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Association between the biofilm formation of *streptococcus mutans*, dental caries experience, and resistance to antibiotics in adult patients

Ahmed Abdullah Howilah [2], Rassam Abdo Saleh Alsubari [3] , Nesreen Fadel Al-Sanabani [2,3]
Fatima Mohammed Abdullah Al-Rohmi [1] , Hassan Abdulwahab Al-Shamahy [3,*]

[1-] Department of Restorative and Esthetic Dentistry, Faculty of Dentistry, Sana'a University, Sana'a, Yemen.
[2-] Department of Prosthodontics, Faculty of Dentistry, Sana'a University, Sana'a, Yemen.
[3-] Department of Basic Sciences, Faculty of Dentistry, Sana'a University, Sana'a, Yemen.

***Corresponding author: shmahe@yemen.net.ye , h.shmahe15@su.edu.ye**

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ABSTRACT

Objectives: The aim of this study was to consider the potential association between the formation of bucall mucusa *streptococcus* biofilms and a high DMFT index, as well as the occurrence of antibiotic resistance among adult patients in Sana'a, Yemen.

Study design: A total of 100; 34-85 year old. Clinical examinations of patients were performed to estimate dental caries experience with the Silness-Loe index, as well as bucall mucusa swabs were collected to assess biofilm production by the phenotypic method, i.e., tissue culture palate methods (TCPM). Finally, the antibiogram susceptibility pattern of isolated *S. mutans* was done by the Kirby-Bauer disc diffusion method for L-Lactam antibiotics (as ampicillin and penicillin) and non-L-Lactam antibiotics (clindamycin, erythromycin, lincomycin, and vancomycin).

Results: When isolated *S. mutans* were exposed to biofilm detection by the TCP method, 1 (1.2%) showed strong biofilm formation capacity, 71 (86.6%) showed moderate biofilm formation capacity, and 10 (12.2%) showed non/weak formation capacity of biofilm. There was an escalation in the rate of formation of *S. mutans* biofilms with an increased degree of caries index. The *S. mutans* biofilms positively showed a higher rate of resistance than non/weak biofilm formation, e.g., ampicillin (91.1% versus 8.9%, *p <0x7E>* 0.0001), tetracycline (87.8% versus 12.2%, *p <0x7E> 0.0001*), and co-trimoxazole (90%% versus 10%, p <0x7E> 0.0001), etc.

Conclusion: The present study proved that *S. mutans* is still the major bacteria isolated from the oral cavity, but few persons might not have a significant number of *S. mutans* in the oral cavity. The *S. mutans* biofilm producers were more able to cause dental caries compared to the *S. mutans* biofilm non-producers. Drug-resistant factor in the *S. mutans* isolates was found to be associated with *S. mutans* biofilm formation.

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1. Introduction:

Any artificial combination of microorganisms in which cells adhere to the surface and each other frequently makes up a biofilm. These adhering cells get enmeshed in an extracellular polymeric substance (EPS)-based sticky extracellular matrix. EPS components are produced by cells in biofilms and are often a polymeric mixture of extracellular DNA, proteins, carbohydrates, and lipids [1]. Dental caries is significantly influenced by *Streptococcus mutans*, an optional Gram-positive anaerobic bacteria that typically occurs in the human oral cavity and forms alpha hemolytic colonies in blood agar [2,3]. Caries is more likely to occur on dental surfaces that have been colonized by *S. mutans* [4]. A recent study conducted in Yemen revealed that only 2% to 4.6% of people had no caries (Score 0), while the remaining 95.4% to 98.4% had caries (Score 1-3), and that the rate of *S. mutans* heavy colonization was significantly increasing as the caries score increased [5, 6]. There has been a documented positive correlation between *S. mutans* levels and dental caries occurrence in subpopulations with a moderately high caries occurrence [7-9]. Compared to individuals in the same population with a lower concentration of *S. mutans*, those with high levels of the mutans also develop coronary and root caries in both temporary and permanent restorations [10-12]. To determine whether there was a direct correlation between the ratio of *S. mutans* in dental plaque development and their strong colonization, researchers looked at the levels of the bacteria in bucall mucus memberane and saliva [6,13]. There is, however, no data about any potential connections between bucall mucusa *streptococcus mutans's* ratio in dental plaque formation and its capacity to create biofilms. It has been said that dental caries is caused by the mouth's environment, which includes bacteria that are contagious and sugar that is readily available in foods and beverages. According to reports, one of the main causes of dental cavities and normal static plaque is *Streptococcus mutans* [13–15]. There has been little research done on the function of biofilms in dental caries. The pathogenesis of dental caries is well known, and the researchers discovered that bacterial colonization appears to be a crucial stage in oral sickness that results in the production of biofilms [2,6,16,17]. Most oral biofilms are made up of several different bacterial strains. More than 700 different bacterial strains have been shown to be present in tooth plaque recently [18].

