JPIS
Journal of Periodontal & Implant Science

Research Article
Periodontal Science

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Efficacy of salivary versus subgingival bacterial sampling for the detection and quantification of periodontal pathogens

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ABSTRACT

Purpose: The aim of this study was to investigate the efficacy and validity of subgingival bacterial sampling using a retraction cord, and to evaluate how well this sampling method reflected changes in periodontal conditions after periodontal therapy.
Methods: Based on clinical examinations, 87 subjects were divided into a healthy group (n=40) and a periodontitis group (n=47). Clinical measurements were obtained from all subjects including periodontal probing depth (PD), bleeding on probing (BOP), clinical attachment loss (CAL), and the plaque index. Saliva and gingival crevicular fluid (GCF) as a subgingival bacterial sample were sampled before and 3 months after periodontal therapy. The salivary and subgingival bacterial samples were analyzed by reverse-transcription polymerase chain reaction to quantify the following 11 periodontal pathogens: *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythus* (*Tf*), *Treponema denticola* (*Td*), *Prevotella intermedia* (*Pi*), *Fusobacterium nucleatum* (*Fn*), *Pavimonas micra* (*Pm*), *Campylobacter rectus* (*Cr*), *Prevotella nigrescens* (*Pn*), *Eikenella corrodens* (*Ec*), and *Eubacterium nodatum* (*En*).
Results: Non-surgical periodontal therapy resulted in significant decreases in PD ($P<0.01$), CAL ($P<0.01$), and BOP ($P<0.05$) after 3 months. Four species (*Pg*, *Tf*, *Pi*, and *Pm*) were significantly more abundant in both types of samples in the periodontitis group than in the healthy group. After periodontal therapy, *Cr* was the only bacterium that showed a statistically significant decrease in saliva, whereas statistically significant decreases in *Cr*, *Pg*, and *Pn* were found in GCF.
Conclusions: Salivary and subgingival bacterial sampling with a gingival retraction cord were found to be equivalent in terms of their accuracy for differentiating periodontitis, but GCF reflected changes in bacterial abundance after periodontal therapy more sensitively than saliva.

Keywords: Gingival crevicular fluid; Microbiota; Periodontitis; Polymerase chain reaction; Saliva

INTRODUCTION

The primary etiology of periodontitis is bacterial infection followed by interactions between biofilm and microbial species that result in an inflammatory reaction in a susceptible host, which can lead to the destruction of periodontal tissues [1]. The initiation and progression

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of the disease is specifically linked to microbial species of the subgingival microbiome, such as *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythus* (*Tf*), and *Treponema denticola* (*Td*) [2]. Given that the abundance of specific periodontal pathogens from the subgingival microbiota is the primary etiological driver of periodontitis [3], screening for these pathogens in plaque, gingival crevicular fluid (GCF), and saliva is vital for diagnosis and treatment [4,5].

GCF is simultaneously a physiological transudate and an inflammatory exudate produced by both an osmotic gradient and the initiation of inflammation [6]. Individuals with experimental gingivitis (a periodontal disease) show an approximately 5-fold increase in GCF [7]; therefore, in patients with periodontitis, GCF can be collected non-invasively to enable the detection and quantification of subgingival microbiota and inflammatory mediators released during progression of the disease [8]. For example, in a study of the relationships among GCF biomarkers, the clinical parameters of periodontal disease, and the subgingival microbiota, Teles et al. [9] revealed that high levels of periodontal pathogens were found in healthy sites adjacent to teeth with periodontitis. Various GCF and subgingival plaque sampling methods exist, including the use of paper points, curettes, and dental floss, and there is no consensus on the superior method. With paper points, the outer layer of subgingival plaque can be successfully cultured, but the deeper layer may be missed [10]. In comparison, curettes can collect subgingival plaque from the entire periodontal pocket, but the active periodontal pathogens in the outer layer of the plaque can be underestimated with this method [11]. Nevertheless, a quantitative real-time polymerase chain reaction (RT-PCR) study demonstrated that paper point and curette sampling provided similar results; therefore, they could each be considered as a suitable sampling method for characterizing the subgingival microbiota, pathogens, and plaque [12]. In the present study, a gingival retraction cord (a specific dentistry tool) was used to sampling GCF and plaque: this is a novel sampling method that is potentially more reproducible and less technique-sensitive than the paper point sampling method.

