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Quantitative detection of periodontal pathogens using real-time polymerase chain reaction with TaqMan probes

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Quantitative analysis, with identification of periodontopathic bacteria, is important for the diagnosis, therapeutic evaluation and risk assessment of periodontal disease. We developed a highly sensitive and specific method using real-time polymerase chain reaction (PCR) to detect and quantify six periodontal bacteria: *Porphyromonas gingivalis, Tannerella forsythia, Actinobacillus actinomycetemcomitans, Treponema denticola, Prevotella intermedia*, and *Prevotella nigrescens*. Species-specific TaqMan probe/primer sets were designed according to 16S ribosomal RNA gene sequences. Plaque and tongue debris specimens were collected from 10 patients with advanced periodontitis and 10 periodontal healthy individuals and analyzed. All species, except for *P. nigrescens*, were detected in samples from diseased sites in significantly greater numbers than in those from healthy sites, whereas greater numbers of *P. nigrescens* were found in the controls. These results suggest that the present real-time PCR method with the designed probe/primer sets enabled sensitive detection of the six periodontal bacteria, and may also assist future microbial studies of periodontal diseases.

Key words: *Actinobacillus actinomycetemcomitans*; *Porphyromonas gingivalis*; *Prevotella intermedia*; *Prevotella nigrescens*; quantitative detection; real-time polymerase chain reaction; *Tannerella forsythia*; TaqMan probe; *Treponema denticola*

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Periodontal diseases are infectious disorders and the pathogenic microbial populations involved are known to be highly complex. Numerous reports have demonstrated a close association between periodontitis and a small subset of microbial species that includes *Porphyromonas gingivalis, Tannerella forsythia, Actinobacillus actinomycetemcomitans, Treponema denticola*, and *Prevotella intermedia* (12, 22, 47, 49, 53). These pathogens are harbored on the tongue surface and their metabolic products have also been suggested as causative factors of halitosis (11, 38, 43). Therefore, quantitative analysis with identification of pathogens in clinical specimens would be helpful for the diagnosis and therapeutic evaluation of periodontitis, as well as to understand the pathogenesis of halitosis.

Currently, several methods of quantitative analysis are used to identify oral pathogens, including flow cytometry (54), a DNA–DNA hybridization (8, 48, 51), and real-time polymerase chain reaction (PCR) (4, 30, 33, 40, 45, 60, 61). Real-time PCR has some advantages, as its detection limit of approximately 10^2 copies is more sensitive than that of a DNA probe (10^3–10^4 copies) (8, 48). In addition, a real-time PCR assay, along with a universal probe/primer set, can be used to quantify an entire bacterial load in a single clinical specimen with a fair degree of precision, which is not possible with a DNA probe. As for flow cytometry, most bacteria are optically too similar to be distinguished from each other or from debris without artificially modifying the target bacteria with fluorescent labeling techniques, such as fluorescent antibodies or dyes (23, 54). Furthermore, coaggregation of bacteria and the presence of different contaminating matrices (e.g. dirt, food, dental plaque) can also make accurate counting difficult with direct or fluorescence microscopy. For the reasons stated above, real-time PCR would currently be more suitable for quantitative detection of microorganisms than the other methods.

A variety of *Prevotella* species are commonly detected in the human oral cavity

(19, 31, 34), with *P. intermedia* and *Prevotella nigrescens* the most prevalent. *P. intermedia* is considered to be a periodontal pathogen, whereas *P. nigrescens* is a marker of relative periodontal health (7–9, 16, 17, 19, 31, 36, 37, 44). These species are phenotypically very similar, and biochemical or serological differentiation is considerably difficult and laborious (7, 10, 14, 17, 29, 34, 46). In light of the postulated different roles of these two species, however, it is essential to identify and quantify them differentially in clinical specimens. A real-time PCR method using SYBR Green I, a double strand DNA binding dye, for detecting five periodontal pathogens (*P. gingivalis, A. actinomycetemcomitans, T. forsythia, T. denticola* and *Treponema socranskii*), has been reported (45). In the TaqMan system, a set of three specific PCR probes, forward and reverse primers, and TaqMan probe, is used. The real-time PCR with TaqMan probe allows continuous measurement of products throughout the reaction in a closed tube and exploits the 5' to 3' exonuclease activity of Taq polymerase in conjunction with fluorogenic DNA probes. In this method, a TaqMan probe, designed to hybridize to the target PCR product, is labeled with a fluorescent reporter dye and a quencher dye. During PCR amplification, the probe is digested by Taq polymerase, separating the dyes and resulting in an accumulation of reporter fluorescence along with a corresponding increased fluorescence intensity (4, 30, 33, 40, 60, 61). Thus, TaqMan hybridization probes are likely to be adopted as more reliable options for distinguishing between periodontal pathogens, especially for such closely related species as *P. intermedia* and *P. nigrescens*. For the same reason, a TaqMan system would also be useful for distinguishing *A. actinomycetemcomitans* (formerly *Haemophilus actinomycetemcomitans*) from *Haemophilus influenzae*, based on their 16s rRNA sequences. In the present study, species-specific TaqMan probe/primer sets were designed for rapid and reliable quantitative identification of *P. gingivalis, T. forsythia, A. actinomycetemcomitans, T. denticola, P. intermedia*, and *P. nigrescens*. Using these probe/primer sets, clinical specimens from individuals with varying clinical conditions were analyzed.

