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Prevalence of Putative Periodontopathogens in Subgingival Dental Plaques from Gingivitis Lesions in Korean Orthodontic Patients

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The objective of this study was to detect and compare the presence of periodontopathogens in the subgingival plaques of gingivitis lesions in adults who wore fixed orthodontic appliances, as opposed to adults who did not wear any orthodontic appliances. Thirty-six individuals participated in this study. Ninteen of these subjects did not wear any orthodontic appliances, and these subjects comprised the control group. The other 17 individuals had been wearing fixed orthodontic appliances for at least 3 months each. After a periodontal examination, we collected subgingival plaque samples from the gingivitis lesions of each patient. Using PCR based on 16S rDNA, we detected the presence of 6 putative periodontopathogenic species, *Treponema denticola*, *Porphyromonas gingivalis*, *Tannerella forsythia* (formerly *Bacteroides forsythus*), *Prevotella nigrescens*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans*. With regard to the presence of individual periodontopathogens, we found that *T. forsythia*, *T. denticola*, and *P. nigrescens* were significantly more common in the samples obtained from the orthodontic patients than in the samples obtained from the non-orthodontic patient controls. Our results indicate that the local changes associated with the wearing of fixed orthodontic appliances may affect the prevalence of periodontopathogens in subgingival dental plaques.

Key words: gingivitis, periodontopathogens, orthodontic patients, PCR, 16S rDNA

One of the most frequently encountered problems during orthodontic treatment is periodontal disease, a category which includes gingivitis. A variety of studies have reported that gingival changes occurring during orthodontic treatment were temporary, and did not normally result in permanent periodontal losses (Zachrisson and Zachrisson, 1972; Kloehn and Pfeifer, 1974; Alstad and Zachrisson, 1979; Trossello and Gianelly, 1979). However, some studies have also reported that losses of clinical attachment increased significantly during orthodontic treatment (Zachrisson and Alnæs, 1973; Zachrisson, 1976). It has also been stated that if good oral hygiene is maintained, orthodontic treatment results in no harmful effects with regard to periodontal health (Kloehn and Pfeifer, 1974). According to these studies, therefore, it would appear that periodontal problems during orthodontic treatment may be primarily attributable to poor oral hygiene.

The primary causative factor of periodontal diseases, including gingivitis, is the colonization of anaerobic microorganisms in the subgingival plaque. Two hypotheses currently exist regarding the development of periodontal diseases. One hypothesis is that an excessive accumulation of microorganisms can cause periodontal diseases, regardless of the specific pathogenic microorganisms involved (Theilade, 1986). The opposing hypothesis holds that specific microorganisms are responsible for specific periodontal diseases (Slots and Listgarten, 1988). The latter hypothesis is supported by the clear relationship between *Actinobacillus actinomycetemcomitans* and localized juvenile periodontitis. Until recently, there were no clearly defined relationships between specific pathogenic microorganisms and specific periodontal diseases. However, a few highly toxic microorganisms have since been shown to be causative of specific diseases. For example, the primary pathogenic microorganisms implicated in destructive periodontitis are as follows: *Porphyromonas gingivalis, A. actinomycetemcomitans, Tannerella forsythia, Treponema denticola, Prevotella intermedia, Prevotella nigrescens, Campylobacter rectus, Peptostreptococcus micros, Fusobacterium nucleatum* subsp. *vincen-*

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tii, Fusobacterium nucleatum subsp. *nucleatum, Selenomonas noxia, Selenomonas flueggeii, and* spirochetes (Darveau *et al.*, 1997). The existence and multiplication of these highly toxic microorganisms clearly results in periodontal disease. The former hypothesis, therefore, may have been postulated before the presence and composition of such specific pathogenic microorganisms had been firmly established.

