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# Biofilm Detection of Staphylococcus Aureus Isolated from Oral Infections

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## Abstract

This work involves identifying biofilm development by *Staphylococcus aureus* isolated from oral infections. Biofilm formation transpires due to bacterial activity influenced by multiple elements that create an oral environment conducive to its development, as biofilm formation is a critical pathogenicity factor for bacteria. This investigation involved the collection of forty clinical samples from the oral cavities of individuals with oral issues. *Staphylococcus aureus* was isolated and identified using several methods, with confirmation via polymerase chain reaction (PCR), yielding 28 positive samples and 12 negative samples. Bacteria can establish biofilms on biological and abiotic surfaces in both natural and clinical settings. Bacterial aggregation within biofilms is created by the extracellular matrix they secrete. *Staphylococcus aureus* is a common pathogen linked to biofilm infection. The assessment of biofilm formation on polymeric surfaces by *Staphylococcus aureus* isolates was conducted utilizing the crystal violet microtiter plate technique. Crystal violet is a negative stain that exhibits affinity for positively charged molecules on the cell surface, nucleic acids, and polysaccharides. The extent of biofilm formation was quantified using optical density (OD) values, which were compared to the control OD (ODc); the classifications resulted in 21 highly adherent samples, 8 lightly adherent, 49 barely adherent and 12 nonadherent.

***Keywords:*** *Biofilm formation, Staph. aureus, and Oral infections.*

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## 1. Introduction

The biofilms are sessile colonies of microorganisms distinguished by cells that attach to a substrate, interface, or for one another, are encased in an arrangement of extracellular polymeric substances, and display altered phenotypes concerning growth, protein production and gene expression. Biofilm size may vary from a monolayer of cells to a significant aggregation enveloped in a viscous polymeric body. Structural examinations indicate that distinct pillar or mushroom-like formations may arise from the micro-colony architecture of thick biofilms; although, alternative forms can develop based on environmental variables. Complex channel networks traverse these structures, facilitating access to vital nutrients despite those deepest areas of the biofilms. While biofilms production usually is not essential for persistent infections, its removal is challenging, often necessitating surgical interventions, thus meriting additional research [1-3]. Staphylococcus aureus

belongs to the Gram-positive commensal bacteria found primarily in the natural bacterial ecology of humans' skin and mucosal surfaces (World Health Organization, 2014). These bacteria can form a mature 3D biofilm, Cells from bacteria follow a surface, or different cells are embedded within a barrier of polymeric extracellular structure [4,26].

*Staphylococcus aureus* frequently causes infection acquired in community as well as hospital settings. It is also one of the major human pathogens, characterized by its resistance to methicillin (MRSA). The bacterial disease may lead to the development of the disease and thus lead to many diseases, including oral diseases and gingivitis, skin infections, infections, gastrointestinal infections, pneumonia, as well as infections of soft tissues and bones, damage and inflammation of heart valves, and even cases of poisoning that may occur that may be fatal to humans and thus end their lives. Streptococcus bacteria are rapidly developing antibiotic resistance, a major problem; it has become clear in recent years [5, 6,27].

There are a lot of sick people and deaths around the world because of *Staphylococcus aureus*. There are many kinds of diseases, like mild skin infection, sepsis and asthma, that can kill. It makes up a huge range of virulence factors. There are many toxins and immune-evasion factors in these, and many protein and non-protein parts that help them spread to different body parts during an illness [7]. People die from bacteremia caused by S. aureus more often than from HIV/AIDS, TB, and viral hepatitis put together. It was first known that S. aureus was resistant to penicillin in the early 1940s. Since then, *S. aureus* has slowly built-up resistance mechanisms, making it immune to almost all modern antibiotics [8].

Biofilms function as barrier, creating an adequate inside atmosphere for bacteria cellular functions and protecting bacteria cell from unfavorable circumstances, as well as extreme temperatures, nutritional deprivation, dehydration, or antibacterial agents. As a result, bacteria can rapidly colonize and shield themselves from host defensive mechanisms, therefore facilitating persistent infection by increasing adherence to the surface of the host. Biofilms serves as the primary defensive barrier for microorganisms. Biofilms-forming bacteria are recognized for their resistance to most antibiotics [9].

