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Quantitative assessment of different bacterial sampling methods in patients with periodontitis using multiplex real-time polymerase chain reaction

다중 실시간 중합효소연쇄반응법을 이용한 치주염 환자의 다양한 세균 샘플링 방법에 대한 정량적 평가

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Abstract

Quantitative assessment of different bacterial sampling methods in patients with periodontitis using multiplex real-time polymerase chain reaction

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Objectives. The aim of the study was to quantitatively compare bacterial profile of patients with different severity of periodontal disease using samples from mouthwash and the subgingival area. Further analysis was performed to evaluate the correlation between mouthwash and two subgingival sampling methods: paperpoint and gingival retraction cord.

Materials and Methods. One hundred and fourteen subjects were enrolled in the study, and were divided equally into three groups according to disease severity. Mouthwash and subgingival sampling were conducted, and the samples were analyzed for 11 target periodontopathic bacteria using multiplex real-time PCR.

Results. The majority of the target bacteria showed increasing tendency in their amount as the severity of periodontal disease deteriorated. The amount of bacterial DNA in mouthwash and that in subgingival sampling methods had a tendency of enhanced correlation as the severity of periodontitis deteriorated. The amount of the DNA of 6 bacterial species had statistically significant correlations with the severity of periodontal disease, but only *Porphyromonas gingivalis* and *Tannerella forsythia* presented fair correlations (ρ = 0.530, 0.438, respectively). In binary logistic regression analysis, *Tannerella forsythia* only demonstrated statistically significant odds ratio both in gingival retraction cord sampling and in mouthwash sampling (OR = 1.206 and 1.581, respectively)

Conclusion. Mouthwash sampling showed significant correlations with two different subgingival sampling methods in the detection of several bacteria. However, the correlation was more prominent as disease severity increased. Bacteria in mouthwash may be more suitable for the diagnosis of severe periodontitis, rather than early diagnosis.

Keywords : Multiplex polymerase chain reaction; Periodontitis; Diagnosis; Bacteria; Mouthwash
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CONTENTS

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I. Introduction

Periodontitis is inflammatory conditions of the tooth-supporting structures, which results in the destruction of periodontium and finally leads to the loss of tooth [1]. Since the inflammation is known to be caused by the host response to oral microbial biofilm [2], the role of oral microbiome in the pathogenesis of the disease has been extensively explored for a long period. Various hypotheses, such as nonspecific plaque hypothesis and specific plaque hypothesis, have been proposed to explain the process of periodontal tissue destruction [3]. More recently, ecological plaque hypothesis was introduced, suggesting that the accumulation of dental plaque around the gingival margin may provide ecological stresses that favor the proliferation of anaerobic Gram-negative bacteria, and finally cause tissue-destructive host response [4]. These ecological shifts in the composition of subgingival microbiota, that is, the proliferation of different Gram-negative anaerobes over facultative Gram-positive species in the periodontal pocket, is well documented to be associated with the development and progression of periodontitis [5].

Socransky and coworkers [6] suggested a detailed analysis of the microbial complexes in the subgingival plaque. The cluster analysis

yielded six closely associated bacterial complexes, and they were designated with color codes. Four complexes mainly consist of early colonizers of the tooth surface, namely, “Blue complex” consisting of *Actinomyces* species, “Yellow complex” consisting of various *Streptococci*, “Green complex” consisting of *Eiknella corrodens* and *Capnocytophaga* species, and “Purple complex” consisting of *Veillonella parvula* and *Actinomyces odontolyticus*. Two additional complexes were recognized. “Orange complex” includes various species of *Prevotella*, *Fusobacterium*, *Campylobacter* and other bacteria. “Red complex” comprises three bacterial species, that is, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. These two complexes have been classified as late colonizer in the development and maturation of subgingival plaque in the periodontal pocket and they have been closely related to the pathological conditions of periodontal tissue. This classification system is still valid in the area of periodontal microbiology.

A variety of sampling techniques have been utilized for the microbiological assessment. To acquire the sample of subgingival microbiota from the periodontal pocket, the insertion of sterile paperpoint or curette into the periodontal pocket were widely utilized. However, they may be invasive and difficult to perform, so a well-trained practitioner is required for sampling procedure [7]. Recently, saliva is

considered to be more appropriate for the diagnostic aid in daily practice due to its easy and non-invasive sampling procedure [8]. Interestingly, the salivary microbiota of periodontitis patients was presented to be different from that of healthy subjects. A microarray-based research by Belstrom and coworkers [9] reported eight bacterial taxa, and four bacterial clusters were observed to be present statistically more frequently in samples from periodontitis. Another study conducted by Chen and colleagues [10] demonstrated that six genera including *Porphyromonas*, *Tannerella*, and *Eubacterium* in the saliva sample of periodontitis patients exhibited significant abundance over that in the saliva of healthy controls using 16S rDNA sequencing. It was also reported that the salivary microbial profile could reflect the periodontopathogens in subgingival plaque sample [11–13]. Moreover, mouthwash sampling has been suggested as an alternative of saliva sampling [32]. However, there are only a limited number of documentations of the correlations of different bacterial sampling methods in detection of bacteria that are correlated with pathologic periodontal conditions.

Various methods have been adopted for the detection of bacteria. Cultivation technique, microscopic evaluation, immunological methods, and DNA hybridization-based methods have elucidated different microbiological characteristics in periodontitis patients. However, the

majority of the detection methods mentioned above are semi-quantitative, expensive, often time-consuming and labor-intensive. Currently, multiplex real-time polymerase chain reaction (PCR) received attention for its ability to detect several target DNA sequences simultaneously, and high-throughput quantification with less sample and input material. Nevertheless, there are only limited numbers of documentations available that adopted multiplex real-time PCR for the identification and quantification of microbiological profiles. For example, Estrela and colleagues [14] reported a prevalence of bacterial species in samples from different intraoral sites of periodontitis patients using multiplex real-time PCR, but there were no data on quantity of different bacterial species. More recently, a research performed by Lochman [15] presented the quantification of cariogenic and periodontopathic bacteria from 30 Czech children who had severe early childhood caries and gingivitis, without including periodontitis of adult subjects.

