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Comparative analysis of putative periodontopathic bacteria by multiplex polymerase chain reaction

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Background and Objective: The polymerase chain reaction (PCR) has been applied for the rapid and specific detection of periodontopathic bacteria in subgingival plaque and is potentially of clinical benefit in the diagnosis and treatment of periodontitis subjects. However, several technical points need to be modified before the conventional PCR detection system can be used by clinicians.

Material and Methods: To develop a PCR-based technique more applicable for clinical use than conventional PCR, we established a multiplex PCR for five putative periodontopathic (*Treponema denticola*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia* and *Tannerella forsythia*) and two nonperiodontopathic (*Streptococcus sanguinis* and *Streptococcus salivarius*) species of bacteria using whole-plaque suspension as templates, and detected bacteria in subgingival plaque taken from 85 subjects at the supportive periodontal therapy stage after active periodontal treatments.

Results: Among putative periodontopathic bacteria, the detection frequency of *T. denticola* and *P. gingivalis* was elevated in parallel with higher probing pocket depth and clinical attachment loss, and had 4.2–14.1 times increasing odds of the clinical parameters tested. Detection of any of the five species of putative periodontopathic bacteria markedly increased the odds ratio of a higher probing pocket depth, clinical attachment loss and bleeding on probing.

Conclusion: The multiplex PCR system developed in this study enabled the detection of all the bacteria under investigation in one reaction tube in a less time- and labor-intensive manner than conventional PCR. These results support the potential clinical use of multiplex PCR for detecting periodontopathic bacteria and for evaluating therapeutic strategies and predicting the prognosis for each subject.

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There are estimated to be more than 600 different species of bacteria present in the oral cavity and approximately 500 species of bacteria in dental plaque (1,2). Many previous studies have focused on the identification and detection of periodontopathic bacteria using anaerobic culture techniques and reported that several species of bacteria may be responsible for the initiation and progression of periodontitis. Therefore, the detection of individual species of bacteria in each patient should help to predict patient prognoses and to provide treatment strategies. However, as the detection of anaerobic bacteria can be problematic because of their specific growth requirements, certain technical barriers need to be overcome before

information about such bacterial species can be provided for routine dental clinics.

Recent advances in molecular biology have enabled the identification of specific bacteria in large numbers of periodontitis subjects. The polymerase chain reaction (PCR) is a commonly used technique and is used for the identification of periodontopathic bacteria in many laboratories. PCR rapidly and specifically identifies the presence of bacteria, and has been used to investigate the involvement of certain bacteria in the pathology of periodontitis in comparison with previous data obtained by bacterial culture (3).

Among bacteria colonized in periodontal pockets, the presence of *Treponema denticola*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*), *Prevotella intermedia* and *Tannerella forsythia* (formerly *Bacteroides forsythus*) are linked to the progression of periodontal disease, and are considered as strong markers for periodontitis in adults (4–7). These bacteria irritate host periodontal tissues and initiate inflammatory reactions, resulting in periodontal breakdown. To develop a more rapid and easier-to-use system for clinicians than conventional PCR, we developed a multiplex PCR system detecting five putative periodontopathic bacteria (*T. denticola*, *P. gingivalis*, *A. actinomycetemcomitnas*, *P. intermedia* and *T. forsythia*) and two nonperiodontopathic bacteria (*Streptococcus sanguinis* and *Streptococcus salivarius*), and examined the presence of bacteria in subgingival plaque taken from subjects at the supportive periodontal therapy stage.

Material and methods

Study population

Eighty-five subjects at the the supportive periodontal therapy stage after active periodontal treatments were randomly selected at The Nippon Dental University Hospital at Niigata (39 men and 46 women; age range: 31–80 years; mean age ± 1 standard deviation: 61.8 ± 9.7 years). Patients had not used antibiotics in the past 3 mo. All plaque samples were collected after obtaining informed consent from the participant and after approval of the study by the Institutional Review Board of The Nippon Dental University, School of Life Dentistry at Niigata.

