursor to the locus surface, while de-acylation and translocation (by *IcaB*) transfer formed or forming gel matrix from cell interiors onto its exterior face [21]. For its significance in encoding PIA, the icaA gene has an important association with forming slime and biofilm in staphylococci, especially S. aureus [22]. The importance of this gene comes from the role played by its product (PIA) in Biofilm production, which happened in three steps: bacterial engagement with the biomaterials or artificial surfaces, formation of an extracellular slime like PIA that mediating cell to cell adhesions and disassembly of biofilms, along with subsequent community expansion [23]. Besides, this gene controls the formation of exopolysaccharide (EPS) of biofilm, which boosts bacterial adhesion and could serve as a refuge against the immune system of hosts and antibiotics therapy [24]. In Kurdistan of Iraq, Hussein and Salih reported that the entire isolates of hospital staff were diagnosed as S. aureus [25]. In another Iraqi investigation, it was found that 36% of isolates from clinical cases have the typical biochemical tests and morphology that are specific to S. aureus, whereas the remaining clinical isolates (64%) belong to other species of staphylococci or other pathogenic bacteria and fungi [26].

Because biofilm-forming organisms may pose inherent challenges in clinical samples, it is important to rapidly identify the presence of biofilm genes within a target organism. Device-associated infection prevention and management therefore necessitates this approach. This study was primarily aimed to determine the responsible genes (*icaA*, *icaB* and *icaD*) with biofilm coding tendencies.

Material and method

Collection and isolation of samples

A total of 425 clinical samples were collected from wounds and urine cases, taking into account the exclusion of some criteria: pregnant women, diabetics and foot ulcer patients, from Al-Kindi and Imam Ali Hospitals for the two months period November and December 2023.

These samples consisted of 217 wound swabs and 208 midstream urine samples from UTI patients were collected under sterile conditions, including sterile test tubes and swabs and then immediately transported to the microbiology laboratory. Every sample was raised in Brain Heart Infusion Broth (BHIB) before being kept for 24 hours at 37°C. Every sample was injected into culture into the selective media blood and mannitol agar for bacterial isolation and identification. After that done microscopically, morphologically and biochemical tests were involved IMVIC, catalase, oxidase, urease and coagulase tests also identified the bacterial colonies of Staphylococci that formed yellow colonies on MSA and were submitted to microscopic examination after staining by gram stain [27],[28]. Finally, the Vitek2 technique

Compact System (Bio Mérieux, Marcy l'Etoile, France) was used to ensure bacterial identification. Later, it was kept at -20°C in glycerol stock.

Biofilm formation by Microtiter plates method

In 96-well polystyrene microtiter plates, 180 L of BHIB with 0.1% glucose and 20 L of bacteria were added to quantify the amount of biofilm development. The dishes were then incubated for 24 hours at 37°C. The experiments were done in triplicates and involved three PBS washes, Crystal Violet staining for 14 minutes, 200 L of Glacial Acetic Acid for 10 minutes, and biofilm production measurement for optical density (O.D). The following describes how the results were arrived at: OD630 (bacteria) equals biofilm and OD630 (bacteria) - OD630 when divided by three (Control) Strains that produce biofilm were given a value of (strong 0.86 / moderate 0.578 or weak 0.220). The following scores were in red: Strong: OD>4xODc, Moderate: 2xODcOD4xODc, and Weak: ODcOD2xODc [29].

DNA Extraction and PCR conditions:

The genomic DNA has been extracted and purified using the Presto TM Mini gDNA Bacteria Kit (GBB004 GBB100/101 GBB300/301 gene aid/Thailand). The meticulously selected staphylococcal isolates were cultured overnight, and then DNA was extracted using the manufacturer's recommended methods. The purity and the concentration of the DNA were estimated by Nanodrop. It was observed that the purity ranged between 1.6 and 1.9 while the concentration fluctuated between 36.3 and 134.7 ng/µl.

