Original Article

Changes in salivary periodontal pathogens after orthodontic treatment: An in vivo prospective study

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ABSTRACT

Objective: To analyze the initial changes in salivary levels of periodontal pathogens after orthodontic treatment with fixed appliances.

Materials and Methods: The subjects consisted of 54 adult patients. The Simplified Oral Hygiene Index, Plaque Index, and Gingival Index were measured as periodontal parameters. Both the plaque and gingival indexes were obtained from the central and lateral incisors and first molars of both arches. Whole saliva and periodontal parameters were obtained at the following four time points: immediately before debonding (T1), 1 week after debonding (T2), 5 weeks after debonding (T3), and 13 weeks after debonding (T4). Repeated measures analysis of variance was used to determine salivary bacterial levels and periodontal parameters among the four time points after quantifying salivary levels of *Aggregatibacter actinomycetemcomitans* (Aa), *Fusobacterium nucleatum* (Fn), *Porphyromonas gingivalis* (Pg), *Prevotella intermedia* (Pi), *Tannerella forsythia* (Tf), and total bacteria using the real-time polymerase chain reaction.

Results: All periodontal parameters were significantly decreased immediately after debonding (T2). The salivary levels of total bacteria and Pg were decreased at T3, while Pi and Tf levels were decreased at T4. However, the amount of Aa and Fn remained at similar levels in saliva during the experimental period. Interestingly, Aa and Fn were present in saliva at higher levels than were Pg, Pi, and Tf.

Conclusion: The higher salivary levels of Aa and Fn after debonding suggests that the risk of periodontal problems cannot be completely eliminated by the removal of fixed orthodontic appliances during the initial retention period, despite improved oral hygiene. (*Angle Orthod.* 2016;86:998–1003.)

KEY WORDS: Debonding; Orthodontic treatment; Periodontal pathogen; Saliva

INTRODUCTION

Gingival inflammation and enlargement are the common side effects of orthodontic treatment.[1] New retentive places for oral bacteria after the placing of fixed appliances are considered the main factors in increasing plaque accumulation and gingival disturbances.[2] Most periodontal pathogens, such *as Aggregatibacter actinomycetemcomitans* (Aa), *Fusobacterium nucleatum* (Fn), *Prevotella intermedia* (Pi), *Porphyromonas gingivalis* (Pg), and *Tannerella forsythia* (Tf), which are strongly related to gingival inflammation and periodontal destruction, are significantly increased in patients after bracket placement.[3] Because periodontal problems are associated with aging and the recent development of esthetic orthodontic appliances placed in adult patients, it is important to understand variations in the pathogenic bacterial levels in adult orthodontic patients.

Previous studies have shown positive associations between the levels of periodontal pathogens in saliva

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Table 1. Demographic Information on the Subjects in This Study

	Brackets		
Sex	Clarity SL	Clippy-C	Damon Q
Male (n = 20)	21.8 ± 2.1 (n = 4)	24.2 ± 4.0 (n = 9)	21.9 ± 3.0 (n = 7)
Female (n = 34)	25.7 ± 7.1 (n = 14)	21.5 ± 3.5 (n = 15)	25.6 ± 12.1 (n = 5)
Total (n = 54)	24.8 ± 6.6 (n = 18)	22.5 ± 3.9 (n = 24)	23.4 ± 8.2 (n = 12)

and in subgingival plaque[4] and between the detection of periodontal pathogens in the saliva and the degree of gingival inflammation.[5] Considering that saliva collection is a simple, safe, economical, and non-invasive method, it should be a useful medium for monitoring oral pathogen levels during orthodontic treatment.

