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Evaluating and Comparing Microbial Biofilm Formation on the Surface of Natural Tooth and Artificial Dental Crown by a Clinical Isolate

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ABSTRACT

The use of traditional antibiotics to treat microbial illnesses associated with biofilm development through quorum sensing has been a significant challenge. Dental plaque is the term for the biofilms that form on the surface of teeth. The sticky, white coating on teeth and around gums results from bacteria colonizing within dental plaque. Biofilm can lead to gum inflammation, resulting in gingivitis and other gum issues if left untreated. In absence of proper care, plaque can harden into tartar, or dental calculus, which is much more difficult to treat with antibiotics. The current study aims to compare and measure biofilm formation on the surfaces of natural teeth and dental crowns, given the ongoing issues with dental plaque. The resulting average percentage of in vitro biofilm formation reached almost 82% on natural tooth surface whereas 60% on artificial crown surface, clearly indicating the decline in the process of progressive deterioration of the artificial tooth.

Biofilm acts as a protective barrier for the bacteria within it, making these microorganisms much more resistant to antibiotics than their free-floating counterparts. This resistance complicates treatment and often makes standard antimicrobial therapies less effective, highlighting the need for preventive care. Additionally, the complex structure of dental biofilms helps a variety of bacterial communities survive and grow, each contributing to oral health problems. Dental crowns, which are commonly used in restorative dentistry, can also be locations for biofilm growth, raising questions about how adherence and microbial colonization differ between natural and artificial dental surfaces. By measuring the extent and characteristics of biofilm formation in these settings, this research aims to identify potential weaknesses that can be addressed for better clinical care. Understanding these differences will not only help in creating more effective preventive methods but also aid in designing materials or treatments that limit plaque buildup. Ultimately, the study seeks to fill current gaps in our knowledge, supporting long-term dental health and improved patient results.

KEY WORDS: Dental plaque, *S. aureus, comparative biofilm* formation

INTRODUCTION

Oral health is an essential component of overall well-being and personal hygiene. Its significance extends beyond the mouth, as maintaining healthy oral tissues can reduce the risk and severity of a broad spectrum of

oral diseases (Dye 2012; Milgrom and Reisine 2000; Petersen 2009). Among the most common oral health concerns are dental caries and inflammatory conditions of the gums both of which, if ignored, may result not only in discomfort but also tooth loss and impaired quality of life (Kornman 2008; Marsh 2012; Milgrom and Reisine 2000). Numerous epidemiological studies have documented the widespread prevalence of these diseases (Bayani M et al, 2023).

Dental plaque, often regarded as the most basic form of oral biofilm, is composed of various microorganisms found on oral surfaces (Socransky and Haffajee 2005). When this plaque accumulates over time without adequate oral hygiene, it becomes a key instigator in the development and progression of oral diseases (Berezow and Darveau 2011; Marsh 2012). The untreated build-up of plaque around the gums triggers an inflammatory response that can become chronic if not addressed early (Kornman 2008; Rüdiger et al. 2002). As oral disease progresses, both protein profiles and the makeup of microbial populations shift, reflecting the dynamic nature of plaque and its interactions with the host (Rüdiger et al. 2002; Socransky and Haffajee 2005).

Interestingly, not all plaque is inherently harmful in a balanced state, it may contribute to the host's defense by preventing the colonization of harmful, external microorganisms. The oral environment is particularly complex, serving as a reservoir for a wide diversity of bacterial species, with over 300 identified to date many of which remain non-cultivable and have only been discovered through molecular techniques. Studies have long focused on bacteria such as *Staphylococcus aureus*, but its role within the mouth is still not well understood, with only a handful of investigations examining the oral cavity as a potential reservoir for this organism.

The presence of *Staphylococci* in the oral cavity remains partially unresolved. While *S. aureus* has been linked to various infections in the mouth and surrounding regions including jaw osteomyelitis, parotitis, and mucositis it's uncertain whether it truly contributes to the onset and progression of oral diseases. Some evidence suggests that deep periodontal pockets may harbor these bacteria, potentially serving as sources for systemic infections, particularly in vulnerable groups like hospitalized or immunocompromised patients (Raghavendran et al., 2007; Paju & Scannapieco, 2007).

