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Communication

Detection of Bacterial Species in Chronic Periodontitis Tissues at Different Stages of Disease Severity

Da-Le Yoon[1§], Shukho Kim[2§], Hyesoon Song[2], Yong-Gun Kim[1], Jae-Mok Lee[1*] and Jungmin Kim[2*]

[1]Department of Periodontology, Kyungpook National University School of Dentistry, Daegu; [2]Department of Microbiology, Kyungpook National University School of Medicine, Daegu, Korea

The goal of this research was to determine the relationship between the stage of chronic periodontitis and the presence of six bacterial pathogens (*Aggregatibacter actinomycetemcomitans*: AA, *Fusobacterium nucleatum*: FN, *Porphyromonas gingivalis*: PG, *Prevotella intermedia*: PI, *Enterococcus faecalis*: EF, and *Parvimonas micra*: PM). Forty-six chronic periodontitis patients visiting a dental hospital were included in this investigation. They were classified into four chronic periodontitis stages based on the sulcus bleeding index value and the probing depth. The tissue samples from the periodontal surgery were used for a direct PCR detection assay. A total of 49 samples from 46 patients were collected and classified into four chronic periodontitis groups (N: 6, P1: 13, P2: 18, P3: 12). The PCR assay showed that FN, PI, and PM were involved from the beginning of chronic periodontitis (P1), while AA and PG existed regardless of the disease stages. EF was strongly linked to the P3 stage of the disease. In order to assess the effect of dental treatments on patients with chronic periodontitis, EF should be a critical marker for P3 patients, while FN, PI, and PM would be good indicators for chronic periodontitis.

Key Words: Chronic periodontitis, Oral bacteria, PCR detection

INTRODUCTION

Chronic periodontitis is an inflammatory disease that results in the destruction of periodontal tissue, including the alveolar bone and connective tissue that support the teeth (1). Periodontal tissue is capable of maintaining a healthy status when there is a balance between the host defense and the toxicity of bacteria. However, there are many pathogenic bacteria involved in periodontal disease, and various kinds of pathological toxic substances are secreted as disease progresses (2). Pathogenic bacteria can either avoid, weaken, and/or neutralize host defense mechanisms and initiate various immune pathological processes that can cause periodontal disease and even worsen disease condition. According to Socransky *et al*., there are various inducing factors for periodontitis, such as a decrease of bacteria in number that play a protective role against pathogenic bacteria, local

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§These authors equally contributed to this work.
*Corresponding author: Jungmin Kim. Department of Microbiology, Kyungpook National University School of Medicine, Daegu 41944, Korea. Phone: +82-53-420-4845, Fax: +82-53-427-5664, e-mail: minkim@knu.ac.kr
*Corresponding author: Jae-Mok Lee. Department of Periodontology, Kyungpook National University School of Dentistry, Daegu 41940, Korea. Phone: +82-53-600-7522, Fax: +82-53-427-3263, e-mail: leejm@knu.ac.kr
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trauma, and the mental instability of the host (3, 4). These factors promote the growth of bacteria that cause periodontal disease, and thereby allow them to overcome host defenses so that periodontitis proceeds. Certain changes in the condition for bacterial growth and in the host body, especially intraoral tissue, inhibit the host from effectively suppressing the growth of bacteria and inevitable tissue destruction that follows. Shift phenomenon of bacteria and periodic increase of bacteria which comes from the periodic weakening in bacterial control incapability of the host bring the tissue destruction. This phenomenon can be defined as cross-reactivity between the defense factor and destructive factor, which causes periodic tissue destruction (5). As a result, oral bacteria irritate epithelial cells, initiating the periodontal disease by triggering innate, inflammatory, and adaptive immune responses (6). *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Campylobacter rectus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, *Parvimonas micra*, *Treponema denticola*, and *Eubacterium* are bacteria that are characteristically observed in chronic periodontitis. Specifically, *A. actinomycetemcomitans*, *C. rectus*, *P. gingivalis*, *P. intermedia*, *F. nucleatum*, and *T. forsythia* are diversely dispersed in the space with high periodontal disease reactivity (7, 8). In addition, in 2012, it was found that oral *Enterococcus faecalis* possibly played a role as a reservoir for the transferable virulence factor and antimicrobial resistance genes and was responsible for the pathogenesis of chronic periodontitis (9, 10).

Therefore, it is critical to determine which bacterial species or strains are the major cause of chronic periodontitis. An analysis of the quantities of periodontal pathogens, depending on the severity or progression of chronic periodontitis, should follow for more accurate diagnosis and prevention of periodontitis progression. The identification of these pathogenic bacterial species will also help us set the main targets for periodontitis therapies. However, specific pathogens that are responsible for each stage in the progression of periodontal disease are not yet fully known.