One possible mechanism for the creation of biofilms is that the *S. mutant* develops glucosyltransferase on the bacterial cell wall, allowing the bacteria to grow from polysaccharides made of sucrose. The ability of bacteria to clump together and adhere to tooth enamel, forming biofilms, is due to these adhesive polysaccharides. By blocking *S. mutans'* capacity to adhere to dental enamel, anti-cellular glucosyltransferase (CA-gtf) immunoglobulin Y prevents the fungus from proliferating. Research has demonstrated that Anti-CA-gtf IgY can effectively regulate *S. mutans* in the oral mucosa [19–21].

Human anxiety has spread due to the rise in bacterial infections' resistance to frequent antibiotic usage. The extent of antibiotic resistance is a major financial issue as well as a cause of mortality. Antibiotic resistance is more common in developing nations than in developed nations, such as Yemen [19, 22]. Furthermore, S. mutans is recognized as an endocarditis cause. Knowing which medicines are effective against S. mutans is crucial for treating endocarditis appropriately [23, 24]. The American Heart Association suggests that one hour before the dental procedure, preventive antimicrobial therapy should be given to high-risk cardiovascular patients, such as amoxicillin (2 g) as a first choice and clindamycin (600 mg) as a second choice [21]. However, beta-lactamase production is unusual for most streptococci, as resistance occurs via a slight change in penicillin-binding proteins [25-27]. Thus, more information is required concerning the distribution of *S. mutans* biofilm formation strains and the correlation of levels of *S. mutans*

biofilm formation with caries in adult Yemeni patients. The present study was planned in an adult population of Sana'a city in Yemen (i) to determine the *S. mutans* biofilm formation levels in their bucall mucosa and (ii) to correlate the dental caries in these individuals with their relation to *S. mutans* biofilm formation and scores of dental caries. Also to reveal antibacterial sensitivity to isolated *S. mutans* and to study the relationship between biofilm formation and antibiotic resistance.

2. Materials and methods

Biofilm production was performed on 82 oral *S. mutans* bacteria isolates from 100 patients who visited dental clinics run by Sana'a University's Faculty of Dentistry and private dental clinics. The ensuing phenotypic identification of isolated bacteria was performed by standard methods following the Clinical and Laboratory Standards Institute (CLSI) 2015 guidelines. Biofilm-forming oral bacteria were detected by the tissue culture plate (TCA) method. The impact of biofilm production was correlated with the DMFT index of the tested patients.

Recording of dental caries

The same examiner performed the examinations on each of the study adults. The caries diagnostic criteria were taken into consideration when doing the intra-examiner calibration. The adult Silness-Loe plague index was completed. This index is based not only on the simple counting of the number of decayed, missing (due to caries solely), and treated teeth, but also on the field clinical evaluation of the research participants using a probe, mirror, and cotton rolls.

Biofilm production detection*:*

Tissue culture/microtiter plate approach (TCA) was used to identify biofilm [28, 29]. After being inoculated with 2 ml of BHI broth, the bacterial isolates from fresh agar plates were cultured for 24 hours at 37°C. Following a 1:40 dilution with fresh medium (BHI broth supplemented with 1% glucose), 200 l of the sample was added to each individual microtitration plate, and the plates were incubated for an additional 24 hours at 37 °C. After lightly tapping the contents, free floating sessile bacteria were eliminated by repeatedly rinsing it with phosphate buffered saline (pH 7.2). For ten to fifteen minutes, adhering bacteria that produced biofilm were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). After removing the unbound crystal violet solution in three separate PBS washes, the plate was set aside to dry. In order to release the dye, 200µl of 95% ethanol was added to each well, and an optical density (OD) reading at 630 nm was taken. Each test strain's OD value as well as that of the negative control were computed, and OD cutoff values (ODc) were evaluated in accordance with earlier instructions [29].