Saliva samples could also be used to measure subgingival microbiota because saliva contains bacteria shed from the tongue, tooth surface, and oral mucosa [13]. However, the accuracy of saliva samples in terms of reflecting the actual levels of subgingival microbiota has been studied because periodontal pathogens are also found in the tongue microbiome of orally healthy individuals [14]. Consequently, the results of a saliva sample analysis may not truly represent the conditions of the subgingival microbiota. Nevertheless, Haririan et al. [15] reported a significant positive correlation between subgingival and salivary microbiota when 11 periodontal pathogens were evaluated in patients with chronic periodontitis. In addition, Masunaga et al. [4] reported a significant difference in the abundance of periodontal pathogens from saliva samples taken from periodontitis patients and healthy individuals. In the present study, we measured the levels of periodontal pathogens before and after periodontal treatment to evaluate how well saliva sampling mirrored subgingival microbiota sampling. We also investigated the efficacy and validity of the subgingival bacterial sampling method when using a retraction cord, and we assessed how well this sampling method reflected changes in periodontal conditions after periodontal therapy.

MATERIALS AND METHODS

Study population and clinical measurements

This study was performed from November 2017 to February 2019 at the Department of Periodontics of Seoul National University Dental Hospital. Subjects who had systemic or medical conditions that would affect the onset or treatment of periodontal disease, such as diabetes, pregnancy, use of local or systemic antibiotics or immunosuppressants within the last 3 months, or who had received a professional dental cleaning within the last 3 months were excluded. Initially, the plan was for 100 subjects to be included, but 13 subjects dropped out during the follow-up period. In total, 87 adults were enrolled in the study, and for each subject, a panoramic radiograph was taken and periodontal probing depth (PD), clinical attachment loss (CAL), bleeding on probing (BOP), and the Quigley-Hein plaque index (PI) were all measured by 1 trained dentist.

Study design

Based on clinical examinations, the 87 subjects were divided into a healthy group (n=40) and a periodontitis group (n=47). The adults in the healthy group (mean age, 40.6 years; standard deviation [SD], 15.04 years) showed no evidence of periodontitis, whereas the adults in the periodontitis group (mean age, 54.9 years; SD, 11.10 years) had untreated periodontitis, and >30% of the teeth in their remaining dentition were periodontally compromised (CAL ≥3 mm).

The subjects in the periodontitis group received non-surgical periodontal therapy, which included scaling and root planing. Their clinical measurements (PD, BOP, CAL, and PI) were recorded, and saliva and GCF as subgingival bacterial samples were sampled before and 3 months after periodontal therapy in the periodontitis group. The subjects in the healthy group underwent the same measurement and sampling procedure, but without periodontal therapy and with only 1 sample. Saliva and GCF samples were analyzed by RT-PCR for quantification of the following 11 periodontal pathogens: *Aggregatibacter actinomycetemcomitans* (*Aa*), *Pg*, *Tf*, *Treponema denticola* (*Td*), *Prevotella intermedia* (*Pi*), *Fusobacterium nucleatum* (*Fn*), *Pavimonas micra* (*Pm*), *Campylobacter rectus* (*Cr*), *Prevotella nigrescens* (*Pn*), *Eikenella corrodens* (*Ec*), and *Eubacterium nodatum* (*En*). The differences in the number of periodontal pathogens between the groups and before and 3 months after periodontal therapy in the periodontitis group were analyzed statistically.

Sampling method

For each subject, 2 mL of unstimulated whole saliva was collected after the use of mouthwash before (baseline) and 3 months after periodontal therapy (in the periodontitis group) via passive drooling into sterile plastic tubes. Subgingival bacterial samples were collected from the gingival sulcus of the tooth that exhibited the highest CAL value: a gingival retraction cord cut to the right size and sterilized by autoclaving was used for 30 seconds and was isolated from the saliva by application of a cotton roll. Subgingival bacterial sampling was performed before clinical measurements were taken to avoid contamination from blood or pus.