The local environmental changes associated with orthodontic brackets and bands can significantly affect the bacterial species present in the plaque. In particular, preformed orthodontic bands that encompass the teeth invariably produce some overhangs, which facilitate the accumulation of plaque, and generate favorable conditions for the development of chronic periodontitis (Lang *et al.*, 1983). In this regard, a host of studies has been conducted to investigate changes in the strain makeup and composition of microorganisms in the subgingival plaque neighboring orthodontic bands (Baer and Coccaro, 1964; Zachrisson, 1976; Alexander, 1991; Boyd and Baumrind, 1992). However, considering that periodontal disease frequently develops around teeth surrounded by orthodontic brackets, it appears necessary that a study be conducted to address changes in the strains and compositions of microorganisms in plaque located below directly-bonded orthodontic brackets. Therefore, we assessed the presence of putative periodontopathogens in the subgingival plaque from the gingivitis lesions of orthodontic patients with directly bonded brackets, and compared them to gingivitis lesion samples from patients who did not wear any orthodontic appliances. Among the putative periodontopathogens encountered, we opted to evaluate the prevalence of *T. denticola, P. gingivalis, T. forsythia, P. nigrescens, P. intermedia,* and *A. actinomycetemcomitans,* via 16S rDNA PCR.

Materials and Methods

Subject selection

A total of 36 individuals participated in this study. Nineteen of these subjects wore no orthodontic appliances, thus comprising the control group. The other 17 individuals had worn fixed orthodontic appliances for at least 3 months each. All of the participants in the study were recent referrals to the Department of Orthodontics at the Chosun University Dental Hospital, Gwangju, Korea. Our selection criteria were as follows: (a) no alveolar bone loss visible to X-ray, (b) no known systemic diseases, (c) not currently pregnant, and (d) no periodontal treatment within the last 6 months. None of the participants had received antibiotic therapy within the previous 3 months, and none were receiving antibiotic therapy during the experimental period. All participants were informed of the objectives of this study, and all provided informed consent prior to participation in the study.

Clinical measurement

We selected the periodontal sites that exhibited the deepest pocket depth (PD) in each quadrant. In cases in which two or more sites with the same PD were present in a given quadrant, the site was selected randomly. PDs were measured at 2 - 9 sites on each tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiodistal). One hundred twenty-eight sites from 32 participants were assigned as study sites. The clinical parameters assessed included the following: plaque index (PI) (Silness and Löee, 1964), gingival index (GI) (Löee and Silness, 1963), PD, clinical attachment level (CAL), and bleeding on probing (BOP). In the latter case, sites that bled within 30 sec after probing were designated BOP. Clinical measurements were conducted both at baseline, and after the initial treatment (Takamatsu *et al.*, 1999).

Subgingival plaque sampling

After clinical measurements had been conducted, each site was isolated with cotton rolls, and was subsequently air-dried. Visible supra-gingival plaque was removed, and a sterile paper point was inserted into the site, remaining there for 30 sec. The paper point was then immediately placed into a microcentrifuge tube containing 1 ml of 1 × PBS. The tubes were mixed thoroughly on a Vortex mixer, and stored at -20°C until analysis. The Chosun University Institutional Research Board granted approval of all study protocols herein.

Polymerase chain reaction (PCR)

For the DNA extraction, a 10 μl aliquot of each stored sample was added to 10 μl of 2 × lysis buffer (2 mM EDTA, 1% Triton X-100). This mixture was then boiled for 10 minutes and placed on ice. PCR was performed using an *AccuPower*® PCR PreMix (Bioneer, Korea), containing 5 nmol of each deoxynucleoside triphosphate, 0.8 μmol of KCl, 0.2 μmol of Tris-HCl (pH 9.0), 0.03 μmol of $MgCl_2$, and 1 unit of *Taq* DNA polymerase. The bacterial genomic DNA and 20 pmol of each primer were then added to a PCR PreMix tube. PCR was carried out in a final volume of 20 μl. Table 1 displays the species-specific primers used in the PCR amplification of the 16S rRNA genes (Ashimoto *et al.*, 1996; Stubbs *et al.*, 1999).