Material and methods

Bacterial strains

P. gingivalis, ATCC33277, ATCC53977, 6/26, HW24D1, W50, and HNA99; *A. actinomycetemcomitans,* ATCC29522, ATCC29523, and FDCY4; *T. forsythia*, ATCC43037; *T. denticola,* ATCC33520; *P. intermedia*, ATCC25611; and *P. nigrescens*, ATCC25261 were used as reference strains. *P. gingivalis, P. intermedia*, and *P. nigrescens* cells were grown in trypticase soy broth as described previously (26), while *A. actinomycetemcomitans* was grown in TSB supplemented with yeast extract (1 mg/ml) and sodium bicarbonate (1 mg/ml) (42). *T. forsythia* and *T. denticola* were also grown under conditions described previously (41, 59). Each species was cultured at 37°C under anerobic conditions (80% N_2, 10% CO_2, 10% H_2) to the late exponential phase, then harvested by centrifugation (12,000 ***g*** at 4°C for 2 min) and washed with phosphate-buffered saline (PBS) (pH 7.4).

Species-specific probe/primer sets for real-time PCR

Multiple alignment analyses of the rRNA genes of 100 major oral bacteria were employed as reference materials for designing the assays.

The 16S rRNA gene sequences (GenBank) from the following bacteria were aligned using the Clustal W program accessed from DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/): *Actinomyces bovis, Actinomyces israelii, Actinomyces naeslundii, Actinomyces odontolyticus, Actinomyces viscosus, A. actinomycetemcomitans, Actinobacillus delphinicola, Actinobacillus seminis, Actinomyces suis, Anaerococcus prevotii, Bacteroides fragilis, Bifidobacterium dentium, Corynebacterium matruchotii, Capnocytophaga gingivalis, Capnocytophaga ochracea, Capnocytophaga sputigena, Campylobacter rectus, Camphylobacter sputorum, Desulfovibrio* sp., *Eikenella corrodens, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Eubacterium nodatum, Eubacterium timidum, Filifactor alocis, Fusobacterium nucleatum* subsp. nucleatum, *Fusobacterium sulci, Fusobacterium simiae, Haemophilus aphrophilus, Haemophilus ducreyi, Haemophilus haemolyticus, Haemophilus influenzae, Haemophilus paracuniculus, Haemophilus parainfluenzae, Haemophilus paraphrophilus, Haemophilus parasuis, Klebsiella pneumoniae, Lactobacillus casei, Lactococcus lactis, Legionella pneumophila, Leptotrichia buccalis, Neisseria cinerea, Neisseria denitrificans, Neisseria flavescens, Neisseria mucosa, Pasteurella avium, Pasteurella multocida, Pasteurella pneumotropica, Peptococcus niger, Peptostreptococcus anaerobius, Peptostreptococcus micros, Porphyromonas cansulci, Porphyromonas endodontalis, Porphyromonas gingivalis, Prevotella heparinolytica, Prevotella intermedia, Prevotella loescheii, Prevotella melaninogenica, Prevotella nigrescens, Prevotella oralis, Prevotella oris, Prevotella veroralis, Propionibacterium acnes, Propionibacterium propionicus, Pseudomonas aeruginosa, Pseudomonas fluorescens, Ruminobacter amylophilus, Selenomonas sputigena, Staphylococcus aureus, Staphylococcus epidermidis*, *Streptococcus anginosus, Streptococcus bovis, Streptococcus constellatus, Streptococcus cricetus, Streptococcus downei, Streptococcus gordonii, Streptococcus intermedius, Streptococcus macacae, Streptococcus mitis, Streptococcus mutans, Streptococcus oralis, Streptococcus pyogenes, Streptococcus rattus, Streptococcus salivarius, Streptococcus sanguis, Streptococcus saprophyticus, Streptococcus sobrinus, Serratia marcescens, Tannerella forsythia, Treponema denticola, Treponema medium, Treponema pallidum, Treponema pectinovorum, Treponema socranskii* subsp. socranskii, *Treponema vincentti, Veillonella atypica, Veillonella dispar, Veillonella parvula*, and *Vibrio cholerae.*