Microbiological analyses: RT-PCR

Bacterial chromosomal DNA in saliva was extracted using a SV Mini Kit (GeneAll, Seoul, Korea). The samples were analyzed for the presence of the 11 aforementioned periodontal pathogens using an RT-PCR kit (Microis Co. Ltd, Seoul, Korea). The DNA from each bacterium as amplified with a specific primer using a functional gene (*rgpB*, *waaA*, or *gtf*). A total bacterial 16s rDNA control was used to detect DNA from the total bacterial load.

In the hot-start Taq DNA polymerase assay, samples were analyzed in a 20-μL reaction mixture containing genomic DNA (2 μL), the specific primer, a probe set, and the PCR reaction buffer. The thermal program was as follows: 40 cycles at 95℃ for 30 seconds, 55℃ for 30 seconds, and 72℃ for 30 seconds, with an initial denaturation at 95℃ for 15 minutes. All data were analyzed using 7500 software (ABI 7500 Fast Real-Time PCR System, Applied Biosystems, Thermo Fisher, Waltham, MA, USA). Standard curves were used to convert cycle threshold scores into the number of bacterial cells using samples with known amounts of bacteria-specific DNA. DNA was 10-fold serially amplified from 10^0 to 10^5 copies and subjected to RT-PCR to create a standard curve in which threshold cycles were plotted against the copy number of the plasmid DNA, as previously described.

Data analysis

For both the healthy group and the periodontitis group, the mean and SD of the clinical measurements and number of periodontal pathogens in saliva and GCF were calculated using individual subjects as the statistical unit. To assess statistical differences, the relative proportions of the 11 periodontal pathogens in saliva and GCF before treatment were first calculated in both groups. The number of these pathogens in saliva and GCF was compared between the groups before treatment using the independent t-test. To assess the effects of periodontal therapy, the number of these pathogens in saliva and GCF before and after treatment was compared using the paired t-test and Pearson correlation coefficients. For all tests, $P<0.05$ was considered to indicate statistical significance. All statistical analyses were performed using SPSS v25 (IBM Corp., Armonk, NY, USA).

The protocol for this study was approved by the Research Ethics Committee of the Seoul National University Dental Hospital (approval No. CRI17009). All participants voluntarily took part in the study and provided informed consent prior to the intervention.

RESULTS

Clinical measurements

Full-mouth clinical measurements for the healthy group and for the periodontitis group before and after periodontal therapy are presented in Table 1. Before treatment, the PD ($P<0.001$), CAL ($P<0.001$), and BOP ($P<0.001$) in the periodontitis group were significantly higher than those in the healthy group. However, the PI was not significantly different between the 2 groups. Non-surgical periodontal therapy reduced PD ($P<0.01$), CAL ($P<0.01$),

Table 1. Full-mouth clinical parameters of all subjects in each group (healthy and periodontitis), before and 3 months after periodontal therapy

Parameter	Healthy group	Periodontitis group	
		Before treatment	3 months after treatment
PD (mm)	2.23±0.22	2.63±0.39[a]	2.49±0.29[b]
CAL (mm)	2.23±0.22	2.65±0.40[a]	2.50±0.30[b]
BOP (%)	58.47±30.84	78.52±25.04[a]	67.98±25.05[c]
Quigley-Hein PI	30.84±24.53	39.65±30.95	35.39±22.05

Data represent the mean±standard deviation.
PD: probing depth, CAL: clinical attachment loss, BOP: bleeding on probing, PI: plaque index.
[a]Statistically significant difference compared to the healthy group at $P<0.001$ using the independent t-test; [b]Statistically significant difference compared to the periodontitis group before treatment at $P<0.05$ using the independent t-test; [c]Statistically significant difference compared to the periodontitis group before treatment at $P<0.01$ using the independent t-test.

and BOP (*P*<0.05) when the periodontitis group was resampled 3 months after the procedure. However, periodontal therapy had no significant effect on the PI.