## 2. Materials and Methods

### 2.1. Materials

Korean Labtech makes an autoclave and a digital camera Sony (Japan), GFL (Germany) for the distillator, Bio Lab (Korea) for the hood, Memmert (Germany) for the oven and incubator, Olympus (Japan) for the light microscope, and others. The ELISA system was made by Hermle Labortechnik in Germany. Mikropipette: Eppiendrof (Germany); Refrigerator: Beko (Korea); Thermocycler: Bio-Rad (USA); UV-Visible Spectrophotometer: Shimadzu (Japan); Vortex Mixer: US Thermolyne, US Agarose, US Crystal Violet Powder, and US Conda English BDH, DNA Ladder 100 bpDNA loading dye from Promega (USA) and ethanol (96%) from Promega (USA).Ethidium Bromide Solution (BDH) from England Canada's Bio Basic and Gram Stain Phosphate Buffer Saline (PBS) from Bioworld (USA), Hydrochloric Acid (0.25 M) Sodium Hydroxide (NaOH) from EMC (Germany), Sucrose from Himedia (India), and Tris-EDTA (TE) Buffer from Bioworld (USA) were used. You can get Bio Basic from Canada, MSA (mannitol salty agar), and Blood Agar Medium.

### 2.2. Method

#### 2.2.1. Identification and Isolation of Staphylococcus aureus

Using clean swabs, 40 clinical samples were taken from people with oral infections. From October 20, 2023, to January 20, 2024, samples were taken from the Al-Hindiyya Teaching Hospital in Karbala and sent straight to the lab. Using mannitol salty agar and blood agar medium, bacterial



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cultures were grown on Petri plates then kept at 37 °C/twenty-four hours to be sure that the bacteria were *S. aureus*, the Gram stain method and biochemical tests (catalase, oxidase, and coagulation tests) were done on cultured cells [10]. Thirty-two samples were positive, and eight were negative due to tests.

### 2.2.2. Genetically detection of Staph. aureus (Genomic DNA Extraction and PCR Experiments)

The Presto $^{Tm}$ Mini gDNA Bacteria Kit was utilized to extract genomic DNA from S. aureus, following the manufacturer's instructions, employing particular primers for the S. aureus gene: forward 5"'-GCATTGATGGTACGGTT -3'" and reverse "5'-AGCCAAGCCTTGACGAACTAAAGC -3' [11]. 50 µl of Master Cycler gradient PCR (Techne, England) and GoTaq® G2 Green Master Mix (Promega, USA)" were utilized for gene amplification. The following settings were used to set up the PCR machine, as shown in table No. **1**:

**Table no.1:** Polymerase Chain Reaction (PCR).

| Steps | "Temperature°C | Time | No. of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 2 minutes | 1 |
| denaturation | 95 | 30 second | 35 |
| Annealing | 54 | | |
| Extension | 72 | | |
| Final extension | 72" | 5 minutes | 1 |

### 2.2.3. Biofilm Formation Detection of *Staph. aureus*

The assessment of biofilm development by Staphylococcus aureus isolates was performed utilizing the microtiter plate crystal violet assay [12, 13].

*Staphylococcus aureus* isolates stored in glycerol were brought back to life on Heart-brain agar medium and kept warm (37°C) in a candles jar about forty-eight hours. A clean plastics loop was inserted into a container containing five milliliters of isotonic saline and observed against the McFarland turbidity norm, which ranges from 0.5 / 0.62.

Subsequently, 100 µl of standardized isotonic saline was added to 10 ml broth of brain heart. Subsequently, 200 milliliters of each reduced solution went to a sterilized flat-bottom 96-well a plate, with each well including 100 milliliters of new medium (brain heart infusion broth + 5% sucrose) in triplicate. The plates underwent incubation at 37ºC in a candle jar for a duration of 24 hours. The negative control wells included all components except microorganisms. The broth was discarded after incubation, and the wells were meticulously rinsed three times with saline solution. 200 µl Phosphate Buffer Solution (PBS) was added to each well except the control (this step is repeated twice and removed immediately). Subsequently, 200 µl sodium acetate was added to each well with control for half an hour (fixation of bacteria).

After removing sodium acetate from each well without washing, the plate was allowed for drying, after which biofilm quantification was conducted using 200 µl of 0.1% crystal violet for 15 minutes. The surplus stain was removed using saline solution, inverted onto tissues, and allowed to dry; subsequently, it was resolubilized using 100 µl of 98% ethanol for fifteen minutes.