There are limited numbers of research studies that evaluated the quantitative assessment of bacterial profile of periodontitis patients using multiplex real-time PCR. The aim of the present study was to quantitatively compare the bacterial profile of patients with different severity of periodontal disease using samples from mouthwash and the subgingival area. An additional purpose was to evaluate the correlation between the salivary and subgingival bacterial profile using multiplex

real-time PCR, ultimately evaluating the microbiological diagnostic performance of mouthwash compared to other sampling methods in patients with periodontal diseases.

II. Material and Method

1. Ethical approval and study population

This study was approved by Institutional Review Board of Seoul National University Dental Hospital (Code: CRI18002, 19 October 2017) and was conducted with strict observance of the Declaration of Helsinki. Sufficient information on the clinical study was given to all participants and written informed consents were obtained at their own free will before enrollment in the study.

The inclusion criteria of study population were presented as follows: age of 20 to 69 years old who visited Seoul National University Dental Hospital from December 2018 to March 2020, having at least 20 natural teeth, and absence of any systemic disease. Subjects were excluded from the study if they had antibiotic therapy within 2 weeks from the study date, if they had periodontal treatment within 6 months from the study date, and if they were under orthodontic treatment.

2. Clinical examination and study group assignment

After enrollment in the study, all participants underwent full mouth recording of various periodontal parameters, including probing pocket

depth (PPD), and clinical attachment loss (CAL), bleeding on probing (BOP), plaque index (PI), and gingival index (GI), which were registered at six sites (mesio-facial, mid-facial, disto-facial, mesio-lingual, mid-lingual, and disto-lingual) of all teeth except third molars and dental implants. Panoramic x-ray was taken to evaluate alveolar bone loss and to screen any other clinically significant pathology.

Additionally, following parameters were recorded: the number of natural teeth, the number of dental implants, smoking status, the number of full veneer crown, the number of restorations, the number of furcation-involved teeth, the number of caries-involved teeth.

Based on the result of clinical examination and radiographic evaluation, all participants were assigned to one of the three study groups. The classification of different study groups was originated and modified from the case definition introduced by the US Centers for Disease Control and Prevention and the American Academy of Periodontology [16].

• Group 1 (Severe periodontitis, SP): presence of 2 or more interproximal sites with ≥6 mm of CAL and 1 or more interproximal site(s) with ≥5 mm of PPD

• Group 2 (Moderate periodontitis, MP): 2 or more interproximal sites with ≥4 mm of CAL or 2 or more interproximal sites with ≥5 mm of PPD

• Group 3 (Gingivitis/Mild periodontitis, G/M): subjects who are not assigned to Group 1 or 2

3. Microbial sampling

All subjects were refrained from eating or drinking anything and from toothbrushing at least 3 hours before sample collection. For salivary sample collection, 12 mL of mouthwash solution provided by analytical company (Periogen, Gyeonggi-do, Korea) was given to all subjects to rinse their mouth for 30 seconds. The mouthwash contains sodium fluoride as main active ingredient, and some other additives, such as menthol, xylitol, sodium citrate, glycerol and ethanol, were also included. After rinsing, the mouthwash solution was spitted into a sample collection tube and the cap of the tube was closed tightly. For subgingival microbial sampling, two teeth from every individual who presented the deepest PPD and similar periodontitis lesions were selected. Before subgingival sampling, supragingival dental biofilm was gently removed and all sampling sites were isolated from saliva. Three sterile ISO #35 paperpoints were inserted into three of six periodontal pocket sites of one representative tooth for 30 seconds. One sterile gingival retraction cord of 10 mm in length was inserted into the periodontal pocket of the other representative tooth for 30 seconds. After retrieval from each periodontal

pocket, both paperpoint and gingival retraction cord samples were immediately transferred to an EP-tube containing 1 mL of the mouthwash solution mentioned above. All samples were stored in a refrigerator at 4◦C before DNA extraction.

4. DNA extraction and multiplex real-time PCR

Bacterial DNA was extracted using Exgene Cell SV mini kit (GeneAll, Seoul, Korea). The extracted DNA samples were stored at −20 ◦C before any further analyses. The samples were analyzed using a real-time PCR kit (GeneAll, Seoul, Korea) to detect the following periodontopathic bacteria: *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythia* (*Tf*), *Treponema denticola* (*Td*), *Prevotella intermedia* (*Pi*), *Fusobacterium nucleatum* (*Fn*), *Parvimonas micra* (*Pm*), *Campylobacter rectus* (*Cr*), *Eiknella corrodens* (*Ec*), *Prevotella nigrescens* (*Pn*), *Eubacterium nodatum* (*En*). Each bacterial DNA sample was amplified by the specific primer that targets functional gene (e.g., rpgB, waaA, gtf) of each species.

For the samples to be analyzed in the Hot-start Taq DNA polymerase assay, all samples were processed in 20 μL reaction mixture, containing 2 μL of extracted DNA solution, periodontal pathogen-specific primers (Periogen, Gyeonggi-do, Korea), and PCR reaction buffer. PCR analyses

were conducted with ABI 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). After initial denaturation at 95◦C for 15 minutes, 40 cycles of amplification were programmed, each amplification being composed of 95◦C for 30 seconds, 55◦C for 30 seconds, and 72◦C for 30 seconds.

Standard curves were constructed with known amounts of bacterial DNA, plotting the relationship between cycle threshold (Ct) values and the numbers of bacterial DNA copies. The obtained Ct value of each bacterial sample was converted into the DNA copy numbers, which were used in the quantitative comparison procedures.

5. Statistical analysis

All statistical analyses were performed using SPSS 25.0 (IBM Corp., Armonk, NY, USA). All data were checked on their normality with the Shapiro–Wilk test. If the data set could be assumed to follow Gaussian distribution, one-way ANOVA and Dunnet T3 test for post hoc analysis was performed. Nonparametric data sets were compared with the Kruskal–Wallis H test, and Bonferroni correction was adopted for multiple comparison. Prevalence of certain bacteria in each sample was compared with the Pearson's Chi-square test. Correlations between data sets were analyzed with Spearman's rank correlation. Logistic regression

analysis was performed to explore which bacterial species had potential to be applied in the diagnostic procedure. Multicollinearity test and goodness-of-fit test were done to construct the model that has the strongest power of explanation. P values < 0.05 were set to indicate statistical significance. All graphs were plotted using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

III. Result

1. Demographics and clinical data

One hundred and fifteen subjects were volunteered to be enrolled in this study, but one subject was excluded due to ongoing orthodontic treatment; 114 participants were divided into 3 groups that were previously described, 38 subjects comprising each study group.