In this study, we examined the presence of six pathogenic bacteria (*Aggregatibacter actinomycetemcomitans*: AA, *Fusobacterium nucleatum*: FN, *Porphyromonas gingivalis*: PG, *Prevotella intermedia*: PI, *Enterococcus faecalis*: EF, and *Parvimonas micra*: PM) in the chronic periodontitis tissue samples from the patients with varying disease severity. PCR is a highly sensitive detection tool that is widely used for the microbiological diagnosis of dental diseases. Many dental studies have been reported on oral bacteria-associated dental diseases and microbiomes of dental patients, which used PCR and/or DNA sequencing (11, 12). We used a direct PCR detection assay to detect the six pathogenic bacteria from tissue sample obtained from the periodontal surgery. We also determined the relationship between the presence of six pathogenic bacteria and the stages of chronic periodontitis.

MATERIALS AND METHODS

Patients

A total of 49 tissue samples were obtained from 46 patients who visited Kyungpook National University Dental Hospital for chronic periodontal treatment. Accounting for the 49 samples, three patients each gave two chronic periodontitis samples that were taken from different regions of the mouth with different severities of chronic periodontitis. Informed consent was obtained, before the surgery, from the all the patients enrolled in this study. The study protocol was approved by the IRB of Kyungpook National University Hospital (IRB Number: 74005-830).

Clinical measurement and sampling

The sulcus bleeding index (SBI) value and probing depth (PD) are the clinical periodontal parameters that can be obtained from six sites per tooth in an initial examination. Clinical criteria to describe gingiva were determined using these parameters (13). The level of bone resorption could be observed in the available radiographic images. Normal and chronic periodontitis were classified according to the following criteria: normal (N) is clinically healthy gingiva without bleeding that shows no evidence of bone resorption or periodontal pockets. Chronic periodontitis has more than one periodontal pocket (≥ 5 mm) with at least one of them

Table 1. Primers used in this study

Primer name	Primer sequence	Target pathogen[1]	Amplicon size (bp)
AA F3	5'-TGCGTAGAGATGTGGAGGAA-3'	AA	165
AA B3	5'-GGCGGTCGATTTATCACGT-3'		
FN F3	5'-AGGCGATGATGGGTAGCC-3'	FN	214
FN B3	5'-AGCCGTCACTTCTTCTGTTG-3'		
PG F3	5'-GGTAAGTCAGCGGTGAAACC-3'	PG	218
PG B3	5'-GCGTGGACTACCAGGGTAT-3'		
PI F3	5'-ACGGCCTAATACCCGATGTT-3'	PI	193
PI B3	5'-CTGCCTCCCGTAGGAGTT-3'		
EF16s-F	5'-CGCTTCTTTCCTCCCGAGT-3'	EF	142
EF16s-R	5'-GCCATGCGGCATAAACTG-3'		
PM16s-F	5'-TCGAACGTGATTTTTGTGGAAA-3'	PM	85
PM16s-R	5'-GGTAGGTTGCTCACGTGTTACTCA-3'		

[1]AA: *Aggregatibacter actinomycetemcomitans*, FN: *Fusobacterium nucleatum*, PG: *Porphyromonas gingivalis*, PI: *Prevotella intermedia*, EF: *Enterococcus faecalis*, PM: *Parvimonas micra*

showing ≥ 4 mm loss of attachment, a gingival sulcus bleeding index of 3, and/or clear evidence of bone resorption. Chronic periodontitis can be classified in more detail according to its probe attachment level (PAL) as follow: If the PAL is 1~2 mm, it is classified as P1 (mild chronic periodontitis); if the PAL is 3~4 mm or ≥ 5 mm, it is classified as P2 or P3, respectively (14, 15).

Samples of subgingival tissue were collected from patients with periodontal disease and from healthy individuals, at the Department of Periodontology, School of Dentistry, Kyungpook National University. The samples were obtained from the patients during periodontal surgery, which included surgical crown lengthening or tooth extraction by internal bevel incision. Before the surgery, informed consent was provided to all of the study participants.

