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ANALYSIS OF ORAL MICROORGANISMS BEFORE AND AFTER PERIODONTAL TREATMENT USING REAL-TIME PCR

Yun-Kyung Kim[1]* and Gey-Pyo Kim[2]

[1]Dept. of Dental Hygiene, Andong Science College, Andong, 36616, Korea.
[2]Dept. of Oral Health, Kosin University, Busan, 49267, Korea.

***Corresponding Author: Yun-Kyung Kim**

Dept. of Dental Hygiene, Andong Science College, Andong, 36616, Korea.

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ABSTRACT

It is important to analyze oral microorganisms in patients with periodontal disease because this disease is caused by the proliferation of specific types and numbers of microorganisms in the oral cavity. The aim of this study was to analyze the changes in the number and type of pathogenic microorganisms in the oral cavity using real-time PCR before or after periodontal treatment in patients aged 50 years or more who visited the D dental clinic in Busan. The levels of pathogenic microorganisms before and after periodontal treatment were 12.44 ± 1.76% and 7.01 ± 0.10%, respectively. Thus, the level after periodontal treatment was significantly decreased compared to before treatment. Analysis of the major pathogens of dental caries revealed that Sm, Ss, and major pathogens of periodontal disease, including Ac, Pg, Tf, Td, Pi, Fn, Pm, Cr, Pn, and En, were significantly decreased compared to before periodontal treatment. Quantitative analysis of periodontal pathogens in the clinical by real-time PCR demonstrated the effectiveness of the treatment, which will help to improve the oral hygiene management ability of patients.

KEYWORDS: Dental clinic, Oral microorganisms, Periodontal disease, Periodontal treatment, Real-time PCR.

1. INTRODUCTION

The intraoral environment provides appropriate conditions for bacterial metabolism and proliferation. Over 700 different types of bacteria exhibit close relationships to form the microecosystem. Most bacteria are not harmful to oral health, and some bacteria are beneficial. However, disturbances in homeostasis in the oral environment or human body may promote specific types of bacteria to proliferate, resulting in the development of dental caries or periodontal diseases.[1,2]

Accurate identification and analysis of causative pathogens is crucial for the prevention and treatment or oral diseases, and previous studies have identified different pathogens driving specific oral diseases. The following bacteria are known to be cause periodontal diseases: Aggregatibacter actinomycetemcomitans (Ac), Porphyromonas gingivalis (Pg), Tannerella forsythia (Tf), Treponema denticola (Td), Prevotella intermedia (Pi), Fusobacterium nucleatum (Fn), Parvimonas micra (Pm), Campylobacter rectus (Cr), Prevotella nigrescens (Pn), Eubacterium nodatum (En), and Eikenella corrodens (Ec).[3] Additionally, Streptococcus mutans (Sm) and Streptococcus sobrinus (Ss) are considered as key pathogenic bacteria in dental caries. A higher level of these dental caries-inducing pathogens was reported to be associated with a higher morbidity of periodontal disease.[4]

Bacteria that induce periodontal disease as well as toxins and inflammatory mediators produced at the lesion can be spread throughout the body via the circulatory system,[5] which may explain the association between periodontal disease and systemic diseases (i.e. cardiovascular diseases and diabetes). Therefore, the prevention and treatment of periodontal disease are important not only for treating the oral condition, but also preventing systemic diseases. Common treatment methods for periodontal disease involve removal of the causative pathogenic bacteria, which includes scaling, antibiotics, gingival curettage, root planning, and periodontal tissue regeneration, based on disease progression or the patient's condition. In the previous studies in patients with periodontal diseases before and after treatment, clinical indices such as tooth mobility[6] or attachment loss and the depth of the periodontal pocket[7] were assessed to determine the effectiveness of periodontal treatments.

Analysis of oral microorganisms before and after periodontal treatment using real- time PCR can reveal the effectiveness of periodontal treatment and compare the types and amounts of periodontal pathogens for appropriate diagnosis and treatment of periodontal diseases.

Real- time PCR is a technique that utilizes pathogen-specific primers to measure gene amplification in real-time, enabling not only detection of the causative pathogen but also quantitative analysis. Thus, real- time PCR is used in clinical (dental) settings to diagnose and prevent periodontal diseases. Recent studies have focused on different cohorts of patients including the elderly,[4] female,[8] and patients with chronic periodontal diseases[9] to investigate the causative pathogen of periodontal diseases and various associated factors using real- time PCR to quantitatively analyze oral pathogenic bacteria. However, few studies have evaluated the changes in pathogenic bacteria after periodontal treatment in real clinical (dental) settings.

Therefore, this study was conducted to compare the prevalence of and quantity 9 periodontal pathogenic bacteria and 2 dental caries-causing bacteria by real-time PCR before and after periodontal treatment in a cohort of patients with periodontal disease.

