Original Research Article

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

ABSTRACT

Objectives: The aim of this study was to consider the possible relationship 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 selected. Clinical examination of females were conducted 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 (penicillin, ampicillin, cefotaxime, amoxicillin, cefazolin and methicillin) and 4 non β-Lactam antibiotics (erythromycin, lincomycin, clindamycin 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. **Conclusion:** 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: Saliva; Dental caries; *streptococcus mutans*; adult females, biofilm formation, oral cavity; antibiotic resistance

INTRODUCTION

Mutans streptococcus has been identified as one of the main causative agents of dental caries[1,2]. Dental surfaces colonized with *S. mutans* are more susceptible to caries [3]. In subpopulations with a relatively high caries experience, a positive association between saliva levels of *S. mutans* and the experience of dental caries [4,5] has been reported. Individuals with high levels of *S. mutans* also develop coronary and root caries in temporary and permanent restorations than individuals in the same population with a lower concentration of *S. mutans* [6,7]. 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[8]. However, there is limited information on the possible relationship between the ability of mutant salivary *streptococcus* to form biofilms and its ratio in the formation of dental plaque. Dental caries has been described as an environmental collision in the mouth, including infectious bacteria and the readily available sugar in drinks and foods. *Streptococcus mutans* has been reported as a major causative agent of dental cavities and normal static plaque [8,9]. 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 established and it appears that bacterial colonization is an important step for oral diseases, which leads to the formation of biofilms [2,10-11]. Oral biofilms predominantly consist of multiple bacterial strains. It has recently been demonstrated that more than 700 bacterial strains are present in dental plaque [12]. The potential mechanism of biofilm formation is that *s.mutant* secretes glucosyltransferase on the cell wall, which allows bacteria to produce polysaccharides from sucrose. These sticky polysaccharides are responsible for the ability 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 adhere to tooth enamel, thus preventing it from reproducing. Studies have shown that Anti-CA-gtf IgY is able to effectively suppress *S. mutans* in the oral cavity [13,14].

The expansion of bacterial pathogens resistance to regular antibiotics use has turned into a general human concern. The spread of antibiotic resistance is causing deaths as well as significant financial inconvenience. In low-economic countries such as Yemen, antibiotic resistance is more prevalent than in developed countries [15]. *S. mutans* is also included as a causative agent of endocarditis. Information about the antibiotic profile of *S. mutans* is of interest for prescribing appropriate treatment in the case of endocarditis [16]. One hour before the dental procedure, the American Heart Association suggests prophylactic antimicrobial therapy for 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[18-20].

Thus, more information is needed regarding 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 present study was conducted in the Department of Medical Microbiology Faculty of Dentistry, Sana'a University. The study protocol was approved by the ethics committee of the Faculty. A written informed consent was obtained from the selected 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 [21] 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

The saliva collection was scheduled after clinical examination. Participants were forced to swallow their pre-existing saliva, in order to clear the mouth of any remaining unstimulated saliva. Then, each participant was asked to chew a standard piece of paraffin wax, for 5 minutes to induce the stimulated saliva needed for collection. The saliva samples of all participants were classified using a code number during the sample collection and processing period.

Microbiological procedure

The sample was transferred to the laboratory immediately after collection with a thioglycolate broth and processed on the same day. The sample was rotated (15 s) and diluted 1: 1000 in isotonic saline before inoculation. One loop (1/1000 ml of sample) was inoculated on Mitis salivarius agar with potassium tellurite medium, bacitracin and 20% sucrose. Plates were incubated at 37 ° C anaerobically. *Mutant streptococci* were detected in 251 (96.2%) saliva samples. The detected *mutant streptococci* were then tested for biofilm formation ability and antibiotic sensitivity.