The optical density was assessed at 570 nm using a "Stat Fax-2600 microplate reader (Awareness Technology, USA)". The results documented in figure 1 were obtained using the microtiter plate method as [14]. The threshold optical density (ODc) for the negative control was determined as following:



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1. OD < ODc signifies non-compliance.
2. ODc < OD < 2 ODc signifies insufficient adherence.
3. 2ODc < OD < 4 ODc signifies moderate adherence.
4. OD > 4 ODc signifies robust adherence.

## 3. Results

The biofilm detection results indicate a wide range of absorbance values across different wells, suggesting varying levels of biofilm formation. Among the wells, the highest absorbance values were observed in wells A8 (0.528), B5 (0.584), C8 (0.672), and H6 (0.634), indicating strong biofilm production in these samples. Moderate biofilm formation was detected in wells such as E2 (0.551), E12 (0.650), and F2 (0.608). Conversely, wells such as A1 (0.095), F9 (0.117), and F11 (0.123) exhibited the lowest absorbance values, indicating minimal biofilm formation. These findings demonstrate a significant variation in biofilm-forming ability among the tested samples, with certain wells showing a pronounced ability to form biofilms, potentially highlighting differences in microbial activity or environmental conditions.

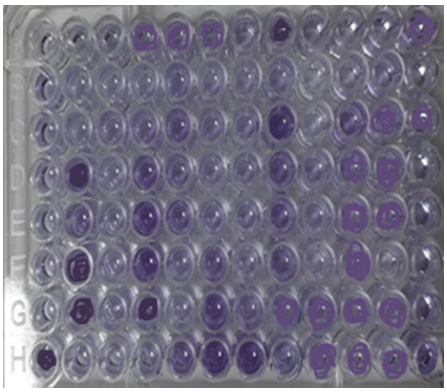

**Figure 1:** Biofilm recorded on a micro-titer plate.

Table 2: Recorded Sheet of biofilm detection.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | **0.095** | **0.109** | **0.119** | **0.141** | **0.172** | **0.138** | **0.133** | **0.528** | **0.138** | **0.133** | **0.147** | **0.162** |
| **B** | **0.181** | **0.133** | **0.322** | **0.125** | **0.584** | **0.125** | **0.123** | **0.157** | **0.103** | **0.143** | **0.167** | **0.107** |
| **C** | **0.574** | **0.163** | **0.184** | **0.503** | **0.255** | **0.173** | **0.164** | **0.672** | **0.131** | **0.257** | **0.171** | **0.325** |
| **D** | **0.138** | **0.215** | **0.125** | **0.485** | **0.177** | **0.147** | **0.156** | **0.274** | **0.164** | **0.488** | **0.563** | **0.452** |
| **E** | **0.144** | **0.551** | **0.149** | **0.499** | **0.367** | **0.175** | **0.184** | **0.270** | **0.168** | **0.190** | **0.168** | **0.650** |
| **F** | **0.137** | **0.608** | **0.136** | **0.491** | **0.161** | **0.276** | **0.128** | **0.235** | **0.117** | **0.146** | **0.123** | **0.133** |
| **G** | **0.133** | **0.548** | **0.174** | **0.187** | **0.156** | **0.504** | **0.192** | **0.204** | **0.202** | **0.174** | **0.165** | **0.475** |
| **H** | **0.558** | **0.188** | **0.178** | **0.285** | **0.306** | **0.634** | **0.591** | **0.233** | **0.189** | **0.176** | **0.202** | **0.535** |



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**Table 3:** Biofilm formation grade as compared with optical density control **ODc**.

| ODc | 5 |
|---|---|
| non-adherence | 12 |
| weak adherence | 50 |
| moderate adherence | 8 |
| strong adherence | 21 |

## 4. Discussion

This study involved the collection of 40 clinical samples from the oral cavities of patients with microbial-induced oral infections, from which *Staphylococcus aureus* was isolated using various techniques, including bacterial culture on Petri dishes and visual assessment via Gram staining methods [15]. The golden colonies were identified on Mannitol Salt Agar (MSA), which transformed color from light red to yellow in the medium, as shown in **figure no.2-A.** On blood agar, it generates yellow, circular, medium-sized colonies encircled by a clear zone, signifying the bacteria's ability to secrete beta-hemolysin, resulting in the whole lysis of red blood cells in the agar, as shown in **figure no.2-B**.