Advances in molecular techniques have increased the detection of periodontal pathogens. Recently, polymerase chain reaction (PCR) tools have been introduced into periodontal research because of their higher sensitivity and specificity compared with the classical culturing procedures. In particular, real-time PCR is simple, rapid, and useful for detecting uncultured or extremely anaerobic microorganisms.[6]

Many studies have reported quantitative changes in bacterial levels related to orthodontic treatment and periodontal pathogens related to oral hygiene during orthodontic treatment.[7,8] However, few studies have investigated quantitative changes in periodontal pathogens after orthodontic treatment, specifically in saliva. The aim of this in vivo prospective study was to analyze the changes in the salivary levels of Aa, Fn, Pi, Pg, and Tf after orthodontic treatment with fixed appliances using quantitative real-time PCR.

MATERIALS AND METHODS

The study population initially consisted of adult patients who finished orthodontic treatment with fixed appliances. Inclusion criteria at the starting point of this experiment were (1) age greater than 19 and 17 years in males and females, respectively, (2) permanent dentition of more than 24 teeth, (3) a longer than 12-month treatment period, and (4) use of the following three bracket types with a 0.022-inch slot: Clarity SL (3M Unitek, Monrovia, Calif), Clippy-C (Tomy, Tokyo, Japan), and Damon Q (Ormco, Orange, Calif). Exclusion criteria were (1) any systematic disease, (2) any active carious lesions, (3) any active periodontal lesions, and (4) topical fluoride application (except for fluoridated dentifrice) or antibacterial therapy within 6 months.

When power analysis to estimate effect size was performed using information from previous studies,[5,7] at least 40 subjects would have been required to analyze the periodontal pathogens. Because a previous study showed that bracket type does not significantly influence time-related differences in salivary bacterial levels,[9] saliva samples from 54 subjects with different brackets were analyzed as one group to evaluate time-related changes in salivary levels of periodontal pathogens after debonding (Table 1). All subjects signed informed consent forms, and the institutional review board approved the study protocol.

All patients received maxillary wraparound and mandibular Hawley removable retainers after debonding and were asked to wear the retainers 24 hours a day. The subjects received oral hygiene instructions, including brushing and flossing, and maintenance methods for the removable retainers with mechanical brushing and rinsing.

Unstimulated whole saliva (UWS) was collected by the spitting method. All subjects were asked to refrain from eating, drinking, brushing, and rinsing for at least 2 hours before saliva collection. UWS was collected at the following four time points, according to common retention protocols previously reported[10]: immediately before debonding (T1), 1 week after debonding when the patients began to wear removable retainers (T2), 5 weeks after debonding (T3), and 13 weeks after debonding (T4). The Simplified Oral Hygiene Index (OHI-S),[11] Plaque Index (PI),[12] and Gingival Index (GI)[12] were measured as periodontal parameters. OHI-S measures oral hygiene status using debris and calculus deposition from two anterior and four posterior teeth at a specific time point. Both PI and GI were obtained from the central and lateral incisors and first molars of both arches and averaged. All parameters were examined by a single investigator at each time point. The data collected at T1 may represent bacterial and hygienic conditions during orthodontic treatment because all subjects were wearing fixed orthodontic appliances at T1.

One milliliter of UWS was centrifuged at 13,000 rpm for 10 minutes. After removing the supernatant, we washed the precipitated pellet three times with 1.0 mL phosphate-buffered saline (PBS, pH = 7.4). The pellet was resuspended with 1.0 mL PBS and homogenized by sonication using three 30-second pulses with 30-second intermittent cooling stages in a refrigerator. Bacterial chromosomal DNA in saliva was extracted using a CellEase Bacteria II Genomic DNA Extraction Kit (Biocosm, Osaka, Japan) according to the manufacturer's instructions. The DNA was

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Table 2. Primers Used in This Study