Clinical measurements

Clinical measurements made included probing pocket depth, clinical attachment loss, plaque index and bleeding on probing (8,9). Measurement data were taken from the periodontal pocket with the deepest probing pocket depth and used for statistical analyses. Third molars were excluded.

Plaque sampling

Subgingival plaque samples from 85 patients and supragingival plaque samples from four healthy subjects were collected. In each, a selected tooth was isolated with sterile cotton rolls followed by the removal of supragingival plaque with curettes and sterile cotton pellets, and a sterilized paper point was carefully inserted at the bottom of the periodontal pocket and held in position for 15 s. Samples of supragingival plaque and tongue plaque were taken with sterile explorers, and sterilized paper points were held in plaque samples for 15 s. The paper points from each subject were immediately placed in a microtube containing 20 μL of sterilized saline and stored at −20°C until use.

PCR primer design

To detect individual bacteria by PCR, we designed PCR primer sets specific for each bacterium: chymotrypsin-like protease for *T. denticola* (10); arg-gingipain for *P. gingivalis* (11); leucotoxin A for *A. actinomycetemcomitans* (12); acid phosphatase for *P. intermedia* (13); ribosomal intergenic spacer region for *T. forsythia* (14); glucosyltransferase for *S. sanguinis* (15); and dextranase for *S. salivalius* (16) (Table 1). The specificities of primers were tested against the following strains cultured under appropriate conditions (17–22): *T. denticola* JCM8225; *P. gingivalis* W83; *A. actinomycetemcomitans* ATCC29522; *P. intermedia* (isolated by the Department of Oral Surgery, Kanazawa University); *T. forsythia* JCM10827; *S. sanguinis* JCM5708; and *S. salivarius* JCM5707. PCR was performed on each strain under the same experimental conditions (described below) and the amplification product was then sequenced.

Multiplex PCR

A multiplex PCR was performed using a mixture of all PCR primer sets (0.25 μM each), 1 μL of plaque suspension and *Taq* DNA polymerase (Qiagen, Tokyo, Japan) in a total volume of 20 μL. To avoid the difference in annealing temperature of primer sets, we performed 30 cycles of touchdown PCR (94°C for 45 s, annealing for 45 s, and 72°C for 1 min) followed by a final extension at 72°C for 5 min. Annealing was started at 64°C, touchdowned by 1°C per cycle and maintained at 56°C when this temperature was reached. PCR samples were then loaded onto 2% agarose gels (15 μL/lane) and visualized by ethidium bromide staining. We also confirmed specific amplification of bacteria in randomly selected plaque samples by sequence analyses.

Statistical analyses

The detection frequency of bacteria was calculated from PCR data of plaque samples and given as the percentage of bacteria-positive subjects. Multiple factorial analysis of variance followed by Bonferroni correction and Scheffe's extract were applied to compare the detection frequency of the bacterial species between groups in probing pocket depth ($\leq$ 3 mm vs. $>$ 3 mm), clinical attachment loss ($\leq$ 3 mm vs. $>$ 3 mm), plaque index (0 vs. 1 or 2) and bleeding on probing (negative vs. positive). In order to take clinical parameters into account, the odds ratio with 95% confidence interval of detecting bacteria was analyzed by multivariate logistic regression analysis. Significance of odds ratios was tested using the Wald score.

