Original Article

Evaluation of *icaA* and *icaD* genes Involved in Biofilm Formation in *Staphylococcus aureus* Isolates from Clinical Sources Using Reverse Transcriptase PCR

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ABSTRACT

Staphylococcus aureus is recognized for its capacity to generate biofilms, which facilitate bacterial adhesion to diverse substrates and present a significant challenge to therapeutic intervention. The process of biofilm formation is dependent on the icaABCD operon, with the icaA and icaD genes playing a pivotal role in this intricate process. The objective of this study is to investigate the role of these genes in the biofilm formation of S. aureus isolates sourced from clinical settings. A total of 100 S. aureus isolates were collected from clinical sources and subsequently subjected to DNA and RNA extraction using a commercial kit from Kiagen Co. To transcribe the RNA samples into cDNA, a commercial kit from Kiagen Co. was employed.The capacity to produce phenotypic and molecular biofilm formation was then measured using the microtiter plate method and PCR, respectively. The expression levels of the icaA and icaD genes were determined via RT-PCR (Reverse transcription polymerase chain reaction). The results indicated that 95% (95%) of the isolates were capable of producing biofilm, with 16 (16%) producing weak, 64 (64%) producing medium, and 15 (15%) producing strong biofilms. Furthermore, the icaA gene was detected in 72% of the isolates, while the icaD gene was detected in 58%. Of these isolates, 70 (97.2%) expressed the icaA gene, and 53 (73.6%) expressed the icaD gene. Conversely, four isolates (5.5%) that possessed the icaA gene but lacked the icaD gene did not form biofilm. One strain did not express either of the genes. The presence of either the icaA or icaD gene is crucial for the development of biofilm. However, further investigation is necessary to fully comprehend the intricacies of biofilm formation.

Keywords: Biofilm, *Staphylococcus Aureus*, *icaA* Gene, *icaD* Gene, Reverse Transcriptase PCR.

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1. Introduction

In recent years, nosocomial infections caused by *Staphylococcus* species have become increasingly prevalent due to several factors, including the rise in patients with compromised immune systems, the emergence of resistant strains, and the overuse of medical devices (1, 2). Among the various types of *Staphylococcus*, *S. aureus* has been identified as the most virulent. While it is a component of the natural microbial flora of many individuals worldwide, S. aureus can cause both common and serious diseases when it enters an area outside of its natural location. S. aureus is an opportunistic bacterium that possesses multiple virulence factors, including hemolysin, lipase, protease, lecithinase, DNase, and various types of toxins (3). This bacterium poses a grave threat due to its ability to express various cell-associated pathogenic factors, which can lead to a wide spectrum of infections. A notable virulence mechanism of S. aureus is its ability to form a biofilm, which facilitates bacterial adhesion to diverse surfaces (4). The strong adhesion of the biofilm is the first stage of bacteremia, which can lead to the presence of bacteria in the bloodstream and the spread of infection throughout the body. The structural integrity of the biofilm is maintained by polysaccharide intercellular adhesion, a complex macromolecular compound comprising N-acetyl glucosamine and non-N-acetylated D-glucosaminyl subunits (5). Polysaccharide Intercellular Antigen (PIA) is composed of N-acetylglucosamine residues linked by beta 1 to 6, and an anionic section that contains fewer non-acetylated D-glucosaminyl residues with ester-linked phosphate and succinate. Research has demonstrated that the presence of *icaA* and *icaD* genes is essential for the formation of PIA-dependent biofilm, particularly in response to anaerobic growth (6). The expression of intercellular adhesive polysaccharides is subject to regulation by the ica operon, which is contingent upon environmental factors and growth conditions. The *icaA*, *icaD*, *icaB*, and *icaC* genes, which are crucial for biofilm formation, are the products of the *ica* operon. The *icaA* and *icaD* genes, in particular, have been identified as playing a primary role in exopolysaccharide synthesis (7). The *icaA* gene is responsible for the synthesis of the IcaA protein, which possesses transferase activity. However, the IcaA protein alone exhibits minimal N-acetylglucosaminyl transferase activity. Concurrent expression of both *icaA* and *icaD* has been observed to lead to an approximately 20-fold augmentation in transferase activity, thereby resulting in the synthesis of IcaA and IcaD proteins (8). In the context of clinical settings, bacterial biofilms have emerged as an increasingly salient concern. Studies have indicated that over 65% of clinical infections are associated with biofilms (9). In response to this crisis, there have been endeavors to develop technologies aimed at controlling and eliminating biofilm-related infections. However, further research is necessary to comprehensively assess the interactions between biofilms, to develop new anti-biofilm compounds, and to optimize measures for preventing and treating biofilm infections. The identification and investigation of genes implicated in biofilm formation and development are paramount, as their expression is indispensable during infection (10, 11). The present study aims to utilize the Reverse Transcriptase PCR (RT-PCR) technique to examine the capacity of *S. aureus* bacteria, isolated from clinical sources, to express genes associated with biofilm formation (*icaA* and *icaD*).