Primer	Primer information and the detail experiment conditions	Size of Amplicon (bp)	Initial Denaturation	Denaturation	Annealing	Extension	Cycles
Universal	F: TGGAGCATGTGGTTTAATTCGA	160	94°C	95°C	60°C	60°C	40
	R: TGCGGGACTTAACCCAACA		30 s	20 s	45 s	10 s	
Aa[a]	F: GGCGAGCCTGTATTTGATGTGCG	113	95°C	95°C	72°C	72°C	40
	R: GTGCCCGGTGCTGCGTCTTTG		10 min	10 s	30 s	30 s	
Fn	F: ACCTAAGGGAGAAAC AGA ACC A	171	95°C	95°C	66°C	66°C	40
	R: CCTGCCTTTAATTCATCTCCAT		10 min	10 s	30 s	30 s	
Pi	F: AATACCCGATGTTGTCCACA	337	95°C	95°C	61°C	72°C	40
	R: TTAGCCGGTCCTTATTCGAA		1 min	5 s	15 s	33 s	
Pg	F: TGCAACTTGCCTTACAGAGGG	344	95°C	95°C	61°C	72°C	40
	R: ACTCGTATCGCCCGTTATTC		1 min	5 s	15 s	33 s	
Tf	F: CGGGCGTGCATCTTGTCGTCTAC	134	95°C	95°C	72°C	72°C	40
	R: CTTAACCGGCCGCCTCTTTGAA		10 min	10 s	30 s	30 s	

[a] Aa indicates *Aggregatibacter actinomycetemcomitans*; Fn, *Fusobacterium nucleatum*; Pi, *Prevotella intermedia*; Pg, *Porphyromonas gingivalis*; and Tf, *Tannerella forsythia*.

purified by phenol-chloroform extraction and ethanol precipitation.[13]

Known specific primers that amplify the RNA polymerase β subunit of Aa, Fn, and Tf were used for the PCR.[14,15] The PCR primers of Pi and Pg were designed based on the 16S rRNA gene (Table 2). A conserved sequence in the 16S rRNA was selected to count the total bacteria.[16] All primers were commercially synthesized (Bioneer, Seoul, Korea).

Bacterial chromosomal DNA was extracted from Aa ATCC 43718, Fn ATCC 10953, Pi ATCC 25611, Pg ATCC 33277, and Tf ATCC 43037. The DNA standard curve consisted of known amounts of molecules of purified PCR products isolated from agarose gels. DNA concentration was estimated by absorbance at 260 nm and a series of 10-fold dilutions were prepared for standard curves as previously described.[17] The amount of bacterial DNA in the samples was estimated from the standard curve.

Real-time PCR was performed using the Bio-Rad iQ5 system (Bio-Rad, Hercules, Calif). The reaction mixtures contained 2 μL purified DNA from saliva samples, 500 nM primers, and 10 μL 2× iQ SYBR Green Supermix (Bio-Rad). Distilled water was added to a final volume of 20 μL. Detailed experimental conditions are described in Table 2. All data were analyzed using iQ5 Optical System Software (Bio-Rad). All the experiments for quantifying bacterial levels were performed in triplicate and independently repeated twice.

Repeated measures analysis of variance was used to determine the time-related differences in OHI-S, salivary levels of total bacteria, Aa, Fn, Pi, Pg, and Tf, and the proportion of Aa, Fn, Pi, Pg, and Tf to total bacteria. Values were considered statistically significant when a P value was less than .05 after applying Scheffé's multiple comparison tests.

RESULTS

Specificity of the real-time PCR primers was tested with the genomic DNAs from the 18 known bacterial species (Table 3). The data demonstrated that amplified DNA was not detected in bacterial genomic DNA other than the target species (data not shown).

The changes in salivary levels of total bacteria and five periodontal pathogens (Aa, Fn, Pi, Pg, and Tf), and periodontal parameters (OHI-S, PI, and GI) are shown in Table 4. There was a significant decrease in the levels of total bacteria, Pi, Pg, Tf, and periodontal parameters after orthodontic treatment. However, no significant changes in the salivary level of Aa and Fn were detected during the experimental period.

