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Original Article

Investigation of Subgingival Profile of Periodontopathic Bacteria Using Polymerase Chain Reaction

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Abstract

Periodontopathic bacteria such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Campylobacter rectus* and *Treponema denticola* play an important role in the initiation and progression of periodontitis. The aim of this investigation was to evaluate the relationship between periodontal clinical parameters and the subgingival profile of periodontopathic bacteria. Twenty-six periodontitis patients (23–62 years of age; mean age, 40.2 ± 13.2) with no systemic disease agreed to participate in the study. Periodontal clinical parameters, including probing depth (PD) and bleeding on probing (BOP) were recorded. Subgingival plaque samples were obtained from deep (PD≧4 mm) and shallow (PD≦3 mm) pockets in each patient for detection of *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia*, *C. rectus* and *T. denticola* by polymerase chain reaction technique. The relationship between the periodontal pathogens and clinical parameters was determined with the Fisher exact test, and a statistically significant association was found between detection of *P. gingivalis*, *T. forsythia*, *C. rectus* and *T. denticola* and PD or BOP. *T. denticola* was the most prevalent pathogen in both shallow PD and deep PD sites. No statistically significant association was found between detection of *A. actinomycetemcomitans* and the clinical parameters examined. A statistically significant association was found between detection of the red complex bacteria and the clinical parameters. These results suggest that the red complex pathogens and *C. rectus* play an important role in the initiation and progression of periodontitis.

Key words: Periodontopathic bacteria—Polymicrobial infection—Red complex—Periodontitis

Introduction

Periodontitis is an oral infectious disease induced mainly by periodontopathic bacteria[18]. Previous studies suggested that periodontopathic bacteria play an important role in the initiation and progression of periodontal disease[13]. Subgingival microorganisms

Table 1 Clinical characteristics of subjects and sampled sites

Subjects (n)		26
Age (mean ± SD, range)		40.2 ± 13.2 (23–62)
Sex (male/female)		16/10
Smoker/Nonsmoker		6/20
	Mean ± SD (mm)	BOP+ (% sites)
(Deep sites = 73)	7.0 ± 1.9	93.2
(Shallow sites = 28)	2.5 ± 0.6	14.3

PD: probing depth; BOP: bleeding on probing; SD: standard deviation.
Deep sites: PD of 4 mm or more
Shallow sites: PD of 3 mm or less

have been detected by several methods: anaerobic culture[15], microscopy[11], enzyme reaction[6], immunohistology[8], DNA probe[9] and polymerase chain reaction (PCR)[2]. The culture method is time-consuming and requires specific techniques. The rapid advancement of molecular biological techniques has facilitated the use of PCR for bacterial detection. Previous studies suggested that microbiological monitoring is a very important tool in the diagnosis and selection of periodontal therapy, as microflora was found to differ between active and inactive sites[4]. Periodontopathic bacteria such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Campylobacter rectus* and *Treponema denticola* have been frequently detected in the subgingival pockets of patients with periodontitis. The purpose of this study was to investigate the relationship between detection of periodontopathic bacteria and periodontal clinical parameters.

Methods

1. Participants

Twenty-six periodontitis patients (23–62 years of age; mean age, 40.2 ± 13.2) visiting Tokyo Dental College, Chiba Hospital participated in this study between 2000 and 2003. Informed consent was obtained from each patient. Table 1 shows age, sex and clinical characteristics. To be included in this study, patients had to have at least two sites with PD≧4 mm with attachment loss. Exclusion criteria included pregnancy, periodontal therapy or antibiotics in previous 3 months, any systemic condition which might have affected the progression or treatment of periodontitis and the need for pre-medication for monitoring or therapy. No patient with localized aggressive periodontitis was included in this study.

2. Clinical parameters

1) Probing depth (PD)

PD was measured from the gingival margin to the bottom of the periodontal pocket at sampled sites with the Williams probe.

2) Bleeding on probing (BOP)

The dichotomous system was used to register the presence or absence of bleeding after PD measurements[1].

3. Microbiological sampling

PD data were categorized into deep PD (≧4 mm) and shallow PD (≦3 mm) sites. At least two deep PD and one shallow PD sites were selected in each patient. Each sampling site was isolated with cotton rolls, and supragingival plaque and saliva carefully removed with sterile cotton pellets and dried with air. Three paper points were inserted into each selected pocket until firm resistance was felt and kept in place for 30 seconds.

