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Original article

Real-Time PCR analysis of microbial composition in white spot lesions associated with fixed orthodontic appliances

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ABSTRACT

Background: Orthodontics is a specialty of dentistry that deals with the diagnosis, prevention, and correction of malpositioned teeth and jaws. It is designed to correct oral health issues and incorrect jaw position, as well as improve the appearance of the teeth and face. Fixed orthodontic appliances are non-removable; hence, the presence of nooks and crannies encourages the accumulation of food and plaque, which makes oral hygiene challenging and even leads to dental pathologies. **Objective**: The study's objective is to molecularly analyze of micro-organisms in white spot lesions caused by fixed orthodontic appliances. **Method**: A total of 100 individuals, including 50(50%) patients and 50 (50%) controls, 36 males and 64 females, aged 15-35 years, underwent fixed orthodontic treatment for those measuring the microbial load in dental plaque around the bracket for at least one month after activation. **Results**: The findings showed that the case group had higher significant difference as opposed to the control group with *p* value = 0.002 in the count and *p* value = 0.003 in presence of *Streptococcus mutans*, which determined by real-time PCR .**Conclusion**: In contrast to the control group, which is free of WSLs, case group had a higher load and existence of *Streptococcus mutans*..

Introduction

Orthodontics is a branch of dentistry that specializes in treating and correcting misaligned teeth and jaws, this may include utilizing orthodontic devices such as braces to straighten teeth and/or to correct any difference in how the teeth bite together.

Orthodontic treatment has become increasingly popular in recent years. However, one significant issue affecting dental aesthetics is the appearance of white chalky spot , known as white spot lesions (WSLs), which often develop after treatment [1].

The mineral loss is because of the acidogenic bacteria, leading to the formation of WSLs, which are subsurface enamel porosities, especially on smooth surfaces[2]. Orthodontic appliances, comprising brackets, bands, molar tubes, and wires, are niches for plaque accumulation, leading eventually to enamel breakdown [3,4] .

The cause of dental caries is due to the formation of bacteria in the biofilm, Specific bacterial species, which make the acid the main cause of caries; Successful management requires early detection and interventions, which aim at arresting or reversing the carious process and maintaining tooth form and function [5,6]. The emergence of early enamel decay is characterized by the acidic degradation of enamel rods and the subsequent expansion of enamel sheath. The disorder of enamel hypoplasia can impact the

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tooth's surface, leading to the development of pits and grooves, resulting in a thinner layer or complete absence of enamel [7]. Under dry conditions, initial carious lesions are observable but virtually cease to exist when the enamel is wet; on the other hand, hypocalcification remains noticeable regardless of hydration status [8].In the presence of fixed orthodontic appliances and plaque accumulation, the oral microbiome is significantly changed with an increased number of acidogenic bacteria, mainly *Streptococcus mutans* (*S.mutans)*, which is the main cause of WSLs, subsequently leading to the decreased PH levels . [9,10] .The relationship between *Streptococcus mutans* and the development of dental caries is intricate. From a traditional perspective, several key traits of *S. mutans* play pivotal roles. These include its capacity to adhere to tooth enamel, its acid-producing ability (acidogenicity), its resilience in acidic environments (aciduricity), and its proficiency in generating extracellular polysaccharides. Collectively, these characteristics enable *S. mutans* to create a localized environment that promotes enamel demineralization and facilitates the progression of caries [11] . Due to the massive role of *Streptococcus mutans* in the initiation of dental caries, the study goal was to evaluate the microbiota of WSLs in orthodontic patients.

Materials and Methods

Study design and patients

This study is a case-control study conducted to compare individuals with WSLs to healthy controls. A Total 100 study samples, (50 case; 50 control), with age ranging from 15-35 years, a dental plaque was obtained from both groups after at least 4 weeks of fixed orthodontic installment near the area of orthodontic bracket. All specimens were obtained from Babil specialized dental center during the time span of the study Between November 2024 and March 2025.