Statistical Analysis: Epi-Info Statistics version 7 was utilized to examine the information. Statistical analysis was performed to consider the degree of biofilm production of 82 *S. mutans* with the mean of DMFT, decayed, missing due to caries, and filled teeth in permanent teeth by calculating the diffrance, 95% CI, and p-value of the level of weak, moderate, and strong biofilm production.

Ethical Consideration: The Contract No. 217 project received ethical authorization on August 21, 2022, from the Medical Ethics and Research Committee of Sana'a University's Faculty of Medicine and Health Sciences. The review committee's established ethical guidelines were constantly adhered to. The selected individuals gave their written and informed consent.

3. Results

The study included 100 individuals, 51 with dental prostheses and 49 controls without dental prostheses, 39% male and 61% female, ranging in age from 34 to 65 years, with a mean ± SD of age equal to 40.1 to 8.4 years old. Most of the

participants were in the age group 35–44 years (40%) (Table 1).

Table 1: General characteristics of participate in the study

Characters	N (%)
Sex	
Male	39 (39)
Female	61 (61)
Ages	
24-34	30 (30)
35-44	40 (40)
45-54	21 (21)
≥55	9 (9)
Mean age	40.1Years
SD	8.4 Years
Mode	40 Years
Median	40 Years
Min-Max	34-65 Years
Type of prosthesis	
No prosthesis	49 (49)
Removable	18 (18)
Fixed	33 (33)
Prosthesis sit	
Upper arch	28 (54.9)
Lower arch	23 (45.1)
Duration of prosthesis	
1-3 years	32 (62.7)
4-6 years	6 (11.8)
< 6 years	13 (25.5)

Regarding the type of prosthesis, 49% are without prosthesis, 18% have removable prosthesis, and 33% have fixed prosthesis. Regarding the duration of the prosthesis, most of the participants have prostheses lasting 1-3 years (62.7%). Regarding the sit of the prosthesis, 54.9% is in the upper arch and 45.1% is in the lower arch. Considering the Streptococcus viridans group, *S. mutans* had 86.6% strains with moderate biofilm production and 1.2% with strong biofilm production (Table 2).

Table 2: Biofilm detection by TCA method among Streptococcus mutans of oral cavity isolated Bacteria, n=82.

Bacteria	Biofilm production by TCA			
	Negative No (%)	Weak No (%)	moderate No (%)	Strong No (%)
S.mutans n=82	0 (0.0)	10 (12.2)	71 (86.6)	1 (1.2)
Total n=294	9 (3.1)	63 (21.4)	213 (72.4)	9 (3.1)

Considering the relationship between the DMFT index and the ability of bacteria to produce biofilms, there was a higher mean ± SD (6.8 ± 2.5) of DMFT for moderate biofilm-producing *S. mutans* bacteria with a difference equal to 2.6, 95% CI = 0.96-4.2, and this result is highly significant (p = 0.0022). This suggests that biofilm production is associated with tooth decay. Also, there was a higher mean ± SD (4.1 ± 1.3) of decayed teeth for moderate biofilm-producing bacteria compared to non-weak-biofilm-producing *S. mutans* bacteria, with a difference equal to 1.3, 95% CI = 0.4-2.2, and this result is significant (p = 0.004) (Table 3).

Table 3– Comparison among the DMFT index and biofilm production of S. mutans oral isolated bacteria.

Biofilm results	**Mean ± SD**			
	DMFT	**Decayed**	**Missed**	**Filled**
Negative n=0 (control)	-	-	-	-
Reference				
Weak n=10	4.2±1.9	2.8±1.4	2.5 ±3.5	1.6±1.5
Test				
Moderate n=71	6.8±2.5	4.1±1.3	0.7±1.8	2.7 ±1.8
Difference	2.6	1.3	-1.8	1.1
95% CI	0.96 -4.2	0.4-2.2	-3.1-0.41	-0.08-2.3
Significance level	0.0022	0.004	0.01	0.06
Strong n=1	-	-	-	-

This procedure calculates the difference between the observed means in two independent samples. A significance value (P-value) and 95% Confidence Interval (CI) of the difference is reported. The P-value is the probability of obtaining the observed difference between the samples if the null hypothesis were true. The null hypothesis is the hypothesis that the difference is 0.

The association between *S. mutans* biofilm growth and antibiotic resistance in isolates from patient buccal mucosa is presented in Table 4.