A ratio of 1.8-2.0 is generally accepted for DNA as pure. If the ratio was significantly lower than the indicated ratio, the presence of protein phenol or other contaminants which absorbed strongly at or close to 280 nm may be defined [30].

The conventional PCR technique was adopted to confirm the identification via amplification of icA gene for S. Aureus isolates. In this technique the references forward and reverse primers have been used, their sequences are displayed in Table (1). The PCR conditions and programs are shown in Table (2).

Statistical Analysis

The SPSS program (SPSS, Inc., Chicago, IL, USA) version 26 has been applied to analyze the results of current investigations. The significance of results was accepted at 0.05 levels. The Chi-square test was employed to compare between percentages (two or more groups); while the difference among means of groups were analyzed using one-way ANOVA analysis and LSD test had employed to detect critical values for comparisons between means.

Results and discussion

Isolation of *S. aurous*

Staphylococcal infections hold significant significance in the field of human medicine. *S. aurous* is a highly important bacterium responsible for producing infections on a global scale It is a major contributor to hospital-acquired infections and is responsible for various diseases in humans, such as food poisoning, mastitis, endocarditis, septicemia, toxic shock syndrome, skin infections, bone infections and soft tissue infections [31].

The samples for the present study were taken between November and December 2023 from 425 patients in Iraq at Al-Kindi, and Imam Ali Hospital. Males and females were given separate sets of wound and urine samples. The age range of individuals with urinary tract infections (UTI) was (8-82) years. The age range of the research group was somewhat in agreement with Al-Mathkhury and Abdul-Ghaffar's findings in Iraq, whereas the wound patients' age range was (18-66) years, as shown in (Table 3) and Figure 1, and this was somewhat in agreement with Almeida.

Managing UTIs is crucial due to the rising prevalence of pathophysiology and the development of resistance to therapies associated with UTIs. Even though urine is not atypically considered as a reservoir for staphylococci, *S. aurous* strains have been increasingly isolated from urine specimens. Consequently, *S. aurous* is now regarded as a urinary pathogen [32]. The results of this study indicated that organism is one of the potential etiologic agents of UTIs. These results aligned with the findings of Elzouki et al. [33], who identified *S. aurous* as the predominant microbe among patients with UTIs, making up 94% of all isolates. Comparable reports were previously documented [34].

A rising number of people around the world are affected by wounds, whether they are chronic or acute. Infections caused by microorganisms are a leading cause of delayed wound healing, and *S. aurous* is the most prevalent pathogenic bacterium identified in various wound samples [35]. The prevalence of *S. aurous* in wound isolates was higher in our investigation compared to

the prevalence estimate of 34.7% recorded in Saudi Arabia [36]. In contrast, Rasmi et al. [37] reported a higher prevalence of 66.8% for *S. aurous* in both females and males. To identify infection-causing and colonizer-specific pathogens, it is necessary to assess the intrinsic virulence characteristics of isolated species, since the virulence potential of different bacterial species varies in wound environments [38].

Detection of 16SrRNA by Polymerase chain reaction (PCR)

The method previously described was used to prepare isolates from overnight cultures used to extract the genomic DNA of bacteria. Nano drop calculated the DNA's purity and quantity. The content varied between 36.3 and 134.7µg/µl, and the purity was found to be between 1.6 and 2.0. Most people agree that DNA is pure if the number is between 1.8 and 2.0. The presence of contaminants such as protein, phenol, or other substances that strongly absorbed light at or near 260\ 280 nm may be determined if the ratio was considerably lower than the suggested ratio [39]. For *S. aurous* isolates, the PCR method was used to amplify a *16S rRNA* gene fragment and validate the identification.

Based on this molecular method, the results revealed that all 29 of the isolates were correctly identified as *S. aurous.* To determine the PCR product's size, which was 108bp, the isolates PCR products were observed on an Agarose gel as outlined in (Figure 2).