4. Detection of periodontal pathogens

Sedimented microorganisms were suspended in 50 μl buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1% tritonX-100) and boiled at 100°C for 10 min. After removal of

Table 2 Primers used

	Primer (5′–3′)	Base position (amplicon length in bp)
P. gingivalis	AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	729–1,132 (404)
A. actinomycetemcomitans	AAA CCC ATC TCT GAG TTC TTC TTC ATG CCA ACT TGA CGT TAA AT	478–1,034 (557)
T. forsythia	GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	120–760 (641)
C. rectus	TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	415–1,012 (598)
T. denticola	TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA	193–508 (316)

cell debris by centrifugation at 15,000×g for 15 min, the supernatant was used for detection of *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia*, *C. rectus* and *T. denticola* by PCR according to the method of Ashimoto *et al.*[2]. Briefly, 5μl sample was added to 45μl reaction mixture consisting of 1×PCR buffer (Takara Biomedicals, Shiga, Japan), 12.5μM dNTP, the specific primer pairs listed in Table 2 and 0.25 U Taq DNA polymerase (Takara Biomedicals). The PCR assay was then performed using a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer Biosystem, Foster City, CA) under conditions of 32 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and primer extension at 72°C for 30 min. The PCR product was electrophoresed on a 2% agarose gel and visualized under UV light following staining with ethidium bromide. The detection level was expressed as the lowest number of target cells detected clearly by PCR.

5. Statistical analysis

The relationship between clinical parameters and detection of each periodontopathic bacterium was determined with the Fisher exact test.

Results

The patients were divided into two groups based on PD values. Mean percentage of bleeding sites out of 72 sites in 26 patients was 93.2% for deep sites and 14.3% for shallow sites (Table 1). The relationship between PD and detection of *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia*, *C. rectus* and *T. denticola* is shown in Fig. 1. A statistically significant association was found between detection of *P. gingivalis*, *T. forsythia*, *C. rectus* or *T. denticola* and PD. A strong association was found between *T. forsythia* and PD. The prevalence of *T. denticola* was highest in both deep and shallow sites. No statistically significant association was found between detection of *A. actinomycetemcomitans* and PD. The detection rate of *A. actinomycetemcomitans* was lower in deep PD sites than in the other four bacteria, and prevalence was 32.1% at shallow sites. A statistically significant association was found between detection of *P. gingivalis*, *T. forsythia*, *C. rectus* or *T. denticola* and BOP (Fig. 2). The most prevalent pathogen was *T. denticola*: 77.8% for BOP-positive sites and 55.2% for BOP-negative sites. However, the relationship between detection of *A. actinomycetemcomitans* and BOP was not significant. The association between detection of the red complex bacteria (*P. gingivalis*, *T. forsythia* and *T. denticola*, as described by Socransky *et al.*[19]) and PD was statistically significant ($p<0.001$, Table 3). The detection rate of red complex bacteria was 34.2% for deep sites and 3.6% for shallow sites. The association between detection of

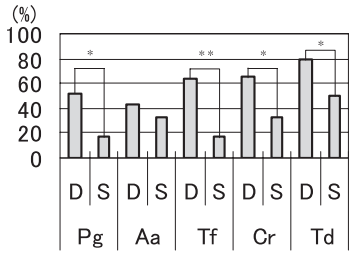

Fig. 1 Percentage of detection sites with Periodontopathic bacteria between Deep PD sites and Shallow PD sites

D = Deep PD (≧4 mm) sites (n = 73)
S = Shallow PD (≦3 mm) sites (n = 28)
*,**: statistically significant between *P.g.*, *T.f.*, *C.r.*, and *T.d.* and Probing depth respectively by Fisher exact test.
* $p<0.01$, ** $p<0.001$
P.g.: *P. gingivalis*, *T.f.*: *T. forsythia*, *C.r.*: *C. rectus*, *T.d.*: *T. denticola*
% sites detected.

