ANALYSIS OF BIOFILMS FOR *STREPTOCOCCUS MUTANS* FROM DENTAL ROOT SURFACES OF ADULT PATIENTS WITH ROOT CARIES.

ABSTRACT

Background and objectives: Knowledge of the pathogenicity of the primary etiological factor of root caries, the microbial biofilm, might provide important information for the development of diagnosis and treatment strategies. This study evaluated the numbers and determined the proportion of *mutans streptococci* , which is possible relevant cariogenic organisms, in biofilms recovered from lesions at root surfaces with active caries lesions (ARC), inactive caries lesions, and sound root surfaces (SRS).
Material and methods: Samples were cultured in MSB agar for *mutans streptococci* counts, and brain-heart infusion agar for total viable anaerobic counts. After incubation, the number of colony-forming units (CFUs) was determined and compared between groups by the Mann-Whitney U test with a significance level set at 95%. The proportion of counts of *mutans streptococci* in the total viable microorganisms was also analyzed by Chi-square test. 108 samples (36 from each surface) from 36 patients were cultured and analyzed. **Results:** The mean ± SD for the counts of active root caries lesions was 7.47 ± 9.89 10, significantly higher than that of inactive root caries lesions (2.5 ± 0.97) and sound root surfaces (3.03 ± 0.71 10). In conclusion, a trend towards higher counts was evident for ARC. In the ARC lesions among the dominant oral anaerobic bacteria, we could not identify *streptococcal* colonies (unspecified) in 11% while in IRC lesions it occurred in 47%, and SRS it occurred in 47%. In addition, in ARC the samples were ≥0.1-≥10 (CFU x10) ≥0.1-≥10 colonies of Streptococcus mutans. C**onclusion**: In conclusion, a trend towards higher counts was evident for ARC and for most samples, the proportion of *Streptococcus mutans* was low relative to the viable number of total viable anaerobic microorganisms.

Keywords: Root caries. Bacterial Load, *Streptococcus mutans*

INTRODUCTION

The mouth inhibited by a variety of oral bacteria, but only a few types of bacteria are believed to cause tooth decay: among them, *Streptococcus mutans*. *Streptococcus mutans* is a Gram-positive bacteria that forms biofilms on the surface of the teeth. These organisms can produce high levels of lactic acid after fermentation of dietary sugars and are resistant to the harmful effects of low pH, which are essential properties of dental caries-causing bacteria[1,2]. Because root surfaces demineralized more readily than enamel surfaces, a variety of bacteria can cause root decay, including *Streptococcus mutans*, *Lactobacillus acidophilus*, *Actinomyces* spp., and *Nocardia* spp.. Bacteria gather around the teeth and gums in a sticky, cream-colored mass called plaque, which acts as a biofilm. Some places gather plaques more frequently than others, e.g. sites with a low rate of saliva flow as in molar fissures. channels on the occlusal surfaces of the molar and premolars teeth offer microscopic retention places for plaque bacteria, the same as do the interstitial places. Plaque may also collect above or below the gum, where it is referred to the same as supra- or subgingival plaque, respectively [1,2]. Improvements in dental health care globally have led to a reduction in tooth loss. As for Yemen, in the year 2019 [3,4], many researches indicated erosion of the gums, and this led to an increase in the number of exposed root surfaces prone to decay. The high prevalence and restoration of this type of cavities poses challenges with regard to the lack of restorative materials that bind well to dental tissues, so dental root caries has become an important problem in dentistry [3, 4].

It is very important that new and effective preventive treatment strategies are required to avoid tooth extraction and maintain oral health. In this regard, knowledge of the pathogenesis of the primary causative agent of root caries, the microbial biofilm, may provide important information for the development of diagnostic and treatment strategies. Of the main factors that contribute to the modulation of germs and the risk of root caries, gum recession, reduced saliva flow rate, and the use of xerostomic medicines are the most important factors, mainly in older adults [2,5]. On the other hand, the current understanding of the bacterial composition of root caries is limited compared to other oral diseases[2,6,7]. Many studies have attempted to identify the pathogenic species that cause root decay [2,8-14], but there is no consensus on naming and identifying the germs associated with root decay in the world due to the limited researches in this aspect.

Separate patterns and individual changes in microbial composition were observed, despite the marked predominance of *Actinomyces* spp. As links it as a potential agent of root decay. However, the *Actinomyces* spp. recently demonstrated to be highly metabolically active in root surface and intact root surface (SRS) biofilms [15], indicating that these organisms are more site-related rather than dysbiosis. Some studies have also suggested a role for *Streptococcus mutans* and *lactobacilli* in root decay.

