مجلة كلية التربية الاساسية
كلية التربية الاساسية – الجامعة المستنصرية
Journal of the College of Basic Education Vol.31 (NO. 132) 2025, pp. 69-78

Detection of Biofilm Formation by *Staphylococcus aureus* Isolated from some Respiratory Tract Infections

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Abstract:
Staphylococcus aureus is a significant pathogen involved in respiratory tract infections (RTIs), and its ability to form biofilms significantly contributes to its persistence and resistance to treatment. This study aimed to evaluate the biofilm-forming capacity of *S. aureus* isolates from RTIs using two phenotypic methods. A total of 55 isolates were collected from respiratory specimens, from different hospitals in Baghdad. Identification was performed using phenotypic characteristics, biochemical tests, and the VITEK 2 system. Biofilm formation was assessed using Congo red agar and the microtiter plate assay (MTP). According to Congo red, 43.63% of isolates were strong biofilm producers, 47.27% intermediate and 9.09% non-producers, whereas the MTP showed 40% strong, 38.18% moderate, and 21.81% weak producers. Notably, all isolates demonstrated some level of biofilm formation by the MTP. These findings highlight the widespread presence of biofilm-forming ability among *S. aureus* isolates from RTIs and underscore the potential role of biofilms in treatment resistance and infection persistence. Consequently, there is a need to develop novel therapeutic strategies that target biofilm eradication.

Keywords: *Staphylococcus aureus*, Biofilm, Respiratory tract infections.

1. Introduction

Respiratory tract infections (RTIs) are a significant public health concern due to their widespread prevalence and association with substantial morbidity and mortality worldwide [1]. They account for approximately 50% of healthcare visits and 40% of hospital admissions [2]. Among the Gram-positive bacteria, *Staphylococcus aureus* is recognized as the third most significant causative agent of pneumonia, following *Streptococcus pneumoniae* and *Haemophilus influenzae* [3]. *S. aureus* is an opportunistic pathogen that colonizes the human nares, skin, and gastrointestinal tract, increasing the risk of infection [4]. It can transition from a commensal organism to a major cause of respiratory infections such as pneumonia, particularly in individuals with weakened immunity or following

viral infections like influenza [5]. Microorganisms associated with lower respiratory tract infections often develop adaptive mechanisms that enhance their persistence and resistance within the host, including biofilm formation [6]. Biofilms are structured microbial communities derived from single or multiple bacterial strains [7]. In *S. aureus* infections, biofilms are characterized by multilayered bacterial aggregates enclosed in an exopolysaccharide glycocalyx [8]. Biofilm development typically proceeds through four stages: adhesion, aggregation, maturation, and dispersion [9]. Its formation and regulation are largely controlled by the accessory gene regulator (agr) system, which modulates the expression of key virulence factors, including surface adhesins that facilitate bacterial attachment and contribute to the synthesis of the extracellular polymeric substance (EPS) matrix [10]. The EPS matrix primarily consists of poly-N-acetyl-β-(1–6)-glucosamine (PNAG or PIA), proteins, and extracellular DNA (eDNA) [11]. PIA synthesis is mediated by the icaADBC operon, which encodes membrane-associated enzymes and extracellular proteins [12]. Biofilms are implicated in the majority of chronic infections and contribute to increased antimicrobial resistance and treatment failure in over 80% of clinical cases [13]. These structures allow *S. aureus* to evade host immune responses such as opsonophagocytosis and tolerate antibiotic concentrations up to 10,000 times higher than those required for planktonic cells [14, 15]. The complete eradication of biofilms remains a clinical challenge, as surviving subpopulations may persist after treatment and repopulate the infection site, leading to recurrence and the spread of resistance [16]. Therefore, this study aims to evaluate the biofilm-forming capacity of *S. aureus* isolates from respiratory tract infections, as biofilm formation is often associated with persistent infections, treatment failure, and antimicrobial resistance.

2. Methodology

2.1. Bacterial isolates Identification

Fifty-five *Staphylococcus aureus* isolates from respiratory tract infections (sputum and nasal swap) of different ages and genders were collected from patients at hospitals in Baghdad city . All isolates from primary cultures were examined by culturing on Mannitol Salt Agar (MSA) and blood agar media incubated for 24 hours . The growth culture was examined for colony morphology, and the isolates were subsequently tested using Gram stain, and some biochemical tests such as, the Oxidase test, Catalase test, Coagulase test, DNase production test and further conformational characterization, are performed using the Vitek 2 system.