Species-specific probe and primer sets were designed from the variable regions of the 16S rRNA gene sequences. Regions of identity were surveyed for possible cross-hybridization with other bacterial genes using the rRNA BLAST program, which was accessed from The European Ribosomal RNA database (http://oberon.fvms.ugent.be:8080/rRNA/), and the BLAST program accessed from National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov./blast/). All probe/primer sets were subjected to Primer Express version 1.0, using the guidelines established by Applied Biosystems (Foster City, CA). A universal probe/primer set was used as described previously (40). The probes and primers were synthesized by Applied Biosystems, except the forward primer of *P. nigrescens* (the mixed primer), which was synthesized by Sigma Genosys (The Woodlands, TX). The oligonucleotide probes were labeled with 6-carboxyfluorescein (FAM) at the 5' end and 6-carboxytetramethylrhodamine (TAMRA) at the 3' end and stored at −20°C.

Clinical specimens

Seventy plaque samples, whose bacterial profiles were analyzed in our previous study (2), were used to confirm the specificities of the newly designed TaqMan probe/primer sets. In addition, subgingival

Table 1. Species-specific primers for conventional PCR

Primer sets	Product size (bp)	Ta (°C)*	Reference
Porphyromonas gingivalis 5′-TGTAGATGACTGATGGTGAAAACC-3′ 5′-ACGTCATCCCCACCTTCCTC-3′	197	60	(35)
Actinobacillus actinomycetemcomitans 5′-CTAGGTATTGCGAAACAATTTG-3′ 5′-CCTGAAATTAAGCTGGTAATC-3′	262	55	(20)
Tannerella forsythia 5′-GCGTATGTAACCTGCCCGCA-3′ 5′-TGCTTCAGTGTCAGTTATACCT-3′	641	60	(5)
Treponema denticola 5′-TAATACCGAATGTGCTCATTTACAT-3′ 5′-TCAAAGAAGCATTCCCTCTTCTTCTTA-3′	316	55	(45)
Prevotella intermedia 5′-TTTGTTGGGGAGTAAAGCGGG-3′ 5′-TCAACATCTCTGTATCCTGCGT-3′	575	55	(5)
Prevotella nigrescens 5′-ATGAAACAAAGGTTTTCCGGTAAG-3′ 5′-CCCACGTCTCTGTGGGCTGCGA-3′	804	55	(5)

*Annealing temperature (Ta) of PCR reaction.

plaque and tongue surface debris specimens were collected from 20 individuals, of whom 10 were patients with advanced periodontitis (mean age 48.3 ± 15.9 years) and 10 were periodontal healthy controls (mean age 56.6 ± 12.4 years). The periodontitis patients possessed active sites in greater than 40% of all their teeth, which were defined by probing depth of 6 mm or greater, bleeding on probing, and the presence of either erythema or suppuration. The controls had probing depths of 3 mm or less and exhibited no clinical signs of alveolar bone loss in dentition, bleeding on probing, or signs of erythema or suppuration. The subgingival plaque samples were collected from the deepest pocket of each subject in a manner described previously (2), while tongue surface debris samples were collected with sterile spatulas as thoroughly as possible and then immediately suspended in sterile PBS. After collection, all samples were kept on ice, and genomic DNA was immediately extracted, as described below.

DNA isolation

Genomic DNA isolation from the specimens was performed using a PUREGENE DNA Isolation Kit (Gentra systems, Minneapolis, MN) according to the manufacturer's instructions. Purified genomic DNA of *H. influenzae* ATCC 33991 was purchased from American Type Culture Collection (ATCC, Manassas, VA). DNA concentrations were determined spectrophotometrically using a GeneQuant II RNA/DNA Calculator (Amersham Pharmacia Biotech, Piscataway, NJ).

Screening by conventional PCR

Bacterial species-specific primers used for conventional PCR are shown in Table 1. PCR amplification was performed in a reaction mixture (25 μl) consisting of Ready-To-Go PCR beads (Amersham Pharmacia Biotech) containing an enzyme and the required reagents, along with 0.8 μM of each primer and 2 μl of the template DNA solution (20–50 μg/ml), as described previously (3). The amplification reaction was performed in a model 2400 thermal cycler (Perkin Elmer, Branchburg, NJ) with the cycling parameters set as follows.