2. Materials and Methods

2.1. Bacterial Samples

The objective of this study was to observe and describe 100 isolates of *S. aureus* collected from clinical sources in Karaj in 2022. These samples were subsequently transferred to a microbiology laboratory for further analysis. The isolates were tested using specific culture media, Gram staining, and a range of biochemical standard tests. The bacterial samples were stored at -20°C for subsequent experimental analysis.

2.2. Investigation of Biofilm Formation

The Christensen et al. (12) method is a viable approach for investigating biofilm formation. The method entails the dilution of an overnight bacterial culture in Tryptic Soy Broth (TSB) medium to a turbidity of 0.5 McFarland, corresponding to a 1% dilution, followed by the addition of 200 µL of this dilution to each of the three wells per strain in a 96-well flat-bottom polystyrene tissue culture plate. The plates are then covered and incubated aerobically at 37°C for 24 hours. Following this incubation period, the wells are subjected to a series of washes with 0.2 mL of phosphate-buffered saline (pH 7.2) to ensure the removal of non-adherent bacterial contaminants. To fix adherent bacteria, 200 µL of 99% methanol per well is added and left for 15 minutes. Subsequently, 0.2 mL of 2% crystal violet is applied and left for 7 minutes. The plates are then air-dried, and the dye bound to the adherent cells is solubilized with 160 µL of 33% (v/v) glacial acetic acid per well. The optical density (OD) of each well is subsequently measured using an ELISA reader (Stat Fax 2100) at a wavelength of 630 nm. The OD cut-off (ODc) method was employed to evaluate the results. To verify the findings, the standard deviation and average OD of the negative control wells were initially calculated. Subsequently, the amount of biofilm was determined using the formula ODc= Average OD of negative control wells+(3×standard deviation of negative control wells). The following classifications were used to determine the biofilm formation ability of the samples: if the OD of the bacterial samples is equal to or less than the ODc, the bacteria are considered to have no biofilm formation ability. If the OD samples are equal to or less than twice the ODc, the bacteria are considered to have weak biofilm formation ability. Finally, if the OD samples exceed four times the ODc and are less than or equal to four times the ODc, the bacteria are considered to have moderate biofilm formation ability. Finally, if the OD samples are equal to or greater than four times the ODc, the bacteria are considered to have strong biofilm formation

ability. The reference strain *S. aureus* ATCC 35556 is utilized as a positive control, while wells containing sterile TSB (Merck) function as negative controls.

2.3. Molecular Detection of *ica*A and *ica*D Gene

Pure genomic DNA was extracted from 24-hour bacterial cultures in Nutrient Broth culture medium using the Qiagen Co. kit. The presence of *icaA* and *icaD* genes in the isolates investigated was determined using specific primers (Table 1) and following the PCR reaction. The PCR reactions contained 25 µL of final volume, with 12.5 µL of Amplicon's master mix (which includes Maxer Mix 1X, Tris-HCl 0.5 M, MgCl2 2 mM, dNTPs 1.6 mM, Taq 0.04 Units/µL, and 0.5 µL), 1 microliter (0.2 µM) of forward and reverse primer for each gene, 2 microliters (20 ng) of template DNA, and double-distilled sterile distilled water. The genes of interest were amplified using a thermal cycler (Applied Biosystem) under the following conditions. Initially, the temperature was set to 95°C for 5 minutes. Subsequently, 33 thermal cycles were executed, with each cycle comprising denaturation at 95°C for 30 seconds, annealing at 62°C for 30 seconds, and amplification at 72°C for 30 seconds per minute. Subsequent to the final amplification, the temperature was maintained at 72°C for a duration of 5 minutes (13-15). In the negative control, all materials were used except for the template DNA. For the positive control, the standard strains of *Staphylococcus epidermidis* ATCC 35984 were utilized for genes *icaA* and *icaD*. Subsequently, a 1% agarose gel and a 100 bp DNA Ladder were employed to verify the presence of the studied genes (16).