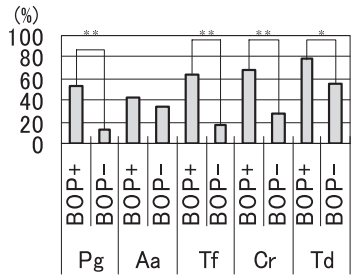

Fig. 2 Percentage of detection sites with Periodontopathic bacteria between positive and negative with bleeding on probing sites

BOP+: bleeding-positive sites on probing (n = 72)
BOP−: bleeding-negative sites on probing (n = 29)
*,**: statistically significant between *P.g.*, *T.f.*, *C.r.*, and *T.d.* and Bleeding on probing by Fisher exact test.
* $p<0.05$, ** $p<0.001$
P.g.: *P. gingivalis*, *T.f.*: *T. forsythia*, *C.r.*: *C. rectus*, *T.d.*: *T. denticola*
% sites detected.

the red complex bacteria and BOP was also statistically significant ($p<0.001$, Table 3). The detection rate of red complex bacteria was 34.7% for BOP-positive sites and 3.4% for BOP-negative sites.

We found that the relationship between co-detection of the red complex bacteria with *C. rectus* and the clinical parameters tested was statistically significant (Table 4).

Discussion

Periodontitis has been considered to be a biofilm infection[3,17,19], and the polymicrobial nature of the periodontopathic bacteria in periodontal lesions has received particular attention[7,19,20]. The results of this study indicate that the relationships between co-existence of *P. gingivalis*, *T. forsythia* and *T. denticola*, designated the red complex, and PD or BOP were significant in periodontitis. This finding is in an agreement with that of an earlier report by Socransky *et al.*[19]. In this study, no significant association was found between *A. actinomycetemcomitans* and the clinical parameters tested, supporting the findings of previous studies[9,19]. This may be due to the fact that *A. actinomycetemcomitans* is not an obligatory anaerobic bacterium. Our findings suggest that the red complex microbial consortium plays an important role in the progression of periodontitis. A relatively strong association was found for *T. forsythia* and the clinical parameters, strongly implicating it in periodontitis.

In this study, we found that the relationship between co-existence of the red complex bacteria with *C. rectus* and the clinical parameters was statistically significant. Some previous studies suggested that *T. forsythia* and *C. rectus* possessed an S-layer on the cell surface, which has been associated with virulence in periodontitis[5,16]. Monitoring of these bacteria may prove to be an important tool in periodontal therapy.

Periodontitis has been associated with multiple virulence factors, with recent study focusing on biofilm. We focused on percentage of sites detected, not percentage of patients. In this study, the criteria of periodontal disease severity were based on a PD of 4 mm, as described in a previous report[9]. Therefore, the subgingival bacteria detection rate in this study was somewhat lower than that in previous studies. Monitoring of periodontopathic bacteria together with clinical parameters may help in our understanding of

Table 3 Relationship between detection of red complex bacteria and probing depth and bleeding on probing

	Sites	Red complex+	Red complex−
*PD	Deep	25 (34.2%)	48 (65.8%)
	Shallow	1 (3.6%)	27 (96.4%)
*BOP	BOP+	25 (34.7%)	47 (65.3%)
	BOP−	1 (3.4%)	28 (96.6%)

red complex+/−: sites with co-existence of *P.g.*, *T.f.* and *T.d.* sites/without
PD: probing depth
Deep: PD (≧4 mm) sites (n=73)
Shallow: PD (≦3 mm) sites (n=28)
BOP: bleeding on probing
BOP+: bleeding-positive sites on probing (n=72)
BOP−: bleeding-negative sites on probing (n=29)
*: statistically significant ($p<0.001$) by Fisher exact test

Table 4 Relationship between detection of red complex bacteria and *C. rectus* and probing depth and bleeding on probing

	Sites	Red complex and *C. rectus*+	Others
*PD	Deep	20 (27.4%)	53 (72.6%)
	Shallow	1 (3.6%)	27 (96.4%)
*BOP	BOP+	20 (27.8%)	52 (72.2%)
	BOP−	1 (3.4%)	28 (96.6%)

red complex and *C. rectus* +/−: sites with co-existence of *P.g.*, *T.f.*, *T.d.* sites and *C. rectus*/without
PD: probing depth
Deep: PD (≧4 mm) sites (n=73)
Shallow: PD (≦3 mm) sites (n=28)
BOP: bleeding on probing
BOP+: bleeding-positive sites on probing (n=72)
BOP−: bleeding-negative sites on probing (n=29)
*: statistically significant ($p<0.01$) by Fisher exact test

the pathogenesis of periodontitis[10,12,14]. Further study is needed to clarify the etiology of periodontitis and its association with the co-existence of periodontopathic bacteria. Early detection of bacteria associated with the development of periodontitis by quantitative PCR would provide important information for subsequent diagnosis and treatment. Monitoring of the red complex and *C. rectus* may be particularly important in periodontal therapy.

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