Within these biofilm communities, bacteria communicate through quorum sensing, a sophisticated signaling process that enables them to coordinate gene expression and regulate the virulence of opportunistic pathogens. *S. aureus*, which is known as a common cause of both hospital and community acquired infections, has also been found in chronic and aggressive forms of periodontitis and peri-implantitis (Leonhardt et al., 2003; Murdoch et al., 2004; Souto et al., 2006; Fu¨rst et al., 2007; Fritschi et al., 2008; Persson et al., 2008). Although often isolated from dental plaque and destroyed tooth roots, *S. aureus* is not typically regarded as a native inhabitant of the oral cavity; rather, its presence there is opportunistic and situation-dependent (Dahle´n & Wikstro¨m, 1995; Colombo et al., 2002; Didilescu et al., 2005; Kouidhi et al., 2010).

Biofilms themselves are highly organized, with microorganisms encased in a self-produced matrix that anchors them to surfaces and shields them from external threats, including antimicrobial agents. Understanding the capacity of these bacteria to produce biofilms is vital both for exploring their potential uses

in applications like bioremediation and for informing strategies aimed at interrupting the pathogenic processes they promote. In this context, the present study aims to quantitatively and comparatively assess the formation and surface attachment strength of biofilms on both natural and artificial dental surfaces. This research not only deepens our knowledge of biofilm development in various oral environments but also sets the stage for designing better preventive and therapeutic measures to enhance oral health

MATERIAL AND METHODS

A Clinical isolate was isolated and identified as S. aureus in a previous study undertaken in the Microbiology department in association with the local Dental clinic, from Mumbai, Maharashtra. The samples in duplicates were obtained and processed, and preserved in an aseptic laboratory condition. All samples were kept at 4^0C until proceeding further. Isolates were grown and maintained in Tryptic Soy Broth (TSB)) medium to give approximately 10 5 to 10 6 CFU/ml. All the solvents and media were obtained from Merck Pvt. Ltd, Mumbai, and Hi Media Pvt. Ltd, Mumbai. The Chemicals, such as 1% Crystal Violet, were obtained from Biolab Diagnostics (I) Pvt. Ltd., 95% Ethanol was prepared from Absolute alcohol made by S D Fine-Chem Ltd., and phosphate buffer saline (pH 7.3) was prepared in the laboratory itself.

Biofilm Formation Assay

To evaluate biofilm formation by clinical isolates of *Staphylococcus aureus*, we employed the established tube assay method as described by Mathur et al. (2006). In order to examine the dynamics of biofilm development, two sets of Tryptic Soy Broth (TSB) tubes were prepared. Each set of tubes received 100 µL of the prepared culture inoculum. The assay was designed to permit both quantitative and comparative analysis of *S. aureus* biofilm development under varying temperature conditions and incubation times. Tryptic Soy Broth (TSB) media was carefully divided into two main groups: one set served as the control, while the other received inoculation with the *S. aureus* test samples. Overall, eight experimental groups were constituted, each consisting of two tubes. In each tube, 100 µL of the bacterial inoculum was aseptically introduced. The tubes were then incubated at two different temperatures, 25°C (room temperature) and 37°C. For each temperature, observations were made following incubation periods of 8h, 24h, 36h, and 48h, respectively.

At the end of each incubation interval, we observations were made closely to the extent to understand the pattern of biofilm formation inside the tubes. For quantification, we utilized the crystal violet colorimetric tube assay, an indirect yet reliable method originally introduced by Christensen et al. This technique relies on staining the biofilm matrix which encapsulates both living and dead bacterial cells with crystal violet dye. Over the years, further refinements have helped to improve the assay's accuracy and reproducibility. After staining and thorough rinsing, the retained crystal violet was subjected to obtained the optical density (OD) of each sample measured at a wavelength between 590 and 595 nm (Kamimura et al., 2022). This absorbance reading serves as an indicator of the overall biofilm biomass.

The percentage of biofilm formation was calculated using the following formula:

OD Sample Biofilm formation (%) = (OD Sample/OD Control) ×100

OD Sample: Absorbance of the test sample (e.g., treated or experimental condition).
OD Control: Absorbance of the control sample (e.g., untreated, negative control, or baseline biofilm)

This approach made it possible to systematically compare the ability of *S. aureus* to form biofilms on two different surface materials under varying environmental conditions, thereby providing insights into the effects of temperature and incubation time on biofilm development.