2.4.The Expression *ica*A and *ica*D Gene

In order to investigate the role of the *icaA* and *icaD* genes in biofilm formation, RT-PCR (Reverse transcription polymerase chain reaction) was conducted to analyze their expression in the studied isolates. The following steps were taken to study gene expression: First, bacterial RNA was extracted from the 24-hour bacterial cultures using a bacterial RNA extraction kit (Kiagen Co). The cDNA was then prepared using the extracted RNA. Polymerase chain reaction (PCR) was then performed using specific primers (Table 1) and following the described protocol. The resulting product was then subjected to electrophoresis using a 1% agarose gel. Standard strains were utilized as positive controls, while all reagents present in the reaction, excluding the template DNA, were employed as negative controls.

2.5. Statistical Analysis

The interpretation of the data was reviewed, and statistical calculations were conducted using GraphPad Prism software version 9 at a statistical level of 95%. A significance level of less than 0.05 was employed to ascertain statistical significance.

3. Results

The diagnostic process involved the analysis of 100 samples, all of which were identified as *S. aureus* by the designated laboratory. To assess the capacity of the bacterial isolates to form biofilm, a microtiter plate method was employed. The results indicated that 5% of the isolates were unable to form biofilm, while the remaining 95% exhibited this capacity. Among the isolates demonstrating biofilm formation, 16% exhibited weak biofilm formation, 64% exhibited moderate strength, and 15% exhibited strong biofilm formation. Statistical analysis at a 95% confidence level revealed a significant difference between the average biofilm formation power and other samples, suggesting that most of the samples can form an average biofilm (p value < 0.05). The results of the polymerase chain reaction (PCR) analysis indicated the presence of the *icaA* and *icaD* genes in 72% and 58% of the isolates, respectively. Statistical analysis indicated no significant difference in gene frequency among the *S. aureus* isolates (p value > 0.05). Subsequent analysis of the five isolates that failed to form biofilm revealed that none of them possessed the *icaD* gene, while 80% had the *icaA* gene (Table 2). The statistical analysis indicated a direct correlation between the absence of the *icaD* gene and the inability to form biofilm (p value < 0.05). The RT-PCR technique was used to examine the expression of *icaA* and icaD genes in *S. aureus* isolates (Table 3). The results demonstrated that 70% of the isolates expressed the *icaA* gene, while 53% expressed the *icaD* gene, indicating a statistically significant difference in gene expression among the *S. aureus* isolates studied. However, subsequent statistical analysis revealed no significance (p value>0.05). However, a significant difference in the expression of *icaA* and *icaD* genes was observed among the isolates incapable of forming biofilm, suggesting a direct relationship between the *icaD* gene and biofilm formation (p value<0.05). Notably, only one non-biofilm producer lacked both *icaA* and *icaD* genes and was unable to express the *icaA* and *icaD* genes in *S. aureus* isolates. A comparative analysis was conducted to investigate the presence and expression of the *icaA* and *icaD* genes in biofilm formation. The results revealed no statistically significant differences between the presence of these genes and their expression (p value>0.05). However, a significant discrepancy was observed in the presence of *icaA* and *icaD* genes (p value<0.05) when examining isolates devoid of biofilm. Among the *S. aureus* isolates examined, the majority exhibited a moderate capacity to form biofilm, with 43 (67.1%) and 40 (62.5%) strains possessing the *icaA* and *icaD* genes, respectively. Of these, 43 (67%) and 38 (59.37%) expressed the genes. Notably, there was no significant difference in gene expression (p value > 0.05). The presence and expression of *icaA* and *icaD* genes were then compared (Figure 1).

4. Discussion

The process of biofilm formation is a critical aspect of bacterial pathogenicity, with the initial stage of colonization or infection involving bacteria attaching to host cells, leading to the production of microcolonies and eventually the formation of biofilms on living or non-living surfaces.

Table 1: The sequence of primers used in this research

Gene primer	Sequence (5′to3′)	Fragment size (bp)	Reference
icaA	F: ACACTTGCTGGCGCAGTCAA	188	(22)
	R: TCTGGAACCAACATCCAACA		
icaD	F: ATGGTCAAGCCCAGACAGAG	198	(22)
	R: AGTATTTTCAATGTTTAAAGCAA		

Table 2: Frequency of presence of *icaA* and *icaD* genes in biofilm-forming and non-forming bacteria

The ability to biofilm formation	Number of isolates	Frequency of *ica*A gene	Frequency of *ica*D gene	*p* value
No biofilm	5	4 (80.0%)	0 (0.0%)	0.001
Weak	16	12 (75.0%)	1 1(68.7%)	0.483
Moderate	64	43 (67.1%)	40 (62.5%)	0.232
Strong	15	13 (86.6%)	9 (60.0%)	0.248
Total	100	72 (72.0%)	58 (58.0%)	0.118