Demographic and clinical data are described in detail in Table 1. Data that follow Gaussian distribution were described with mean ± standard deviation, and nonparametric data were expressed with median and interquartile range in parentheses. There were statistically significant differences on age (SP group: 58.5 (51.5–61.0), MP group: 42.5 (37.0–55.0), G/M group: 27.5 (23.0–37.25)), PPD (SP group: 2.894 ± 0.378 mm, MP group: 2.490 ± 0.265 mm, G/M: 2.274 ± 0.167 mm) and CAL (SP group: 3.382 (3.015–3.619), MP: 2.651 (2.453–2.835), G/M: 2.266 (2.196–2.393)) between all study groups. GI was significantly different between MP group (0.143 (0.038–0.594)) and G/M group (0.417 (0.278–0.656)). Sex distribution, BOP%, and PI were not significantly different between all study groups.

2. Prevalence of target bacteria

Prevalence of target bacteria is presented according to the different sampling strategies, that is, paperpoint (Table 2), gingival retraction cord (Table 3), and mouthwash (Table 4), respectively. *Fn* were detected with high frequencies among all samples, regardless of the sampling methods. In paperpoint sample, six bacteria (*Pg, Tf, Td, Pm, Cr,* and *En*) presented significantly heterogenous prevalence between study groups. Similarly, six bacteria (*Pg, Tf, Td, Pi, Cr,* and *En*) showed unequal distribution of prevalence between study groups. However, in mouthwash samples, significantly different prevalence was detected only from two bacteria (*Pg, Tf*).

3. Quantitative profiles of target bacteria

Bacterial DNA copy numbers were converted from Ct values that were obtained from multiplex real-time PCR analyzer. Different profiles were depicted to compare the difference between study groups in the same sampling method (Figure 1).

Obtained bacterial DNA exhibited highly diverse quantities. The majority of the target bacteria showed increasing tendency in their amount as the severity of periodontal disease deteriorated. In paperpoint

samples, DNA copy numbers of 7 bacteria (*Pg, Tf, Td, Fn, Pm, Cr,* and *En*) presented significant differences between study groups. In gingival retraction cord samples, similar profiles were observed, indicating significant differences in 8 bacterial species (*Pg, Tf, Td, Pi, Fn, Pm, Cr,* and *En*). In mouthwash samples, 6 bacterial (*Pg, Tf, Td, Pm, Cr,* and *En*) exhibited statistical difference in their DNA copy numbers. Regardless of the sampling methods, the majority of the statistical differences were observed between severe periodontitis and moderate periodontitis, and between severe periodontitis and gingivitis/mild periodontitis.

4. Correlations between sampling methods

The DNA copy number in mouthwash samples was correlated with that in the paperpoint sample and that in the retraction cord sample in each study group using Spearman's correlation analysis. Spearman's correlation coefficients (ρ) of each bacterial species in SP, MP, and G/M groups were summarized below (Table 5, Figure 2).

Both subgingival sampling methods presented similar correlations with the mouthwash sampling method in broad outlines. In the G/M group, statistically significant correlation coefficients fall into the range of 0.3–0.5, which indicates fair correlation [17]. However, in the SP and MP groups, compared to the G/M group, many correlation coefficients

that present statistical significance belong in the range of 0.4–0.8, suggesting fair to moderately strong correlation. The results may imply that mouthwash samples were more strongly correlated with site-specific subgingival samples as the severity of periodontal disease increases.

5. Correlations between sampling method and periodontal disease severity

The severity of periodontal disease was correlated with various clinical parameters and bacterial DNA counts in mouthwash samples using Spearman's correlation analysis (Table 6). The severity of periodontal disease presented statistically significant correlation with mean PPD, mean CAL ($\rho = 0.664, 0.792$, respectively). In bacterial DNA counts, *Pg, Tf, Td, Pm, Cr* and *En* in mouthwash samples were significantly correlated with the severity of periodontal disease ($\rho = 0.530, 0.438, 0.209, 0.276, 0.283, 0.311$, respectively). However, only *Pg* and *Tf* in mouthwash exhibited fair correlation with the severity of periodontal disease.

6. Binary logistic regression analysis

Considering that bacterial profile did not present significant

difference between moderate periodontitis and gingivitis/mild periodontitis, binary logistic regression model was constructed between SP group and MP + G/M group according to the different sampling methods. Multicollinearity test was performed and as a consequence, several bactrerial species were excluded from the statistical model.

In paperpoint sampling, none of the bacteria showed statistically significant odds ratio after adjustment of various parameters (Table 7). In contrast, after adjusting various parameters, only one bacterial species, *Tf*, demonstrated statistically significant odds ratio both in gingival retraction cord sampling and in mouthwash sampling (OR = 1.206 and 1.581, respectively) (Table 8, 9).

IV. Discussion

In the present study, 11 target periodontopathic bacteria, which were mainly ‘Red complex’ and ‘Orange complex’, were selected and tested for their feasibility of diagnostic application, as bacteria were closely related to the pathogenesis of periodontitis. Our data demonstrated that the majority of target bacteria exhibited increased counts both in mouthwash and in subgingival samples as the severity of periodontal disease increased. Regardless of the sampling methods, *Pg, Tf, Td, Pm, Cr*, and *En* presented significant differences between study groups. This result is similar to that of a previous study presenting higher *Pg* and *Tf* level in periodontitis patients [18].