The association between these species approved an increased risk of root surface caries when it occurred they were existing together [12]. Most of the relevant culture-based studies showed a strong relationship between root caries and *Streptococcus mutans* due to higher and/or higher isolation frequency percentage of carious root surfaces[14,16,17]. Nevertheless, these studies were developed prior to the 1990s, and there are only a few studies discussing this topic recently [2], and no contemporary studies have looked back at root decay bacteria. While there is evidence that the microbiota of root surface caries lesions changes with lesion activity, few studies have characterized the microbiota of active and inactive root caries lesions[14, 17.]

Investigation and survey of dental health problems in Yemen despite everything is small and somewhat limited, although there are studies that dealt with the problems of dental caries, periodontal infections, causes of permanent tooth extraction, and the prevalence and pattern of third molar impaction in adults and children[18-26] but no researches into the association of *Streptococcus mutans* with root caries with different caries activity. Hence, the aim of this study was to evaluate the numbers and determine the proportions of *Streptococcus mutans*, which could be a related cariogenic, in root caries lesions with different caries activity.

MATERIALS AND METHODS

Patients: This study included 36 patients who tested positive for active root caries (ARC) and inactive root caries (IRC), who were admitted to the dental clinic of the Faculty of Dentistry, Sana'a University, Sana'a, during a six-month period, which started in January 2021 and it expired in June 2021, the time the Faculty of Dentistry provided for this study. The sample size was 36 patients, whose ages ranged from 21 to 58 years (median 38 years).The inclusion criteria consist in presenting at least one active root caries lesion, one inactive root caries lesion and another root surface without a caries lesion (sound).

Definition:

Active root caries lesion (ARC): lesions not resistant for probing, light-brown to light-yellow color, cavitated or not cavitated, and opaque.

Inactive root caries (IRC): resistant for probing, brown to black in color, and shiny.

These patients were not a part of any caries control program and did not control their dietary intake. The patients do not receive any instruction of oral hygiene.

Sample collection:

Dental plaque biofilms were collected from different sites from the same patient. The first was a root surface with an active caries lesion (ARC); the second is a root surface with an inactive caries lesion (IRC); the third had SRS as a control in the same ARC or IRC patients. Biofilm samples were collected in the morning, after drying and isolation, with cotton rolls using a number 17 sterile dentin excavator were immediately transferred to a sterile container containing 1 mL of reducing transport fluid medium. The amount of biofilm collected corresponds to a complete dentin borer. Samples were kept on ice and processed within 2 hours. Cultivation was performed in the Microbiology Department of the National Center for Public Health Laboratories (NCPHL) Sana'a, Yemen.

Microbiological procedures

Samples were vortexed with glass beads for 60 seconds and 5-fold serially diluted in 0.005 M potassium phosphate buffer (pH 7.3). Subsequently, 25 µL aliquots of 0 to 10^{-3} were cultured in duplicate on the MSB agar (Difco) supplemented with 20% sucrose, 0.2 units/mL bacitracin, and 1% potassium telurite for *streptococci mutans* counts. MSB plates were incubated under microaerophilic conditions at 37 °C for 48 hours. The brain-heart infusion agar (Difco) supplemented with 4% blood and enriched with k-hemin vitamin (BHI) for total viable anaerobic microorganisms counts. BHI agar plates were cultured anaerobically (Gas Packed anaerobic system), at 37 °C for 120 hours. After incubation, the number of colony-forming units (CFUs) was determined. The counts derived from the selective media included only colonies with the relevant characteristic morphology. In case of doubt, two or three representative colonies from each culture medium were selected for Gram staining and biochemical activities.

Statistical analysis

The quantity of count of *streptococci mutans* in the total viable microorganisms was investigated by Chi-square test. The bacterial counts are expressed at $\log^{10}$ and the constant 1 was added to the CFUs.

To compare the counts of *streptococci mutans* in ARC, inactive root caries, and SRS biofilms, the Mann-Whitney U test was used. The significance level was set at 5% for both tests.

Ethical approval

The written consent in all cases were obtained. Approval was obtained from the participants prior to including in the study. Ethical approval was obtained from the Medical Research and Ethics Committee of the Faculty of Medicine and Health Sciences, Sana'a University with reference number (2001) on 01/01/2021.