Table 3: Expression of *ica*A and *ica*D genes in biofilm-producing and non-producing *S. aureus* isolates

The ability to biofilm formation	Number of isolates	Expression of *ica*A gene	Expression of *ica*D gene	*p* value
No biofilm	5	4 (80.0%)	0 (0.0%)	0.001
Weak	16	12 (75.0%)	9 (56.2%)	0.392
Moderate	64	43 (67.2%)	38 (59.3%)	0.180
Strong	15	11 (73.3%)	6 (40.0%)	0.248
Total	100	70 (70.0%)	53 (53.0%)	0.129

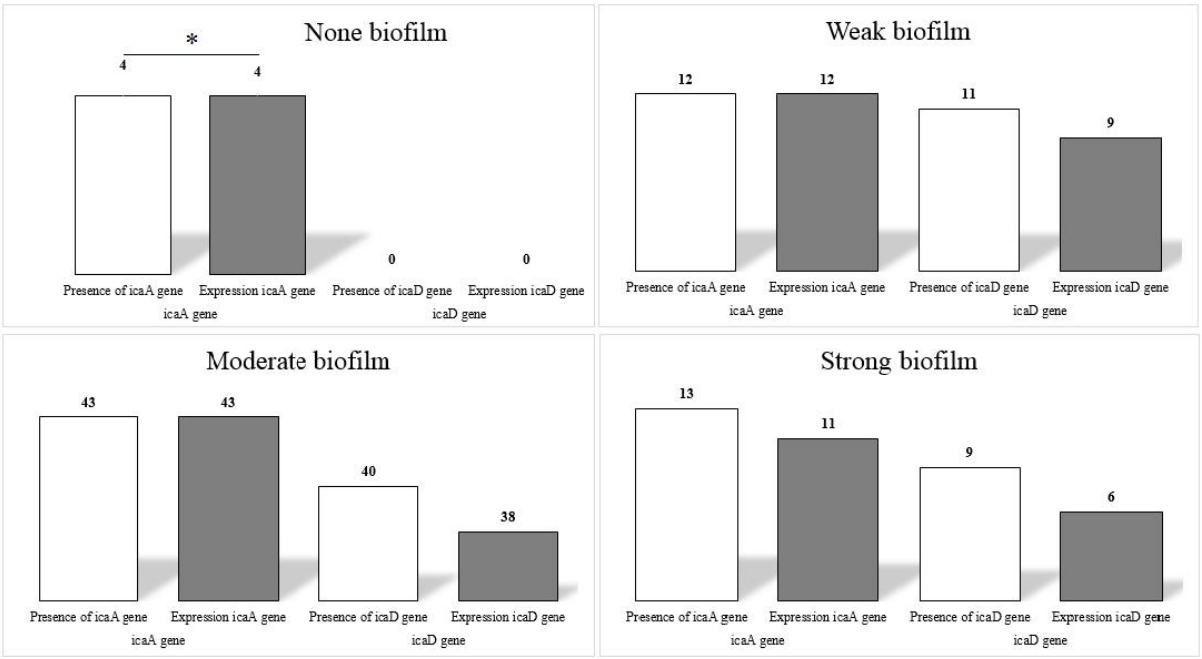

Figure 1: Compares the presence or absence of the *icaA* and *icaD* genes and their expression in biofilm and non-biofilm isolates. The asterisk (*) indicates the significance level.