From our data, the prevalence and quantity of periodontopathic bacteria in subgingival samples tend to increase as the disease severity increases. Notably, *Fn* was detected with high frequency and quantity from all samples. This result is in line with previous study demonstrating that *Fn* was the most abundant in both conditions of healthy and periodontitis [19]. *Fn* is known as a bridging species linking early colonizers on tooth surface and late-colonizing pathogens, such as ‘Red complex’ [20,21]. Significant increase of *Fn* in severe periodontitis was observed in subgingival samples, but the differences in amount between

study groups were insignificant in mouthwash samples. This may be explained by the ubiquity of *Fn* in the oral cavity, as it is capable of binding to oral epithelial cells [22]. Despite the increase of *Fn* in the subgingival area and its flush-out into oral cavity, it may be masked by previously populated *Fn* in oral cavity.

Not only *Fn* but also many other bacterial species presented higher quantity in mouthwash sample than in paperpoint sample or gingival retraction cord sample. This could be explained by the fact that periodontopathic bacteria may be colonized in other oral sites as well as subgingival area. Mager *et al*. [23] reported that *Ec*, and *Pg* was observed from saliva, lateral and dorsal tongue surfaces. In addition, Cortelli *et al.* [24] demonstrated that 5 bacterial species (*Cr, Pg, Aa, Pi*, and *Tf*) were found to exist on tongue and cheek mucosa. Considering that mouthwash sampling may reflect the microbiota in the whole oral cavity, including tongue, cheek, or other mucosal surface, it might be possible to explain that more bacterial count could be observed from the mouthwash sample. Further research is required on the quantity and distribution of periodontopathic bacteria in other oral sites to examine this idea.

Various sampling techniques were adopted for the analysis of subgingival plaque. Curette [6,25] or paperpoint [26,27] was the most frequently utilized in the subgingival sampling procedure. Previous

studies demonstrated the relationship between the subgingival sampling methods. It was reported that although curette sampling could represent higher total bacterial counts than paperpoint sampling, the plaque composition of target bacteria was similar for both sampling methods, suggesting that both sampling techniques can be used in microbial assessment [28]. Belibasakis and colleagues [29] reported similar profiles of 'Red complex' both from paperpoint and from curette samples. In another study, ligature that induced experimental periodontitis and paperpoint subsequently inserted in the same sites after ligature removal were compared with checkerboard DNA-DNA hybridization [30]. Considering that similar bacterial profiles between the ligature and paperpoint, it is suggested that a ligature on a tooth may also be used as a sampling method. In the present study, the gingival retraction cord, which resembles the ligature used in the animal experiment, was utilized for subgingival sampling. Gingival retraction cord was assumed to be more reproducible than paperpoint, because it would cover a larger surface of subgingival area, resulting in effective reflection of subgingival microbiota. Moreover, paperpoint would be deformed or folded after absorption of gingival crevicular fluid. This might hinder the insertion of paperpoint deep into the periodontal pocket, and gingival retraction cord would be less technique-sensitive than paperpoint [31]. However, as the result of the present study exhibited, there was no

significant difference in the amount of target bacterial counts between two subgingival sampling methods. This implies that the gingival retraction cord may also be utilized in the subgingival biofilm sampling procedure.

Saliva was featured as a promising source of periodontopathic bacteria because of its easiness and non-invasiveness of sampling. Instead of saliva, mouthwash sampling was suggested for the detection of bacterial DNA since it was more straightforward and faster than saliva collection [17]. Since mouthwash contains several antiseptics and alcohol, it can prevent bacterial growth during the entire sampling procedure and cooling step for storage. This was supported by one study reporting that overall bacterial composition was not significantly different between mouthwash sample and saliva sample [32]. Another study demonstrated that the utilization of commercially available mouthwash yielded a significant amount of human genomic DNA from buccal cells with high quality [33]. Taken together, it could be suggested that the mouthwash sampling would not significantly degrade bacterial DNA in the mouthwash solution. Hence, mouthwash sampling may be considered an alternative to the saliva sampling.

In our data, *Pg, Tf, Td, Pm, Cr,* and *En* in the mouthwash presented a significant difference between study groups. Among them, *Pg* only

presented a significant difference between all study groups. In addition, *Pg* in mouthwash was correlated with the severity of periodontal disease, exhibiting greatest correlation coefficient of 0.530. This indicates that the *Pg* in mouthwash may serve as a bacterial biomarker for periodontitis. This is consistent with previous studies demonstrating different salivary *Pg* profiles in periodontitis patients and healthy subjects [34,35]. More recently, salivary microbiota was analyzed with sequencing-based method, and also demonstrated that the amount of salivary *Pg* was more prominent in periodontitis patients than that in healthy subjects [36,37]. Based on these results, it may be suggested that *Pg* in saliva or mouthwash has potential to be utilized as a diagnostic marker of periodontitis.

The bacterial profile of mouthwash samples was correlated with that of subgingival samples in this study. The majority of the correlation coefficients of target bacteria presented significant correlation between mouthwash and subgingival samplings. Notably, as the severity of periodontal disease increased (from gingivitis/mild periodontitis to severe periodontitis), the correlations became stronger. This can be explained by the combinatorial effect of bacterial proliferation and the flow of gingival crevicular fluid. The subgingival periodontopathic bacteria increase in deep periodontal pocket as the severity of periodontal disease deteriorates [38]. In addition, the flow of gingival crevicular fluid

increases and its flushing action is reinforced in the inflammatory conditions [39]. As a result, the increased bacterial DNA is washed out to oral cavity more abundantly, and finally is detected with mouthwash sampling.

The tendency of enhanced correlation between mouthwash sample and subgingival sample in the severe periodontitis group was demonstrated in the present study. In addition, the quantitative profile did not significantly discriminate moderate periodontitis and gingivitis/mild periodontitis in the majority of targeted bacterial species. These may imply that the microbiological diagnosis using mouthwash sampling may be more suitable for diagnostic application in severe periodontitis, rather than mild or moderate periodontitis. This may represent the difficulty of early diagnosis of periodontitis using microbiological assessment. Although microbiological examination with multiplex real-time PCR may not be suitable for early diagnosis of periodontitis, it still has some values in the management of periodontitis. First, it can be adopted in the decision-making procedure of antibiotics use. Aggressive periodontitis or periodontitis with refractory characteristics which did not respond to mechanical debridement well can be managed with the administration of antibiotics. Second, it may be utilized in patient monitoring for the recurrence of periodontitis. Since it is reported that an increase of some periodontopathic bacteria over a

certain threshold in regular recall check-up was correlated with 2.5 times greater risk of disease recurrence [40], microbiological test may be helpful in the maintenance care of periodontitis-susceptible patients.

