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Prevalence of *Porphyromonas gingivalis* in relation to periodontal status assessed by real-time PCR

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Many studies have examined the presence of *Porphyromonas gingivalis* in periodontal pockets. However, monitoring the number of bacterial cells is difficult. In this study, we performed quantitative analyses of *P. gingivalis* to clarify the relationship between the numbers of this organism and periodontal status. Using the TaqMan real-time PCR system, we found a significant positive correlation ($P < 0.0001$) between the number of *P. gingivalis* and pocket depth. The slope of the regression line indicated that for every 1-mm increase in pocket depth, the number of *P. gingivalis* increased 10- fold. There was also a significant reduction ($P < 0.01$) in the numbers of *P. gingivalis* before and after treatment. These results suggest that the absolute and relative numbers of *P. gingivalis* are closely associated with periodontal status, and that quantitative analysis of this organism is important for the evaluation of periodontal therapy.

Key words: periodontitis; pocket depth; *Porphyromonas gingivalis*; real-time polymerase chain reaction; TaqMan assay

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Periodontitis is an infectious disease that causes destructive changes leading to the loss of bone and connective tissue attachment (13, 19). Of the bacterial species suspected of playing an important role in the pathogenesis of adult-onset periodontitis, *Porphyromonas gingivalis*, a black-pigmented gram-negative anaerobic rod, has been strongly implicated as a major pathogen in this disease (12, 25), as well as in the development of oral malodor (9, 24).

In the diagnosis and evaluation of therapy for periodontal diseases, clinical attention has focused on the detection of *P. gingivalis* in periodontal pockets. Culture techniques have been used in several studies to estimate the bacterial cell numbers in subgingival plaque samples (1, 7, 15). However, compared to other bacterial species, it is difficult to quantify the cell numbers. Monitoring how the *P. gingivalis* population changes over the course of periodontal treatment (18), and the relationship between the *P. gingivalis* population and the progression of periodontitis (15), is necessary for an understanding of periodontitis.

We previously developed a method of quantifying the relative amounts of *P. gingivalis* and *Actinobacillus actinomycetemcomitans* in subgingival plaque and saliva samples using TaqMan real-time polymerase chain reaction (PCR) (22). With this technique, we examined the relationship between the number of *P. gingivalis* and periodontal status, and evaluated the effect of periodontal therapy.

Material and methods

Bacterial strain and culture conditions

P. gingivalis ATCC33277 was cultured as previously described (24).

Study subjects

Forty patients who visited the Department of Preventive Dentistry, Kyushu University Dental Hospital, were examined for the presence of *P. gingivalis* in the saliva using conventional PCR; 26 patients were positive. The protocol for detecting *P. gingivalis* has been described in a previous study (23). All of the patients were given a full-mouth periodontal examination. After the periodontal examination, nine *P. gingivalis*-positive patients with moderate to severe periodontitis (three males and six females, 28–72 years old; mean ± SD of age, 54.1 ± 26.1), having at least four teeth with ≥4 mm pocket depth, were selected for further examination using real-time PCR. None of them had taken antibiotics, or undergone scaling or root planing within the 6 months prior to the study. All of the

subjects who participated in this study understood the nature of the research project and provided informed consent; the protocol was reviewed and approved by the Ethics Committee of Kyushu University Dental Hospital.

Clinical examination and preparation of subgingival plaque

All patients received a full-mouth periodontal examination. Pocket depth was measured at six sites of each tooth (distal, mid, and mesial sites for the buccal and lingual surfaces) using a periodontal probe. Following the World Health Organization (WHO) criteria (17), six segments (sextants) were evaluated for each mouth. The deepest periodontal pockets in each segment were selected for microbial sampling from each patient, for a total of 119 samples. Subgingival plaque samples were obtained by inserting a sterile endodontic paperpoint into the subgingival site for 10 s. The paperpoint was transferred into 200 μl of cell lysis buffer (1.0% Triton X-100, 20 mM Tris-HCl, and 2 mM EDTA [pH 8.0]), boiled at 100°C for 5 min; the supernatant served as the PCR template (16).