Relative proportions of periodontal pathogens

Figure 1 illustrates the relative proportions of periodontal pathogens in saliva and GCF in both groups. Similar proportions of periodontal pathogens were found in saliva and GCF samples from the healthy group; the only difference was that the proportion of *Pm* was significantly higher in saliva (12.42%) than in GCF (4.40%). In the periodontitis group, the proportion of *Pm* was also higher in saliva (21.84%) than in GCF (7.15%), but the proportions of *Tf* and *Pn* were also significantly higher in GCF (2.61% and 11.84%, respectively) than in saliva (1.19% and 2.95%, respectively).

Comparison of bacterial abundance between the periodontitis group and the healthy group before periodontal therapy

The number of periodontal pathogens found in saliva and GCF samples before periodontal therapy is shown in Tables 2 and 3, respectively. Compared to the healthy group, the salivary and subgingival abundance of periodontal pathogens was higher in the periodontitis group for all 11 species of pathogen except *Pn.* Furthermore, saliva and GCF had equivalent power in differentiating the groups statistically, as 4 species (*Pg*, *Tf*, *Pi*, and *Pm*) were significantly higher in the periodontitis group than in the healthy group in both saliva and GCF samples.

Change in bacterial abundance before and after periodontal therapy

The number of bacteria detected in the periodontitis group before and after periodontal therapy in saliva and GCF samples is illustrated in Figure 2. After periodontal therapy, GCF samples reflected the change in bacterial abundance more sensitively than saliva samples: the mean number of the 11 periodontal pathogens decreased in GCF samples, whereas only 9 periodontal pathogens showed decreased numbers in saliva samples. After periodontal
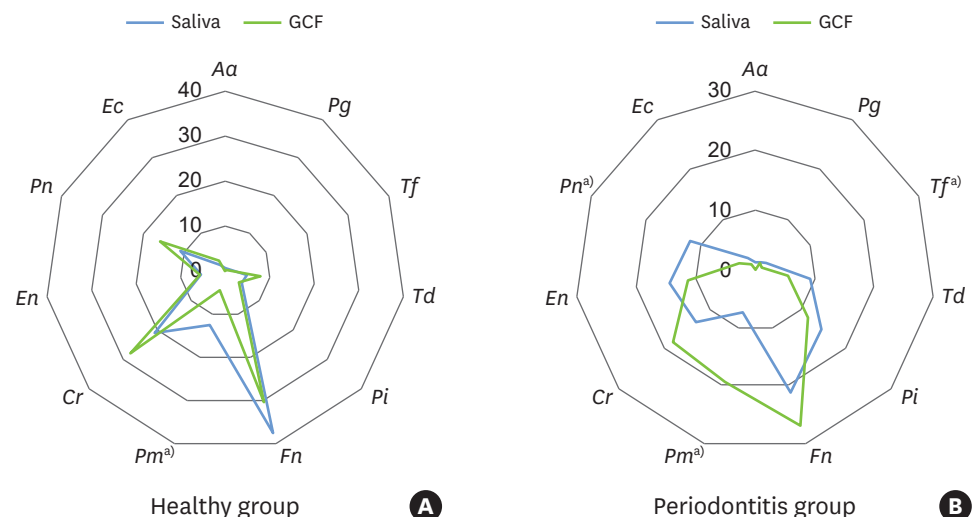

Figure 1. Relative proportions of periodontal pathogens in saliva and GCF samples of the healthy group (A) and the periodontitis group (B). Periodontal pathogens: *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythus* (*Tf*), *Treponema denticola* (*Td*), *Prevotella intermedia* (*Pi*), *Fusobacterium nucleatum* (*Fn*), *Pavimonas micra* (*Pm*), *Campylobacter rectus* (*Cr*), *Prevotella nigrescens* (*Pn*), *Eikenella corrodens* (*Ec*), and *Eubacterium nodatum* (*En*).
GCF: gingival crevicular fluid.
[a]*P*<0.05 using the independent *t*-test.