V. Conclusion

Using multiplex real-time PCR, it was demonstrated that major periodontopathic bacteria presented different bacterial profiles and prevalence among the study groups with different severity of periodontal disease. Many bacterial species tested in mouthwash exhibited significant correlation with that in subgingival samples, demonstrating the possibility that bacteria in mouthwash can reflect that in the subgingival area. This may imply that bacterial count in mouthwash as a potential biomarker for the diagnosis of periodontitis. However, considering the correlation was enhanced as the severity of periodontitis deteriorates, microbial assessment might be more suitable to be utilized to the patients with severe periodontitis, rather than early stage of periodontitis. In future, the discovery of biomarkers that can report early diagnosis of periodontitis may be desired, and bacterial biomarkers might be concomitantly utilized with the newly developed biomarkers in the diagnosis of the periodontitis.

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Table 1. Demographic and clinical data of study groups.

	SP Group	MP Group	G/M Group	*p*
Participants number	38	38	38	
Age (years) [1]	58.5 (51.5–61.0)	42.5 (37.0–55.0)	27.5 (23.0–37.25)	<0.001
Female : Male	22 : 16	22 : 16	25 : 13	0.719
Number of smokers	4	5	2	0.494
Number of natural teeth [5]	25.61 (±2.32)	26.37 (±2.63)	27.26 (±1.27)	0.005
Number of implants [6]	1.00 (0.00-1.25)	0.00 (0.00-2.00)	0.00 (0.00-0.00)	0.001
Number of restorations	7.66 (±4.89)	7.24 (±6.19)	7.08 (±5.29)	0.893
Number of furcation -involved teeth [1]	3.00 (1.75-4.00)	1.00 (0.00-2.00)	0.00 (0.00-0.00)	<0.001
Number of caries -involved teeth [4]	1.211 (±1.398)	0.553 (±0.724)	0.868 (±1.070)	0.037
PPD (mm) [2]	2.894 (±0.378)	2.490 (±0.265)	2.274 (±0.167)	<0.001
CAL (mm) [1]	3.382 (3.015–3.619)	2.651 (2.453–2.835)	2.266 (2.196–2.393)	<0.001
BOP%	53.910 (±26.826)	47.126 (±20.334)	49.015 (±20.190)	0.394
PI	0.275 (0.194–0.664)	0.211 (0.122–0.451)	0.280 (0.176–0.414)	0.196
GI[3]	0.295 (0.086–0.436)	0.143 (0.038–0.594)	0.417 (0.278–0.656)	0.009

[1] Significant difference between all study groups. Kruskal–Wallis H test and Bonferroni correction. [2] Significant difference between all study groups. One-way ANOVA and Dunnet T3 test. [3] Significant difference between MP group and G/M group. Kruskal–Wallis H test and Bonferroni correction. [4] Significant difference between SP group and MP group. One-way ANOVA and Dunnet T3 test. [5] Significant difference between SP group and G/M group. One-way ANOVA and Dunnet T3 test. [6] Significant difference between SP group and G/M group. Kruskal-Wallis H test and Bonferroni correction. SP: severe periodontitis, MP: moderate periodontitis, G/M: gingivitis/mild

periodontitis, PPD: probing pocket depth, CAL: clinical attachment loss, BOP: bleeding on probing, PI: plaque index, GI: gingival index.

Table 2. Prevalence of target bacteria in paperpoint samples of different study groups.

	Aa	***Pg***	***Tf***	***Td***	***Pi***	***Fn***	***Pm***	***Cr***	***En***	***Pn***	***Ec***
SP (%)	5.26	76.32	78.95	52.63	28.95	97.37	86.84	55.26	63.16	60.53	65.79
MP (%)	2.63	36.84	44.74	21.05	18.42	97.37	65.79	39.47	26.32	71.05	60.53
G/M (%)	0	15.79	23.68	15.79	10.53	94.74	47.37	7.89	7.89	52.63	55.26
p [1]	0.358	<0.001	<0.001	0.004	0.124	0.772	0.007	<0.001	<0.001	0.317	0.644

SP: severe periodontitis, MP: moderate periodontitis, G/M: gingivitis/mild periodontitis. [1] *p*-values were calculated by Pearson's Chi-square test.

Table 3. Prevalence of target bacteria in gingival retraction cord samples of different study groups.

	Aa	***Pg***	***Tf***	***Td***	***Pi***	***Fn***	***Pm***	***Cr***	***En***	***Pn***	***Ec***
SP (%)	10.53	65.79	76.32	39.47	21.05	94.74	86.84	52.63	65.79	60.53	68.42
MP (%)	2.63	26.32	42.11	15.79	13.16	100	78.95	21.05	26.32	57.89	65.79
G/M (%)	5.26	10.53	18.42	5.26	7.89	92.11	76.32	10.53	7.89	63.16	55.26
p [1]	0.345	<0.001	<0.001	0.001	0.037	0.231	0.481	<0.001	<0.001	0.896	0.712

SP: severe periodontitis, MP: moderate periodontitis, G/M: gingivitis/mild periodontitis. [1] *p*-values were calculated by Pearson's Chi-square test.

Table 4. Prevalence of target bacteria in mouthwash samples of different study groups.

	Aa	***Pg***	***Tf***	***Td***	***Pi***	***Fn***	***Pm***	***Cr***	***En***	***Pn***	***Ec***
SP (%)	13.16	89.47	97.37	63.16	36.84	100	100	81.58	73.68	84.21	100
MP (%)	10.53	63.16	94.74	55.26	47.37	100	100	65.79	57.89	84.21	100
G/M (%)	5.26	23.68	81.58	57.89	31.58	100	100	57.89	50.00	89.47	100
p [1]	0.494	<0.001	0.033	0.867	0.355	-	-	0.077	0.099	0.748	-

SP: severe periodontitis, MP: moderate periodontitis, G/M: gingivitis/mild periodontitis. [1] *p*-values were calculated by Pearson's Chi-square test.