In-vitro Biofilm Formation Assay on Tooth Surface

A quantitative test was performed to evaluate the extent of biofilm formation on natural teeth compared to artificial crown. The in-vitro test conducted was similar to the that of biofilm formation tube assay with inclusive of natural and artificial crown inside the test tubes. This evaluation enabled to understand the amount of biofilm that can develop on different dental surface materials. For the experiment, Tryptic Soy Broth (TSB) was placed in 10 ml of culture media tubes, each set with control and test samples, separately for natural and artificial teeth. To each tube, 100 µL of *Staphylococcus aureus* culture was added. All the tubes were then incubated for 24 hours at 37°C. Each test was performed in triplicate to ensure accuracy. After incubation, the liquid broth was removed, and 5 ml of crystal violet stain was added to each tube to color the biofilm formed on surface of sample material. The stain was left on for 30 minutes. Then, the stain was poured out, and 5 ml of methanol was added to remove the stain from areas with biofilm, after 30 minutes. Finally, the absorbance of each sample was measured at 600 nm using a colorimeter. The obtained optical density (OD) values were recorded to evaluate the amount of biofilm formed on both natural and artificial teeth.

Surface Attachment Strength

Surface attachment strength is a standard way to measure how strongly bacteria can stick to a surface over time. This method is commonly performed same manner as the biofilm tube assay. It helps us understand how well a microbe, like *Staphylococcus aureus*, can attach to natural and non-living (abiotic) surfaces. Originally, this method was developed to study how coagulase-negative *Staphylococcus* species attach to plastic surfaces, but now it is used for many other types of *Staphylococcus* bacteria. When the pathogenic bacteria incubated for a short time (about 1 to 2 hours), this makes it possible to assess the efficiency of bacterial adhesion during the early stages of growth on the various surface materials. If continued the incubation for longer (around 20 hours), enabled to measure the actual biofilm that slimy layer developed by bacteria.

This study employed an in-vitro assay to evaluate the biofilm-forming capacity of *S. aureus* on natural tooth versus artificial crown surface. First, the natural tooth and artificial crown were disinfected and placed separately into two tubes, each containing 10 ml of Tryptic Soy Broth (TSB). Then, 1 ml of bacterial culture was added to each tube. These tubes were incubated for 6 hours at 37°C to allow bacteria to attach to the tooth surfaces. After incubation, the liquid broth was removed, and phosphate-buffered saline (PBS) was added to wash the tubes. The tubes were gently tilted, and 1 ml of the washed suspension was taken and diluted with

99 ml of fresh PBS in new tubes. From this diluted solution, 1 ml was taken and spread onto nutrient agar plates, which were then incubated at 37°C for 24 hours to allow bacterial colonies to grow. Next, the tubes were washed again by removing the liquid, adding 10 ml of PBS, and shaking them vigorously to detach any remaining bacteria. Then, 1 ml of this suspension was taken and diluted again with 99 ml of PBS. Another 1 ml was plated on nutrient agar plates and incubated under the same conditions. After 24 hours, the number of colony forming units (CFU) on all plates were calculated. This allowed us to compare strong or weak surface adhesion of S. *aureus* and grew as biofilms on natural versus artificial tooth surfaces.

RESULTS & DISCUSSION

Biofilm Formation Assay

Tryptic Soy Broth (TSB) was prepared under aseptic conditions to maintain sterility and prevent contamination. Once prepared, the media was divided into two equal parts of 10 ml each: one set designated as the blank and the other as the test sample. A total of four sets were arranged, each consisting of two test tubes. Equal quantity of inoculum was added to each test tube. The tubes were then incubated at two different temperatures: room temperature (approximately 25°C) and 37°C. Observations were made at multiple time points 8 h, 24 h, 36 h, and 48 h respectively and for each of two defined temperature set. After the incubation, the broth medium from each tube was separated and subjected to crystal violet staining to evaluate quantitative biofilm formation. The resulting optical density (OD) values were recorded. As each test was performed in triplicate the resulting OD was taken as an average of three values obtained. Although the qualitative appearance of biofilm formation was generally similar across average samples, differences were noted in the intensity of biofilm developed with OD values obtained. Specifically, the turbidity intensity, indicating biofilm density, increased in a specific order that reflected the growth conditions and incubation durations.