Table 1. Bacterial primer sequences for multiplex polymerase chain reaction (PCR) and predicted size of the PCR products

Species of bacteria	Genbank accession number	Primer sequence	Size (bp)
Treponema denticola	AJ272339	Forward 5′-CAAATAATGCCGATTACGGGCTTT-3′ Reverse 5′-GCCTTCGTTACCCATCGCAA-3′	653
Porphyromonas gingivalis	D26470	Forward 5′-CGAAGTCTTCATCGGTCGTT-3′ Reverse 5′-GTACCTGTCGGCTTACCATCTT-3′	498
Aggregatibacter actinomycetemcomitans	X16829	Forward 5′-GAAGGCGACGACCACTTAGC-3′ Reverse 5′-GTGCACGATCCTTTTCAGGT-3′	400
Prevotella intermedia	AB017537	Forward 5′-CAAAGACGCACGTACCAATC-3′ Reverse 5′-CTCTGGTGTTGTTTCCTTGCT-3′	262
Tannerella forsythia	AY546489	Forward 5′-CTGGAGCAGTCTTGGAATCTG-3′ Reverse 5′-GCAGCCTGAGTCAGGCTTTTT-3′	168
Streptococcus sanguinis	AB056712	Forward 5′-GGCGCCTGTTAATACTGAGC-3′ Reverse 5′-GTTTTTCCATCCTTGAGGATAGC-3′	330
Streptococcus salivarius	D29644	Forward 5′-CGGTCAAGATAACGTTGACCT-3′ Reverse 5′-CTGCTACGATACCGTAACGTG-3′	212

Results

Development of multiplex PCR system

For optimization of the multiplex PCR system, as reported by Markoulatos *et al*. (23), we initially validated the PCR conditions: the concentration of each primer set, dNTP, $MgCl_2$ and DNA polymerase; cycles and temperature of the PCR; the setting of the touchdown program; selection of DNA polymerase; reaction volume; and preparation of the PCR template. Database analysis for primer specificity indicated that all primers were specific and showed a 100% match to different strains of each species (data not shown). Multiplex PCR amplified genomic fragments, ranging from 168 to 653 bp, from the mixture of genomic DNA isolated from seven bacterial strains (Fig. 1). Seven different size bands were detected and we confirmed specific amplification of the genomic region by DNA sequence analysis (data not shown). Subsequently, subgingival and supragingival plaque samples were used as the template, and each PCR band was cloned and applied for sequence analysis. Every primer set specifically augmented the corresponding bacterial genomic region (data not shown). We used this system to detect five putative periodontopathic and two nonperiodontopathic bacteria in plaque samples of subjects at the supportive periodontal therapy stage.

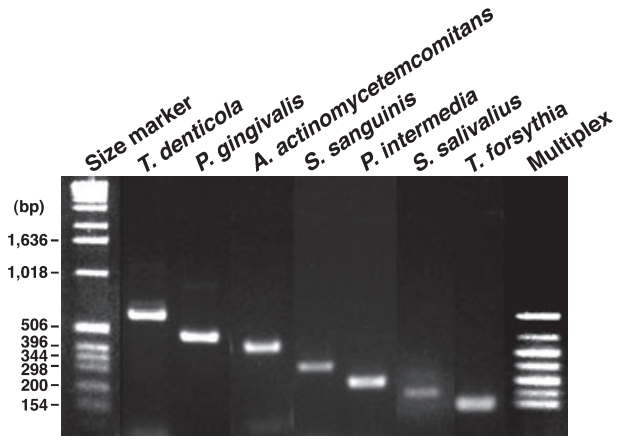

Fig. 1. Development of the multiplex polymerase chain reaction (PCR) system and primer specificity. A mixture of isolated genomic DNA from five putative periodontopathic bacterial strains and two nonperiodontopathic bacterial strains was used as a template and amplified by PCR using a primer set specific to each strain. Right lane: seven different size bands corresponding to each strain were amplified; 653 bp for *Treponema denticola* (*T. denticola*), 498 bp for *Porphyromonas gingivalis* (*P. gingivalis*), 400 bp for *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), 330 bp for *Streptococcus sanguinis* (*S. sanguinis*), 262 bp for *Prevotella intermedia* (*P. intermedia*), 212 bp for *Streptococcus salivalius* (*S. salivalius*) and 168 bp for *Tannerella forsythia* (*T. forsythia*).