OHI-S and PI were significantly decreased after orthodontic treatment compared with baseline levels (before debonding) (T1 $>$ T2, T3, T4, $P < .001$). GI was significantly decreased at T2 and T3 (T1 $>$ T2 $>$ T3, T4, $P < .001$) (Table 4). These findings indicate that patient oral hygiene improved immediately after debonding compared with that during treatment. The amount of Pi and Tf was significantly decreased 13 weeks after debonding (T1, T2 $>$ T4, $P < .01$; T1 $>$ T4, $P < .05$, respectively), while the salivary level of Pg was significantly decreased 5 weeks after debonding (T1 $>$ T3, T4, $P < .05$).

There was no significant difference in salivary levels of Aa between different time points ($P > .05$). The number of Fn tended to decrease from T1 to T4, but this difference was not statistically significant. In addition, Aa and Fn were present in saliva at higher levels than were Pg, Pi, or Tf during the whole experimental period (Table 4).

There were no significant differences in the proportion of Pi, Pg, or Tf to total bacteria as well as the proportion of Aa and Fn to total bacteria during the experimental period (Table 4).

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Table 3. Bacteria Used for Primer Specificity Testing

Gram-Positive Bacteria	Gram-Negative Bacteria
Actinomyces naeslundii KCOM 1472	*Aggregatibacter actinomycetemcomitans* ATCC 33384
Lactobacillus rhamnosus ATCC 7469	*Fusobacterium nucleatum* ATCC 10953
Streptococcus gordonii ATCC 10558	*Neisseria subflava* ATCC 49275
Streptococcus mutans UA159	*Porphyromonas gingivalis* ATCC 33277
Streptococcus oralis ATCC 9811	*Prevotella intermedia* ATCC 25611
Streptococcus rattus BHT	*Prevotella nigrescens* ATCC 33563
Streptococcus salivarius CCUG 50207	*Tannerella forsythia* ATCC 43037
Streptococcus sanguinis CCUG 17826	*Treponema denticola* ATCC 33521
Streptococcus sobrinus SL1	*Veillonella dispar* KCOM 1864

DISCUSSION

We examined changes in the salivary levels of periodontal pathogens and periodontal parameters using the data at T1 (immediately before debonding) as baseline data, because the presence of fixed appliances at T1 can simulate bacterial and hygienic conditions during orthodontic treatment. The results of this study show an immediate improvement of oral hygiene and periodontal conditions after debonding and the improvements maintained at the end of the experiment (Table 4). This is due to the fact that removing the orthodontic appliances eliminates their plaque-retentive effect, which may make practicing good oral hygiene easier. In addition, oral hygiene procedures, such as prophylaxis and scaling at debonding, may immediately improve oral hygiene status and periodontal conditions.

The salivary levels of total bacteria, Pg, Pi, and Tf were significantly decreased after appliance removal, although the decreasing pattern was somewhat later than were periodontal parameters. In general, total bacteria and Pg in saliva were significantly decreased 5 weeks after debonding (total bacteria, T1, T2 > T3, T4; Pg, T1 > T3, T4), while Pi and Tf in saliva were significantly decreased 13 weeks after debonding (Pi, T1, T2 > T4; Tf, T1 > T4). The decreased number of salivary bacteria may be due to the fact that improved oral hygiene had significantly reduced the possibility of dental plaque formation around the teeth and appliances. Improved oral hygiene can also decrease the levels of Pg, Pi, and Tf in both supragingival and subgingival plaque, which may significantly reduce the salivary levels of these bacteria. As a result, only small amounts of total bacteria, Pg, Pi, and Tf remained in the saliva 13 weeks after debonding (Table 4). This is consistent with previous studies, which have shown that higher numbers of periodontal pathogens at the completion of orthodontic treatment were significantly decreased after appliance removal, and a reduction in periodontal pathogens was correlated with improvement in oral hygiene and periodontal health.[18,19]

Although Pg, Pi, and Tf are members of the normal oral flora, decreased levels of these microbes are clinically important. Pg, Pi, and Tf have been