PCR detection

For the detection of six bacterial pathogens (*A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *E. faecalis*, and *P. micra*), PCR primers targeting species-specific 16S rRNA coding regions of these pathogens were chosen, synthesized, and used (16~18) (Table 1). DNA was extracted from samples using a Genomic DNA Preparation Kit (HIGene Genomic DNA Prep Kit, BIOFACT, Korea) according to the manufacturer's instructions. PCR amplification was performed in 50 μl of final solution containing 5 μl of 10X PCR buffer (ExTaq, TaKaRa, Japan), 400 μM of each deoxynucleotide triphosphate (TaKaRa), 400 nM of each primer, 1 U of Taq polymerase (ExTaq, TaKaRa), and 2 μl of the extracted DNA samples. For the positive controls, clinical isolates of *Aggregatibacter actinomycetemcomitans* (AA: KCOM 1299, 1304, 1306), *Fusobacterium nucleatum* (FN: KCOM 1276, 1322, 1323), *Porphyromonas gingivalis* (PG: KCOM 2796, 2797, 2798), *Prevotella intermedia* (PI: KCOM 1101, 1104, 1106), and *Parvimonas micra* (PM: KCOM 1037, 1533, 1535) were kindly provided by the Korean Collection for Oral Microbiology (KCOM, Korea). An ATCC (American Type Culture Collection) *E. faecalis* (EF) strain 29212 was used for the positive PCR control. These positive control strains were harvested from growth media, prepared by boiling, and used for the PCR. Autoclaved, distilled water was used for the negative PCR control.

For the detection of AA, FN, PG, and PI, 35 PCR cycles of 30 sec at 95℃, 30 sec at 52℃, and 1 min at 72℃ were

Table 2. PCR detection results of 49 samples including their clinical information

Sample number	Sampling data (yyyy.mm.dd)	Sex[1]/Age	Chronic periodontitis[2]	AA[3]	FN	PG	PI	EF	PM
1	2014.05.30	M/54	P2	+[4]	+	+	+	−	+
2	2014.04.02	F/45	P2	+	+	+	+	−	+
3	2014.03.18	M/52	P1	+	+	+	+	−	+
4	2014.04.21	F/53	N	+	−	+	−	−	−
5	2014.03.25	M/48	P2	+	+	+	+	−	+
6	2014.04.09	F/18	N	+	+	+	−	−	−
7	2014.06.13	M/60	P2	+	+	+	−	−	+
8	2014.06.13	F/57	P2	+	+	+	+	−	+
9	2014.05.16	M/58	P1	+	+	+	+	−	−
10	2014.01.28	M/52	P2	+	+	+	+	−	+
11	2014.06.27	M/42	P2	+	+	+	−	−	−
12	2014.04.09	F/68	N	+	+	+	−	−	+
13	2014.04.03	M/35	P2	+	+	+	−	−	+
14	2014.04.25	M/51	P2	+	+	+	+	+	+
15	2014.04.02	F/59	P2	+	+	+	+	−	+
16	2014.03.07	M/39	N	+	+	+	+	−	+
17	2014.06.16	M/54	P2	+	+	+	+	−	+
18	2014.02.24	F/42	P2	+	+	+	+	−	+
19	2014.04.16	M/53	N	−	−	−	−	−	−
20	2014.05.09	F/48	N	+	+	+	−	−	−
21	2014.02.07	M/60	P2	+	−	+	−	−	−
22	2014.04.11	F/64	P2	+	+	+	+	−	+
23	2014.04.07	M/49	P1	+	+	+	−	−	−
24	2014.03.19	F/43	P2	+	−	−	−	−	−
25	2014.03.28	M/46	P3	+	+	+	+	+	+
26	2014.06.16	F/53	P3	+	+	+	+	−	+
27	2014.04.11	F/51	P3	+	+	+	+	−	+
28	2014.04.11	F/35	P3	+	+	+	−	−	+
29	2014.06.17	F/53	P3	+	+	+	+	+	+
30	2014.04.07	F/59	P3	−	−	−	−	+	+
31	2014.04.08	M/53	P3	+	+	+	+	+	+
32	2014.06.20	M/45	P3	+	+	+	+	+	+
33	2014.03.14	F/52	P3	+	+	+	+	+	+
34	2014.04.07	F/42	P3	+	+	+	+	+	+
35	2014.04.02	F/55	P3	+	+	+	+	+	+
36	2014.04.21	F/47	P3	+	+	+	+	+	+
37	2014.05.09	M/49	P1	+	+	+	+	−	+

Table 2. Continued

Sample number	Sampling data (yyyy.mm.dd)	Sex[1]/Age	Chronic periodontitis[2]	AA[3]	FN	PG	PI	EF	PM
38	2014.03.28	M/61	P1	+	+	+	+	−	+
39	2014.04.03	M/47	P1	+	+	+	+	−	+
40	2014.06.10	F/55	P1	+	+	+	−	−	+
41	2014.04.30	F/46	P1	−	+	+	+	−	+
42	2014.04.11	F/51	P1	+	+	+	+	−	+
43	2014.02.28	M/38	P1	+	+	+	+	−	+
44	2014.03.26	M/48	P2	+	+	+	−	−	+
45	2014.06.09	M/54	P1	+	+	+	+	−	+
46	2014.04.28	F/48	P1	+	+	+	+	−	+
47	2014.04.02	M/37	P2	+	+	+	+	−	+
48	2014.04.08	M/48	P2	−	+	+	+	−	+
49	2014.04.11	M/52	P1	+	+	+	+	−	+

