Available online at *www.ujpronline.com*
Universal Journal of Pharmaceutical Research
An International Peer Reviewed Journal
ISSN: 2831-5235 (Print); 2456-8058 (Electronic)

RESEARCH ARTICLE

ASSOCIATION BETWEEN THE *STREPTOCOCCUS MUTANS* BIOFILM FORMATION AND DENTAL CARIES EXPERIENCE AND ANTIBIOTICS RESISTANCE IN ADULT FEMALES

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Article Info:

Article History:
Received: 10 October 2020
Reviewed: 14 November 2020
Accepted: 22 December 2020
Published: 15 January 2021

Cite this article:
Alhasani AH, Ishag RA, Yahya Al-Akwa AY, Al Shamahy HA, Al-labani MA. Association between the *Streptococcus mutans* biofilm formation and dental caries experience and antibiotics resistance in adult females. Universal Journal of Pharmaceutical Research 2020; 5(6):18-23.
https://doi.org/10.22270/ujpr.v5i6.507

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Abstract

Objectives: The aim of this study was to consider the potential association between the formation of salivary *streptococcus* biofilms and the incidence of caries, as well as with the occurrence of antibiotic resistance among adult mothers in Sana'a, Yemen.
Study design: A total of 261; 25-35 year old females were chosen. Clinical examination of females were performed to estimate dental caries experience with the Silness-Loe index, as well as stimulated saliva were collected to assess biofilm production by the phenotypic method i.e. Tissue Culture Palate Methods (TCPM). Finally, antibiogram susceptibility pattern of isolated *S. mutans* was done by Kirby-Bauer disc diffusion method for 6 β-Lactam antibiotics (ampicillin, penicillin, amoxicillin, cefotaxime, methicillin and cefazolin) and 4 non β-Lactam antibiotics (clindamycin, erythromycin, lincomycin and vancomycin).
Results: When isolated *S. mutans* were exposed to biofilm detection by TCP method, 31 (12.2%) showed high biofilm formation capacity, 46 (18%) showed moderate biofilm formation capacity, while 184 (72.2%) showed non/weak formation capacity of biofilm. The overall rate of biofilm formation was 30.2%. There was an escalation in the rate of formation of *S. mutans* biofilms with an increased degree of caries index. The *S. mutans* biofilms positve showed a higher rate of resistance than non/weak biofilm formation e.g. ampicillin (28.6% versus 12.9%, p=0.002), amoxicillin (77.9% versus 18%, p<0.0001), and penicillin (79.2 % versus 23%, <0.0001) etc.
Conclusions: The present study proved that *S. mutans* is still the major bacteria isolate from the oral cavity, but few persons might not have significant number of *S. mutans* in 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.
Keywords: Adult females, antibiotic resistance, biofilm formation, dental caries; oral cavity, saliva; *Streptococcus mutans*.

INTRODUCTION

The biofilm consists of any synthetic union of microorganisms in which cells stick to each other and often also to the surface. These adherent cells become embedded within a sticky extra-cellular matrix made up of extracellular polymeric materials (EPSs). Cells within biofilms produce EPS components, which are usually a polymeric conglomeration of sugars, proteins, fats and extracellular DNA[1]. *Streptococcus mutans* is an elective Gram-positive anaerobic bacteria, alpha hemolytic colonies in blood agar commonly found in the human oral cavity and is a significant contributor to dental caries[2,3]. Dental surfaces colonized with *S. mutans* are more susceptible to caries[4]. In Yemen recent study found that the only 4.6% of adults were caries free (Score 0) and 249 (95.4%) presented with caries (Score 1-3) with significant grow in the rate of *S. mutans* heavy colonization with growing caries score[5]. In subpopulations with a moderately high caries occurrence, a positive relationship between saliva levels of *S. mutans* and the occurrence of dental