Specimens Collection Methods

The samples were taken from patients who had fixed orthodontic appliances. Following the protocol of collection dental plaque, samples were taken for at least two hours after eating or drinking in the morning. A sterile cotton swab was used to collect the dental plaque around the orthodontic bracket. Each sample then preserved in 5 mL of phosphate buffer saline (PBS) and frozen -20 C.[12]

Genomic identification of *Streptococcus mutans*

Statistical analysis

All statistical analyses were conducted using Microsoft Excel 2016 and IBM SPSS Statistics version 26. The Chi-square test of independence was applied to evaluate associations between categorical variables like presence or absence of *S.mutans*. A p-value of < 0.05 was considered statistically significant throughout all analyses.

Results

The study includes 100 individuals with fixed orthodontic appliances ;technically, seven samples have been destroyed ,subsequently resulted in 47 cases and 46 controls comprising 32 males,61 females *p* value = 0.298 . The control group samples were collected through swabs from individuals free of WSLs with fixed orthodontic appliances, whereas the case group with WSLs. The samples have been diagnosed by real- time PCR. In this study found that there was no significant correlation between age and *S. mutans* copy number of *p* value =0.871.The sex been also had been investigated which had no significant correlation to *S. mutans* presence and copy number. The presence of *S. mutans* is significantly higher in the case group compared to the control group of *P* value = 0.00253, suggesting a strong association with WSLs. The concentration and purity of extracted DNA were assessed using a nanodrop spectrophotometer. DNA bands were validated by gel electrophoresis. The results showed that twenty-four positive samples exhibited a detectable gene, represented by 144 bp as shown in figure 5. The gel was captured using gel documentation imaging figure5. The copy number of *S. mutans* is markedly elevated in the case group (16,120.47) compared to the control (1,591.4), which is statistically significant *P* value = 0.00333. This suggests that *S. mutans* may play a key role in WSLs.

Table1. Primer sequence of *Streptococcus mutans*

Primer	**Sequence(5-3)**	**product**
16S r RNA *Streptococcus mutans*	Smf GCCTACAGCTCAGAGATGCTATTCT Smr GCCATACACCACTCATGAATTGA	144bp [13]

Table 2. Master mix for real time PCR.

No	Contents of reaction mixture	concentration	volume
1-	SYBER green I	2.5x	20 µl
2-	Forward primer	10pmol/ µl	1 µl
3-	Reverse primer	10pmol/ µl	1 µl
4-	Mgcl2	25mM	1 µl
5-	DNA sample	10-20 ng/ µl	2 µl
6-	Nuclease free water		25 µl
7-	Total volume		50 µl

Table3. Real time PCR reaction

Step	Stage	Temperature	Time	No. of cycle
1	Initial denaturation	95 °C	1 min	1
2	DNA denaturation	95 °C	10 sec	40°C
3	Primer annealing	55 °C	30 sec	
	Extension and(reading on green channel)	72 °C	30 sec	
4	Melt	60-95 °C	5 sec	

Table4. Distribution of *Streptococcus mutans* concentration

status	negative	positive	p-value	Mean CN	STD CN	p-value CN
case	28	19	0.0025	16120.47	18714.07	0.0033
control	41	5		1591.4	714.05	

Figure1. Identification of *S.mutans* by real-time PCR A- Amplification curve, B- Melting curve.Red colour: positive, blue colour: negative.