Table No. 1: Intensity of Biofilm formation at different experimental conditions (Visual Observation)

Duration of Incubation	Intensity of Biofilm formation at Temp	
	25^0C	37^0C
8h	+	+
24h	++	+++
36h	++	+++
48h	+++	++++

+ Minimum; ++ Medium; +++++ Highest

A change in pH is observed, which resulted in a color change, and intense biofilm formation was observed by the *S. aureus* clinical isolate. The highest intensity of biofilm formation is found at 37^0C 48 hrs.

Table No. 2: Average percentage of Biofilm development at different temperatures

Duration of Incubation	Average % of Biofilm formation at Temp	
	25^0C	37^0C
8h	56	63
24h	66	76
36h	72	80
48h	85	90

The biofilm tube assay, which utilizes test tubes as vessels for biofilm development and thus quantification, offers a simple yet relatively high-throughput method for assessing biofilm formation. This assay enables the evaluation of biofilm development on both the inner wall and the bottom of the tube, making it especially valuable when comparing multiple strains or growth conditions in parallel. Its straight forward and scalable nature support its widespread use in genetic screening experiments as well as in the routine analysis of microbial adherence. The standard protocol involves staining the attached biofilm with crystal violet (CV), a dye that binds to biomass and allows for spectrophotometric quantification. However, other colorimetric and metabolic stains have also been employed to measure biofilm quantity using this format, depending on the specific research objective.

The primary aim of this method is to quantify the degree to which a microorganism can adhere to and colonize an abiotic surface, such as the glass or plastic walls of a tube. In addition to crystal violet, colorimetric assays targeting the constituents of the biofilm matrix such as exopolysaccharides (EPS), total proteins, and carbohydrates have been adapted for use in the tube assay format. These assays enable a more detailed biochemical analysis of biofilm biomass. However, it is important to note that the measurement of individual components, such as specific EPS fractions, does not always correlate directly with the total biofilm mass. Therefore, while such methods enhance the assay's versatility, crystal violet staining remains the standard for rapid and general biofilm quantification.