Table 2. The number of periodontal pathogens found in saliva samples (before periodontal therapy)

Periodontal pathogen (copy, ×10⁴)	Healthy group	Periodontitis group (before periodontal therapy)	P value
Aa	29.41±95.54	10.42±48.95	0.250
Pg	99.71±264.55	473.26±1,240.90	0.044[a]
Tf	90.86±209.48	367.34±762.26	0.027[a]
Td	339.33±390.83	1,349.98±4,962.27	0.202
Pi	1,229.06±4,228.56	4,139.97±8,474.83	0.049[a]
Fn	5,607.99±7,690.96	18,344.95±66,359.03	0.231
Pm	1,894.07±3,238.67	5,630.02±10,882.48	0.039[a]
Pn	1,206.42±1,794.20	819.01±2,808.86	0.455
Cr	3,914.79±6,980.73	4,614.79±7,660.50	0.659
En	1,083.88±2,652.00	3,536.14±8,376.21	0.079
Ec	216.21±343.99	548.07±2,063.37	0.318

Data represent the mean±standard deviation. Periodontal pathogens: *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythus* (*Tf*), *Treponema denticola* (*Td*), *Prevotella intermedia* (*Pi*), *Fusobacterium nucleatum* (*Fn*), *Pavimonas micra* (*Pm*), *Campylobacter rectus* (*Cr*), *Prevotella nigrescens* (*Pn*), *Eikenella corrodens* (*Ec*), and *Eubacterium nodatum* (*En*).
[a]Statistically significant difference compared to the healthy group at *P*<0.05 using the independent *t*-test.

Table 3. The number of periodontal pathogens found in gingival crevicular fluid samples (before periodontal therapy)

Periodontal pathogen (copy, ×10⁴)	Healthy group	Periodontitis group (before periodontal therapy)	P value
Aa	47.64±292.19	37.68±152.52	0.840
Pg	57.27±168.19	1,409.11±3,328.45	0.011[a]
Tf	222.18±696.59	1,859.02±3,629.16	0.006[b]
Td	421.65±798.71	7,154.65±25,475.21	0.099
Pi	1,263.10±4,992.79	14,993.53±39,636.60	0.032[a]
Fn	6,119.83±12,096.83	9,620.92±13,973.34	0.212
Pm	1,316.50±4,068.30	4,292.83±9,842.34	0.043[a]
Pn	2,822.06±5,048.51	3,434.74±5,392.14	0.582
Cr	3,479.12±5,978.87	6,658.30±13,140.35	0.051
En	1,549.54±4,450.55	12,232.47±23,938.38	0.075
Ec	332.58±1,221.25	351.45±516.72	0.922

Data represent the mean±standard deviation. Periodontal pathogens: *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythus* (*Tf*), *Treponema denticola* (*Td*), *Prevotella intermedia* (*Pi*), *Fusobacterium nucleatum* (*Fn*), *Pavimonas micra* (*Pm*), *Campylobacter rectus* (*Cr*), *Prevotella nigrescens* (*Pn*), *Eikenella corrodens* (*Ec*), and *Eubacterium nodatum* (*En*).
[a]Statistically significant difference compared to the healthy group at *P*<0.05 using the independent *t*-test;
[b]Statistically significant difference compared to the healthy group at *P*<0.01 using the independent *t*-test.

therapy, the decrease in *Cr* was statistically significant in saliva samples, whereas the decreases in *Cr*, *Pg*, and *Pn* were statistically significant in GCF samples.

The results of the Pearson correlation analysis of the change in abundance in periodontal pathogens in saliva and GCF samples before and after periodontal therapy are presented in Table 4. The change in the abundance of all 11 periodontal pathogens was positively correlated between saliva and GCF before and after treatment, and significantly so for 5 pathogens *Tf* (r=0.421, *P*<0.01), *Pi* (r=0.331, *P*<0.05), *Cr* (r=0.325, *P*<0.05), *Pn* (r=0.304, *P*<0.05), and *Ec* (r=0.294, *P*<0.05).