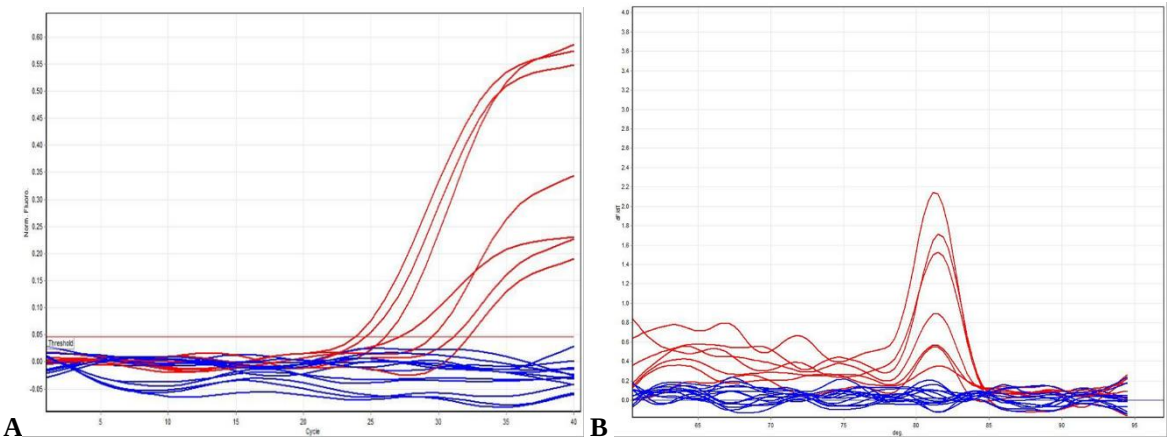


Figure 2. Sex and age Distribution

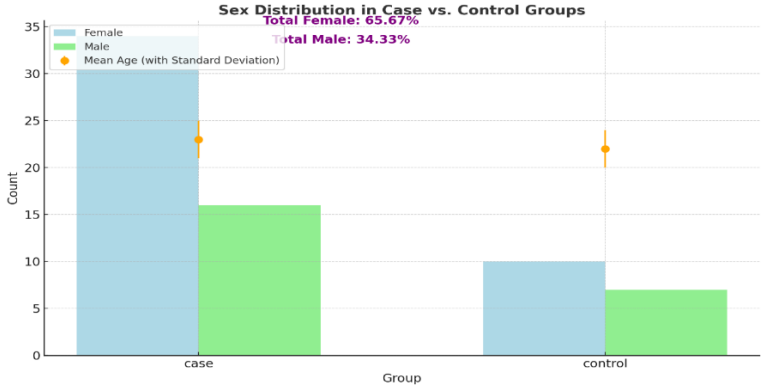


Figure 3. *S. mutans* copy number by status

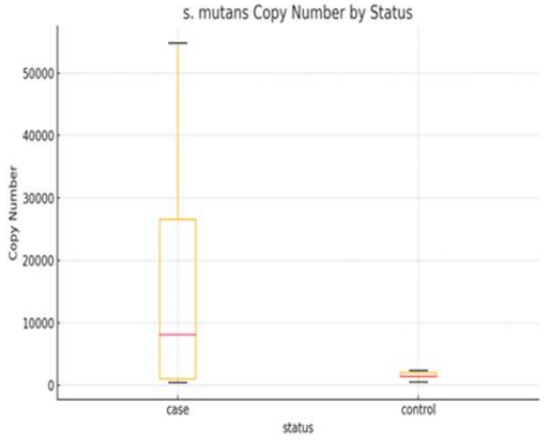


Figure 4. *S. mutans* presences by status

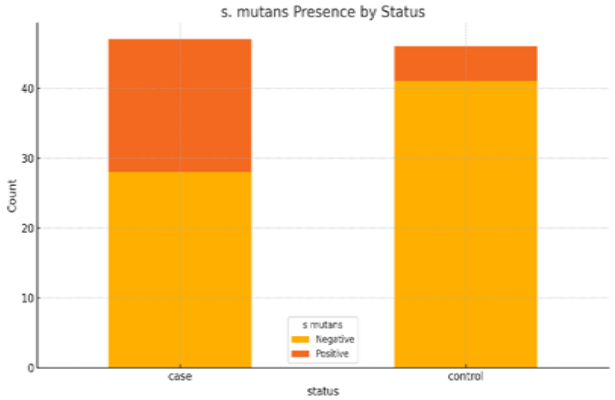
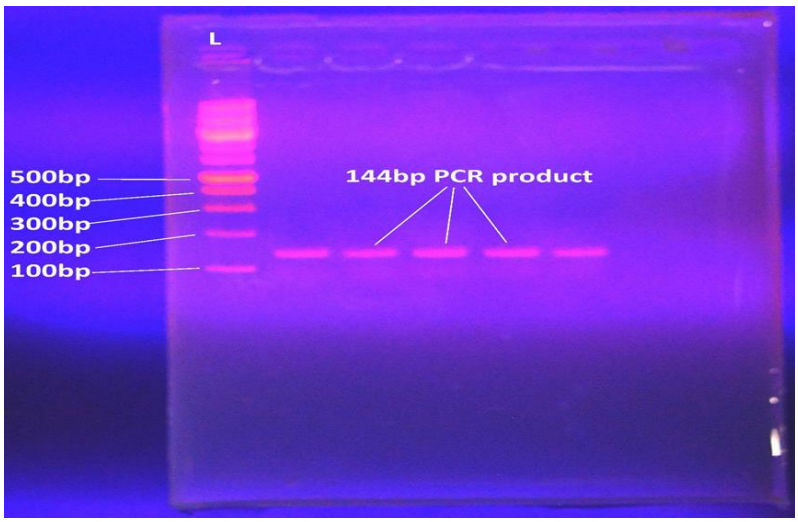