Table 5. Correlations between different sampling methods for the detection of each bacterial species in different study groups. Mouthwash samples (M) were correlated with paperpoint samples (P) and gingival retraction cord samples (C).

		Aa	***Pg***	***Tf***	***Td***	***Pi***	***Fn***	***Pm***	***Cr***	***En***	***Pn***	***Ec***
SP	$\rho_{P\text{-}M}$	0.284	0.640	0.591	0.823	0.606	0.449	0.581	0.665	0.585	0.555	0.293
	p	0.084	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	<0.001	<0.001	<0.001	0.075
	$\rho_{C\text{-}M}$	0.656	0.793	0.705	0.753	0.534	0.444	0.661	0.807	0.734	0.480	0.238
	p	<0.001	<0.001	<0.001	<0.001	0.001	0.005	<0.001	<0.001	<0.001	0.002	0.149
MP	$\rho_{P\text{-}M}$	0.464	0.743	0.513	0.552	0.438	0.306	0.415	0.556	0.660	0.361	0.256
	p	0.003	<0.001	0.001	<0.001	0.006	0.062	0.010	<0.001	<0.001	0.026	0.120
	$\rho_{C\text{-}M}$	0.492	0.506	0.723	0.631	0.576	0.496	0.508	0.625	0.742	0.617	0.289
	p	0.002	0.001	<0.001	<0.001	<0.001	0.002	0.001	<0.001	<0.001	<0.001	0.078
G/M	$\rho_{P\text{-}M}$	-	0.463	0.424	0.434	0.380	0.431	0.242	0.206	0.418	0.063	0.496
	p	-	0.003	0.008	0.006	0.019	0.007	0.143	0.215	0.009	0.705	0.002
	$\rho_{C\text{-}M}$	0.500	0.237	0.442	0.351	0.316	0.412	0.372	0.308	0.326	0.334	0.182
	p	0.001	0.152	0.006	0.031	0.053	0.010	0.021	0.060	0.046	0.041	0.274

SP: severe periodontitis, MP: moderate periodontitis, G/M: gingivitis/mild periodontitis. $\rho_{P\text{-}M}$: Spearman's correlation coefficient between paperpoint sample and mouthwash sample, $\rho_{C\text{-}M}$: Spearman's correlation coefficient between gingival retraction cord sample and mouthwash sample.

Table 6. Correlations between the severity of periodontal disease and clinical parameters and bacterial DNA counts in mouthwash samples.

Clinical parameters		**PPD**		**CAL**		**BOP%**		**PI**		**GI**	
	ρ	0.664		0.792		0.089		0.048		-0.181	
	p	<0.001		<0.001		0.345		0.613		0.054	
Bacterial DNA counts in mouthwash sample		***Aa***	***Pg***	***Tf***	***Td***	***Pi***	***Fn***	***Pm***	***Cr***	***En***	***Pn***	***Ec***
	ρ	0.100	0.530	0.438	0.209	0.076	-0.104	0.276	0.283	0.311	-0.091	0.147
	p	0.290	<0.001	<0.001	0.026	0.419	0.272	0.003	0.002	0.001	0.337	0.118

SP: severe periodontitis, MP: moderate periodontitis, G/M: gingivitis/mild periodontitis. ρ: Spearman's correlation coefficient between the severity of periodontal disease and various parameters.

Table 7. Binary logistic regression analysis between severe periodontitis and moderate periodontitis + gingivitis/mild periodontitis in paperpoint sampling.

	OR	**95% CI**	***p***
Age	1.105	0.985 - 1.239	0.090
Male	0.310	0.032 - 2.972	0.310
Smoking	1.234	0.101 - 15.024	0.869
Natural teeth (n)	1.408	1.081 - 1.833	0.011
Full veneer crown (n)	0.805	0.542 - 1.197	0.284
Teeth with restoration (n)	0.808	0.649 - 1.007	0.057
Teeth with caries (n)	2.136	1.149 - 3.971	0.016
Bone level	0.773	0.675 - 0.885	<0.001
log *Tf*	1.152	0.863 - 1.538	0.336
log *Pi*	1.080	0.902 - 1.293	0.402
log *Aa*	1.115	0.823 - 1.512	0.482
log *Pn*	0.894	0.777 - 1.028	0.116
log *Ec*	1.012	0.856 - 1.197	0.889
log *Td*	1.185	0.984 - 1.428	0.073

OR: odds ratio, CI: confidence interval, $p < 0.05$ was set to be statistical significance.

Table 8. Binary logistic regression analysis between severe periodontitis and moderate periodontitis + gingivitis/mild periodontitis in gingival retraction cord sampling.

	OR	**95% CI**	***p***
Age	1.055	0.985 - 1.130	0.129
Male	0.745	0.180 - 3.086	0.685
Natural teeth (n)	1.174	0.935 - 1.475	0.168
Bone level	0.777	0.628 - 0.960	0.020
log *Tf*	1.206	1.025 - 1.419	0.024
log *Pi*	1.024	0.905 - 1.159	0.702
log *Aa*	1.056	0.930 - 1.200	0.401
log *Pn*	0.962	0.845 - 1.095	0.554
log *Ec*	0.952	0.803 - 1.130	0.575
log *Td*	1.039	0.913 - 1.184	0.560

OR: odds ratio, CI: confidence interval, $p < 0.05$ was set to be statistical significance.

Table 9. Binary logistic regression analysis between severe periodontitis and moderate periodontitis + gingivitis/mild periodontitis in mouthwash sampling.

	OR	**95% CI**	***p***
Age	1.100	1.006 - 1.202	0.037
Male	0.196	0.030 - 1.319	0.094
Smoking	1.118	0.056 - 22.460	0.942
Natural teeth (n)	1.156	0.842 - 1.587	0.370
Full veneer crown (n)	0.833	0.672 - 1.033	0.096
Teeth with restoration (n)	0.867	0.724 - 1.038	0.120
Teeth with caries (n)	2.445	1.007 - 5.934	0.048
Bone level	0.739	0.626 - 0.872	<0.001
log *Tf*	1.581	1.136 - 2.201	0.007
log *Pi*	0.984	0.889 - 1.089	0.757
log *Aa*	0.963	0.851 - 1.088	0.543
log *Pn*	1.088	0.923 - 1.283	0.314
log *Ec*	0.993	0.657 - 1.501	0.972
log *Td*	1.043	0.950 - 1.145	0.380

OR: odds ratio, CI: confidence interval, $p < 0.05$ was set to be statistical significance.