2. MATERIALS AND METHODS

2.1. Subjects

The subjects of this study included patients who were ≥50 years old and were admitted to the "D" dental clinic in Yeonsan-dong, Busan, with periodontal diseases. Consent was obtained from the patients to utilize their data. The study period was between June 1 and December 30, 2015. After excluding patients who were undergoing treatment or those who were not analyzed real- time PCR after gingival curettage, 51 patients were included in the final analysis.

2.2. Study design

The subjects were analyzed by real- time PCR before periodontal treatment and were trained for proper tooth-brushing after reviewing the examination results with the clinicians. Next, the subjects underwent 4–6 gingival curettages over ~2 months based on their individual oral conditions. On the last day of treatment, specimens were collected for real- time PCR examination.

2.3 Methods

2.3.1 Sample acquisition

The subject was asked to perform mouth-washing with 10 mL sterilized saline for 1 min after brushing the teeth. The mouth-wash solution was collected in a sterilized 1.5-mL tube containing 0.2 mL phosphate-buffered saline, which was used as the sample. This collected specimen was stored at 4℃ and transferred to the lab on the next day.

2.3.2 DNA extraction of bacterial genome and real- time PCR

After DNA extraction, the 16s ribosomal DNA fragment was amplified, and bacteria-specific primers were used to amplify a DNA fragment of ~200 base pairs to perform microbial detection. To calculate the optimal annealing temperature of the primers, denaturation and extension were performed at 95°C and 72°C for 30 s and 1 min, respectively, and gradient annealing temperatures between 59 and 72°C for 1 min were tested. The reaction mixture for real-time PCR was 20 µL, which was distributed into a 96-well plate and amplified in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). After an initial denaturation step at 95°C for 10 min, the reaction was conducted for 45 cycles of denaturation (95°C for 15 s), annealing (55°C for 15 s), and extension (72°C for 30 s), followed by a final extension step at 72°C for 10 min. The reaction mixture was separated in a 1.0% agarose gel to detect the amplified products.

2.4 Statistical analysis

Data analysis in this study was performed using SPSS Statistics 22 software (SPSS, Inc., Chicago, IL, USA). General characteristics of the subjects are shown based on frequency analysis, and the expression of pathogenic bacteria before and after treatment was assessed by real-time PCR and analyzed with the paired *t*-test. $P < 0.05$ was considered to indicate a statistically significant outcome.

3. RESULTS

3.1 General characteristics

A total of 51 subjects participated in this study, among which 29 were male (56.9%) and 22 were female (43.1%). There were 14 patients in the <50 years old group (27.5%), 24 patients in the 51–60 years old group (47.1%), and 13 patients in ≥61 years old group. Additionally, 21 patients (41.2%) had systemic disease and 30 patients (58.8%) did not have systemic disease (Table 1).

Table 1: General Characteristics N = 51

Characteristics		N	%
Gender	Male	29	56.9
	Female	22	43.1
Age (years)	≤50	14	27.5
	51–60	24	47.1
	≥61	13	25,5
Systemic disease	Yes	21	41.2
	No	30	58.8

3.2 Changes in pathogenic microorganism after periodontal disease

Real- time PCR analysis performed in the patients with periodontal disease showed that the proportion of pathogenic microorganism was 12.44 ± 1.76%. After tooth-brushing training and periodontal treatment, the proportion of pathogenic microorganism was significantly reduced to 7.01 ± 0.10%, compared to the proportion before the treatment (Fig. 1).

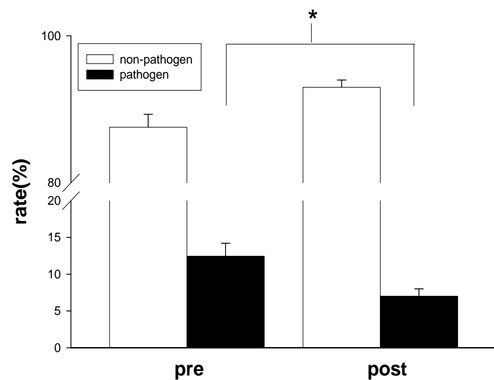

Fig. 1: Changes of pathogenic microorganisms after periodontal treatment.

3.3 Changes in 11 types of oral microorganism after periodontal disease real- time PCR was utilized to analyze the changes in oral microorganism, before and after periodontal treatment. All 9 types of periodontal disease-causing bacteria (Ac, Pg, Tf, Td, Pi, Fn, Pm, Cr, Pn, En) and 2 types of dental caries-causing bacteria (Sm, Ss) exhibited significantly reduced level after the periodontal treatment, compared to pre-treatment values (Table 2).

Table 2: Changes in 11 Oral Microorganisms after Periodontal Treatment Unit: copy/mL.