Detection of periodontopathic bacteria in subgingival plaque

As shown in Fig. 2, both *S. sanguinis* and *S. salivarius* were the bacteria most frequently detected in supragingival and tongue plaque. Subgingival plaque samples ($n = 85$) from individual subjects at the supportive periodontal therapy stage who had previously received active periodontal treatments were applied for the analysis. Putative periodontopathic bacteria were detected in 47.1% of patients: *T. denticola* in 28.2%; *P. gingivalis* in 31.3%; *A. actinomycetemcomitans* in 1.2%; *P. intermedia* in 9.3%; and *T. forsythia* in 8.1%. Two nonperiodontopahtic bacteria, *S. sanguinis* and *S. salivarius*, were detected in 3.5% and 2.3% of subjects, respectively.

Implications for clinical parameters

We determined the statistical correlations between each bacterium and the clinical parameters (probing pocket

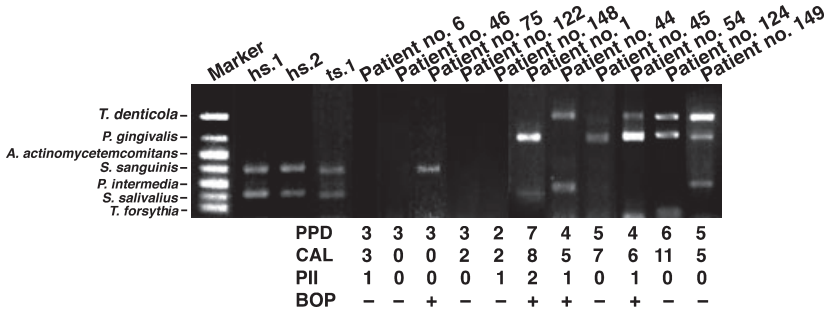

Fig. 2. Detection of bacteria by multiplex polymerase chain reaction (PCR). Multiplex PCR was used to detect putative periodontopathic and nonperiodontopathic bacteria in subgingival, supragingival and tongue plaque. Nonperiodontopathic bacteria (*Streptococcus sanguinis* and *Streptococcus salivalius*) were detected in the supragingival plaque (hs.1 and hs.2) and tongue plaque (ts.1) of healthy subjects. Putative periodontopathic bacterial DNA fragments were exclusively amplified in the subgingival plaque of subjects at the supportive periodontal therapy. The clinical parameters probing pocket depth (PPD), clinical attachment loss (CAL), plaque index (PlI) and bleeding on probing (BOP) were obtained for 11 subjects, as indicated.

depth, clinical attachment level, plaque index and bleeding on probing). The average ± standard deviation for each parameter at the sample sites was 3.37 ± 1.82 mm for probing pocket depth, 4.68 ± 2.50 mm for clinical attachment loss, 0.37 ± 0.60 for plaque index and 1.41 ± 0.54 for bleeding on probing. *A. actinomycetemcomitans* was not correlated with any of the clinical parameters in this study as a result of the lack of subjects in which this bacterium was detected (only one patient was positive). The detection frequencies of *T. denticola* and *P. gingivalis* were elevated in subjects with a higher probing pocket depth or clinical attachment loss (> 3 mm) (Fig. 3). *T. forsythia* was detected at a high frequency in subgingival plaque samples collected from periodontal pockets with a clinical attachment loss of > 3 mm. The detection frequencies of all of the five putative periodontal pathogens (*T. denticola*, *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *T. forsythia*) showed a parallel with the probing pocket depth, clinical attachment loss and bleeding on probing. *P. intermedia* was not statistically correlated with the clinical parameters in this study. The age and gender of subjects was not correlated with any specific bacterial species (data not shown).