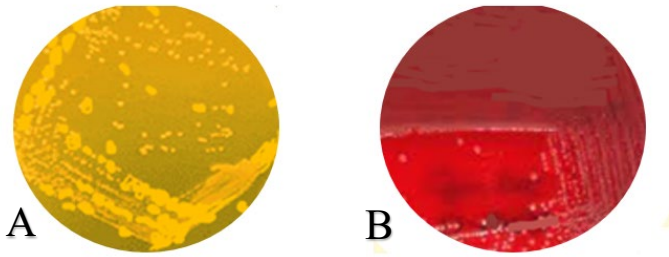


**Figure 2:** *Staphylococcus aureus* growth on Mannitol salt agar and Blood agar (colonies formation at 37C after 24 hours) [16].

The bacteria were diagnosed, and the culture results were validated via genetic testing using PCR, confirming that the isolated bacteria were *Staphylococcus aureus*. The isolates were previously recognized using culture characteristics (mannitol salty agar/ blood agar) and biochemical tests. Additionally, *Staphylococcus* aureus was confirmed through molecular identification by amplifying a conserved portion of the nuc gene, which encodes the thermostable nuclease enzyme, with particular primers (nuc primers) to verify species-level identity. Only *Staphylococcus aureus* can be identified by the nuc primer, among other staphylococci. The polymerase chain reaction result of the nuc gene manifests as a singular DNA band, approximately 279 bp in molecular size, as compared to the DNA ladder [17], as shown in **Figure no.3**.



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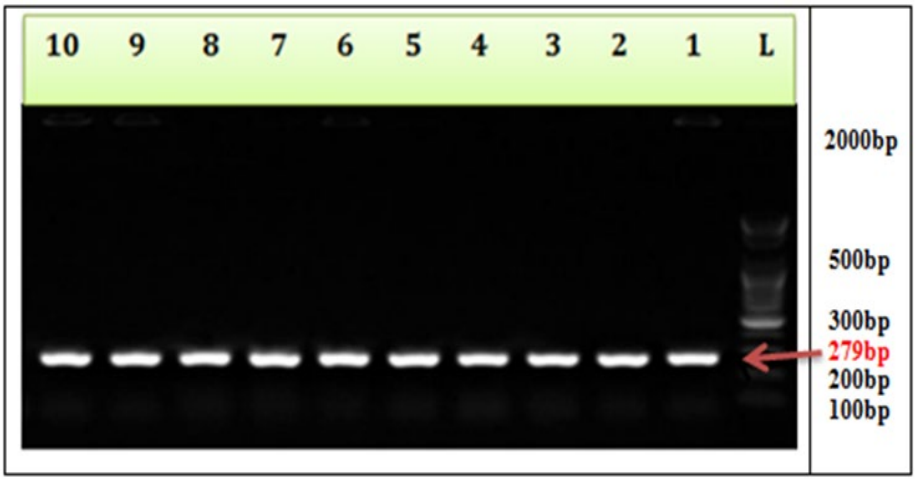


**Figure 3:** Agarose gel electrophoresis of a specific PCR product measuring 279bp was conducted using 1.5% agarose gel at 90 V for 1 hour in 1x TBE buffer, followed by visualization under a transilluminator with UV light after staining with Red Safe. Lane L: bp DNA ladder. All lines exhibit a good result (279bp fragment) of *Staphylococcus aureus*.

Staphylococcus aureus isolates assessed biofilm formation on polymeric surfaces using the microtiter plate crystal violet assay [18]. Crystal violet is a negatively charged dye that binds to positively charged molecules on the cell surface, nucleic acids, and polysaccharides, hence offering a thorough evaluation of biofilm formation in gram-positive organisms [19]. The biofilm development grades, represented as OD values in **Figure 1** and **Table no.2**, were compared with ODc, resulting in the following estimates: "12 non-adherent, 49 weakly adherent, 8 moderately adherent, and 21 strongly adherent", as shown in **Figure no.4**, and **table no.3.**