2.2. Biofilm Formation Assay

In this study, the ability of *S. aureus* to form biofilm was estimated in two methods:

2.2.1. Congo Red (CR) method

In this method, Congo red agar was prepared by mixing (37 g/L) of brain heart infusion broth, (50 g/L) of sucrose and (10 g/L) of agar dissolved in 900 ml of distilled water and then sterilized by autoclave. In 100 ml of distilled water, 0.8g of Congo red stain was dissolved and sterilized using an autoclave. When the medium and dye cooled to 50ºC the dye was added to the medium and poured into sterile petri dishes . In this medium, colonies from each isolate were incubated at 37 ºC for 24 hours [17].

2.2.2. Micro-titer method

Staphylococcus aureus isolates were quantitatively assessed for their biofilm-forming ability using the microtiter plate method as described in [18] with some modification. In 96-well flat-bottom polystyrene microtiter plate (180 µL) of brain heart infusion (BHI) broth with (2%) sucrose was added and then inoculated with (20 µL) of bacterial suspension which was prepared by taking a few colonies from overnight bacterial culture and suspending in 5 ml of normal saline and adjusting the turbidity to match 0.5 McFarland standard and as control wells, 200 µl uninoculated broth was added. The micro-titer plate was covered and incubated at 37°C for 48 h. After that, unattached bacteria were washed with phosphate-buffered saline (PBS) (pH=7.2). The plates were left to dry for 15 minutes, then (200) µL of 0.1% crystal violet was added and left for 15 minutes. Then PBS (pH=7.2) was used to wash the wells three times and left to dry at room temperature; 200 µl of ethanol was added to each well. The optical density (absorbance) was measured at 630 nm by using a microtiter plate reader. The cut-off value (ODc) was evaluated according to the (Table 1).

Table 1: Group adherence capabilities for biofilm formation

OD value	Biofilm formation
OD≤ODc	Non-adherent
2 ODc > OD > ODc	Weakly adherent
4 ODc > OD > 2 ODc	Moderately adherent
OD > 4 ODc	Strongly adherent

OD= optical density, ODc= cut-off value.

2.3. Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) version 22.0 was utilized to analyze the findings.

3. Results and Discussion

3.1. Bacterial isolates Identification

The result showed that all bacterial isolates produced round, smooth, and Yellow colonies with yellow zones around them after 24 hours of incubation on MSA at 37°C indicating a positive result that confirmed fermentation of mannitol; this medium contained high salt concentration that other bacteria will be inhibited. On blood agar medium (containing 5% blood), semi-quantitative screening showed that all isolates were hemolysin producers, but in different effectiveness (Figure 1). The gram-staining result showed that all the bacterial isolates were gram-positive. Biochemical tests were used for further determination, and all isolates of *S. aureus* showed positive results for catalase through the formation of bubbles due to its ability to break down hydrogen peroxide. All of the isolates showed positive results for the coagulase test due to the ability of bacteria to produce coagulase enzymes, which convert fibrinogen to fibrin . None of the isolates showed any purple color, indicating a negative result for oxidase . DNase test for the isolates showed positive results, which appeared as a colourless zone around bacterial colonies after inoculation on the DNase plate due to its ability to produce the enzyme DNase, which can hydrolyze nucleic acid in this medium.

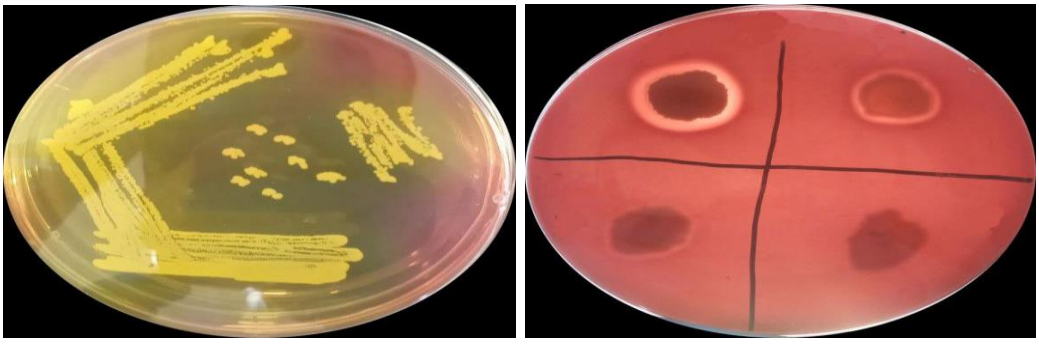

Figure 1: Growing colonies of *S. aureus* on: A- Mannitol salt agar B- Blood agar