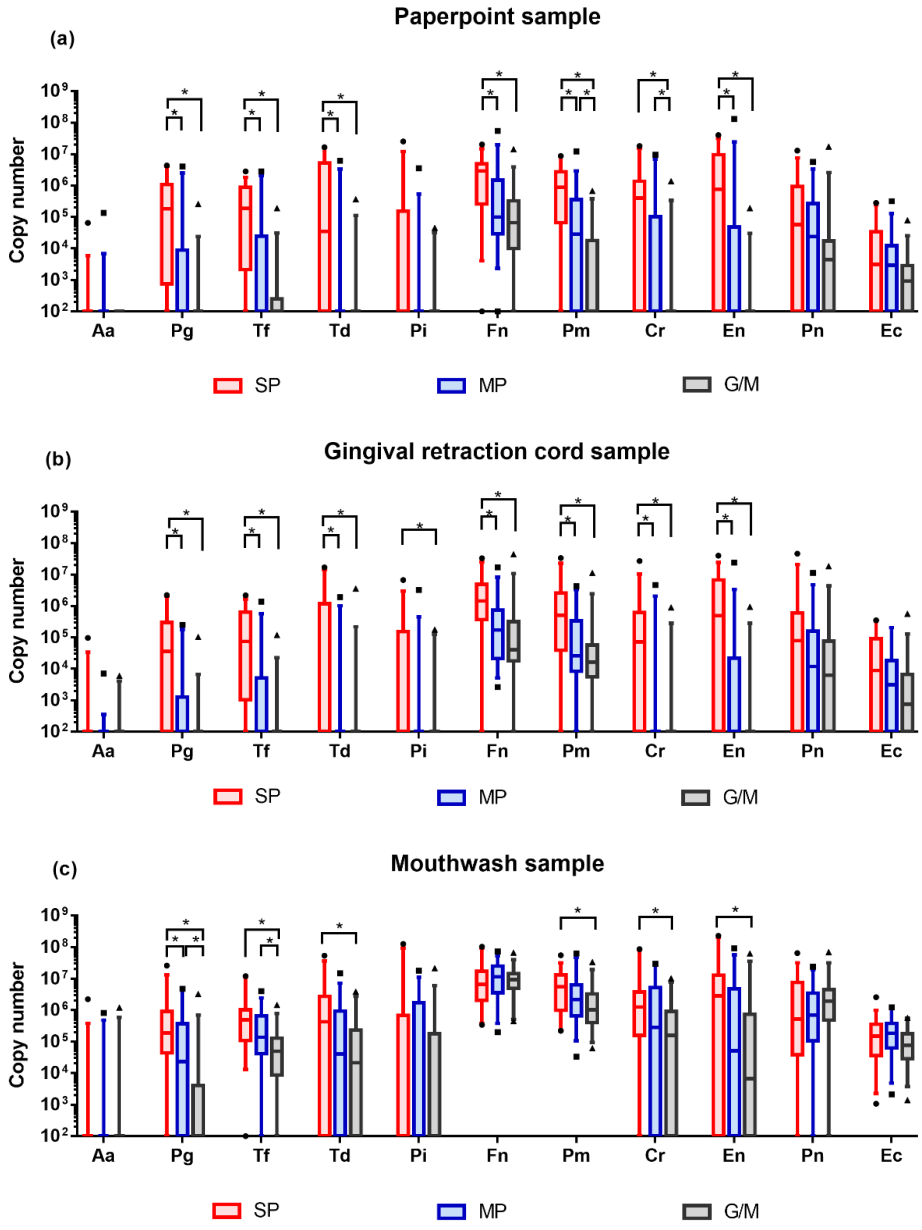

Figure 1. Quantitative profiles of target bacteria. DNA copy numbers are reported on a $\log_{10}$ scale. DNA copy numbers are plotted with box and whisker plot, indicating interquartile range and 5–95 percentile, respectively. All comparisons were conducted with Kruskal–Wallis H test and Bonferroni correction. Adjusted *p*-value = 0.0167. (**a**) Bacterial

profile in the paperpoint samples; (**b**) Bacterial profile in the gingival retraction cord samples; (**c**) Bacterial profile in the mouthwash samples. SP: severe periodontitis, MP: moderate periodontitis, G/M: gingivitis/mild periodontitis.

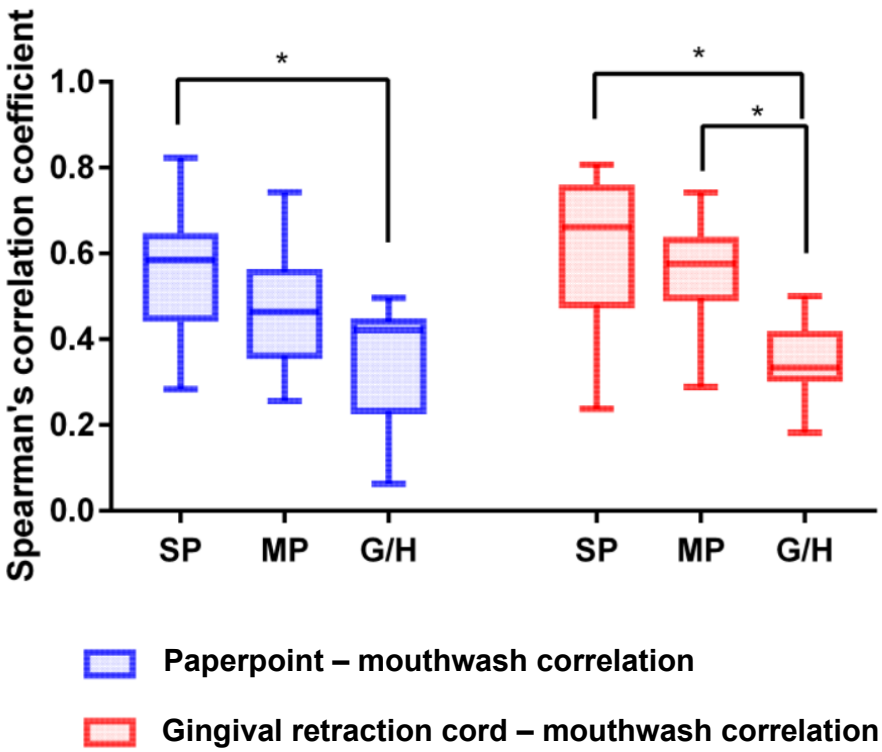

Figure 2. Distribution of Spearman's correlation coefficients of different sampling methods. Correlation coefficients are plotted with box and whisker plot, indicating interquartile range and 5–95 percentile, respectively. The data were compared with Kruskal–Wallis H test and Bonferroni correction. Adjusted *p*-value = 0.0167. SP: severe periodontitis, MP: moderate periodontitis, G/M: gingivitis/mild periodontitis.