Gene Expression of Biofilm Producing Isolates

Three *S. aurous* isolates (98, 67, 146) chosen based on varying levels of biofilm formation (strong, weak) were subjected to a quantitive PCR assay (qPCR) to measure the expression levels of the icaA gene, which is implicated in biofilm formation Strains that produce biofilm were given a value of (strong 0.86 / moderate 0.578 or weak 0.220). Table 4 shows that there is a highly significant variation between isolate 67's expression and isolates 98 and 146 at ($P<0.01$).

Quantitative of biofilm formation by Micro-titer plate method

S. aurous is a significant bacterium that can potentially adhere to and generate biofilms on exterior surfaces, leading to community-acquired illnesses. The rising antibiotic resistance observed in biofilm-producing bacteria in hospital settings poses a significant global challenge for treating staphylococcal infections. Numerous investigations have demonstrated the biofilm development and genetic features of various isolates of *S. aurous* [40].

The current research found that although 388 (91.3%) isolates could produce biofilm, their production levels varied from strong to moderate to weak. The findings from 388 isolates revealed that only 146 isolates (37.6%) produced strong biofilms, while 160 isolates (41.3%) produced moderate biofilms and 82 isolates (21.1%) produced weak biofilms (Fig.3). Similar results were reported by Azmi et al. [33], who indicated that all the *S. aurous* isolates formed biofilm, with 46.4% producing moderate biofilm on microtiter plates. In another recent study in South Africa, 90 % of *S. aurous* from clinically isolated were biofilm former [41]. However, in contrast to our results, a majority (52.8%) of their isolates exhibited strong biofilm generation [42]. The variations in the classification of the biofilm phenotypes may arise due to disparities in the interpretation of the findings. Therefore, it is essential to standardize the techniques and understanding of biofilm development.

Our research concentrated on studying the expression of certain genes linked to binding factors and the production of biofilms in a controlled laboratory setting. Among these genes, we examined the *icaA* gene. Biofilm formation necessitates the aggregation of microbial cells into multilayered cell clusters that are enveloped in a slimy material. The expression of *ica*, which results in the synthesis of the PIA, performs an essential function in the adherence and initiation of staphylococcal biofilms. It was intriguing to ascertain the expression levels of the *icaA*, which is responsible for production of PIA, in the biofilm of strains. Additionally, identify the specific phase of biofilm growth in which this gene is most active. Many studies have also reported the results of RT-PCR and DNA microarray analysis and for *S. aurous* biofilm gene expression [43-44]. However, there is still a limited understanding of the involvement of specific genes in the production of biofilms.

The findings showed that *icaA* gene expression dropped with increasing surface binding forces and PIA production, but it returned to normal after 3-6 hours. The process by which the adhesion force signals the organism to enter its adhering state should be viewed as causing nanoscale cell wall deformation and membrane stress [45]. It has been

suggested that the deformation of lipid bilayers results in the activation of mechanosensitive channels that are responsible for sensing binding forces. These channels convert mechanical stress into signals of chemicals [46]. Furthermore, Biofilm formation and maintenance rely on signaling events that are triggered by gating events, which in turn release auto-inducers, Which contributes to creating the problem of antibiotic resistance at the cellular level. Most antibiotics are unable to kill S. aurous biofilm bacteria because they can form biofilms that block the introduction of drugs into the membrane [47-48]

The first layer that adheres to the surface is different from the second layer that will interact and merge with the first layer to form a biofilm. The PIA's operations The synthesis of PIA via proteins encoded by icaA regulates the biofilm formation process in S. aurous [49], the primary component facilitating the accumulation phase of adhesive connections among microbial cells in a biofilm, is anticipated to result in a drop in the level of expression of *icaA* as the force of adhesion intensifies within the initial hour, followed by an increase after three hours. This time period can describe how bacteria adapt to their environment on the surface (1-3 h) [50].