As summarized in Table 2, the presence of *T. denticola* and *P. gingivalis* increased the odds ratio for a higher probing pocket depth and clinical attachment loss. Subjects positive for *T. forsythia* had a 9.4 times higher odds of having a probing pocket depth of > 3 mm. *P. intermedia* did not show any correlation with the clinical parameters tested. Detection of any putative periodontopathic bacteria increased the odds ratio for a larger probing pocket depth (19.50, 95% confidence interval: 6.37–59.68, $p < 0.0001$), higher clinical attachment loss (15.07, 95% confidence interval: 2.97–27.44, $p < 0.0001$), and more bleeding on probing (4.53, 95% confidence interval: 1.76–11.66, $p = 0.0017$). Plaque index was not statistically associated with bacteria in this analysis.

Discussion

Recent advances in genome sequence information have enabled the identification of bacteria by PCR more rapidly and in a less labor-intensive manner. Accumulating evidence has established the benefits of PCR-based techniques to detect periodontopathic bacteria in subgingival plaque samples (3,24–30). However, at present, these PCR techniques are generally performed on a single species of bacteria, making it a laborious process with problems of false-negative results. To address this problem in the clinical situation, we developed a multiplex PCR system with the ability to detect five putative periodontopathic bacteria – *T. denticola*, *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *T. forsythia* – and analyzed the presence of these bacteria in the plaque of patients at the supportive periodontal therapy stage.

Among the five species of bacteria analyzed, *P. gingivalis* was most frequently detected (30.6%) followed by *T. denticola* (28.2%). The detection frequencies of both *P. gingivalis* and *T. denticola* were significantly increased in subgingival plaque taken from periodontal pockets with > 3 mm probing pocket depth and/or > 3 mm clinical attachment loss (*P. gingivalis*, 55.6%; and *T. denticola*, 52.8%). The presence of *P. gingivalis* and *T. denticola* also increased the odds ratio for a higher probing pocket depth (*P. gingivalis*, 6.71; and *T. denticola*, 14.06) and clinical attachment loss (*P. gingivalis*, 4.22; and *T. denticola*, 9.82). These results confirm previous studies demonstrating an increase of their detection frequency in association with increasing odds for clinical parameters (24–27). Although the detection frequencies of *T. forsythia* and *P. intermedia* are known to be increased in parallel with the odds ratio (24–26), we only observed an association between *T. forsythia* and a higher clinical attachment loss. The involvement of *A. actninomycetemcomitans* in periodontitis is controversial (24–26,31–34). In the present study, only one subject was positive for *A. actinomycetemcomitans*. The prevalence of *A. actinomycetemcomitans* may be influenced by differences in detection methods and sample populations. We also determined the significance of the presence of any of the five putative periodontopathic bacteria relative to the clinical parameters. The detection frequency of periodontopathic bacteria was increased in subgingival plaque taken from subjects showing higher probing pocket depth and clinical attachment loss and presence of bleeding on