[1]M: male, F: female
[2]Refer Materials & Methods for the abbreviation
[3]AA: *Aggregatibacter actinomycetemcomitans,* FN: *Fusobacterium nucleatum,* PG: *Porphyromonas gingivalis,* PI: *Prevotella intermedia,* EF: *Enterococcus faecalis,* PM: *Parvimonas micra*
[4]+: PCR positive, −: PCR negative.

carried out. For the detection of EF, 35 PCR cycles of 30 sec at 95℃, 30 sec at 62℃, and 30 sec at 72℃ were performed. For the detection of PM, the latter PCR conditions, except with an annealing temperature of 60℃, were accomplished. An initial DNA denaturation step of 2 min at 95℃ was completed before the amplification cycles began, and for each PCR reaction, the 35 cycles were followed by a final extension step of 7 min at 72℃. PCR was performed using the TaKaRa PCR Thermal Cycler Dice (TaKaRa Bio). The PCR products were analyzed by 2% agarose gel electrophoresis containing RedSafe™ nucleic acid staining solution (Intron Biotech. Korea) in TRIS-borate-EDTA buffer with a Mupid electrophoresis apparatus (Advance, Japan). The DNA bands were visualized using a Gel Doc XR system (Bio-Rad, USA). If the DNA band of the sample is visible and corresponds to that of the positive PCR control, the decision would be PCR positive. Otherwise would be judged as PCR negative (non-matching or invisible).

Statistical analysis

The statistical analysis was performed using the Pearson's Chi-squared test in SPSS 12.0 (SPSS Inc., USA).

RESULTS & DISCUSSION

The clinical data and characteristics of the recruited patients and specimens were summarized in Table 2. Of the 46 patients, 26 patients were male and 23 were female. No participants were related to each other. Enrolled patient showed past medical history including diabetes mellitus (10 patients), hypertension (8 patients), and hyperlipidemia (3 patients). The others had no specific systemic diseases. The mean age of all patients was 49.5 yr with an 8.6 yr standard deviation. The maximum age was 68 yr and the minimum was 18 yr. There were no apparent differences in the mean ages between the groups (N: 46.50 yr, P1: 50.77 yr, P2: 49.94 yr, and P3: 49.25 yr).

Table 3. Detection of six pathogenic bacteria from dental tissue samples by PCR

Bacteria[1]	No. of PCR positive samples/No. of samples used for PCR (%)			
	Sample groups classified by clinical measurement			
	N, Normal gingiva	P1, Mild chronic periodontitis (PAL[2] ≤ 2 mm)	P2, Moderate chronic periodontitis (PAL ≤ 4 mm)	P3, Severe chronic periodontitis (PAL > 4 mm)
AA	5/6 (83.3)	12/13 (92.3)	17/18 (94.4)	11/12 (91.6)
FN	4/6 (66.7)	13/13 (100)	15/18 (83.3)	11/12 (91.6)
PG	5/6 (83.3)	13/13 (100)	16/18 (88.8)	11/12 (91.6)
PI	1/6 (16.7)	11/13 (84.6)	12/18 (66.7)	10/12 (83.3)
EF	0/6 (0)	0/13 (0)	1/18 (5.5)	9/12 (75.0)
PM	2/6 (33.3)	12/13 (92.3)	15/18 (83.3)	12/12 (100)

[1]AA: *Aggregatibacter actinomycetemcomitans*, FN: *Fusobacterium nucleatum*, PG: *Porphyromonas gingivalis*, PI: *Prevotella intermedia*, EF: *Enterococcus faecalis*, PM: *Parvimonas micra*
[2]PAL: probe attachment level.