DISCUSSION

This study was designed to evaluate how well the subgingival bacterial sampling method, using a retraction cord, represented patients' overall periodontal conditions. Our results suggest that salivary and subgingival bacterial sampling have equivalent power to
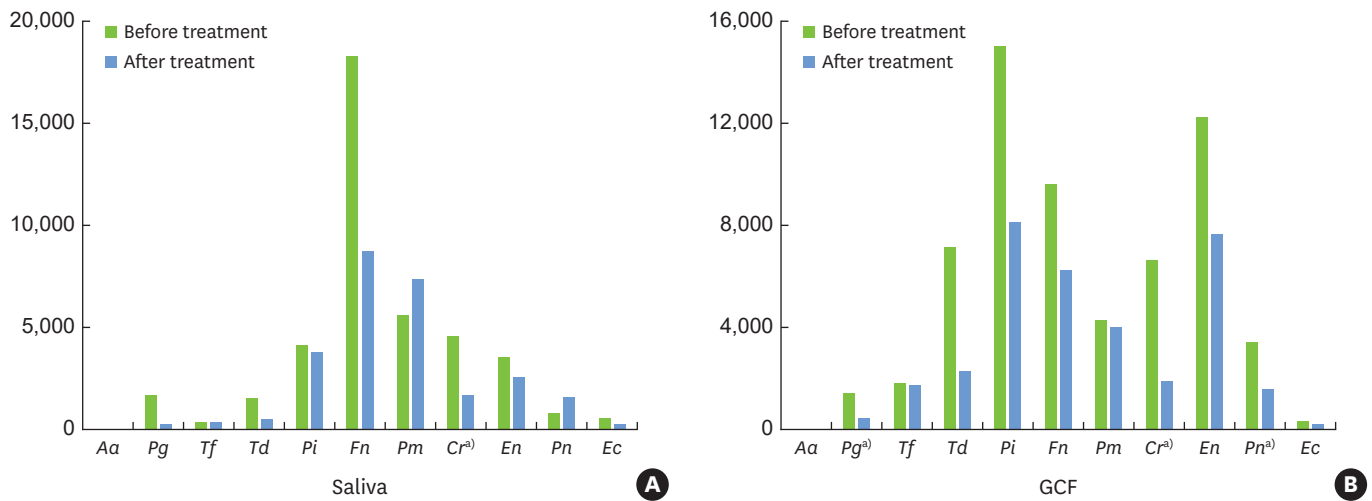
Figure 2. Change in the number of bacteria detected in the periodontitis group before and after periodontal therapy in saliva (A) and gingival crevicular fluid samples (B). Periodontal pathogens: *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythus* (*Tf*), *Treponema denticola* (*Td*), *Prevotella intermedia* (*Pi*), *Fusobacterium nucleatum* (*Fn*), *Pavimonas micra* (*Pm*), *Campylobacter rectus* (*Cr*), *Prevotella nigrescens* (*Pn*), *Eikenella corrodens* (*Ec*), and *Eubacterium nodatum* (*En*). Data represent the mean.
GCF: gingival crevicular fluid.
[a]*P*<0.05 using the paired *t*-test.

Table 4. Pearson correlation analysis of the change in abundance in periodontal pathogens in saliva and gingival crevicular fluid samples before and after periodontal therapy

Periodontal pathogen	Pearson coefficient	*P* value
Aa	0.199	0.180
Pg	0.216	0.145
Tf	0.421	0.003[b]
Td	0.202	0.174
Pi	0.331	0.023[a]
Fn	0.205	0.168
Pm	0.203	0.171
Cr	0.325	0.026[a]
En	0.051	0.731
Pn	0.304	0.036[a]
Ec	0.294	0.045[a]