caries[6,7] has been reported. Those with high levels of *S. mutans* as well arise coronary and root caries in impermanent and permanent restorations than those in the similar population with a lower concentration of *S. mutans*[8,9]. The salivary levels of *S. mutans* were studied to see if there was a direct relationship to the heavy colonization of *S. mutans* and their ratio in the formation of dental plaque[10]. However, there is limited information on the possible relationship between the capability of salivary *streptococcus mutans* to form biofilms and its ratio in the formation of dental plaque. Dental caries has been described as an environmental impact in the mouth, including infectious bacteria and the promptly available sugar in foods and drinks. *Streptococcus mutans* has been reported as a major causative agent of normal static plaque and dental cavities[10,11]. The role of biofilms in dental caries has been studied in a limited way. The researchers found that the etiology of dental caries is well recognized and it appears that bacterial colonization is a significant step for oral illness, which leads to the formation of biofilms[2,12,13]. Oral biofilms predominantly comprise multiple bacterial strains. It has recently been demonstrated that more than 700 bacterial strains are present in dental plaque[14]. The potential mechanism of biofilm formation is that *S. mutant* produces glucosyltransferase on the bacterial cell wall, which permits bacteria to generate from sucrose polysaccharides. These adhesive polysaccharides are accountable for the capacity of bacteria to clump together and stick to tooth enamel, forming biofilms. The use of an anti-cellular glucosyltransferase (CA-gtf) immunoglobulin Y disrupts the ability of *S. mutans* to stick on tooth enamel, thus inhibiting it from reproducing. Studies have revealed that Anti-CA-gtf IgY is capable to efficiently control *S. mutans* in the oral cavity[15,16,17]. The expansion of bacterial pathogens resistance to regular antibiotics use has turned into an universal human apprehension. The extent of antibiotic resistance is reason of deaths as well as significant financial problem. In countries with a low economy like Yemen, antibiotic resistance is more prevalent than in developed countries[15]. *S. mutans* is moreover integrated as a causative agent of endocarditis. Information about knowledge of antibiotics for S. mutans is important for prescribing appropriate treatment in the case of endocarditis[18,19]. 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[17]. However, beta-lactamase production is unusual for most streptococci, as resistance occurs via a slight change in penicillin binding proteins[20,21,22]. Thus, more information is required concerning the distribution of *S. mutans* biofilms formation strians and correlation of levels of *S. mutans* biofilms formation with caries in adult females. The present study was planned in an adult population of Sana'a city, in Yemen (i) to determine the *S. mutans* biofilms formation levels in their stimulated saliva and (ii) to correlate the dental caries in these individuals with their relation to *S. mutans* biofilms 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 resistant.

SUBJECTS AND METHODS

The current study was carried out in the Department of Medical Microbiology Faculty of Dentistry, Sana'a University. The study proposal was permitted by the ethics committee of the Faculty. A written informed consent was taken from the chosen participants.

Study participants

The third and fourth authors visited the families of the study sample members residing in the various sectors of Sana'a city. Females between 25 and 35 years of age were selected and the purpose of the study explained. The sample size required for the study was calculated on the basis of the prevalence of caries in adult females obtained on the basis of a pilot study of 50 subjects and a statistical consultation. The inclusion criteria were that the participants were elderly, had no systemic debilitating disease, and had not taken or had taken antibiotics in the past three months. Individuals who underwent orthodontic treatment with dentures, crowns, or bridges were not included in the study. Thus, the interested participants were randomly selected to form a study group of 261 adult females. The selected individuals were instructed not to eat/drink, brush their teeth, use mouthwash, or smoke one hour before their appointment. Households were reviewed by author (AM) on time and tooth decay was recorded and saliva sample collected. Prior to the commencement of the studies, the Registrar (AM) was trained through frequent calibration sessions conducted in the faculty department.

Tooth decay recording

All study females were examined by the same examiner. Calibration was performed within the examiner regarding the diagnostic criteria for dental caries. The Silness-Loe maternal plague index[23] was performed. This indicator is based on the study participants' field clinical examination using probe, mirrors and cotton rolls, in addition to simply counting the number of decayed and missing teeth (due to caries only) and restored teeth.

Salivary analysis

Method of saliva collection

After the clinical examination, the date for saliva collection was determined. Participants were forced to swallow their pre-existing saliva, in order to clear the mouth of any remaining un-stimulated saliva. Then, each participant chewed a standard piece of paraffin wax for 5 minutes to stimulate the production of saliva that needs to be collected. The saliva samples of all participants were categorized using a code number during the sample collection and processing period.

Microbiological procedure

The sample was collected and transferred to the laboratory immediately in a thioglycolate culture medium and processed on the same day. The sample was then rotated (15 s) and diluted 1: 1000 in isotonic saline before inoculation. One loop (1/1000 ml of

sample) was inoculated on the culture medium Mitis salivarius agar with potassium tellurate, bacitracin and 20% sucrose. Plates were incubated at 37°C anaerobically. *Streptococci mutans* were detected in 251 (96.2%) saliva samples. The detected *Streptococci mutant* were then tested for biofilm formation ability and antibiotic sensitivity.

Biofilm production detection

The biofilm was detected by the tissue culture method/microtiter plate method (TCA)[24,25]. *S. mutans* isolates were inoculated from fresh agar plates in 2 mL of Brain Heart Infusion (BHI) broth and incubated for 24 h at 37°C. Then the cultures were diluted 1:40 with fresh medium (BHI broth with 1% glucose added); 200 µl of the sample was dispensed in the individual microtitration plate and incubated further 24 h at 37°C anaerobically. The content was removed again with subsequent washing with phosphate saline (pH 7.2) three times to remove free-floating sessile bacteria with gentle pecking - then the adherent bacteria, a biofilm product, was fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/w). Volume for 10-15 minutes. The liberated violet crystal solution was removed with triplicate wash with PBS, and then the plate was kept for drying. Finally, with 200 µl ethanol (95%) all wells were filled to release the dye from the well and at the wavelength of 630 nm the optical density (OD) was performed. The OD value was calculated for each negative test and control strain, and the OD cutoff values (ODc) were evaluated as previously described[25].