Periodontal treatment

Nine subjects (three males and six females) received professional oral hygiene instructions, which were reinforced at each session, and scaling. Three months after full-mouth scaling, the clinical effects of this treatment were evaluated by measuring periodontal pocket depth.

Primers and probes for real-time PCR

P. gingivalis-specific and ubiquitous primer-probe sets were designed using Primer Express 1.5 software (Applied Biosystems, Foster City, CA), as previously described (22). Briefly, a fluorescent probe was used to monitor PCR product formation continuously during PCR. This oligonucleotide probe was double labeled with a reporter dye (FAM: 6-carboxyfluorescein) covalently attached at the 5′ end, and a quencher dye (TAMRA: 6-carboxytetramethylrhodamine) covalently attached at the 3′ end. The *P. gingivalis*-specific primers and probe were designed from the 16S rRNA gene to be specific for *P. gingivalis*. This primer-probe set included the forward (Pg1198-F, 5′-TACCCATCGTCGCCTTGGT-3′) and reverse (Pg1323-R, 5′-CGGACTAAAACCGCATACACTTG-3′) primers and a probe (Pg1238T, 5′-FAM GCTAATGGGACGCATGCCTATCTTACAGCT-TAMRA-3′). The oligonucleotide primers and probe used to detect a broad range of bacteria included the forward (Uni152-F, 5′-CGCTAGTAATCGTGGATCAGAATG-3′) and reverse (Uni220-R, 5′-TGTGACGGGCGGTGTGTA-3′) primers and a probe (Uni177T, 5′-FAM-CACGGTGAATACGTTCCCGGGC-TAMRA-3′), which were complementary to highly conserved regions within the 16S rRNA gene, as previously described (3).

TaqMan real-time PCR assay for quantifying *P. gingivalis*

Real-time PCR conditions for the quantitative detection of *P. gingivalis* were set up as previously described (22). A standard curve was plotted for each primer-probe set, with Ct values obtained from the amplification of known quantities of DNA extracted from samples containing 1.5×10^0–1.5×10^9 CFU. The number of colony-forming units (CFU) was determined by plating culture dilutions on Tryptic Soy Agar (Difco Laboratories, Detroit, MI) plates. To determine the linearity and detection limit of the assay, solutions of lysed *P. gingivalis* were amplified for successive 10-fold dilutions in a series of real-time PCRs so that the correlation coefficient could be calculated from the standard curve of Ct values. For the relative quantification, the *P. gingivalis* DNA copies were normalized to the 16S rRNA gene copies using a simplification of the comparative threshold cycle (ΔΔCt) method, as previously described (21, 22).

Statistics

Logarithmic transformation was performed for the *P. gingivalis* levels detected in patients to improve normality, and the results were compared with periodontal pocket depth in a linear regression analysis. The difference between the mean pocket depth before and after treatment was assessed using a paired *t*-test. Wilcoxon's signed-rank test was used for statistical comparison of the *P. gingivalis* levels detected in the periodontal pockets before and after treatment. The level of statistical significance was set at $P < 0.05$.

Results

Real-time PCR assay for the quantitative detection of *P. gingivalis*

With the real-time PCR assay, using 10-fold serial dilutions of *P. gingivalis,* it was possible to detect bacterial DNA in mixtures containing DNA over a linear range from 1.5×10^0 to 1.5×10^6 CFU per reaction mixture, with Ct values ranging between 40.9 and 22.6 (Fig. 1). The presence of PCR inhibitors in dental plaque was assessed using the levels of fluorescence for serial dilutions of lysed *P. gingivalis* cells ranging from 1.5×10^0 to 1.5×10^6 copies. Lysates containing approximately 10 μg (wet weight) of *P. gingivalis*-negative dental plaque in each mixture did not show any inhibition (data not shown).