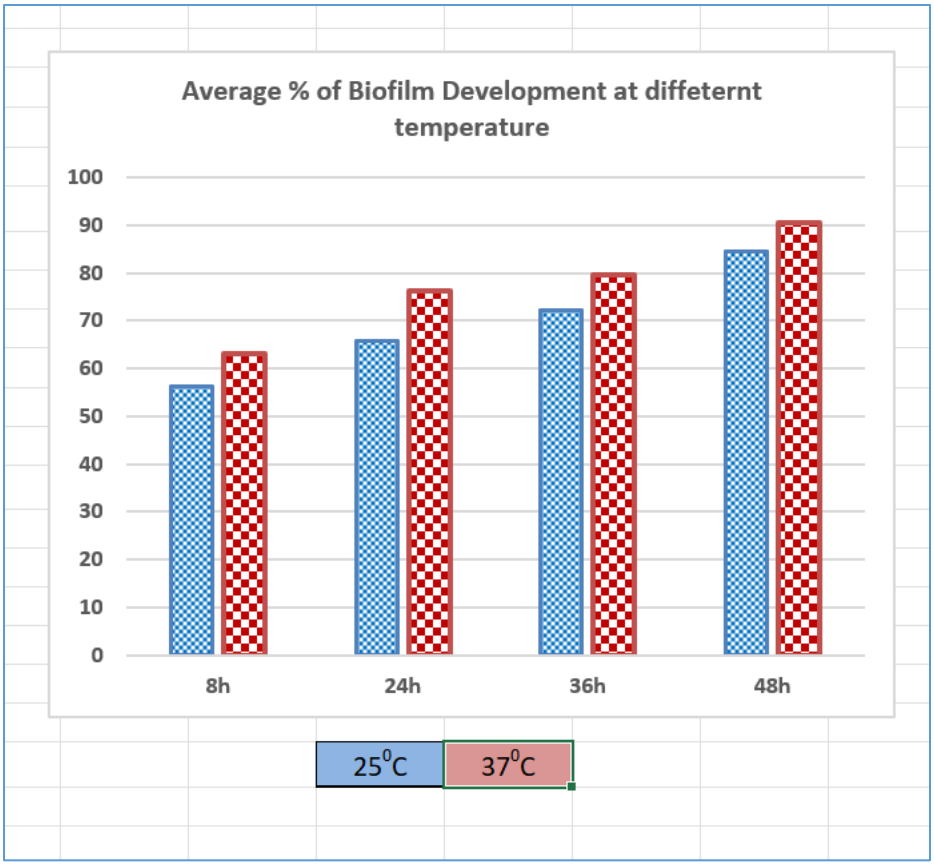

Fig. 1. Graph Average % of In Vitro Biofilm development at various temperatures

Biofilm Formation Assay on Natural Tooth and Artificial Crown Surface

After following the assay process as described above, the test samples were inoculated with natural tooth and artificial crown specimens. Following 24 hours of incubation, the formed biofilms were de-stained using a methanol solution. The optical density (OD) values were then measured using a UV spectrophotometer, and the recorded average of three samples was calculated as average percentage of the biofilm formation as presented in Table No.3.

Table No.3 Average % of Biofilm Formation

#	Test Samples	Average % Biofilm Formation
1	Natural T	82%
2	Artificial C	60%

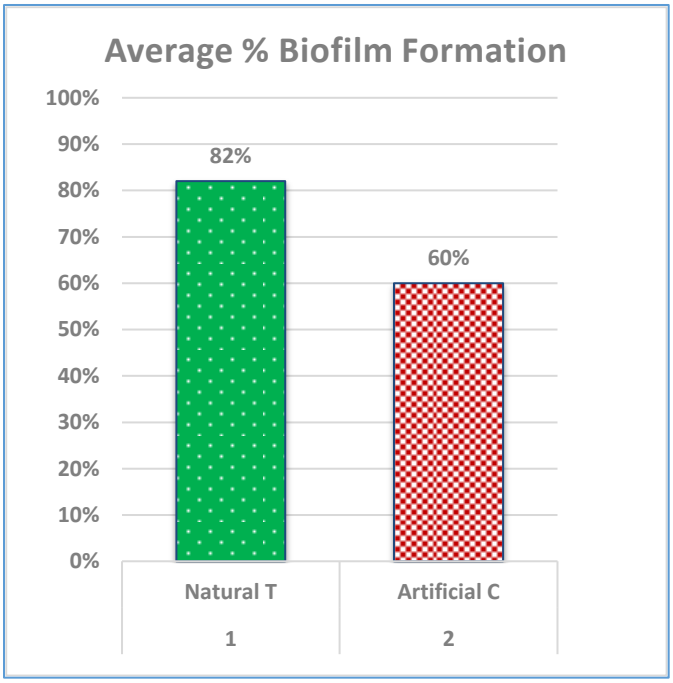

Fig. 3. Graph Average % of Biofilm Formation

The resulting average percentage of biofilm formation is 82% and 60% respectively, on natural tooth and artificial crown, clearly indicating the slow deterioration of the artificial tooth.

Surface Attachment Strength

After sterilizing all equipment and preparing the medium, approximately 30 ml of Tryptic Soy Broth (TSB) was made under aseptic conditions. 10 ml of this broth was then dispensed into each tube across two separate sets corresponding to three experimental conditions: control, natural tooth, and artificial tooth. Almost 1 ml of a *Staphylococcus aureus* inoculum was aseptically added to each tube to ensure standardized microbial exposure. The tubes were then incubated at 37°C for 24 hours under suitable conditions to allow bacterial growth and interaction with the respective substrates. Following the incubation period, the broth was carefully discarded from each tube. Fresh phosphate-buffered saline (PBS) was added to rinse the tubes, and 1 ml of this PBS solution was collected from each tube. This sample was then diluted with 99 ml of PBS, and the mixture was gently tilted to achieve uniform dilution. An aliquot from this first dilution was plated onto agar plates, creating the first set of plates (Set 1). Subsequently, the medium in the tubes was discarded once more. A new aliquot of 1 ml was taken, diluted again with 99 ml of PBS, and vigorously shaken to ensure thorough mixing. From this second dilution, aliquots were plated onto agar plates forming the second set of plates (Set 2). All four sets of plates were incubated for an additional 24 hours at 37°C. After this incubation, the colony-forming units (CFU) on each plate were counted to quantify bacterial growth. These results were carefully recorded and presented in an accompanying table No. (4 and 5) for comparative analysis.