Antibiotic sensitivity

The antibiotic sensitivity profile was determined by the disc diffusion method. Inoculations were adjusted to match the turbidity of 0.5 McFarland criteria, swabbed on Brian Heart infusion agar and left to dry for 10 minutes[26]. The antibiotics employed in this study were: ampicillin (AMP) 10 µg, cefotaxime (CTX) 30 µg, penicillin-G (P) 10 units, erythromycin (E) 15 µg, methicillin (MET) 5 µg, lincomycin (L) 2 µg, cefazolin (CZ) 30 µg, vancomycin V (30 µg) and clindamycin (CC) 2 µg (Oxide, USA). The zone of inhibition was measured after 24 h of anaerobic incubation at 37°C. Each antibiotic was tested in triplicate. The results were interpreted according to the methodology of the Clinical and Laboratory Standards Institute (CLSI)[27].

Statistical analysis

Statistical analysis: Epi Info version 7 was used for analysis data. Difference in proportions and associated odds ratio and test of significance were calculated using 2X2 tables and selected uncorrected statistical test for chi square and 2 tailed p values for significance. Level of statistical significance was assumed at $p<0.05$.

RESULTS

The study, which included 261 saliva samples subjected to bacteriological culture of *S. mutans* isolate, showed 255 (97.7%) significant growth in *S. mutans*. When isolated *S. mutans* were exposed to biofilm detection by TCP method, 31 (12.2%) showed high biofilm formation capacity, 46 (18%) showed moderate biofilm formation capacity, while 184 (72.2%) showed non/weak formation capacity of biofilm. The overall rate of biofilm formation was 30.2% (Table 1).

Table 1: Biofilm detection by TCP method for *S. mutans* isolated from adult females.

Biofilm formation	Number	Percentage
High*	31/255	12.2
Moderate *	46/255	18.0
Total biofilm	77/255	30.2
Non/weak	184/255	72.2
Total *S. mutans* isolates	255/261	97.7
Total saliva cultured specimens	261	100

TCP-High OD ≥ 0.24; Moderate OD =0.127-0.24, Non/weak OD < 0.12

Table 2: The association between biofilm formation of *S. mutans* with Silness-Loe indix for dental caries for adult mothers.

Index	Positive Biofilm N=77		OR	95% CI	X^2	*p*
	No	%				
Score 0 n=12	0	0		Undefined	5.4	0.01
Score 1 n=120	25	20.8	0.4	(0.2-0.7)	9.4	0.002
Score 2 n=90	34	37.8	1.3	0.77-2.3	1.1	0.28
Score 3 n=39	18	46.2	2.2	1.1-4.8	5.5	0.01
Total n=261	77	29.5		Undefined	6.7	0.009

There was an escalation in the rate of formation of *S. mutans* biofilms with an increased degree of caries index (Table 1). Overall, 12 (4.6%) of the females were caries-free (Score 0) and 249 (95.4%) of the females had caries (Score 1-3). With regard to study females who underwent caries; 120 (45.9%) got score 1, 90 (34.5%) got Score 2 and only 39 (14.9%) got Score 3, moreover, there was a significant increase in the ability of *S. mutans* biofilms formation with an increasing degree of decay. For example in score 1, 20.8% of *S. mutans* isolated had positive biofilm formation, in score 2, 37.8% of *S. mutans* isolated had positive biofilm formation; and at score 3, 46.2% of isolated *S. mutans* had positive biofilm formation, with

significant correlated OR = 2.2, 95% CI =1.1-4.8, X^2 = 5.5 and p=0.01 (Table 2). Table 3 presented the antibiotic sensitivity pattern of *S. mutans*. The *S. mutans* biofilms positive showed a higher rate of resistance than non/weak biofilm formation: ampicillin (28.6% versus 12.9%, p=0.002), amoxicillin (77.9% versus 18%, p<0.0001), penicillin (79.2% versus 23%, <0.0001), cefotaxime (32.5% versus 7.3%, p<0.0001), cefazolin (24.7% versus 12.9%, p=0.019), methicillin (27.2% versus 12.4%, p=0.002), Lincomycin (81.8% versus 30.3% , p<0.0001), clindamycin (29.9% versus 13.5 %, p<0.0001), and erythromycin (40.3% versus 26%, p=0.02). While there was no significant difference between the biofilm formation of *S. mutans* and the non-one in vancomycin resistance (39% VS 39.3%, p=0.96) (Table 3).