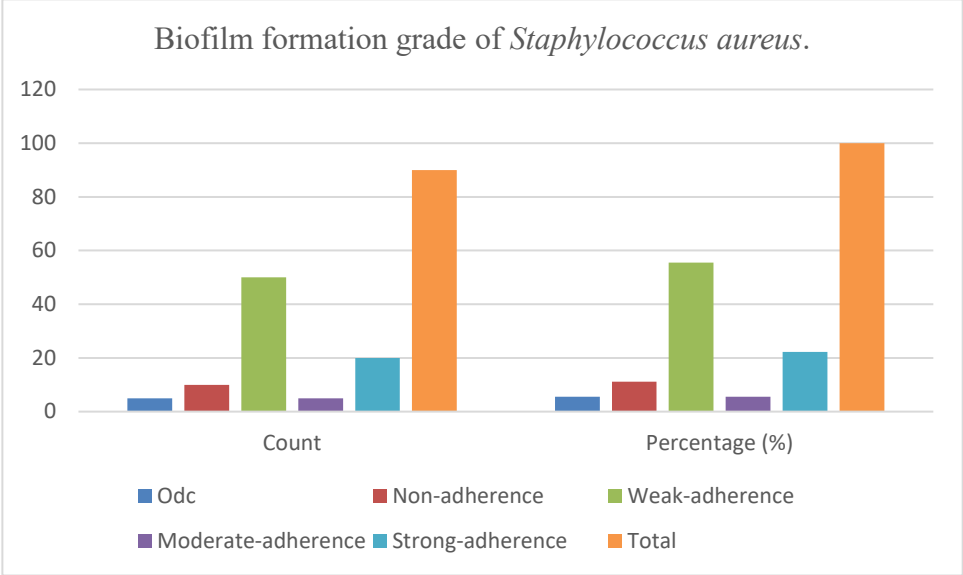


**Figure 4:** Biofilm formation grade of *Staphylococcus aureus*.

The finding of this study reveal a significant disparity in biofilm formation capacities among the Staphylococcus aureus isolates, classified as strong, moderate, weak, and non-producers of biofilm. The disparities in bacterial adherence to dental surfaces and the oral cavity are affected by *Staphylococcus aureus's* capability to generate biofilm which can be linked to differences in patient medication protocols and growth circumstances, such as ionic forces, pH levels, and sub-culturing frequency [20]. Bacteria can rapidly colonize and shield themselves from host defensive mechanisms, facilitating persistent infection through increased adherence to the host surface. Biofilm

serves as the primary protective barrier for microorganisms. Bacteria that produce biofilms are recognized for antibiotic resistance [21].

Biofilms are microbial formations that depend on a solid substrate and extracellular product, including "extracellular polymeric substances" (EPSs) [22]. Bacteria adhere reversibly to the surface, and their fast proliferation leads to the formation of a mature biofilm. Bacteria aggregate at this juncture, creating a barrier that can withstand drugs and serve as a source of systemic, persistent infections. Consequently, biofilms pose a significant health risk [23].

Also, biofilm bacteria can generate super-antigens to circumvent the immune system. Consequently, bacterial infections pose a significant threat despite the plethora of antimicrobial agents. Chronic infections associated with planktonic bacteria and biofilms are consistently challenging to treat due to their intrinsic resistance to pharmaceuticals and the human immune response. Upon attachment to surfaces, bacterial cells commence division and proliferation in the presence of sufficient nutrients. During the aggregation stage, bacteria influence biofilm development by sensing ambient cues that trigger networks of regulation and intracellular communications molecules. The bacteria gradually proliferate and aggregate to form a biofilm. The produced biofilm can provide resistance against the immune system of humans and pharmaceuticals [23]. Bacterial cell multiplying within matrix could lose immediate interaction with the grafting surface and host proteins, primarily relying on cell-cell and cell–EPS adhesion. The biofilm has a highly organized architecture throughout the development phase, culminating in a robust three-dimensional configuration resembling a mushroom or tower [24]. A network of pipes surrounding the microcolony is designed to facilitate the delivery of nutrients to the deeper layers of the biofilm. Mature biofilms display varied and distinctive metabolic systems that withstand detrimental environmental conditions and pressures [25].

## Conclusion

The existence of *Staphylococcus aureus* and several microorganisms as biofilms poses a significant difficulty for the medical industry. Biofilm serve as a survival mechanism for bacteria, rendering them particularly challenging to treat due to their intrinsic immune response and antibiotic resistance. Consequently, it is imperative to do additional research on the production and regulatory mechanisms of *Staphylococcus aureus* biofilms to facilitate the development of anti-biofilm agents that prevent biofilms formation. Forming biofilms is a complex process that entails the co-expression of several genes. *Staphylococcus aureus* depends on an extensive array of regulatory systems that orchestrate biofilms development in complementary or antagonistic ways. Notwithstanding advancements in the regulatory mechanisms governing *Staphylococcus aureus* biofilms formation, researchers now encounter difficulties comprehending the linkages across diverse regulatory networks.

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