RESULTS

Table 1shows the counts of *Streptococcus mutans* (CFU $\log^{10}$) cultivated in selective media from biofilms from active root caries lesions(ARC), inactive root caries lesions(IRC), and sound root surfaces (SRS)from 36 root Caries patients. The mean ±SD for counts of the active root caries lesions was 7.47 ± 9.89^{10} and the counts ranged from $1\text{-}49^{10}$ with 95% margin of error equal to ±43.2%. The mean ±SD for counts of the inactive root caries lesions was 2.5 ± 0.97^{10} and the counts ranged from $1\text{-}37^{10}$ with 95% margin of error equal to ±63.9%. The mean ±SD for counts of the sound root surfaces was 3.03 ± 0.71^{10} and the counts ranged from $1\text{-}24^{10}$ with 95% margin of error equal to ±46.1%. The variance in the numerical amount of bacteria between the three sites was statistically significant with $p < 0.01$. In conclusion tendency towards higher counts was evident for ARC. For active root caries lesions, a 95% confidence interval with a margin of error of 43.2% means that our statistics would be within 43.2% points of the true count value of *S.mutans* in root caries patients. Table 2shows the proportion of *mutans streptococci* of the total viable anaerobic microorganisms counts (total CFU) cultured from biofilms from active (ARC) root caries lesions, inactive (IRC) root caries lesions and sound root surfaces (SRS). In active root caries lesions among the dominant oral anaerobic bacteria, we could not or would not identify streptococcal colonies (not determined) in 11% of the total samples, while in 33.3% of samples counts of *streptococci mutans* colonies was ≤ 0.1 and 55.6% of samples counts of *streptococci mutans* colonies was ≥0.1-≥10 (CFU x^{10}). Inactive root caries lesions among the dominant oral anaerobic bacteria, we could not or would not identify streptococcal colonies (not determined) in 47% of the total samples, while in 50% of samples counts of *streptococci mutans* colonies was ≤ 0.1 (CFU x^{10}) and only 3% of samples counts of *streptococci mutans* colonies was ≥0.1-≥10 (CFU x^{10}). In sound root surfaces among the dominant oral anaerobic bacteria, we could not or would not identify *streptococcal* colonies (not determined) in 16% of the total samples, while in 78% of samples counts of *streptococci mutans* colonies was ≤ 0.1 (CFU x^{10}) and only 6% of samples counts of *streptococci mutans* colonies was ≥0.1-≥10 (CFU x^{10}). In conclusion, for most of the samples the proportion of *mutans streptococci* were low relative to the viable count of total viable anaerobic microorganisms. While in ARC 55.6% of samples counts of *streptococci mutans* colonies was ≥0.1-≥10 (CFU x^{10}).

DISCUSSION

Dental plaque was collected from the root surfaces of three different groups in the same patient (ARC, IRC, SRS) and cultured in media supporting the growth of *Streptococcus mutans*, in which the relevant organisms were optimized to be associated with root caries. These bacteria are considered the most studied in dental caries, but the study of their relationship and spread in root surfaces with active or inactive caries, as well as natural root surfaces is limited. In this study there were differences in the cultured microbiota of active or inactive lesions where the mean ± SD of active root caries lesions counts was 7.47 ± 9.89^{10} significantly higher than that of inactive root caries lesions (2.5 ± 0.97^{10}) and sound root surfaces (3.03 ± 0.71^{10}). The results indicate that the microenvironment can be both in dysbiosis in the patient and in the lesions.

A higher quantity of *Streptococcus mutans*, particularly in active root surfaces, was found in the results of the current study. This is emphatic evidence for the relationship between the proliferation of a predicted cariogenic species (heavy colonization) with root decay, the literature showing a very low or very variable proportion of this species in other culture-based studies, with the collective proportion of *S. mutants* 10% of the total cultivable bacteria [2, 12]. Ellen and colleagues found that including *Lactobacillus* and *Veilonella* raises the value to only 20% of the total cultivable microbiota [27]. Also Van Houte *et al.* found that total *Streptococcus* + *Enterococcus* + *Actinomyces* + *Lactobacilli* account for 47% of the total cultivable microbes in SRS biofilms, 60.1% in non-hollow root lesions, and 63% in hollow lesions [28]. In this study, the mean ± SD of active root caries lesions count was 7.47 ± 9.89^{10}, significantly higher than that of inactive root caries lesions (2.5 ± 0.97^{10}) with 55.6% of the ARC sample populations for *streptococcal mutans* colonies were ≥ 0.1-≥10 (CFU x^{10}) of total bacteria, indicating a more complex composition of dental plaque in active or inactive root caries lesions. Thus,

we can speculate that these organisms are related pathogens whose activity is important for disease progression, and are present as a low percentage of the total population of normal oral flora[2, 27, 28].