The PCR was run on a Peltier thermal cycler (Model PTC-200 DNA engine™, MJ Research, USA) under the following conditions: for *T. forsythia* and *P. gingivalis,* denaturing at 94°C for 1 min, annealing at 60°C for 30 sec, and an extension step at 72°C for 1 min; for *A. actinomycetemcomitans* and *P. intermedia,* denaturing at 94°C for 30 sec, annealing at 55°C for 1 min, and an extension step at 72°C for 1 min. Each of these thermal cycles was performed 36 times. Prior to the first cycle, the DNA was denatured at 95°C for 2 min. After the completion of the final cycle, the PCR products were fully extended for 10 min at 72°C.

Table 1. Species-specific primers used for PCR

Primer pairs (5'-3')	Size of amplicon (bp)	Reference
A. actinomycetemcomitans		
AAA CCC ATC TCT GAG TTC TTC TTC	557	Ashimoto *et al.*[a]
ATG CCA ACT TGA CGT TAA AT		
T. forsythia		
GCG TAT GTA ACC TGC CCG CA	641	Ashimoto *et al.*[a]
TGC TTC AGT GTC AGT TAT ACC T		
P. gingivalis		
AGG CAG CTT GCC ATA CTG CG	404	Ashimoto *et al.*[a]
ACT GTT AGC AAC TAC CGA TGT		
P. intermedia		
CAA AGA TTC ATC GGT GGA	307	Stubbs *et al.*[b]
GCC GGT CCT TAT TCG AAG		
P. nigrescens		
ATG AAA CAA AGG TTT TCC GGT AAG	804	Ashimoto *et al.*[a]
CCC ACG TCT CTG TGG GCT GCG A		
T. denticola		
TAA TAC CGA ATG TGC TCA TTT ACA T	316	Ashimoto *et al.*[a]
TCA AAG AAG CAT TCC CTC TTC TTC TTA		

[a]Ashimoto *et al.* 1996; [b]Stubbs *et al.* 1999.

Table 2. Prevalence of periodontopathogens in subgingival plaque collected from gingivitis lesions in patients with fixed orthodontic appliances, and those without any type of fixed orthodontic appliances

	Prevalence of periodontopathogens (%)						
	Td[a]	Pg	Tf[a]	Cr[a]	Pn[a]	Pi	Aa
With bracket n = 93	36.6	24.7	48.4	88.2	47.3	15.1	10.8
No bracket n = 76	17.1	27.6	22.4	55.3	3.95	11.8	17.1

Td, *Treponema denticola*; Pg, *Porphyromonas gingivalis*; Tf, *Tannerella forsythia*; Cr, *Campylobacter rectus*; Pn, *Prevotella nigrescens*; Pi, *Prevotella intermedia*; Aa, *Actinobacillus actinomycetemcomitans*.
[a]Chi square test indicated significant difference ($p < 0.05$) with respect to the wearing of brackets at frequency of seven periodontopathogens.

The amplified PCR products were then electrophoresed on 1.5% agarose gel in Tris-acetate buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0). The amplification products were stained with ethidium bromide and visualized by UV transillumination.

Statistical analysis

The differences in the prevalence of each bacterial strain were also analyzed using the Chi square test. Differences in BOP, PD, GI, and PI values were analyzed using the Fisher's exact test and trend test. All statistical analyses were carried out with a statistical software package (SPSS for Windows 10.0 SPSS Inc., Korea).