According to PAL measurements, number of samples classified into P1, P2, and P3 were 13, 18, and 12 samples, respectively. Six normal samples, from persons with no evidence of bone resorption or periodontal pockets, were also evaluated in the study. Table 3 shows the frequencies of detecting six bacterial species from 49 samples (normal, P1, P2, and P3 chronic periodontitis tissue). AA, FN, and PG were the most frequently detected bacteria, regardless of a normal status or the level of chronic periodontitis. These bacteria were detected in more than 80% of all chronic periodontitis patients and more than 60% in healthy normal individuals included in the study. FN, PI and PM were highly prevalent in chronic periodontitis patients regardless of the disease stage, but these bacteria were detected less frequently in normal samples (FN, PI and PM, 66.7%, 16.7% and 33.3%, respectively. $p < 0.05$). EF was not detected both in the normal and in P1 stage samples, but only one P2 sample was PCR positive for EF (5.5%), unlike the other bacteria tested. However, 75% of P3 samples was PCR positive for EF that was statistical significance relation between detection rate of P3 and those of the other samples ($p < 0.01$). Pearson's Chi-squared testing suggested that FN, PI, and PM were associated with the beginning of chronic periodontitis (P1) while AA and PG were present in chronic periodontitis tissues regardless of the disease stage. EF was strongly linked to the P3 stage in this study (Table 4).

Table 4. Result of statistical analysis to determine the association of six bacteria with the states of chronic periodontitis

Bacteria[1]	*P* value		
	N versus P1	P1 versus P2	P2 versus P3
AA	0.554	0.811	0.765
FN	0.028*	0.121	0.511
PG	0.130	0.214	0.803
PI	0.004*	0.259	0.311
EF	–	0.387	0.0000772*
PM	0.007*	0.462	0.136

[1]AA: *Aggregatibacter actinomycetemcomitans*, FN: *Fusobacterium nucleatum*, PG: *Porphyromonas gingivalis*, PI: *Prevotella intermedia*, EF: *Enterococcus faecalis*, PM: *Parvimonas micra*
*Statistical significance ($p < 0.05$).

AA, FN, and PG are well-studied bacterial pathogens in the field of chronic periodontitis pathogenesis. Fine *et al*. reported that AA is frequently associated with localized aggressive chronic periodontitis (LAP) and can serve as a risk marker for the initiation of LAP (19, 20). FN, on the other hand, adheres to and invades human epithelial cells,

thus, has been detected in subgingival plaque samples (21). PG is the strongest bacterial marker for chronic periodontitis and is highly associated with periodontal bone loss (22, 23). These three pathogens were detected in 83% of P1 to P3 patients, as well as 66.7~83.3% of normal participants included in this study. These results indicate that these bacteria might be associated with early onset of chronic periodontitis in these patients, or may not play roles in the pathogenic process of periodontitis.

PI and PM were less prevalent in normal samples, but occurred with high frequencies in P1 to P3 samples ($p < 0.01$), suggesting that these two pathogens might be critically associated with the disease, by triggering and/or worsening their chronic periodontitis. Interestingly, EF was detected with relatively high frequency (75%) only in P3 samples in this study. EF is a human commensal which lives in the nutrient-rich, anaerobic, and ecologically variable environment of the oral cavity and intestine (24, 25). Similar to our results, Souto *et al*. reported that EF was detected more often in saliva and subgingival samples of chronic periodontitis patients than those of healthy individuals (26). EF harbors various virulence factors that may subsidize other bacteria present in chronic periodontitis tissues (27). In addition, the deeper chronic periodontitis pockets in P3 patients have wider epithelial surface areas with preexisting dental bacteria biofilms where EF may attach easily. However, the potential pathogenic role of EF in oral infections is still unknown due to limited number of study addressing this question.

According to a study performed in 2012 by Lee H *et al*. (28), to analyze the change of bacterial flora based on the stage of periodontitis, the relative levels of *A. actinomycetemcomitans* and *C. rectus* were positively correlated with the progression of periodontal disease. In this study, large amounts of EF, which was not a focus of the previous study, were detected in severe chronic periodontitis (P2, P3) samples, and it is equally important to note that EF was not detected in healthy periodontal tissue.

Unlike most previous studies, which used subgingival plaque samples to evaluate the prevalance of periodontal pathogens (29), in this study, we identified bacterial pathogens from subgingival tissues. Since bacteria found in chronic periodontitis lesions are a more direct immunological source compared to subgingival plaque, using subgingival tissue instead of plaque samples could be a more accurate and reliable source for determining the impact of pathogens on host immunity.

In summary, this is the first report on six chronic periodontitis-associated pathogens and their association with stages of chronic periodontitis in Korea. Although the sample size of the study was small and target bacteria were restricted to only six microorganisms, our findings could suggest a direction for assessment of treatment of chronic periodontitis through the recognition of pathogens. Further analysis of the quantities of periodontal, depending on the progression of chronic periodontitis, should follow for more accurate diagnosis. In order to assess the effect of dental treatments on chronic periodontitis patients, EF would be a critical marker for P3 patients, while FN, PI, and PM would be good indicators for chronic periodontitis patients overall.

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