Obviously, different exopolysaccharide-producing genes are expressed at various levels by the genetic background of bacterium cell. The failure of bacterial isolates that exhibit reduction in biofilm formation to upregulate icaA might simply reflect more accurately their planktonic nature, since they would presumably produce lower levels of *icaA* comparable with the original 'parent' strains

Kot had found in an alternative research that summed up the expression level of *icaA* between biofilm and planktonic state for different isolates of *S. aurous* as well. The results showed *icaA* expression in biofilm was significantly higher than that under planktonic growth conditions. In addition, differential expression level comparison between strong and weak biofilm producers of isolates revealed that it was maximum in the strong ones [51].

Marques [52], where it was mentioned that *ica* genes were highly expressed by strong biofilm isolates compared to weak production after 24 hours. The results of this study concerning the icaA gene expression are in accordance with another investigation that biofilm high producer isolate had a higher level compared to weak promoter. The major contributors argue the differential biofilm formation by these strains is at part a reflection of different levels of metabolic activity and other results obtained from reference strains may have some differences from that of clinical isolates[49]. Another study by Abdel-Shafi [53] stated that most isolates which are strong biofilm former were observed to have *icaA* or *icaB*, on the other hand they did not identified these genes in only one-third of their patient isolates. These data suggest possible biofilm biogenesis pathways that are not dependent on the *ica* determinants. Therefore, the identification of these yet unknown pathways may be critical to discovering new drugs for treating such infections. Although additional research is necessary to validate the current findings using other clinical samples and methods.

Interestingly, Cells in biofilms typically express more attachment factors—proteins that help cells stick to one another and proteins in the extracellular matrix—than cells in planktonic environments. Not all known biotic surface-attachment factors were expressed in biofilms until 24 hours after attachment, including several that induce cell-to-cell adhesion [54].

The fact that *S. aurous* expresses the *icaA* operon at extremely low levels when grown in vitro suggests that its expression is tightly regulated. This poses the crucial inquiry of how bacteria that emerge afterward in a biofilm, either through proliferation or increased adherence, perceive the adhesive forces that originate from a substrate. Subsequent creatures cannot directly detect surfaces since all repulsive or attracted forces emanating from a substratum surface is constrained to some tens of nanometers. Even more obviously, they undergo adhesion pressures when they adhere to nearby species [55].

Even though the *ica* operon has been extensively researched, it is noted in the literature that only 30% of biofilm-producing strains exhibit high levels of PIA in laboratory settings. The absence or undetectable levels of PIA in these strains may indicate that the bacterial biofilms primarily consist of teichoic acid along with additional protein components [56].

Table 1. The list of the primers used in this research.

Primer name	Primer sequence 5′→3′	Amplicon size bp	reference
16SrRNA-F	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG	108	Martineau et al 1998
16SrRNA-R	CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA	108	Martineau et al 1998

Table 2. PCR Programs

Steps	No. of cycles	Time	Temperature
Initial Denaturation	**1**	**3 min**	**95°c**
Denaturation	**35**	**30 sec**	**95°c**
Anneling	**35**	**30 sec**	**78°c**
Extension	**35**	**25 sec**	**72°c**
Final Extension	**1**	**3 min**	**72°c**

Table 3. Distribution of sample study according to Source

Source	No.	Percentage
UTI	217	51.1%
Wound infection	208	48.9%
Total	425	100%
Chi-square- χ^2 (P-value)	---	2.7 NS (0.101)
NS: Non-Significant.		

Table 4. *S. aurous* strains with different biofilms exhibit *icaA* expression.

S. aurous isolate	Biofilm formation (No.)	icA gene expression mean ± SD
Isolate 98	Weak (82)	1.231 ± 0.07 b
Isolate 67	Moderate (160)	1.624 ± 0.11 b
Isolate 146	Strong (146)	6.508 ± 0.52 a
LSD Value		1.373 **
P-value		0.00294**
The difference between means with distinct letters in the same column was significant, ** (P 0.01) by ANOVA test		

Figure 1. The distribution of the sample analysis by source.