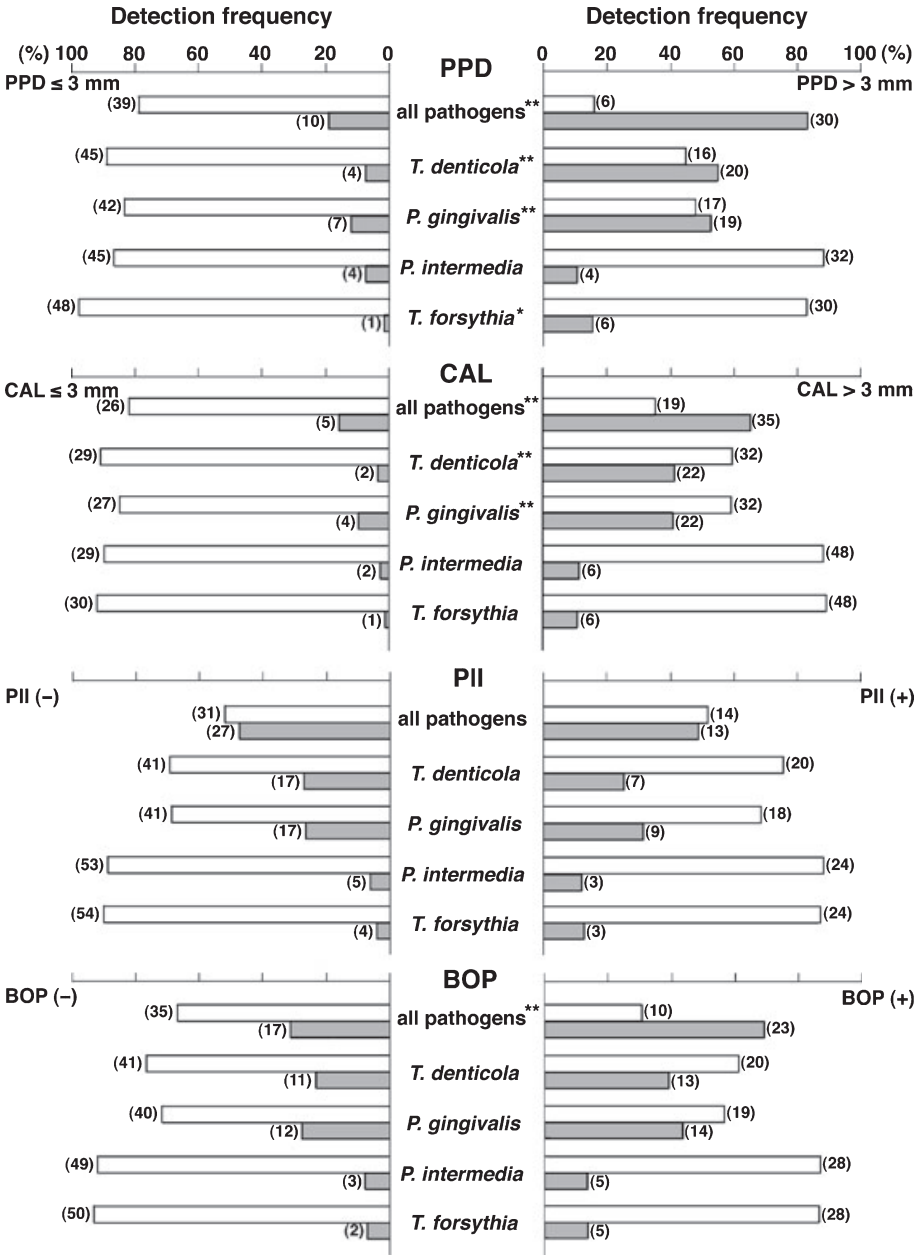

Fig. 3. Prevalence of periodontopathic bacteria in 85 subjects at the supportive periodontal therapy stage. The absence (unfilled bars) and presence (filled bars) of each or all bacteria [any five putative periodontopathic bacteria: *Treponema denticola* (*T. denticola*), *Porphyromonas gingivalis* (*P. gingivalis*), *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), *Prevotella intermedia* (*P. intermedia*) or *Tannerella forsythia* (*T. forsythia*)] were compared with the clinical parameters probing pocket depth (PPD), clinical attachment loss (CAL), plaque index (PlI) and bleeding on probing (BOP). The numbers of subjects observed are given in parentheses. Significant difference for detection frequency was as follows: probing pocket depth $\leq$ 3 mm vs. $>$ 3 mm; clinical attachment level $\leq$ 3 mm vs. $>$ 3 mm; plaque index (–) vs. (+); bleeding on probing (–) vs. (+). A plaque index of (–) indicated a score of 0, and a plaque index of (+) indicated a score of 1 or 2. Bleeding on probing scores of (–) or (+) indicated the absence or the presence of bleeding, respectively. A significant difference with $p < 0.01$ is marked as **, and with $p < 0.05$ as *.

probing. Therefore, the multiplex PCR system is useful for monitoring the presence of periodontopathic bacteria and for predicting disease status. Patients positive for periodontopathic bacteria should be carefully evaluated for signs of relapse, if they are maintained in favourable clinical condition.

