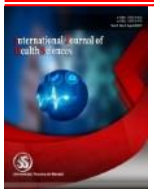

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Biofilm Formation and Associated Gene Detection in Staphylococcus spp. Isolated from Urinary Tract Infection Patients

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Keywords

Biofilm formation;
Staphylococcus;
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Abstract

Objectives: The study aimed to investigate the ability of Staphylococcus species isolated from urinary tract infection (UTI) patients to form biofilms, and to detect the presence of the biofilm regulatory genes icaA and icaD. **Methods:** Urine samples were collected from 100 UTI-diagnosed patients at [Baladrouz Hospital] over six months under aseptic conditions. The collected specimens were inoculated onto Mannitol Salt Agar (MSA) and Blood Agar. Biofilm formation was evaluated using the crystal violet staining method. The presence of the icaR gene was detected by polymerase chain reaction (PCR) in Staphylococcus aureus isolates obtained from urine samples of patients with inflammatory conditions. Antimicrobial susceptibility was assessed by the disc diffusion method. **Results**: Different Staphylococcus species exhibited varying biofilm-forming capacities, with the highest observed in S. capitis and the lowest in S. lugdunensis. All tested S. aureus strains were positive for the icaR gene. Most isolates displayed broad-spectrum antibiotic resistance, particularly among coagulase-negative Staphylococci (CoNS). **Conclusion**: These findings highlight the role of biofilm formation in antibiotic resistance among Staphylococcus species, especially in CoNS. Understanding these mechanisms is crucial for developing effective treatment strategies for UTIs and reducing the incidence of persistent infections.

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Contents

1 Introduction

Urinary tract infections (UTIs) affect over 150 million people worldwide annually and are a major cause of morbidity (Stamm & Norrby, 2001). While Escherichia coli remains the primary pathogen, Gram-positive cocci such as coagulase-negative staphylococci (CoNS) and Staphylococcus aureus have emerged as significant uropathogens, particularly in catheter-associated infections (Flores-Mireles et al., 2015). Biofilms—communities of microorganisms encased in a self-produced extracellular polymeric substance— contribute to the long-term survival of the bacteria during infection (Donlan & Costerton, 2002). In Staphylococcus spp., biofilm formation confers increased resistance to antibiotics and evasion of host immune responses, complicating treatment (Lewis, 2005). Staphylococcus saprophyticus is a major factor in the formation of biofilm, which is largely responsible for the pathogenesis of urinary tract infection (UTI). Typical agent of UTIs, especially in young women (Djawadi et al., 2023), The fact that a large majority of S. saprophyticus isolates are biofilm producers has been established (Hashemzadeh et al., 2021). For instance, in a comprehensive study of many isolates of S. saprophyticus from human infections, colonization, and food environments, we found all isolates produced biofilms (91%), and 91% biofilm producing contracts were good biofilm makers. The fact that S. saprophyticus forms biofilms appears to have several clinical implications. However, UTIs are difficult to cure because biofilms act as barriers to antibiotics and biocides (Lila et al., 2023).

Genetically, biofilm formation in Staphylococcus is governed by various genes: icaA and icaD, which belong to the ica operon involved in biofilm formation, essential for polysaccharide intercellular adhesin production (Arciola et al., 2002). bap (biofilm-associated protein), promotes cell aggregation (Cucarella et al., 2001). fnbA (fibronectin-binding protein A), facilitates adhesion to host tissues and abiotic surfaces (Dziewanowska et al., 1999).

Antimicrobial resistance correlated with the biofilm formation by S. saprophyticus and other staphylococcal species. Biofilms are protective environments for bacteria, and conventional treatments work less well because the bacteria are in biofilms and the antibiotics don't work on bacterial biofilms (Nourbakhsh et al., 2022; Arciola et al., 2012). Antimicrobial expression of S. saprophyticus biofilms to ciprofloxacin, vancomycin, oxacillin, trimethoprim/sulfamethoxazole, and norfloxacin was also studied. It has been shown in these studies which showed that biofilm cells of S. saprophyticus resist these antibiotics better than do their planktonic counterparts. This resistance emphasizes the need for other alternative therapeutic strategies that can hit biofilm-forming bacteria. Biofilm-related antibiotic resistance is a serious clinical problem. Recurrent infections and relapses from genitourinary tract biofilms. Moreover, prosthetic devices like urinary catheters (Lawal et al., 2021). predisposes patients to UTIs with biofilms. The first step is to tackle this problem with a range of different approaches, such as creating new drugs to target or break up the biofilms themselves.