Results and Discussion

In the experimental group, which consisted of 17 gingivitis patients with fixed orthodontic appliances, and the control group, which consisted of 19 gingivitis patients without any orthodontic appliances, the prevalence of seven putative periodontopathogens in the subgingival plaque was detected, and is shown in Table 2. *T. denticola, T. forsythia, C. rectus,* and *P. nigrescens* were significantly more prevalent in the experimental group than in the controls ($p < 0.05$) (Table 2). In terms of their prevalence, *P. gingivalis, P. intermedia,* and *A. actinomycetemcomitans* were not found to be statistically significantly different between the two groups (Table 2). These results indicate that the presence of orthodontic brackets may increase the prevalence of specific periodontopathogens in the subgingival plaque of gingivitis lesions. *T. denticola* and *T. forsythia* were more prevalent in the experimental group than in the controls. *T. denticola* and *T. forsythia* are both strict anaerobic microorganisms, which are present only in the deeper regions of periodontal pockets (Kigure *et al.*, 1995). Recently, *P. nigrescens* was taxonomically

Table 3. Prevalence of periodontopathogens in subgingival plaque collected from gingivitis lesions in patients wearing fixed orthodontic appliances, and those without any type of fixed orthodontic appliances, according to the clinical parameters

Clinical parameters (n)		Prevalence of periodontopathogens (%)						
		Td	Pg	Tf	Cr	Pn	Pi	Aa
Bleeding on probing								
With bracket	+ (52)	46.2[a]	30.8	50.0	88.5	38.5	19.2	7.7
	− (41)	24.4[a]	17.1	46.3	87.8	58.5	9.8	14.6
No bracket	+ (45)	28.9[a]	42.2[a]	31.1[a]	60.0	6.7	20.0[a]	26.7[a]
	− (31)	0.0[a]	6.5[a]	9.7[a]	48.4	0.0	0.0[a]	3.2[a]
Gingival index								
With bracket	1 (40)	35.0	35.0[b]	50.0	87.5	45.0	15.0	15.0
	2 (53)	37.7	17.0[b]	47.2	88.7	49.1	15.1	7.6
No bracket	1 (18)	0.0[b]	5.6[b]	11.1[b]	44.4	0.0	0.0	0.0[b]
	2 (54)	18.5[b]	29.6[b]	20.4[b]	57.4	5.6	14.8	22.2[b]
	3 (4)	75.0[b]	100.0[b]	100.0[b]	75.0	0.0	25.0	25.0[b]
Pocket depth[c]								
With bracket	≤ 2 (18)	33.3	0.0	55.6	83.3	66.7	16.7	11.1
	2 <, ≤ 3 (71)	38.0	29.6	46.5	91.5	43.7	15.5	11.3
	3 < (4)	25.0	50.0	50.0	25.0	0.0	0.0	0.0
No bracket	< 2 (42)	11.9	23.8	23.8	59.5	4.8	9.5	14.3
	2 <, ≤ 3 (26)	19.2	26.9	15.4	34.6	0.0	7.7	11.5
	3 < (10)	30.0	40.0	30.0	80.0	10.0	30.0	40.0
Plaque index								
With bracket	1 (90)	37.8	25.6	48.9	88.9	46.7	15.6	11.1
	2 (3)	0.8	0.0	33.3	66.7	66.7	0.0	0.0
No bracket	1 (51)	9.8[d]	19.6[d]	13.7[d]	47.1	1.96	5.9[d]	11.8
	2 (20)	25.0[d]	35.0[d]	30.0[d]	75.0	10.0	25.0[d]	30.0
	3 (5)	60.0[d]	80.0[d]	80.0[d]	60.0	0.0	20.0[d]	20.0

Td, *Treponema denticola*; Pg, *Porphyromonas gingivalis*; Tf, *Tannerella forsythia*; Cr, *Campylobacter rectus*; Pn, *Prevotella nigrescens*; Pi, *Prevotella intermedia*; Aa, *Actinobacillus actinomycetemcomitans*.

[a]Fisher's exact test indicated significant difference ($p < 0.05$) with respect to BOP at frequency of seven periodontopathogens.

[b]Fisher's exact test or trend test indicated significant difference ($p < 0.05$) with respect to GI at frequency of seven periodontopathogens.

[c]Fisher's exact test indicated no significant difference ($p > 0.05$) with respect to PD at frequency of seven periodontopathogens.