Table 4: Association of biofilm formation and Antibiotic resistant of *S. mutans* isolated from buccal mucosa of prosthesis and non-prosthesis patients n= 82 isolates

	Resistance	**biofilm formation**		**DF (95% CI)**	**p value**
Antibiotic name	**Total N (%)**	**Negative/weak N=10**	**Moderate/strong N=72**		
Tetracycline	82 (100)	10 (12.2)	72 (87.8)	75.6 (43.4-86.8)	<0.0001
Erythromycin	0 (0.0)	0 (0.0)	0 (0.0)	-	-
Ampicillin	79 (96.3)	7 (8.9)	72 (91.1)	82.2 (50.6-90.9)	<0.0001
Cephalothin	40 (48.8)	1 (2.5)	39 (97.5)	95 (65.5 -97.9)	<0.0001
Co-trimoxazole	10 (12.2)	1 (10)	9 (90)	80 (48.3-89.6)	<0.0001
Amoxicillin-Clavulanic Acid	3 (3.7)	0 (0.0)	3 (100)	100 (71.7 - 100)	<0.0001
Gentamicin	6 (7.3)	0 (0.0)	6 (100)	100 (71.7 - 100)	<0.0001
Oxacillin	5 (6.1)	0 (0.0)	5 (100)	100 (71.7 - 100)	<0.0001
Penicillin	0 (0.0)	0 (0.0)	0 (0.0)	-	-
Ciprofloxacin	4 (4.9)	0 (0.0)	4 (100)	100 (71.7 - 100)	<0.0001
Cloxacillin	5 (6.1)	0 (0.0)	5 (100)	100 (71.7 - 100)	<0.0001
Cefoxtine	7 (8.5)	0 (0.0)	7 (100)	100 (71.7 - 100)	<0.0001
Amikacin	0 (0.0)	0 (0.0)	0 (0.0)	-	-
Clindamycin	16 (19.5)	0 (0.0)	16 (100)	100 (71.7 - 100)	<0.0001
Vancomycin	0 (0.0)	0 (0.0)	0 (0.0)	-	-

For instance, the difference in tetracycline resistance was 75.6%, meaning that biofilm-producing germs (strong/moderate) are 75.6% more resistant to tetracycline than negative/weak strains, with a p-value of <0.0001, this rate falls between 43.4 and 86.8%, and the outcome is statistically significant (p<0.0001). In conclusion, moderate/strong biofilm-producing strains of *S. mutans* exhibited a higher rate of drug resistance against the tested antibiotics than did negative/weak biofilm-producing strains. In addition, for other antibiotics, the rate of drug resistance was higher in *S .mutans* strains that

produced moderate or strong biofilms compared to strains that produced weak or negative biofilms. Table 5 shows prevalence of DMFT the sum, Decayed, Missing due to caries, and Filled Teeth in the permanent teeth per sex. Male patients showed higher rate of decayed, missed, filled and DMFT.

Table 5 – Prevalence of DMFT the sum, Decayed, missing due to caries, and Filled Teeth in the permanent teeth per sex.

Variables	Male N (%)	Female N (%)	Total N (%)
	39 (39%)	**61(61%)**	**100 (100)**
Decayed	39 (100)	57 (93.4)	96 (96)
Missed	27 (69.2)	30 (49.2)	57 (57)
Filled	33 (84.6)	36 (59)	69 (69)
DMFT	39 (100)	59 (96.7)	98 (98)

4. Discussion

Biofilms are known to occur on a variety of implant medical equipment, including pacemakers, dentures, heart valves, catheters, and artificial joints. These implants offer a superficial and secure environment for the establishment of biofilms [30]. A device-related infection can have serious and even fatal effects on a person's health [31]. Eighty-two samples in this investigation displayed considerable growth in *S. mutans*, according to a bacteriological culture of the organism. When isolated *S. mutans* were exposed to biofilm detection by the TCP method, 1.2% showed a high biofilm formation capacity, 86.6% showed moderate ability to form biofilms, and 12.2% showed non/weak ability to form biofilms. This high rate of colonization and biofilm production of *S. mutans* in adults may lead to mouth infections in used subjects or transmission to other parts of the body, especially the circulatory system. This suggestion can be confirmed by NHI analysis, which indicates that biofilms generally (as well as bacterial and fungal biofilms) are accountable for more than 80% of all microbial infections [30**]**. For structural and physiological causes, biofilms are inherently resistant to antimicrobial therapy and host immune defenses. Biofilms cause many infections, ranging from superficial mucosal infections to severe and extensive bloodstream infections. This infection often starts from biofilms on mucous surfaces or implanted medical devices. In the current study, there was an escalation in the rate of formation of *S. mutans* biofilms with an increase in the degree of caries index (Table 3).