Relationship between the number of *P. gingivalis* cells and pocket depth

In this study, the number of *P. gingivalis* cells from 119 samples in 26 patients before periodontal treatment were analyzed using the TaqMan assay. Of the 119 periodontal sites, 56 sites (47.1%) were positive for *P. gingivalis* using conventional PCR (data not shown). The absolute numbers of *P. gingivalis* in subgingival plaque were determined using standard curves (Fig. 1). The relationship between the number of log-transformed *P. gingivalis* cells and pocket depth is shown in Fig. 2A. In the simple regression analysis, the number of log-transformed *P. gingivalis* cells was linearly correlated with pocket depth ($\log(y) = 1.14x - 2.01$; $r^2 = 0.37$; $P < 0.0001$), as shown in Fig. 2A. The simple regression analysis of the percentage of *P. gingivalis* in relation to pocket depth also showed a positive correlation ($y = 4.69x - 13.4$; $r^2 = 0.40$; $P < 0.0001$), as shown in Fig. 2B.

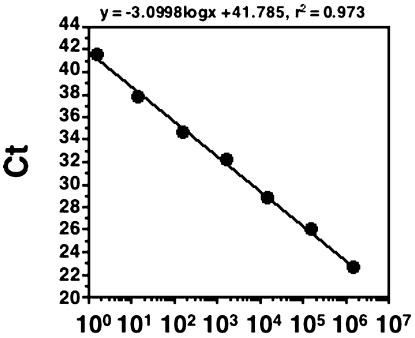

Fig. 1. The standard calibration curves for the *P. gingivalis* real-time PCR assay. The threshold cycle was measured and plotted against the $\log_{10}$ of the dilution. The standard calibration curve was generated from the known CFU of cell lysates. The threshold cycle was measured and plotted against the $\log_{10}$ of the copy number. Each point represents the average of triplicate PCRs.

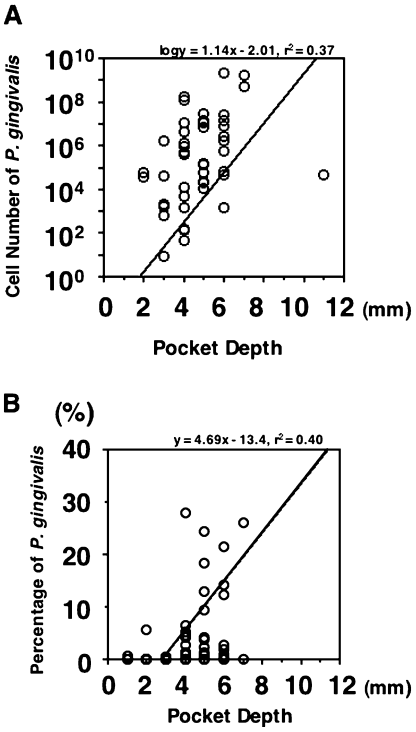

Fig. 2. The relationship between the amount of *P. gingivalis* and pocket depth. The correlation between the log-transformed absolute number of *P. gingivalis* and the depth of 119 periodontal pockets is shown (A). The correlation between the percentages of *P. gingivalis* and the depth of 119 periodontal pockets is shown (B). The five samples with a prevalence exceeding 40% were not included in this figure.