Table 3: Antibacterial resistance pattern of *S. mutans* associated with biofilm formation in *S. mutans* isolated from adult females.

Antibiotics	Total N=255		Biofilm producing *S. mutans* N=77		Non-Biofilm producing *S. mutans* N=178		*p*
	No	%	NO	%	No	%	
Ampicillin	45	17.6	22	28.6	23	12.9	0.002
Amoxicillin	92	36	60	77.9	32	18	<0.0001
Penicillin	102	40	61	79.2	41	23	<0.0001
Cefotaxime	38	14.9	25	32.5	13	7.3	<0.0001
Cefazoline	42	16.5	19	24.7	23	12.9	0.019
Methicillin	43	16.9	21	27.2	22	12.4	0.003
Lincomycin	117	45.9	63	81.8	54	30.3	<0.0001
Clindamycin	47	18.4	23	29.9	24	13.5	<0.0001
Vancomycin	100	39.2	30	39	70	39.3	0.96
Erythromycin	51	20	31	40.3	20	26	0.02

DISCUSSION

Biofilms are recognized for their formation on many implanted medical devices, comprise: heart valves, catheters, dentures, pacemakers and artificial joints, which provide a superficial and safe haven for the growth of biofilms[28]. The consequences of a device-related infection on human health can be severe and life-threatening[29]. In this study, 261 saliva samples subjected to bacteriological culture of *S. mutans*, showed that 97.7% had significant growth in *S. mutans*. When isolated *S. mutans* were exposed to biofilm detection by the TCP method, 12.2% showed a high biofilm formation capacity, 18 % Showed moderate ability to form biofilms, while 72.2% showed non/weak ability to form biofilms. This high rate of colonization and biofilm production of *S. mutans* in adult females 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[28]. 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 1). With regard to study females who underwent caries; 120 (45.9%) got score 1, 90 (34.5%) got score 2 and only 39 (14.9%) got score 3, moreover, there was a significant growth in the rate of formation of *S. mutans* biofilms with increasing degree of decay. For example in score 1, 20.8% of *S. mutans* isolated had positive biofilm formation, in score 2, 37.8% of *S. mutans* isolated had positive biofilm formation; and at score 3, 46.2% of isolated *S. mutans* had positive biofilm formation, with significant association OR=2.2, 95% CI=1.1-4.8, X^2=5.5 and p=0.01 (Table 2). 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*), 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)[31-33]. 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[31-33].

Dentists usually prescribe most of the antibiotics used in this study[34]. 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[34]. *β-lactam* antibiotics are the most frequently prescribed chemo prophylactic agent's in general dental practices. In spite of this, penicillin resistance is increased among oral *streptococcus*[35,36]. The number of resistant oral *streptococci* is greater in people who are frequently exposed to antibiotics[37], even though these bacteria can also be established in healthy people who have not been recently treated with antimicrobials[38]. Also, a significant level of penicillin resistance (40%) in S.mutant isolates in current study are higher than those of Al-Shami *et al.*, (14.9%)[36] and Pasquantonio *et al.*, (13.4%)[39] to oral streptococcal clinical isolates. Additionally, current result is comparable to the

average of a 2014 study by Dhamodhar *et al.*,[40] 38% of *S. mutans* isolates showed complete resistance to penicillin and ampicillin. Production of *β-lactamase* is, however, unusual for most of *streptococci*, where resistance is happening by slightly altered of penicillin binding proteins[20-22]. Though, in current study we observed a significant level of amoxicillin resistance [17.6%) of *S. mutans*; and 18.4% 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 become essentially resistant to drugs due to their changed metabolic states and their constitutive up regulation of drug pumps[28].

CONCLUSIONS

The present study proved that *S. mutans* is still the major bacteria isolate from the oral cavity, but few persons might not have significant number of *S. mutans* in 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 penicillin, erythromycin, amoxicillin, clindamycin, and lincomycin in *S. mutans* isolates. Further study is needed to find out the minimum inhibitory concentration of *β-Lactam* and non *β-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 and 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.

ACKNOWLEDGEMENTS

Authors acknowledge the financial support of Sana'a University, Yemen.

AUTHOR'S CONTRIBUTION

This research work is part of a Master's thesis. **Alhasani AH:** writing original draft, clinical, laboratory, field work. **Ishag RA:** methodology, formal analysis, conceptualization. **Yahya Al-Akwa AY:** formal analysis, review. **Al Shamahy HA:** data curation, investigation. **Al-labani MA:** methodology, formal analysis, literature survey. All authors revised the article and approved the final version.

DATA AVAILABILITY

The data supporting the findings of this study are not currently available in a public repository but can be made available upon request to the corresponding author.

CONFLICT OF INTEREST

No conflict of interest associated with this work.

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