Dental plaque is an oral biofilm that adheres to the teeth and is made up of many types of bacteria and fungi (such as *Streptococcus mutans* and *Candida albicans*) [32–34] and is an integral part of salivary polymers and extracellular microbial products. Accumulation of microorganisms exposes teeth and gum tissues to high concentrations of bacterial metabolites that lead to dental disease. The biofilm on the surface of teeth is often subjected to oxidative stress and acid stress. Dietary carbohydrates can cause a significant decrease in the pH of oral biofilms to values 4 and below (acid stress) [35-37]. A pH of 4 at a body temperature of 37 °C leads to DNA purification, leaving apurinic (AP) sites in the DNA, especially a loss of guanine [35-37].

Dentists usually prescribe most of the antibiotics used in this study [38]. The number of *streptococci* resistant to oral mutations is greater in people who are frequently exposed to antibiotics, although resistant bacteria may also be found in healthy people who have not been recently treated with antibiotics [38]. *L-lactam* antibiotics are the most frequently prescribed chemoprophylactic agents in general dental practices. In spite of this, penicillin resistance is increased among oral *streptococcus* [39,40]. The number of resistant oral *streptococci* is greater in people who are frequently exposed to antibiotics [41], even though these bacteria can also be established in healthy people who have not been recently treated with antimicrobials [42]. Also, an abscent penicillin resistance (0%) in *S. mutant* isolates in the current study is lower than those of Al-Shami *et al.* (14.9%) [40] and Pasquantonio *et al.* (13.4%) [43] to oral streptococcal clinical isolates. Additionally,

the current result is completely different from the average of a 2014 study by Dhamodhar *et al.* [44]. 38% of *S. mutans* isolates showed complete resistance to penicillin and ampicillin. Production of *<0xD3>-lactamase* is, however, unusual for most *streptococci*, where resistance is happening by slightly altered penicillin-binding proteins [25-27]. Though in the current study we observed a significant level of ampacillin resistance [96.3%] of *S. mutans* and 19.5% for clindimycin. In the current study, *in vitro* antibiotic sensitivity to various *S. mutans* strains showed that *S. mutans* biofilms positive had a higher rate of resistance to tested antibiotics. This result can be explained by the facts that *S. mutans* positive biofilms are resistant to standard antibiotics for Gram-positive bacteria medications due to the availability of biofilms that are considered physical protection of *S. mutans* from medications, as well as cells in biofilms becoming essentially resistant to drugs due to their changed metabolic states and their constitutive upregulation of drug pumps [30].

5. Conclusion

The present study proved that *S. mutans* is still the major bacteria isolated from the oral cavity, but few persons might not have a significant number of *S. mutans* in the oral cavity. The *S. mutans* biofilm producers were more able to cause dental caries compared to the *S. mutans* biofilm non-producers. Drug-resistant factor in the *S. mutans* isolates was found to be associated with *S. mutans* biofilm formation. The current study demonstrates significant levels of resistance to ampicillin, tetracycline, cephalothin, and co-trimoxazole and clindamycin in *S. mutans* isolates. Further study is needed to find out the minimum inhibitory concentration of *<0xC8>-Lactam* and non *<0xC8>-Lactam* antibiotics for both biofilm formation *S. mutans* and non-biofilm formation *S. mutans*. These results as well call for enhanced evaluation of antibiotic susceptibility testing for the period of prophylaxis. There is likely to be an alternative to antibiotics, such as herbal extract, that may be better than antibiotics in the coming years to avoid the coming bacterial resistance to antibiotics. Additionally, the increase in the rate of antibiotic resistance in *S. mutans* isolates suggested that more precautions be taken while prescribing antibiotics will preserve the bacteria with less resistance.

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A dispute of interest

Regarding this project, there is no conflict of interest.

Author's contributions

Nesreen Fadel Al-Sanabani did the fieldwork for this study as part of a PhD in the department of Medical Microbiology, Faculty of Medicine and Health Sciences, Sana'a university. Additional authors assisted with data analysis, drafting and reviewing the manuscript, and giving final clearance to the study.

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