[d]Fisher's exact test indicated significant difference ($p < 0.05$) with respect to PI at frequency of seven periodontopathogens.

separated from *P. intermedia,* and was classified as an independent species (Shah and Gharbia, 1992). After this classification, a variety of epidemiological investigations were conducted in order to detect and identify *P. nigrescens* and *P. intermedia* (Teanpaisan *et al.*, 1995; Conrads *et al.*, 1996; Bae *et al.*, 1997; Matto *et al.*, 1997; Teanpaisan *et al.*, 1998). These studies were performed on periodontal lesions, endodontic lesions, and healthy periodontium. According to the results of these studies, the prevalence of *P. intermedia* was elevated in the periodontal lesions, but the prevalence of *P. nigrescens* was elevated in the endodontic lesions. In this study, we determined the prevalence of *P. nigrescens* in the experimental groups was triple that of *P. intermedia*, but that the reverse was true in the control group (Table 2). This implies that local environmental changes in the experimental group may have disrupted the normal microorganismic balance inherent to the oral cavities of the orthodontic patients in this study.

With regard to BOP status, *T. denticola* was significantly more prevalent in the BOP-positive samples than in the BOP-negative samples from the experimental group. *T. denticola, P. gingivalis, T. forsythia, P. intermedia,* and *A. actinomycetemcomitans* were found to be significantly more prevalent in the BOP-positive samples from the control group ($p < 0.05$) (Table 3). This suggests that a greater variety of periodontopathogens exist in the experimental group, even in the BOP-negative samples. Regarding the GI in the experimental group, the prevalences of periodontopathogens, except *P. gingivalis*, did

not increase with increasing GI values. However, the prevalences of most periodontopathogens did increase directly with increases in the GI values of the control group (Table 3). This also indicates that a greater variety of periodontopathogens are present in the experimental group, even in the samples with low GI values. With regard to PD, we detected no statistically significant difference between the groups (Table 3). With regard to the PI, we detected no statistically significant differences within the experimental group (Table 3). However, in the control group, *T. denticola, P. gingivalis, T. forsythia,* and *P intermedia* proportions increased directly with increases in the PI values ($p < 0.05$) (Table 3). This result can be interpreted as indicating that a greater variety of periodontopathogens existed in the experimental group, even in the samples with low PI values. These results reveal a high prevalence of periodontopathogens in the experimental group, even in the samples exhibiting favorable indices. This implies that the local environmental changes associated with the wearing of orthodontic brackets in the experimental group prompted the attachment and growth of a variety of periodontopathogens, even before the onset of periodontal inflammation and plaque accumulation.

Major pathogens need to be detected in order to assess the epidemiology, diagnosis, treatment, and prognosis of infectious diseases. Many studies have reported that several putative pathogens could be detected by PCR (Ashimoto *et al.*, 1996; Lee *et al.*, 1998; Wang and Lee, 2003; Lee *et al.*, 2004). The use of PCR allows considerable savings in time, cost, and experimental effort when compared to other bacterial identification methods such as cell culture, DNA probe method, 16S rDNA sequencing, ribotyping, *etc.* Therefore, PCR has been widely applied for the identification of various bacterial species. The 16S rDNA can be used effectively for PCR assays because 16S rDNA is universally found in prokaryotic organisms, and a comparative analysis of 16S rDNA has shown that the variable sequence regions are interspersed with highly conserved regions (Woese, 1987). Therefore, in this study, we performed PCR for the detection of putative periodontal pathogens from the subgingival plaques.

Clinically, our results imply that gingivitis can readily become exacerbated in orthodontic patients who are treated with orthodontic brackets. Therefore, when gingivitis is observed in orthodontic patients with orthodontic brackets, a more thorough gingivitis management strategy is indicated than would be in a patient who does not wear orthodontic brackets. Further inquiries into the specific local environmental changes caused by orthodontic brackets and their effect on the attachment and growth of specific periodontopathogens are clearly warranted. Also, future studies should focus on the effects of these specific periodontopathogens with regard to the progress of periodontal inflammation.

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