The detection frequency of periodontopathic bacteria in the present study was low compared with previous studies using PCR-based detection systems (3,24–30). Several reasons may exist for this discrepancy. First, in this study, subgingival plaque was randomly collected from subjects at the supportive periodontal therapy stage

Table 2. Odds ratios of clinical parameters for the presence of periodontal bacteria (n = 85)

Parameters	Pathogens	Odds ratio	95% CI[a]	*p*-value[b]
PPD	All pathogens	19.50	6.37–59.68	< 0.0001
	T. denticola	14.06	4.17–47.43	< 0.0001
	P. gingivalis	6.71	2.39–18.85	0.0003
	P. intermedia	1.41	0.33–6.05	0.6468
	T. forsythia	9.40	1.08–82.02	0.0426
CAL	All pathogens	15.07	2.97–27.44	< 0.0001
	T. denticola	9.82	2.11–45.65	0.0036
	P. gingivalis	4.22	1.28–13.86	0.0177
	P. intermedia	1.89	0.36–10.01	0.4535
	T. forsythia	2.55	0.27–23.96	0.4118
PlI	All pathogens	0.98	0.39–2.49	0.9730
	T. denticola	0.72	0.25–2.12	0.5546
	P. gingivalis	1.07	0.39–2.93	0.8925
	P. intermedia	1.12	0.10–12.93	0.9277
	T. forsythia	1.17	0.20–6.87	0.8588
BOP	All pathogens	4.53	1.76–11.66	0.0017
	T. denticola	2.24	0.84–5.94	0.1065
	P. gingivalis	2.28	0.88–5.93	0.0909
	P. intermedia	3.03	0.67–13.65	0.1499
	T. forsythia	3.50	0.60–20.34	0.1629

[a]Confidence interval.
[b]Probability of statistical difference (*p*) was analyzed by multivariate logistic regression analysis.
BOP, bleeding on probing; CAL, clinical attachment loss; PlI, plaque index; PPD, probing pocket depth.
P. gingivalis, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *T. denticola*, *Treponema denticola*; *T. forsythia*, *Tannerella forsythia*.

after active periodontal treatments. Second, we designed PCR primers and used PCR conditions different from those in the other studies, to develop a multiplex PCR system. Third, we used untreated whole-plaque suspension as the PCR template, not isolated genomic DNA as used in the other studies; this is the most probable cause of the low detection frequency in our study. In fact, Tanner *et al.* (35) performed PCR directly using a whole-plaque template and reported similar detection frequencies of *P. gingivalis* and *T. forsythia* (13.0–38.0% and 10.0–25.0%, respectively).

Recent advances in PCR technology have enabled the rapid detection of periodontopathic bacteria. However, several aspects need to be modified before the PCR system is suitable for clinicial use. The present multiplex PCR system directly utilizes a whole-plaque suspension as a template, but does not require isolation of bacterial genomic DNA, and allowed us to monitor all seven species of bacteria within a few hours in one reaction tube. We also confirmed that whole-plaque suspension samples can be stored at −20°C for longer than 7 d without any decrease in the detection sensitivity (data not shown), and that the use of tongue plaque as a template can be used in the PCR reaction as a positive control. These assets of the system strongly support the possibility that dental clinicians themselves may routinely monitor the presence of pathogenic bacteria. A small percentage of subjects at the maintenance stage show recurrence of periodontitis despite periodical evaluation. Identification of bacteria in subgingival plaque provides valuable information on the periodontopathic background. If, clinically, nonperiodontitis subjects and subjects at the supportive periodontal therapy stage are positive for periodontopathic bacteria, dental clinicians can select therapeutic strategies for disease prevention that are appropriate for each subject and condition.

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