The aims of this study

Evaluates the phenotypic formation of biofilm and the existence of the ica operon genes in Staphylococcus spp. isolated from UTI patients (Oliveira et al., 2018).

2 Materials and Methods

Sample Collection and Bacterial Isolation

Urine samples were collected from 100 UTI-diagnosed patients at [Baladrouz Hospital] over six months. under aseptic conditions. The collected specimens were inoculated onto Mannitol Salt Agar (MSA) and Blood Agar, and suspected colonies were confirmed through standard biochemical tests and confirmed via 16S rRNA PCR.

Biofilm Formation Assay

Biofilm formation was determined using a microtiter plate assay as documented by Christensen et al. (1982): Isolates were grown in tryptic soy broth (TSB) supplemented with 1% glucose. Cultures were incubated in 96-well plates for 24 hours. Wells were washed, stained with 0.1% crystal violet. The biofilm density was determined by spectrophotometrically measuring the optical density of the alcoholic solution and categorized as low, intermediate, and high optical density, and OD was measured at 570 nm.

Biofilm formation was categorized as:

1. high: OD > 0.6
2. intermediate: 0.3–0.6
3. low/None: < 0.3

DNA Extraction and PCR

DNA was extracted using the boiling method. PCR was performed using gene-specific primers targeting: icaA, icaD, bap, fnbA. PCR amplicons were resolved on a 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV

Table 1
Primers used for PCR

Gene	Oligonucleotide sequence	Gene Bank accession number	Annealing temp. (°C)	Size	Gene ID	Information
fnbA	f:5'-GGAGAAGGAATTAAGGCG-3' r:5'-GCCGTCGCCTTGAGCGT-3'	**NC_007795.1**	66	179	(S. aureus N315), 31003153	(Strain USA300 FPR3757)
bap	f:5'-AAC GCC AGA CAA CAA CAA GC-3' r:5'-TGC TGA CAC TAA TGC TAA CG-3'	**NC_007795.1**	88	971	(S. aureus N315), 31003220	(Strain USA300 FPR3757)
icaD	f5,- ACCCAACGCTAAAATCATCG-3, r5,- GCGAAAATGCCCATAGTTTC-3,	**NC_002745.2**	57	230	(S. aureus N315), 933019	Intercellular adhesion
icaA	f5,- CTTGCTGGCGCAGTCAATAC-3, r5,- GTAGCCAACGTCGACAACTG-3,	**NC_002745.2**	64	456	(S. aureus N315), 933020	Intercellular adhesion

Statistical Analysis

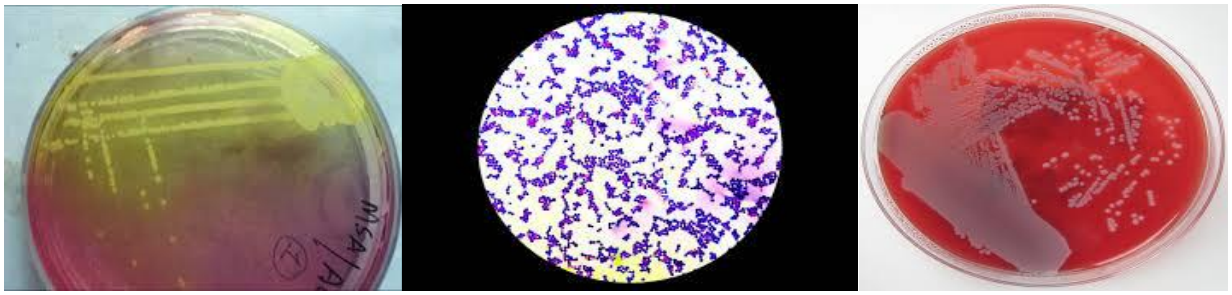
Analysis of the data was performed through SPSS v25.0. The Chi-square test was used to determine the association between biofilm categories and gene presence. A p-value < 0.05 was regarded as statistically significant.

3 Results and Discussions

3.1 Results

Sample collection
A total of 100 clinical urine specimens, 35 specimens (53.8 %) were identified as *Staphylococcus. aureus* based on biochemical tests, and 30 specimens (46.2%) were identified as coagulase-negative staphylococci**.**

1) Isolation and identification
The culture results on mannitol salt agar showed that 65 of the isolates were responsible for causing mannitol fermentation and phenol red indicator changed to the yellow color, as shown in Figure 2 (a). As for culturing on blood agar, the isolates showed hemolysis around the colonies, as shown in Figure 2-c). The results of microscopic examination appeared as Gram-positive cocci, arranged in grape-like clusters, as can be seen in Figure 2-b).