The formation of biofilms is influenced by several factors, including nutrients, bacterial appendages, surface compounds, quorum sensing, and secretory molecules of bacteria. Our study found that 95 out of 100 S. aureus isolates were capable of producing biofilm, which accounts for 95% of the total isolates.Among these isolates, 16 strains produced biofilm that was classified as weak, 64 produced medium biofilm, and 15 produced strong biofilms.Several studies have explored the biofilm of S. aureus clinical isolates. In a particular study, Chen et al. found that approximately 72% of the isolates produced biofilms, with 54.64% of these isolates classified as weak biofilm producers, 14.43% as moderate biofilm producers, and 3.09% as strong biofilm producers (17).A study conducted in Brazil reported results similar to ours, indicating a high rate of biofilm formation. The study found that all the S. aureus isolates had the ability to form biofilm, and the results of another study align with our findings, as it also reported a high rate of biofilm formation and the ability of all methicillin-sensitive and methicillin-resistant S. aureus isolates to produce biofilms (18). A study conducted in Iraq reported that all isolates (100%) formed biofilms, with 47.7% of the isolates producing strong biofilms, 38.6% producing moderate biofilms, and 13.6% producing weak biofilms. This finding is consistent with the results of our study, which also reported a high rate of biofilm formation (19). However, a contrasting viewpoint is presented by certain studies that documented a reduced frequency of biofilm formation. For instance, Harika et al. found that 78.2% of the isolates were biofilm producers, which is lower than the rate observed in our study (20). A similar observation was reported by Mahmood et al., who found that only 24.1% of the isolates were capable of producing biofilms (21). Despite the numerous studies demonstrating the high prevalence of biofilm production in S. aureus isolates, discrepancies in the frequency of biofilms are also observed across studies. These discrepancies can be attributed to various factors, including the type of isolated samples, geographical regions, sources of sample isolation, and the number of samples examined. Following a thorough analysis of 100 isolates, it was ascertained that 72 of them contained the *icaA* gene. Of these 72 isolates, 4, 12, 43, and 13 were detected in no biofilm, weak, moderate, and strong biofilm producers, respectively. Furthermore, our analysis revealed that 58 isolates possessed the *icaD* gene. Of these 58 isolates, 0, 11, 40, and 9 were detected in no biofilm, weak, moderate, and strong biofilm producers, respectively. A parallel study reported that 70% of S. aureus biofilm producers contained the *icaA* and *icaD* genes, while non-biofilm producers lacked these genes (17). In a separate study, it was observed that 66.6% of the *S. aureus* isolates contained the *icaA* gene, while 58.4% contained the *icaD* gene. The co-occurrence of both genes was observed in 58.4% of the isolates, a finding that aligns with the results of our study (22). Furthermore, the *icaD* gene demonstrated a higher frequency compared to the *icaA* gene. Specifically, the frequencies of the *icaA* and *icaD* genes were 77.1% and 97.1% in MSSA isolates, respectively, compared to 86.4% and 100% in MRSA isolates (18). In a separate study, it was determined that 66.7% of *S. aureus* biofilm-forming isolates contained the *icaA* and *icaD* genes. Furthermore, it was determined that 44.4% of the isolates possessed all four genes (*icaA*, *icaB*, *icaC*, and *icaD*) in conjunction (21). In the present study, 70 isolates were found to express the *icaA* gene, while 53 isolates expressed the *icaD* gene. The expression of the *icaA* gene was observed in 4, 12, 43, and 11 of the isolates that produced no biofilm, weak, moderate, and strong biofilms, respectively. Conversely, the expression of the *icaD* gene was observed in 0, 9, 38, and 6 of the isolates that produced no biofilm, weak, moderate, and strong biofilms, respectively. Marques et al. sought to identify the phenotypic expression of biofilm formation in 20 S. aureus strains and to evaluate the expression and regulation of the genes involved, namely *icaA* and *icaD*. The study revealed that all 20 strains exhibited the capacity to produce biofilms, and the genes *icaA* and *icaD* were expressed in all of them. These findings substantiate the pivotal function of the *icaADBC* operon genes in the process of biofilm formation and their contribution to the initial phases of bacterial growth (23). This finding aligns with the findings of the study by Arciola et al., which indicated a correlation between the expression of *icaA* and *icaB* genes and the formation of biofilms in *Staphylococci* spp. (24). Another study utilized real-time reverse transcriptase PCR (RT-qPCR) to investigate the expression dynamics of the ica operon. The results indicated that the expression of the ica operon was high during the initial stages of biofilm formation, but decreased as the biofilm aged. This finding suggests that the *ica* operon plays a crucial role in regulating biofilm formation in *Staphylococcus* species (25). *S. aureus* has been demonstrated to produce a substantial quantity of biofilms, with the genes *icaA* and *icaD* playing a pivotal role in this process. However, it is imperative to acknowledge that additional factors contribute to biofilm formation, and their study is imperative for a comprehensive understanding of this phenomenon. While biofilm formation may not always be indispensable for persistent infections, its formidable resistance to eradication underscores the imperative for effective detection and prevention strategies.

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Authors' Contribution

Writing and Investigation: M. A.
Writing, Revision and Supervision: E. B.
Revision, Methodology, and Visualization: R. M.

Statistical analysis and Interpretation of data: M. B.
Writing and Revision: M. T. M.

Ethics
It has been asserted that all ethical considerations were duly considered during the preparation of the submitted manuscript.

Conflict of Interest
There is no conflict of interest among the authors of this article.

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Data Availability
The article presents a comprehensive overview of the available data.

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