	Pre	Post	P-value
Aa	10,296,609 ± 7,882,627	5,118,471 ± 3,209,970	0.40
Pg	25,519,087 ± 9,338,210	2,889,783 ± 1,156,167	0.02
Tf	5,566,857 ± 1,597,144	1,581,360 ± 415,202	0.01
Td	5,824,379 ± 1,795,479	1,202,922 ± 303,727	0.01
Pi	27,354,362 ± 7,691,760	12,017,415 ±3,735,77 3	0.05
Fn	101,484,400 ± 31,601,515	34,177,014 ± 5,280,630	0.04
Pm	46,475,406 ± 17,339,972	11,035,195 ± 3,341,023	0.04
Cr	10,101,200 ± 3,123,460	2,245,665 ± 865,879	0.02
Ec	1,012,196 ± 309,876	374,046 ± 103,702	0.05
Sm	847,928 ± 293,647	200,185 ± 126,045	0.02
Ss	695,031 ± 573,654	0 ± 0	0.23

Aggregatibacter actinomycetemcomitans (Ac), *Porphyromonas gingivalis* (Pg), *Tannerella forsythia* (Tf), *Treponema denticola* (Td), *Prevotella intermedia* (Pi), *Fusobacterium nucleatum* (Fn), *Parvimonas micra* (Pm), *Campylobacter rectus* (Cr), *Prevotella nigrescens* (Pn), *Eubacterium nodatum* (En), *Eikenella corrodens* (Ec).

4. DISCUSSION

Periodontal disease is an endogenous infectious disease caused by multiple factors (i.e. salivation ability, age, oral hygiene, host defense, food consumption, and systemic disease)[10] driving changes in the distribution of oral microbes. Although bacterial culture tests are widely used to isolate and identify pathogens for diagnosis and evaluation of treatment efficacy, few of these bacteria can be cultured and counting the bacteria is difficult.[11] In contrast, quantitative analysis using real-time PCR allows for accurate identification of the type and number of oral bacteria, and thus can be applied in actual clinical settings for monitoring and diagnostic purposes.[12] Therefore, our study assessed the numbers of 11 different types of oral bacteria in patients with periodontal disease, before and after periodontal treatment, by real- time PCR.

The types and numbers of oral pathogenic microorganisms may differ depending on the location or type of periodontal disease. Moreover, these microorganisms are strongly associated with each other.[13] In this study, high levels of Fn, Pm, Pi, and Pg were detected in the patients before periodontal treatment,

which were associated with the following conditions: Fn with acute necrotic gingivitis and ulcerative gingivitis; Pm with recurrent or intractable periodontal diseases; Pi with hormone-related gingivitis; and Pg with adult periodontal patients. Additionally, these patients were classified as the high-risk group (red complex) or intermediate-risk group (orange complex) based on color classification in the Socransky complex.[14] Therefore, quantitative analysis of oral microorganism using real-time PCR can be utilized for both clinical findings and periodontal treatment. More specifically, the technique is useful for identifying specific pathogens to remove or reduce their levels.

The index of periodontal health for diagnosing periodontal disease includes the dental plaque index, gingival index, periodontal probing depth, bleeding at probing, and tooth motility.[15] Utilizing these indices, the effectiveness of periodontal health promotion has been reported in previous studies. According to Pang,[6] tooth motility was significantly reduced at 12 weeks after gingival curettage. Jo et al. reported that repeated direct training of tooth-brushing increased the patient's ability to manage dental plaque.[16] These clinical values represent the improved state of oral health after periodontal treatment or tooth-brushing but are not useful for accurately analyzing the causes of periodontal diseases. Therefore, analysis of causative pathogens of periodontal disease via real- time PCR is effective for identifying the cause and establishing a treatment plan.

To treat patients with periodontal disease, real- time PCR was performed before and after tooth-brushing training and periodontal treatment. The level of pathogenic microorganisms was significantly reduced after treatment (Fig. 1), and the levels of all 11 types of microorganisms analyzed were significantly reduced (Table 2). These findings support the effectiveness of periodontal disease treatment via changes in oral pathogenic microorganisms.

Park[17] described the association between periodontal disease and diabetes, and a previous study by Hong[18] similarly demonstrated that patients with systemic disease have a 4.1-fold higher risk of *P. intermedia* detection compared to the normal population. These previous studies suggest that systemic disease is a high-risk factor for oral microorganism detection. In our study, although the patients with both periodontal and systemic diseases exhibited higher proportions of pathogenic microorganisms compared to patients with only periodontal disease, the difference was not significant (data not shown). We did not analyze the association between the presence of systemic disease, rather than a specific type of systemic disease, and pathogenic microorganisms, and thus additional studies are needed to assess the association between pathogenic microorganisms and different types of systemic diseases.

CONCLUSIONS

This study revealed the changes in oral microorganisms in patients with periodontal disease before and after treatment by real- time PCR. Pre-treatment assessment of pathogenic microorganisms is useful for identifying the cause of periodontal disease. Additionally, comparison of the quantitative pre- and post-treatment levels of pathogenic microorganisms can provide objective data or evidence to demonstrate treatment efficacy in patients.

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