(a) (b) (c)
Figure 1. (a) Staphylococcus aureus on MSA. (b) Staphylococcus aureus under microscope. (c) Staph.aureus on blood agar

2) Biochemical tests
Coagulase test: (28) % of the isolates were positive to coagulase, as shown in fig. (2.b).
Catalase test: (22) % of the isolates were positive to catalase, as shown in fig. (2.a)

(a) **(b)**
Figure 2. Biochemical tests a) catalase test b) coagulase test

3) Biofilm formation assay
The current research showed that all isolates were able to produce biofilm, but they ranged from strong, moderate, and weak producers (Figure 3). It was observed that 20(30.8) % of the isolates were strong biofilm producers, 22(33.8) % had moderate ability to produce biofilm, and 23(35.4) % were weak\none producers.

Figure 3. Biofilm formation by Staphylococcus aureus isolates

4) Gene detection

Figure 4 displays the results of PCR amplification of the *icaA* gene from 10 *S. aureus* isolates (lanes 1-10) obtained from the urinary tract of patients with inflammatory disease. The presence of visible bands in all lanes (1-10) confirms the successful amplification of the *icaR* gene, which is a regulatory gene involved in biofilm formation. This suggests that these *S. aureus* isolates harbor the *icaA* gene and may have the potential to form biofilms. The consistent amplification of the *icaA* gene across all tested isolates indicates a high prevalence of this gene in *S. aureus* strains associated with inflammatory disease in the oral cavity. While the figure confirms the presence of the gene, it doesn't provide information on gene expression levels or the actual formation of biofilms, which warrant further investigation.

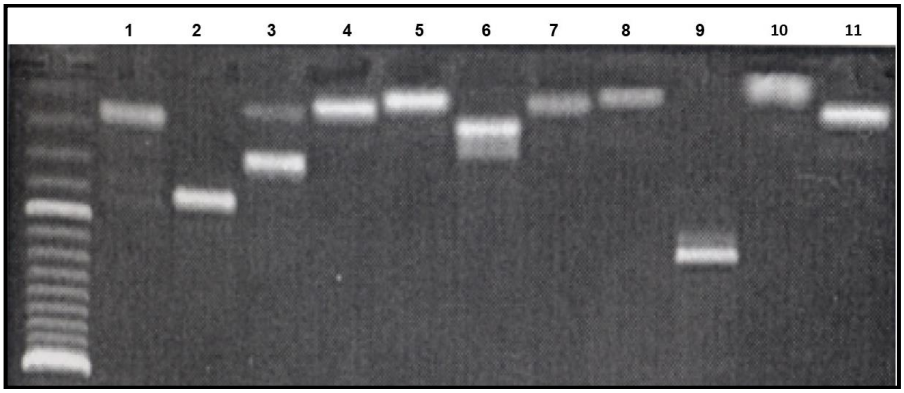


Figure 4. ica A gene S. aureus isolates' amplified DNA fragments from the urinary tract infections of inflammatory disease patients are visualized (lanes 1–11)

4) Correlation of Gene Presence with Biofilm Formation

The presence of icaA and icaD was significantly associated with moderate to strong biofilm production ($p < 0.05$), while fnbA and bap showed a positive trend but were not statistically significant.

Table 2
Association of Biofilm-Related Genes with Biofilm Formation Strength

Gene	Biofilm Formation	No. of Positive Isolates	Statistical Significance
icaA	Moderate–Strong	35	$p < 0.05$ (Significant)
icaD	Moderate–Strong	32	$p < 0.05$ (Significant)
fnbA	Moderate–Strong	15	$p > 0.05$ (Not significant)
Bap	Moderate–Strong	12	$p > 0.05$ (Not significant)

Statistical significance was determined using the Chi-square test. Strong correlation was observed for icaA and icaD, while fnbA and bap showed only a non-significant positive trend.

3.2 Discussion

The study confirms a high frequency of biofilm production among Staphylococcus spp. from UTI patients, with a significant association with the ica operon genes. The presence of bap and fnbA in a subset of isolates underscores their auxiliary role in enhancing adhesion and persistence. These findings align with previous research by Arciola et al. and Cerca et al., who reported the critical role of icaA and icaD in biofilm formation (Djawadi et al., 2023; Cucarella et al., 2001). The detection of biofilm-forming genes in non-biofilm producers also indicates the potential role of regulatory factors and environmental conditions (Dziewanowska et al., 1999). Identifying biofilm-related genes may provide valuable insights for managing chronic and recurrent UTIs, especially those involving indwelling devices where biofilms are prominent.