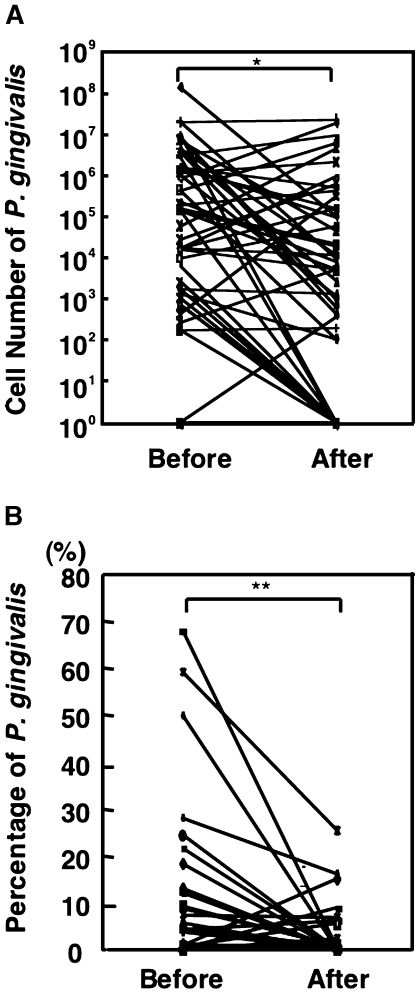

Fig. 3. The *P. gingivalis* population before and after periodontal treatment. The changes in the absolute number (A) and percentage (B) of *P. gingivalis* cells after treatment compared with that before treatment are shown. $*P < 0.01$, $**P < 0.001$.

Monitoring the *P. gingivalis* population before and after periodontal treatment

Changes in pocket depth and the number of *P. gingivalis* cells before and after treatment were monitored. The deepest pocket in each segment was significantly shallower after treatment (paired *t*-test, $P < 0.0001$, data not shown). There was a significant reduction in the absolute number of *P. gingivalis* cells after treatment compared to before treatment (Wilcoxon's signed-rank test, $P < 0.01$, Fig. 3A). A significant reduction in the percentage of *P. gingivalis* after treatment was also observed (32 of the 52 periodontal sites, 61.5%, Wilcoxon's signed-rank test, $P < 0.001$, Fig. 3B).

Discussion

To better understand periodontitis, it is important to examine the relationship between the periodontopathic bacteria population and periodontal status. To do this, both relative and absolute quantification are essential and a suitable quantification procedure must be chosen, according to the purpose or situation. Many investigations have revealed a qualitative relationship between the presence of periodontopathic bacteria and pocket depth (6, 8, 14). However, reports on the quantitative relationship between the relative/absolute numbers of periodontal bacteria and pocket depth are very rare (15).

Using the TaqMan assay and simplified ΔΔCt method (21, 22), we evaluated the number of *P. gingivalis* cells in relation to pocket depth. Simple regression analysis showed a highly significant positive correlation between these parameters, with a correlation coefficient of 0.61 ($P < 0.0001$). The slope of the regression line for the log-translated number of *P. gingivalis* plotted against pocket depth was 1.14. This implies that the number of *P. gingivalis* cells increased approximately 10-fold for each 1-mm increase in pocket depth. This is the first investigation to reveal a quantitative relationship between the number of *P. gingivalis* cells and pocket depth using the TaqMan assay. From our results, we conclude that the number of *P. gingivalis* cells is an important factor affecting the progression of this disease.

Since we found a positive correlation between the number of *P. gingivalis* cells and pocket depth, we used our system to evaluate periodontitis treatment. There were significant differences in the absolute ($P < 0.01$) and percentage ($P < 0.001$) of *P. gingivalis* cells before and after periodontal treatment. This is consistent with previous work using other procedures (4, 18). In addition, there was also a significant difference ($P < 0.0001$) in pocket depth before and after treatment. This indicates that the number of *P. gingivalis* cells reflects the periodontal pocket depth.

The PCR technique is theoretically capable of detecting a single copy of the target DNA. However, this procedure sometimes lacks sensitivity, reproducibility, and specificity when applied to clinical specimens. This is believed to be due to the presence of polymerase inhibitors in clinical materials (2). But when we examined the inhibitory effect of dental plaque, it was negligible (data not shown).

There has been a recent focus on periodontitis because of its relationship to cardiovascular disease (5, 11), obesity (10), and rheumatoid arthritis (20). Our technique could be useful for evaluating periodontal conditions in these general health conditions. Although our investigation demonstrated only one possible application, the real-time PCR assay for periodontal bacteria can be used to determine the amount of bacteria for a broad range of purposes in the study of periodontal disease.

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