Table No. 4 Surface Attachment Strength on Natural Tooth

Surface Attachment Strength (Natural Tooth)	
Type	CFU (colony forming unit)
Weak Adhesion Strength	22
Strong Adhesion Strength	62

Table No.5 Surface Attachment Strength on Artificial Crown

Surface Attachment Strength (Artificial Crown)	
Type	CFU (colony forming unit)
Weak Adhesion Strength	74
Strong Adhesion Strength	more than 100

After calculating the colony-forming units (CFU) on all four of plates from each set, it can be concluded that biofilm production is greater on the natural tooth surfaces as compared to that on the artificial crown surface. The surface attachment of a clinical isolate was observed to be more loosely attached on the surface of artificial crown surface as compare to that on natural tooth surface even after 24 hours of incubation. This suggests that if biofilm remains on the natural teeth with a longer period, it could become harder and attach more firmly to the tooth surface. Typically, such deposition occurs in areas that are difficult to reach with a toothbrush, such as the spaces between the periodontal (gum) socket and the teeth. These regions often remain unexposed to regular brushing and are consequently the primary sites where infection pertains.

CONCLUSION

Staphylococcus aureus is not normally part of the regular oral cavity flora. However, it has evolved the ability to occasionally colonize the human mouth. Its presence in the oral cavity varies and often increases due to factors like poor oral hygiene and medical conditions that weaken the immune system. Because of this, *S. aureus* is considered an opportunistic bacterium it takes advantage of certain conditions to thrive. Infection by *S. aureus* in the mouth mainly occurs due to the continuous buildup of bacteria in periodontal pockets combined with poor oral hygiene. This leads to changes in the oral environment such as shifts in pH and oxygen levels which contribute to infection and damage to the tooth enamel. These bacteria attach to surfaces, form colonies, and multiply, creating complex structures with channels for nutrients and water. This entire process is coordinated through a bacterial communication system called quorum sensing. Targeting quorum sensing could provide a promising future strategy to prevent plaque formation and control oral infections. Quorum quenchers and quorum sensing inhibitors may play a key role in stopping biofilm development.

According to the biofilm formation results presented (Table No. 3), biofilms tend to form more extensively on natural teeth than on artificial teeth. When restoring oral problems, various biomaterials are used, but these materials differ from natural enamel in surface roughness, chemical makeup, and surface energy. These differences can disrupt the balance of oral microorganisms and alter factors like pH and oxygen levels.

Natural teeth consist of two main parts: the enamel, which is the hard, outermost layer with a high degree of mineralization and very little organic content, and the dentin, the tougher, inner region that contains collagen,

fluids, and mineral crystals. Enamel is hydrophilic (water-attracting) in nature. Artificial teeth are typically made from porcelain or resin composites. Porcelain is preferred because of its strength and durability. Porcelain is a ceramic material with glass-like properties; it is generally hydrophobic (repels water), but at high temperatures, it can become hydrophilic. Resins are also hydrophobic but unlike porcelain, they do not dissolve in water under any conditions. The goal of using biomaterials in dental treatments is to benefit the patient without causing harm. One important factor for clinical success is the biomaterial's ability to reduce or prevent biofilm formation. Therefore, improving the materials used for artificial teeth to inhibit biofilm growth is crucial. With the increase in oral surgeries and dental implants, controlling plaque growth and preventing infections has become more important than ever. Dental implants often use materials like porcelain or resin composites that help reduce biofilm formation.

In this study, the ability of biofilm formation and surface adhesion strength of *S. aureus* were evaluated on both natural tooth and artificial crown surfaces. The findings revealed that biofilms developed more strongly and adhered more firmly to natural tooth surfaces. In contrast, biofilms on artificial crown exhibited weaker surface attachment, potentially limiting bacterial biofilm development due to reduced adhesion strength. These findings emphasize the importance of optimizing and selecting dental biomaterials to better manage biofilm formation and support oral health.

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