Table 1 lists the primers used for PCR amplification of genes related to biofilm formation and virulence in *Staphylococcus aureus*. The table provides the primer sequences, gene bank accession numbers, gene IDs, associated functions, annealing temperatures, and expected product sizes. The targeted genes include *fnbB*, *bap*, *icaD*, and *icaA*, covering functions such as adhesion, biofilm regulation, and toxin production. The selected genes and their corresponding functions are consistent with existing literature on the molecular mechanisms of biofilm formation and virulence in S. aureus (Ratajczak et al., 2021). The chosen annealing temperatures and expected product sizes are also within the typical range for PCR amplification, ensuring the specificity and efficiency of the reaction (Zhao et al., 2021).

Table 2 shown the icaA and icaD genes, which encode proteins involved in the synthesis of polysaccharide intercellular adhesin (PIA), were significantly associated with moderate to strong biofilm production **($p < 0.05$)**. This is consistent with their well-documented role in the initial attachment and accumulation phases of biofilm development in Staphylococcus aureus and S. epidermidis.

Interestingly, all isolates classified as **strong biofilm producers** carried either icaA or icaD, reinforcing their essential role in biofilm architecture. However, a subset of weak or even non-biofilm-producing isolates also carried these genes, suggesting that gene presence alone is not sufficient for biofilm expression. This could be due to gene regulation, environmental conditions, or the involvement of other regulatory pathways (Abdel-Shafi et al., 2022; O'Toole et al., 2000).

The fnbA and bap genes, although detected in a portion of the isolates, showed **no statistically significant correlation** with strong biofilm formation. Nevertheless, their presence indicates possible auxiliary roles in biofilm maturation or structural stability. The bap gene, in particular, has been linked to biofilm enhancement under stress conditions in bovine and human strains, and its role in urinary isolates warrants further investigation (Bowler et al., 2020). Overall results suggest that biofilm formation is common among various isolated strains in UTI and should be taken into consideration when devising an effective treatment strategy for UTI.

In conclusion, this analysis highlights the variability of biofilm formation in MRSA and suggests a potential link between specific genes and biofilm-forming capacity. Further investigation is needed to elucidate the precise role of these genes and their expression levels in influencing this crucial virulence factor.

Results of a PCR analysis targeting the icaA gene for 10 Staphylococcus aureus isolates from the urinary tract of patients with inflammatory disease are shown in Figure 4. All 10 tested isolates have the ica A gene, visible as bands in lanes 1-10 of the figure, which demonstrates PCR products. The transcriptional repressor gene icaR encodes a repressor of the icaADBC operon, which is responsible for PIA synthesis, a key component of the biofilm matrix in S. aureus. The finding is in agreement with previous studies that S. aureus isolates have a high prevalence of the icaR gene, particularly when it is accompanied by biofilm development and infection (Morales-Laverde et al., 2022). However, the presence of icaA in these isolates is consistent with the potential for biofilm formation, but the presence of a gene, icaA, does not imply active biofilm formation. Possibly due to its role in biofilm formation, the icaA gene may not be present because it represses PIA production in some conditions but also can go along with the switch from planktonic to biofilm growth in response to environmental cues (François et al., 2023).

4 Conclusion

In this study, we investigated the biofilm-forming capacity of Staphylococcus species isolated from urinary tract infections (UTI) and whether they contained genes related to this process. Further, Staphylococcus species were prone to forming biofilm, particularly clinically relevant S. capitis and S. saprophyticus. Interestingly, the icaR gene appeared to occur with great frequency in S. aureus isolates isolated from the oral cavities of patients suffering from diseases characterized by inflammation, suggesting that biofilms may also play a role in these infections. Furthermore, our analysis showed that the rates of resistance to antibiotics of common use, such as penicillin and oxacillin, for Coagulase-positive Staphylococci were not acceptable, nor were those of Coagulase-negative Staphylococci. The implications of these observations play a significant role in the concern about antibiotic choice in susceptibility testing. Despite the encouraging susceptibility profiles of rifampin and chloramphenicol, high prevalence of antibiotic resistance, as well as the ability of Staphylococci to form protective biofilms, pose great difficulties in the treatment of staphylococcal infections.

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