Data represent the mean±standard deviation. Periodontal pathogens: *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythus* (*Tf*), *Treponema denticola* (*Td*), *Prevotella intermedia* (*Pi*), *Fusobacterium nucleatum* (*Fn*), *Pavimonas micra* (*Pm*), *Campylobacter rectus* (*Cr*), *Prevotella nigrescens* (*Pn*), *Eikenella corrodens* (*Ec*), and *Eubacterium nodatum* (*En*).
[a]Statistically significant difference at *P*<0.05; [b]Statistically significant difference at *P*<0.01.

differentiate between healthy patients and those with periodontitis. Specifically, 4 bacterial species (*Pg*, *Tf*, *Pi*, and *Pm*) were present at significantly higher levels in the periodontitis group using both sampling methods. This result is similar to that of a previous study, which showed a higher number of *Pg* and *Tf* in periodontal patients than in healthy patients [4].

In the present study, the change in the level of periodontal pathogens before and after periodontal therapy was also measured to evaluate how well saliva and subgingival bacterial sampling could represent overall periodontal conditions following treatment. In saliva, only the abundance of *Cr* decreased significantly after treatment; however, in subgingival bacterial sampling, *Pg*, *Cr*, and *Pn* all decreased significantly after treatment. This result indicates that the subgingival bacterial sampling reflected changes in the number of periodontal pathogens after periodontal therapy more sensitively than saliva sampling. This result is in accordance with a previous study, which reported that the relative abundance of periodontal pathogens

was less strongly influenced by periodontal treatment when measured in saliva than when measured in subgingival plaque [16]. This finding implies that measurements of salivary microbiota could be influenced by bacteria from the tongue, tonsils, and pharynx, as well as from subgingival plaque [13].

RT-PCR analysis of saliva and subgingival bacterial samples is a more sensitive and accurate technique for quantifying periodontal pathogens than the conventional culture method, which enables the detection and quantification of only a small number of pathogens [17-20]. In a study in which the RT-PCR and culture methods were compared, the number of *Aa, Pg, Tf,* and *Pi* from both plaque and mouthwash samples determined by RT-PCR was somewhat correlated with the results of detection by the culture method; the sensitivity and specificity of RT-PCR for detection of *Aa, Pg, Tf,* and *Pi* from mouthwash samples was 93%-100% and 75%–89%, respectively [5]. This indicates that RT-PCR is a reliable method for the detection and quantification of periodontal pathogens from saliva and subgingival bacterial sampling. In a previous study, the rate of *Pg, Tf, Pi,* and *Aa* detection was 48%, 44%, 31%, and 27%, respectively, in whole saliva [21], while the rate was 33.3%, 85.7%, 76%, and 9%, respectively, in subgingival bacterial samples (sampling with paper points) [5]. In the present study, the rate of *Pg, Tf, Pi,* and *Aa* detection in the periodontitis group was 67.7%, 81.5%, 78.5%, and 38.5%, respectively, in whole saliva, while the rate was 66%, 80.9%, 74.5%, and 31.9%, respectively, for subgingival bacterial sampling (using a gingival retraction cord).

In the present study, a gingival retraction cord was used as a novel sampling method: this is potentially more reproducible and less technique-sensitive than the paper point sampling method. In a previous study using RT-PCR, paper point and curette sampling showed similar results, and therefore both could be considered as suitable sampling methods for microbiological and plaque diagnoses [12]. These sampling methods have also been considered suitable for microbiological diagnoses when using subgingival bacterial sampling [5]. However, with the paper points method, the results can show high levels of variation even in the same tooth, as the subgingival bacterial load is distributed differently depending on the subgingival environment, which includes factors such as the depth of the periodontal pocket or the degree of the inflammatory reaction. In the present study, subgingival bacterial sampling with a gingival retraction cord was found to be a useful method for sampling subgingival bacteria because it represented the overall subgingival condition well.

In conclusion, saliva and subgingival bacterial sampling with a gingival retraction cord were confirmed to be equivalently accurate for differentiating patients with periodontitis, but GCF sampling reflected changes in bacterial abundance after periodontal therapy more sensitively than saliva sampling.

ACKNOWLEDGEMENTS

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