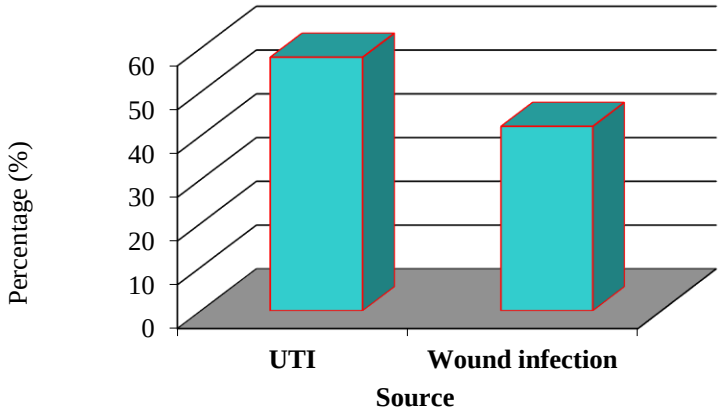


Figure 2. 1.0% agarose gel visualization of the *S. aurous 16S rRNA* gene labeled with red safe stain. The bands on show correspond to the 108 bp PCR product with a 50 bp DNA ladder.

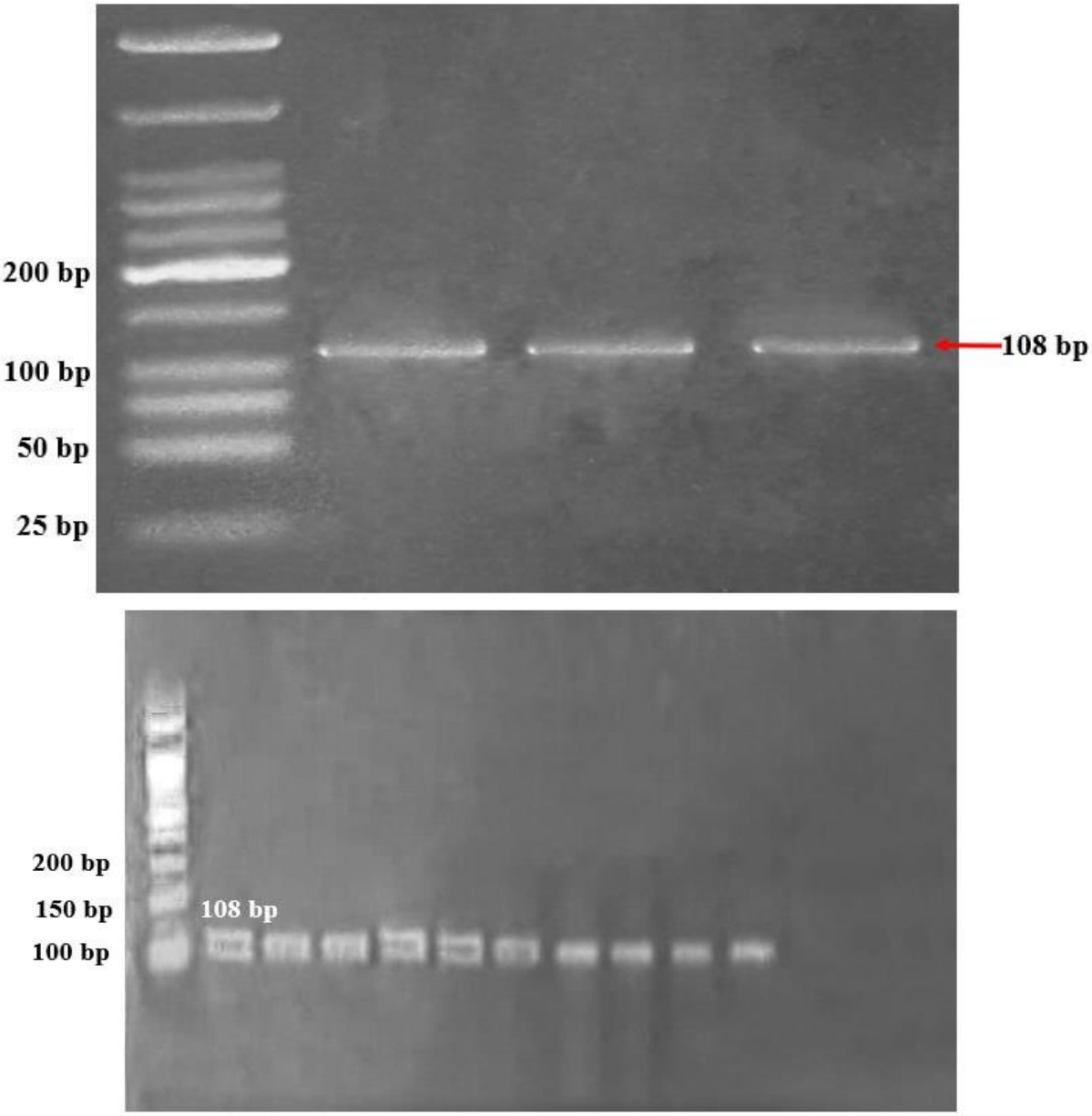
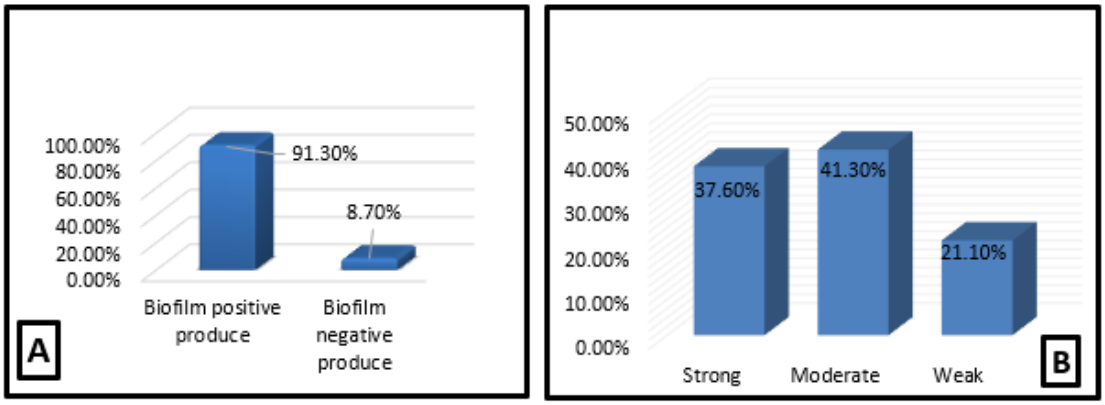


Figure 3. A: Percentage of biofilm produce. B: Score of biofilm produce



Conclusion

The findings of the current study indicated that the levels of gene expression related to the production of Adhesion factors and PIA were notably elevated in biofilm compared to planktonic settings, suggesting that bacterial cells initially form significant quantities of virulence factors that aid contact with extracellular ligands present in the host to enable them to invade host tissues. The initial phase of biofilm development exhibited a decline in the expression level of a gene, likely due to decreased metabolic activity caused by nourishment deprivation and unfavorable oxygen levels. Finally, the recent findings indicate a substantial disparity in the expression level between isolates that produce strong biofilms and those that produce weak to moderate biofilms.

The intensive use of qRT-PCR to investigate the expression levels of the genes implicated in the adhesion and production of biofilm by *S. aureus* strains was valuable in gaining a deeper comprehension of the temporal mechanism underlying biofilm development.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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