Emilson et al. found a relationship between heavy colonization of Streptococcus mutans and an increased risk of root surface caries [12]. Emilson *et al.* [12] showed that subjects with >5 x 10^5 *Streptococcus mutans* per mL of saliva had approximately five times more superficial root lesions than subjects with low or free of these bacteria. The current observation of lower counts of *Streptococcus mutans* in SRS compared with higher counts in ARC (Table 3) is consistent with the results reported by Beighton *et al*. [14], which showed that the higher activity of the lesions led to an increase in the population of total anaerobes, gram-positive rods (*Bifidobacteria*), *Streptococcus mutans*, and *lactobacilli* [14].

Many advances in understanding dental caries are currently related to culture-independent methods. These tools of molecular biology have greatly contributed to the determination of the composition and diversity of microorganisms. Among the advantages of molecular techniques for characterizing oral biofilms, the most important is the evaluation of underestimated non-culturable microorganisms in oral biofilms. Despite the strong relationship of *S. mutans* and root caries microbiota shown in culture-based studies, some studies that used the culture-independent approach did not show any significant difference in the prevalence of *S. mutans* between healthy and different stages of coronal caries [29,30].

Suggesting that the virulence of *S. mutans* is tightly controlled by the presence of health-associated competitors [31]. Nevertheless, Preza *et al.*[6] described a root caries microbiota dominated by *S. mutans* , *Actinomyces* spp., and others. *Lactobacilli* were absent and *mutans streptococci* were rarely observed, while *Actinomyces* sp. were present in 50% of the healthy root surfaces samples[6]. A study by Chen *et al.* using 454-pyrosequencing confirmed that *S. mutans* and *Lactobacillus* spp. are more likely to be root caries pathogens than are other species[7]. Suggesting that the virulence of *S. mutans* is tightly controlled by the presence of health-related competitors [31]. However, Preza *et al.* [6] described a root decay bacteria dominated by *S. mutans, Actinomyces* spp. , and others. *Lactobacilli* were absent and *Streptococcus mutans* was rarely observed, while *Actinomyces* sp. were present in 50% of healthy root surface samples[6]. A study by Chen *et al*. using 454-pyrosequencing confirmed that *S. mutans* and *Lactobacillus* spp. are more likely to be root caries pathogens than are other species[7].

Finally, it is important to acknowledge the limitations of culture-based studies, although we believe that these studies are still reliable for identifying viable organisms and examining species that are already associated with diseases. DNA-based studies have explored the full microbial classification in the caries lesion, but can overestimate some species since DNA from dead or transient cells can be present, and this may be the reason for the difference between the results of these studies.

CONCLUSION

In conclusion, a trend towards higher counts was evident for ARC and for most samples, the proportion of *Streptococcus mutans* was low relative to the viable number of total viable anaerobic microorganisms.

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CONFLICT OF INTEREST

No conflict of interest associated with this work.

AUTHOR'S CONTRIBUTIONS

All authors co-wrote the articles and reviewed the results. Clinical and field works were performed by Khaled Al-gafari, Hamzah Al-Sharafi and Hassan Abdel-Wahab Al-Shamahy.

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Table 1: Counts of *mutans streptococci* (CFU log^{10}) cultivated in selective media from biofilms from active root caries lesions(ARC), inactive root caries lesions(IRC), and sound root surfaces (SRS)from 36 root Caries patients.

Sites of specimens	Counts of *mutans streptococci* (CFU log10)				
	Mean	SD	P value	Range	95% Margin of error #
Active root caries lesions	7.47	9.89	<0.01*	1-49	7.4±3.3 (±43.2%)
Inactive root caries lesions	2.5	0.97	<0.01*	1-37	2.5±1.6 (±63.9%)
Sound root surfaces	3.03	0.71	Reference	1-24	3.03±1.39(±46.1%)

*Mann-Whitney U Test

A **margin of error** tells, how many percentage points your results will differ from the real population value.

Table 2: Proportion of *mutans streptococci* of the total viable anaerobic microorganisms counts (total CFU) cultured from biofilms from active (ARC) root caries lesions, inactive (IRC) root caries lesions and sound root surfaces (SRS).

Total CFU x^{10}		Active root caries lesions	Inactive root caries lesions	Sound root surfaces
Not determined	No	4	17	6
	%	11	47	16
≤ 0.1	No	12	18	28
	%	33.3	50	78
≥0.1-≥10	No	20	1	2
	%	55.6	3	6