Table 4. The Salivary Levels of Bacteria and Periodontal Parameters at the Following Four Time Points: At the Time of Debonding (T1), 1 Week After Debonding (T2), 5 Weeks After Debonding (T3), and 13 Weeks After Debonding (T4)[a]

	T1	T2	T3	T4	Significance[b]
OHI–S[c]	1.45 ± 0.7	0.32 ± 0.4	0.35 ± 0.4	0.28 ± 0.5	T1 > T2, T3, T4***
Plaque index	1.34 ± 0.6	0.28 ± 0.4	0.34 ± 0.4	0.26 ± 0.5	T1 > T2, T3, T4***
Gingival index	1.34 ± 0.4	0.90 ± 0.4	0.39 ± 0.4	0.30 ± 0.4	T1 > T2 > T3, T4***
Total bacteria (log_{10})	7.97 ± 0.5	7.97 ± 0.6	7.77 ± 0.6	7.64 ± 0.5	T1, T2 > T3, T4*
Aa (log_{10})	2.76 ± 0.6	2.80 ± 0.6	2.66 ± 0.7	2.71 ± 0.7	NS
Fn (log_{10})	3.44 ± 0.7	3.37 ± 0.7	3.29 ± 0.7	3.15 ± 0.7	NS
Pi (log_{10})	1.57 ± 1.5	1.54 ± 1.3	1.33 ± 1.2	1.05 ± 1.1	T1, T2 > T4**
Pg (log_{10})	1.93 ± 1.1	1.74 ± 1.0	1.52 ± 0.9	1.58 ± 0.7	T1 > T3, T4*
Tf (log_{10})	2.45 ± 1.6	2.15 ± 1.4	2.03 ± 1.5	1.86 ± 1.5	T1 > T4*
Aa/total (%)	0.002 ± 0.004	0.005 ± 0.016	0.003 ± 0.006	0.007 ± 0.026	NS
Fn/total (%)	0.013 ± 0.026	0.012 ± 0.023	0.021 ± 0.039	0.027 ± 0.083	NS
Pi/total (%)	0.005 ± 0.015	0.002 ± 0.008	0.001 ± 0.001	0.001 ± 0.001	NS
Pg/total (%)	0.002 ± 0.010	0.002 ± 0.002	0.001 ± 0.001	0.001 ± 0.001	NS
Tf/total (%)	0.006 ± 0.016	0.004 ± 0.011	0.007 ± 0.017	0.006 ± 0.017	NS

[a] The unit of bacterial adhesion is the cell number in logarithm per 1.0 mL. The proportion of each periodontal pathogen is determined by dividing the number of periodontal pathogen to number of total bacteria.

[b] Repeated measure ANOVA was used to determine time-related differences at $\alpha = 0.05$; NS, not significant; *$P < .05$; **$P < .01$; ***$P < .001$.

[c] OHI–S, Simplified Oral Hygiene Index; Aa, *Aggregatibacter actinomycetemcomitans*; Pg, *Porphyromonas gingivalis*; Pi, *Prevotella intermedia*; Fn, *Fusobacterium nucleatum*; and Tf, *Tannerella forsythia*.

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mentioned as possible etiologic agents for periodontal lesions.[20] In particular, Pg and Tf are closely related to periodontal breakdown and have been reported to play significant roles in the onset and progress of periodontal disease as the most periodontopathic species.[21] Therefore, decreased levels of Pg, Pi, and Tf might imply a reduced risk for developing gingival or periodontal inflammation.