국문초록

다중 실시간 중합효소연쇄반응법을 이용한 치주염 환자의 다양한 세균 샘플링 방법에 대한 정량적 평가

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1. 목적

본 연구의 목적은 서로 다른 치주질환 심도에 따라 구강세정제를 이용한 세균 샘플과 치은연하의 세균 샘플의 세균 분포를 정량적으로 비교해 보는 것에 있다. 추가적인 분석을 시행하여 구강세정제와 두 가지 치은연하 샘플링 방법, 즉 페이퍼포인트 및 치은압배사 사용 방법 사이의 상관관계를 평가하고자 하였다.

2. 재료 및 방법

114명의 환자가 연구에 등록하였으며 각 환자는 파노라마 방사선 사진 촬영 및 치주 임상 검사를 시행하였다. 이를 통하여 얻은 치주 질환 심도에 따라 각 환자를 중증치주염군, 경증치주염군, 치은염/경미치주염군 세 군으로 같은 수만큼 배정하였다. 각 환자의 2개 치아에서 각각 페이퍼포인트, 치은압배사를 이용한 치은연하 샘플링을 시행하고 구강세정제를 이용한 세균 샘플링을 시행하였다. 그 후 치주 병인 세균으로 알려진 11종의 세균(*Aggregatibacter actinomycetemcomitans; Aa*, *Porphyromonas gingivalis; Pg*, *Tannerella forsythia; Tf*, *Treponema denticola; Td*, *Prevotella intermedia; Pi*, *Fusobacterium nucleatum; Fn*, *Parvimonas micra; Pm*, *Campylobacter rectus; Cr*, *Eiknella corrodens; Ec*, *Prevotella nigrescens; Pn*, *Eubacterium nodatum; En*)에 대하여 DNA를 추출하고 다중 실시간 중합효소연쇄반응법을 이용한 정량 분석을 시행하였다.

얻어진 정량적 데이터에 대하여 통계학적 분석을 시행하였다. 모든 데이터는 정규성 검정을 시행하였으며 정규성이 있을 경우 일원분산분석 및 Dunnet T3 사후검정을 시행하였고 정규성이 없을 경우 Kruskal-Wallis H test 및 Bonferroni 사후검정을 시행하였다. 얻어진 데이터들의 상관관계 분석을 위하여 Spearman’s rank correlation 분석법이 사용되었다. 추가적으로 중증치주염군과 나머지 두 군을 합친 군 사이에 로지스틱 회귀분석을 시행하였다.

3. 결과

각 샘플링에서 얻은 세균 DNA의 양은 다양한 양을 보였다. 페이퍼포인트 샘플에서는 *Pg, Tf, Td, Fn, Pm, Cr, En*의 7종에서 치주 질환 심도가 악화될 때 그 양이 증가하였고 치은압배사 샘플에서는 *Pg, Tf, Td, Pi, Fn, Pm, Cr, En*의 8종에서 치주 질환 심도의 악화에 따라 양이 증가하였다. 한편 구강세정제 샘플에서는 *Pg, Tf, Td, Pm, Cr, En*의 6종에서 그 양이 증가하였다.

페이퍼포인트와 구강세정제, 그리고 치은압배사와 구강세정제의 샘플링 방법 간의 상관관계 분석 결과 치은염/건강군에서 11종의 세균에 대하여 Spearman 상관계수(ρ)가 0.3-0.5의 범위의 값을 나타내었으며, 중증치주염군 및 경증치주염군에서는 0.4-0.8의 범위의 값을 나타내어 치주 질환 심도가 증가하면 치은연하 샘플링과 구강세정제 샘플링 사이의 상관관계가 더욱 강해지는 것을 알 수 있었다.

이러한 결과를 토대로 치주 질환 심도와 구강세정제 내의 세균에 대한 상관관계 분석을 추가로 시행한 결과 *Pg, Tf* 2종의 세균의 구강세정제 내 DNA 양이 치주 질환 심도와 통계학적으로 유의미한 중등도의 상관관계를 나타내었다(각각 ρ = 0.530, 0.438). 한편, 구강 내 여러 요인들을 통제한 상태에서 중증 치주염군과 그 외 군으로 나눠서 로지스틱 회귀분석을 시행한 결과, 여러 세균들 중 *Tf*만이 유일하게 구강세정제 및 치은압배사를 이용한 샘플링에서 통계학적으로 유의미한 Odds ratio를 나타내었다 (각각 OR = 1.581, OR = 1.206).

4. 결론

구강세정제를 이용한 세균 샘플링은 몇 종류의 세균을 탐지함에 있어 치은연하 샘플링을 하는 두 가지 방법과 유의미한 상관관계를 가졌다. 그러나 그 상관관계는 치주 질환의 심도가 증가할수록 더욱 강하게 나타났다. 구강세정제 속에 검출된 세균은 치주염 진단에 있어서 바이오마커로 사용될 수 있는 잠재력이 있으나, 이는 치주염의 초기 진단보다는 중증 치주염의 진단에 더 적합할 것으로 생각된다. 앞으로 추가적인 연구를 통하여 치주염의 조기 진단에 이용될 수 있는 바이오마커의 발굴이 필요할 것으로 보인다.

주요어 : 다중 중합효소연쇄반응; 치주염; 진단; 세균; 구강세정제

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