- For *T. forsythia, P. intermedia*, and *P. nigrescens*: an initial denaturation at 95°C for 5 min; 30 cycles consisting of 95°C for 30 s, 55 or 60°C for 30 s and 72°C for 1 min; and a final extension at 72°C for 7 min.
- For *P. gingivalis, A. actinomycetemcomitans*, and *T. denticola*: an initial denaturation at 95°C for 5 min; 30 cycles consisting of 95°C for 30 s, 55 or 60°C for 30 s and 72°C for 45 s; and a final extension at 72°C for 7 min.

The annealing temperature (Ta) varied depending on the primer sets (Table 1). For negative and positive controls, the PCR assays were also performed with or without the isolated genomic DNA from the reference strains of the targeted organisms. The PCR products were subjected to electrophoresis on a 2% agarose gel with Tris acetate EDTA buffer. The gel was stained with 0.5 μg/ml of ethidium bromide and photographed under ultraviolet illumination. An EZ load 100 bp (Bio-Rad, Hercules, CA) was used as the molecular size standard.

Quantitative analysis by real-time PCR

Real-time PCR was carried out using a LIGHTCYCLER™ system (Roche Diagnostics, Mannheim, Germany) and the designated capillaries. Duplicate samples were routinely used for determination. Each PCR was performed in a total volume of 20 μl containing 2 μl of ×10 LIGHTCYCLER-DNA Master Hybridization Probes (Roche Diagnostics), 0.2 μl each of forward and reverse primers (final concentration, 500 nM each), an appropriate dose of the TaqMan probe (final concentration 200 nM; Applied Biosystems), an appropriate amount of $MgCl_2$ (final concentration 3–6 mM), 2 μl of template DNA solution and an appropriate dose of sterilized DNase–RNase-free water. The optimized $MgCl_2$ final concentration in each species-specific reaction solution was determined as follows: 4 mM for *P. gingivalis*, 5 mM for *A. actinomycetemcomitans*, 3 mM for *T. forsythia*, 6 mM for *T. denticola*, 3 mM for *P. intermedia*, 4 mM for *P. nigrescens*, and 5 mM for the universal probe/primer reaction solution. Each amplification reaction was performed in the LightCycler with the cycling parameters set as follows.

- For *P. gingivalis, T. forsythia, T. denticola*, and *P. nigrescens*: an initial denaturation at 95°C for 1 min, 50 PCR cycles at 95°C for 5 s, 57°C for 15 s, and 72°C for 5 s, and a final cooling at 40°C for 8 min.
- For *P. intermedia*: an initial denaturation at 95°C for 1 min, 50 PCR cycles at 95°C for 5 s, 56°C for 15 s and 72°C for 8 s, and a final cooling at 40°C for 8 min.
- For *A. actinomycetemcomitans*: an initial denaturation at 95°C for 1 min, 50 PCR cycles at 95°C for 5 s, 57°C for 15 s and 72°C for 35 s, and a final cooling at 40°C for 8 min.
- For Universal: an initial denaturation at 95°C for 1 min, 50 PCR cycles at 95°C for 5 s, 58°C for 15 s and 72°C for 20 s, and a final cooling at 40°C for 8 min.

Fluorescence intensity was monitored at the annealing temperature in single acquisition mode. The dye signals generated during a run were measured in fluorimeter channel 2 (F2, 640 ± 30 nm) and channel 1 (F1, 530 ± 30 nm) and the results were indicated as the F2/F1 ratio, which was considered adequate for a TaqMan probe conjugated with FAM and TAMRA. Fluorescent data were analyzed with LightCycler Data Analysis (LCDA) software version 3.5 (Roche Diagnostics).

Calculation of theoretical cell numbers by real-time PCR

The bacterial DNA levels were quantified by real-time PCR and converted to theoretical cell numbers by the following method. TaqMan technology provided by the manufacturer determines the PCR cycle at which the increase in fluorescence of the reporter dye reaches a threshold cycle (C_T). C_T is proportional to the log of the amount of the target gene and, hence, the log of the number of bacteria in the sample, provided there are the same copy numbers of the reported sequence within a genome of various bacteria. When using a gene as a detecting target for the accurate quantification of cell numbers of certain bacteria (not only in real-time PCR, but also in DNA hybridization), the genome weight and targeted gene copy numbers per cell must be known, though they are also affected by doubling time. There are numerous types of bacteria in the oral cavity and it is impossible to accurately know each of their genome weights and 16s rRNA copy numbers. In the present study, therefore, the total bacterial load in the clinical specimens was calculated on the assumption that the 16s rRNA gene copy numbers of the oral anaerobes were not significantly different from each other (22, 40). *P. gingivalis*, whose genome has recently been sequenced and for which the exact genome size (2.2 Mb) and weight (2.37 fg) are known (33), was used as the representative bacterium for the reasons mentioned above.