Biofilm production detection

The detection of biofilm was done by tissue culture method/microtiter plate method (TCA) [22,23]. The *S.mutans* isolates from fresh agar plates were inoculated in 2 mL of Brain heart infusion (BHI) broth and incubated for 24 h at 37°C. The cultures were then diluted 1:40 with fresh medium (BHI broth supplemented with 1% glucose); 200 µl of the sample was dispensed in the individual microtitration plate and incubated further 24 h at 37°C anaerobically. With a gentle tapping, the content was removed

further with a subsequent washing with phosphate buffer saline (pH 7.2) three times to remove free floating sessile bacteria. The adherent bacteria, biofilm producer, were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v) for 10–15 min. The unbound crystal violet solution was removed with a triplicate washing with PBS, and the plate, then, was kept for drying. Finally, all wells were filled with 200 µl of ethanol (95%) to release dye from the well and Optical Density (OD) was performed at the wavelength of 630 nm. OD value of each test strain and negative control were calculated, and OD cutoff values (ODc) were assessed as described previously[23] .

Antibiotic sensitivity

The antibiotic susceptibility profile was determined by disc diffusion method. The inoculums were adjusted to match the turbidity of 0.5 McFarland standards, and was swabbed on Brian heart infusion agar and allowed to dry for 10min [24]. The antibiotics employed in this study were: penicillin-G (P) 10 units, ampicillin (AMP) 10µg, cefotaxime (CTX) 30µg, erythromycin (E) 15µg, cefazolin (CZ) 30µg, methicillin (MET) 5µg, lincomycin (L) 2µg, clindamycin (CC) 2µg and vancomycin V (30µg) (Oxide, USA). Inhibition zone was measured after 24h of anaerobically incubation at 37 °C. The experiments of each antibiotic were performed in triplicate. The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) methodology[25].

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). 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 positve 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).

DISCUSSION

Biofilms are recognized for their formation on many implanted medical devices, including catheters, pacemakers, heart valves, dentures, and artificial joints, which provide a superficial and safe haven for the growth of biofilms[26]. The consequences of a device-related infection on human health can be severe and life-threatening [27]. 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 our 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 in general (including bacterial and fungal biofilms) are responsible for more than 80% of all microbial infections [28]. For structural and physiological reasons, biofilms are inherently resistant to antimicrobial therapy and host

immune defenses. Biofilms cause many infections, ranging from superficial mucosal infections to severe and widespread 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 assocation 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) [29-31]. 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 [29,31].

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 [32]. *β-lactam* antibiotics are the most commonly prescribed chemo prophylactic agent's in general dental practices. In spite of this, penicillin resistance is increased among oral *streptococcus* [33, 34]. The number of resistant oral *streptococci* is greater in people who are frequently exposed to antibiotics[37], although these bacteria can also be found in healthy people who have not been recently treated with antimicrobials[36]. Also, a significant level of penicillin resistance (40%) in S.mutant isolates in our study are higher than those of Al-Shami *et al.* (14.9%) [34] and Pasquantonio *et al.* (13.4%) [37] to oral streptococcal clinical isolates. Additionally, our result is comparable to the average of a 2014 study by Dhamodhar *et al.* [38] 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[18-20]. Though, in our 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] .

CONCLUSION

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. Our study demonstrates significant levels of penicillin, erythromycin, amoxicillin, clindamycin and lincomycin-resistance in *S. mutans* isolates in adult females. Further study is required to know 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 also, call for improved the assessment of antibiotic susceptibility testing during prophylaxis. The alternative of antibiotic such as herbal extract is most likely preferable for the coming years to avoid the upcoming bacterial resistance to the antibiotics. In addition, the rise in the rate of antibiotic resistance in *S. mutans* isolates suggested taking extra precaution while prescribing antibiotics will maintain the bacteria with less resistance.

AUTHOR'S CONTRIBUTION

This research work is part of a Master's thesis. The candidate is Arij Lutf Abdulrhman Abdul Majeed to conduct clinical, laboratory, field work and thesis. Corresponding author (HAA), and other authors supervised the work, revised and edited the thesis draft and the manuscript.

ACKNOWLEDGMENTS

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

CONFLICT OF INTEREST

No conflict of interest associated with this work.

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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 index 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

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