3.2. Biofilm Formation Assay

3.2.1. Congo Red (CR) test

All isolates were streaked on a congo red medium and checked to detect biofilm formation. 24 (43.63%) of isolates appeared as black colonies pigmentation with a dry crystalline, indicating strong biofilm producer; 26(47.27%) showed blackening of the colonies without dry crystalline, indicating intermediate

biofilm, while 5 (9.09%) of isolates did not produce biofilm which showed as a pink colony (Figure 2).

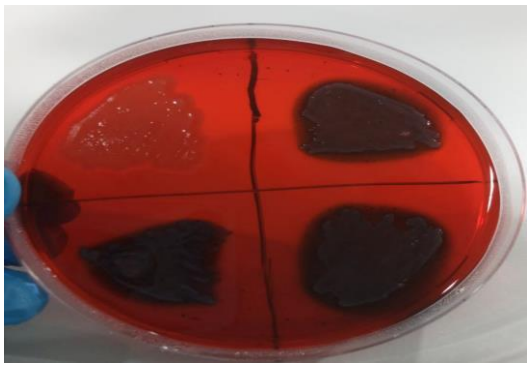

Figure 2: Biofilm formation on Congo red agar by *S.aureus* isolates.

3.2.2. Micro-titer method

It is the most frequent method used for the detection of biofilm formation. The obtained results were categorized into three groups (weak, moderate, and strong producers) (figure 3). The current study demonstrated that out of the 55 isolates, 12 (21.81%) were weak biofilm producers**,** 21(38.18%) produced moderate biofilm, and 22 (40%) produced strong biofilm **(**Table 2). Several previous studies have reported varying rates of biofilm formation by *S. aureus* isolates . In a local study conducted by AlKhfaji et al. [19] among 31 isolates of *S. aureus* from different clinical sources, 9(29.03%) were strong, 12(38.7%) were moderate biofilm producers and 10 (32.25%) were weak and non-producers. Another local study reported by Ali & Abdallah [20] showed that among 100 isolates of *S. aureus* from different clinical sources, 20 were non-producer, 32 formed moderate biofilm, and 48 formed strong biofilm . The result of the current study demonstrates that all *S. aureus* isolates were able to produce biofilm. Another study showed that all *S. aureus* isolates were biofilm producers conducted by Alkhafajy & Al-Mathkhury [21] out of 58 isolates from different clinical sources, eight (13.79%), 28 (48.28%), and 22 (37.93%) isolates, produced weak, moderate and strong biofilms, respectively. Bacterial aggregation and biofilms formation are key mechanisms of adaptation to the lung environment, and *S. aureus* rapidly forms aggregates within a very short period (one hour after nasal inoculation), facilitating its colonization of pulmonary epithelial surfaces [22]. Biofilm formation is the major virulence factor of S aureus, as biofilms can limit bacterial clearance by antimicrobial agents and host immune responses [23]. Additionally, biofilms play a crucial role in the development of severe chronic illnesses [21]. Omidi, Firoozeh, Saffari, et al. [24] reported that the variation in biofilm production among the *S. aureus* strains

could potentially be attributed to differences in gene expression. Biofilm formation also strongly depends on environmental conditions [25]. The variation in sucrose concentration in the media used in the current study could explain the variation in the result of the two tests, the Congo Red (CR) test and the Micro-titer method [26].

Table 2: Strength of biofilm produced by *S. aureus* isolates.