Figure 5. 2% Agarose gel electrophoresis at 100 volts for 50 min for SpaP gene PCR products visualized under ultraviolet light after staining with ethidium bromide ;Lane|1000] were positive for this gene SpaP of *Streptococcus mutans*, the size of product is144 bp molecular weight fragment.



Discussion

The results of this study showed that the prevalence of females is 65.67% in comparison of males is 34.33%, as illustrated in figure2, with a *P* value = 0.298, reflecting that there is no significant association between orthodontic treatment and sex. These outcomes are in good alignment with [14,15], supporting the notion that sex does not significantly influence the need of orthodontic treatment. In this research, the samples were selected from individuals aged 15 to 35 because most of their permanent teeth were fully developed. This selection aligns with the findings of Olkun and Sayar [16] and partially agrees with [17] who observed that the age of patients ranged from 13–35 years. Because there has been a constant pressure on teeth over many years, many orthodontic issues can be addressed in adults, children and teens alike. Although adult bone is denser than child bone, contributing to slightly longer treatment time and fewer orthodontic cases in patients of an older age group, the impact of age groups on the orthodontic number of cases seems to be irreversible, this finding our study revealed and support the conclusion of [18] which noted no differences in the overall interval of treatment with fixed appliances between adults and adolescent.

WSLs are a common problem in orthodontic treatment, primarily occurring due to demineralization of the enamel around orthodontic brackets. The prevalence of WSLs has been widely researched, with several studies inspecting the possible differences between male and female patients, in our study we found no significance correlation in WSLs between male and female with case-gender p value = 0.12 ,control gender *p* value = 0.67 ,and that agrees with [19] [20,21].but [22] reported that male patients exhibited increased in severity of enamel opacities compared to female patients;. But this apparent discrepancy did not reach statistical significance.

This study found that the number of *Streptococcus mutans* is highly significant in number with case group than control group as shown in figure3, of *p* value = 0.003 and this finding is consistent with the finding of [23] and [24] also agreed the fact that orthodontic appliances have been found to cause significant changes in dental plaque, such as increasing the amount of *Streptococcus mutans* along with other bacterial species and lowering the PH of biofilm. In this study was found that the presence of *Streptococcus mutans* in the case group is statistically significant that control group as present in figure4 with *p* value = 0.002 that Increased occurrence and quantities of cariogenic streptococcal species following the application of orthodontic appliances has been well-documented with [25].In this study revealed that no significant correlation between *Streptococcus mutans* copy number and age with *p* value = 0.87,This suggests that *S. mutans* count , do not reliably predict caries severity or experience across different age groups that agreed with [26,27]

Conclusion

There was an over-representation of females in this study sample. Patients aged less than 20 years were predominant compared to other age groups. *Streptococcus mutans* copy number and presence determined by real time PCR were significantly higher in patients with WSLs compared to the control group.

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Ethical approval

The study was performed in line with the ethical principles of the Helsinki declaration. Written and verbal consents were obtained from the patients before sampling. The study protocol and the subject information and consent documents were approved by a local ethics committee (Ref NO.72 on June 1,2025).

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Nil.

Conflict of Interest

The authors declare that there is no competing interest.

Data availability

Data available on request

Authors' contribution

The first author conceived and designed the study, acquired the data, and drafted the manuscript. The second and third authors contributed to the critical revision of the manuscript for important intellectual content. All authors read and approved the final version of the manuscript..

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