Despite a significant decrease in the salivary levels of total bacteria, Pg, Pi, and Tf, the salivary levels of Aa and Fn remained unchanged after appliance removal compared with the amounts measured before appliance removal (Table 4). Aa is a Gram-negative facultative anaerobe that has been implicated in localized, aggressive periodontitis. The facultative anaerobic characteristic may facilitate longer survival in the oral cavity even after the removal of fixed appliances and contribute to maintaining the higher salivary levels of Aa than did the obligate anaerobes such as Pg, Pi, and Tf. Fn is a common human dental plaque species that can aggregate with a wide range of other plaque bacteria.[22] Considering that salivary levels of mutans streptococci are not significantly decreased after debonding,[9] the interaction of Fn with other bacteria including mutans streptococci around periodontal tissues may explain the higher salivary levels of Fn after debonding.

The above hypothesis partly supports why Aa and Fn existed in higher numbers than did Pg and Pi during the entire experimental period (Table 4). A previous study also reported that the detection rate of Aa and Fn was higher than that of Pg and Pi in the saliva of prosthodontic patients.[23] Future long-term studies including subjects with more than a 3-month retention period will be needed to verify changes in the levels of Aa and Fn in orthodontic patients with fixed appliances.

This study demonstrated that there were no significant differences in the proportion of Pi, Pg, and Tf to total bacteria nor in the proportion of Aa and Fn to total bacteria during the experimental period (Table 4). This is due to variations in the number of salivary bacteria among the different time points as well as the small proportion of periodontal pathogens (less than 0.02%) relative to total bacteria in saliva. Compared with a previous study that showed a salivary level of periodontal pathogens from 10^4 to 10^7 cells per mL in patients with periodontal disease,[5] most subjects in this study had fewer than 10^4 periodontal pathogens in their saliva. This might be explained by the fact that the subjects in this study practiced relatively good oral hygiene (OHI-S was 1.45 ± 0.7 at T1) without any active periodontal lesions.

All subjects in the present study were supplied with removable retainers after debonding, which might have influenced the salivary level of periodontal pathogens. Their oral hygiene was significantly improved and salivary levels of total bacteria were significantly decreased after debonding, which remained at similar levels during removable retainer wear. This suggests that the presence of removable retainers did not significantly influence salivary levels of periodontal pathogens compared with the presence of fixed appliances. Although a direct comparison is not possible, our results are similar to those of a previous study showing that lower numbers of anaerobic bacteria, less plaque accumulation, and improved periodontal conditions were found with removable aligners compared with fixed appliances.[24]

The present study shows that removal of fixed appliances does not significantly reduce all periodontal pathogens during the initial retention period. Therefore, our null hypothesis was partly accepted. These findings indicate that changes in periodontal pathogens associated with orthodontic treatment was not effected solely by the removal of orthodontic appliances. Although removal of orthodontic appliances induced significant reductions in total bacteria, Pg, Pi, and Tf in saliva, the salivary levels of Aa and Fn remained unchanged 3 months after the removal of fixed orthodontic appliances. Because Aa and Fn can act as triggering or supporting factors for disease progress, a high prevalence of these microorganisms may indicate that the risk of gingival or periodontal problems cannot be completely eliminated immediately after the removal of fixed orthodontic appliances, despite improved oral hygiene. This study suggests that careful hygienic procedures are needed to restore periodontal health after orthodontic treatment with fixed appliances.

This study has a limitation. Although the presence of fixed appliances at T1 can simulate bacterial and hygienic conditions during orthodontic treatment, there are no data on the presence of periodontopathogens prior to orthodontic treatment. The pretreatment bacterial data would have provided more valuable information on the changes in periodontopathic bacteria during orthodontic treatment. Further long-term studies from the pretreatment to postretention periods are needed to investigate the effects of periodontal pathogens on the periodontal health of orthodontic patients.

CONCLUSIONS

- Removal of orthodontic appliances induced significant reductions in total bacteria, Pg, Pi, and Tf in saliva associated with a significant improvement in oral hygiene status.
- The salivary levels of Aa and Fn remained unchanged 3 months after the removal of fixed

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orthodontic appliances despite improved oral hygiene.
- Both Aa and Fn were present at higher levels than were Pg, Pi, or Tf during the entire experimental period.

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