Biofilm formation	**No. of isolates**	**The percentage (%)**
Weak	12	21.81
Moderate	21	38.18
Strong	22	40

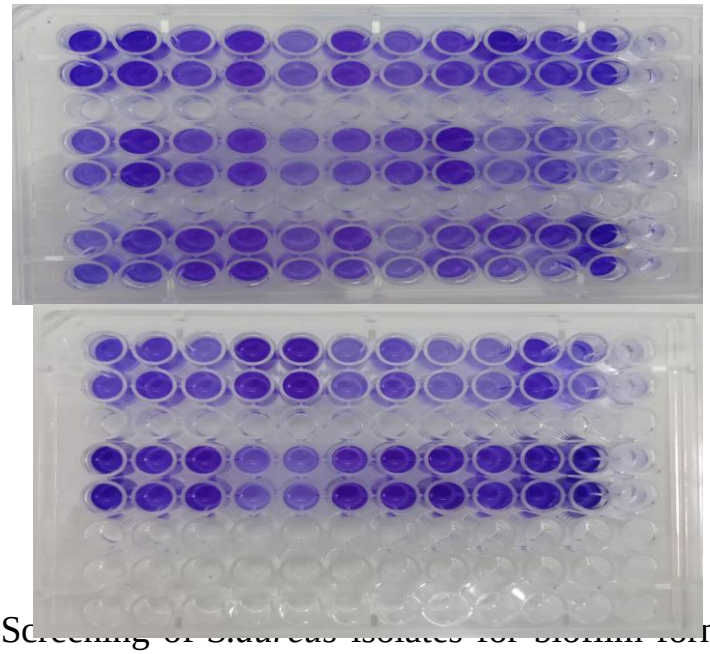

Figure 3: Screening of *S.aureus* isolates for biofilm formation using MTP method.

Conclusion

This study highlights the high prevalence of biofilm-forming ability among *Staphylococcus aureus* isolates from respiratory tract infections. Using two phenotypic methods, Congo red agar and microtiter plate assay—it was found that 43.63% and 40% of isolates, respectively, were strong biofilm producers, while moderate biofilm formation was observed in 47.27% (Congo red) and 38.18% (microtiter plate). Notably, all isolates demonstrated some level of

biofilm formation by the microtiter plate method, indicating that biofilm production is a widespread trait in these clinical isolates. These results underscore the potential role of biofilm formation in the persistence and treatment resistance of *S. aureus* infections in the respiratory tract. Therefore, there is a need to develop therapeutic strategies that specifically target biofilm eradication.

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Journal of the College of Basic Education Vol.31 (NO. 132) 2025, pp. 69-78

الكشف عن تكوين الأغشية الحيوية لبكتريا المكورات العنقودية الذهبية المعزولة من بعض التهابات الجهاز التنفسي

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مستخلص البحث:
تُعد المكورات العنقودية الذهبية من العوامل الممرضة المهمة المتورطة في التهابات الجهاز التنفسي، وتساهم قدرتها على تكوين الأغشية الحيوية بشكل كبير في استمرارية العدوى ومقاومتها للعلاج. هدفت هذه الدراسة إلى تقييم القدرة على تكوين الأغشية الحيوية لعزلات المكورات العنقودية الذهبية المعزولة من بعض التهابات الجهاز التنفسي، باستخدام طريقتين مظهريتين. تم جمع 55 عزلة من عينات تنفسية من عدد من المستشفيات في بغداد. جرى التعرف على العزلات اعتمادًا على الخصائص المظهرية والاختبارات الكيميائية الحيوية ونظام VITEK 2.تم تقييم تكوين الأغشية الحيوية باستخدام وسط أجار الكونغو الأحمر وطريقة الأطباق الدقيقة .أظهرت نتائج وسط الكونغو الأحمر أن 43.63% من العزلات كانت منتجة قوية للأغشية الحيوية، و47.27% متوسطة، و9.09% غير منتجة. أما باستخدام طريقة الأطباق الدقيقة، فقد تبيّن أن 40% من العزلات كانت قوية الإنتاج، و38.18 % متوسطة، و21.81% ضعيفة. ومن الجدير بالذكر أن جميع العزلات أظهرت درجة من تكوين الأغشية الحيوية باستخدام طريقة الأطباق الدقيقة. تشير هذه النتائج إلى الانتشار الواسع لقدرة العزلات السريرية من المكورات العنقوديه الذهبيه على تكوين الأغشية الحيوية في التهابات الجهاز التنفسي، وتُبرز الدور المحتمل لهذه الأغشية في مقاومة المضادات الحيوية واستمرار العدوى. وعليه، تبرز الحاجة إلى تطوير استراتيجيات علاجية مبتكرة تستهدف إزالة الأغشية الحيوية بفعالية لتحسين نتائج العلاج.

الكلمات المفتاحيه: المكورات العنقوديه الذهبيه ، الأغشيه الحيويه ، التهابات الجهاز التنفسي
ملاحظة: البحث مستل من رسالة الماجستير.