First, serial dilutions of *P. gingivalis* genomic DNA were analyzed using the universal probe/primer set. Serial dilutions of the other five bacterial genomic DNA were then analyzed using the universal probe/primer set and their theoretical cell numbers calculated based upon standard curves derived from the genomic DNA of *P. gingivalis*. Adjusted in this manner, serial dilutions comparable to 10^2–10^7 colony-forming units were used for quantitative specific detection of each targeted pathogen.

Statistical analysis

A Mann–Whitney *U*-test was used for comparative analysis among groups of clinical specimens.

Results

Design of the species-specific probe/primer sets

A multiple alignment among the 16S rRNA gene sequences of 100 oral bacteria was performed using Clustal W. The species-specific probe/primer sets were designed according to the sequence of identity determined with Primer Express ver.1 (Table 2). The specificities of the newly designed probes and primers were further confirmed by multiple alignment of the relevant sequences of closely related species and a BLAST homology search program (NCBI). Probe/primer sets for *P. intermedia* and *A. actinomycetemcomitans* were designed according to the sequences of the complementary strand. The 16S rRNA sequences of the closely related *P. intermedia* and *P. nigrescens* showed a high similarity (92% homology), thus, distinctly variable regions between these two sequences were successfully identified for designing the species-specific probe/primer sets (Fig. 1).

Quantitative sensitivity of species-specific probe/primer sets

The total number of bacterial cells was determined using the TaqMan PCR procedure with the universal probe/primer set. The standard curve was analyzed with the universal probe/primer set against a serial dilution of *P. gingivalis* genomic DNA, which corresponded to 10^2–10^7 cells and

Table 2. Species-specific primers /TaqMan probes for real-time PCR

Primers/TaqMan Probe sets	Length	Tm*	%GC	Product size (bp)	Ta (°C)	Reference
Universal						
5'-TCCTACGGGAGGCAGCAGT-3'				466	58	(33)
5'-GGACTACCAGGGTATCTAATCCTGTT-3'						
5'-FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA-3'						
Porphyromonus gingivalis						
5'-ACCTTACCCGGGATTGAAATG-3'	21	59	48	83	57	This study
5'-CAACCATGCAGCACCTACATAGAA-3'	24	60	46			
5'-FAM-ATGACTGATGGTGAAAACCGTCTTCCCTTC-TAMRA-3'	30	69	47			
Actinobacillus actinomycetemcomitans						
5'-CCCATCGCTGGTTGGTTA-3'	18	56	56	696	57	This study
5'-GGCACGTAGGCGGACC-3'	16	57	75			
5'-FAM-CCTCTGTATACGCCATTGTAGCACGTGTGT-TAMRA-3'	30	68	50			
Tannerella forsythia						
5'-AGCGATGGTAGCAATACCTGTC-3'	22	57	50	88	57	This study
5'-TTCGCCGGGTTATCCCTC-3'	18	59	61			
5'-FAM-TGAGTAACGCGTATGTAACCTGCCCGC-TAMRA-3'	27	70	56			
Treponema denticola						
5'-CCGAATGTGCTCATTTACATAAAGGT-3'	26	60	38	122	57	This study
5'-GATACCCATCGTTGCCTTGGT-3'	21	60	52			
5'-FAM-ATGGGCCCGCGTCCCATTAGC-TAMRA-3'	21	70	67			
Prevotella intermedia						
5'-TCCACCGATGAATCTTTGGTC-3'	21	58	48	98	56	This study
5'-ATCCAACCTTCCCTCCACTC-3'	20	57	55			
5'-FAM-CGTCAGATGCCATATGTGGACAACATCG-TAMRA-3'	28	69	50			
Prevotella nigrescens						
5'-CCGTTGAAAGACGGCCTAA-3'	19	57	53	82	57	This study
5'-CCCATCCCTTACCGGRA-3'	17	55	59			
5'-FAM-CCCGATGTGTTTCATTGACGGCATC-TAMRA-3'	25	69	52			

*Melting temperature of DNA (Tm) was analyzed with Primer Express version 1.0 (Applied Biosystems).

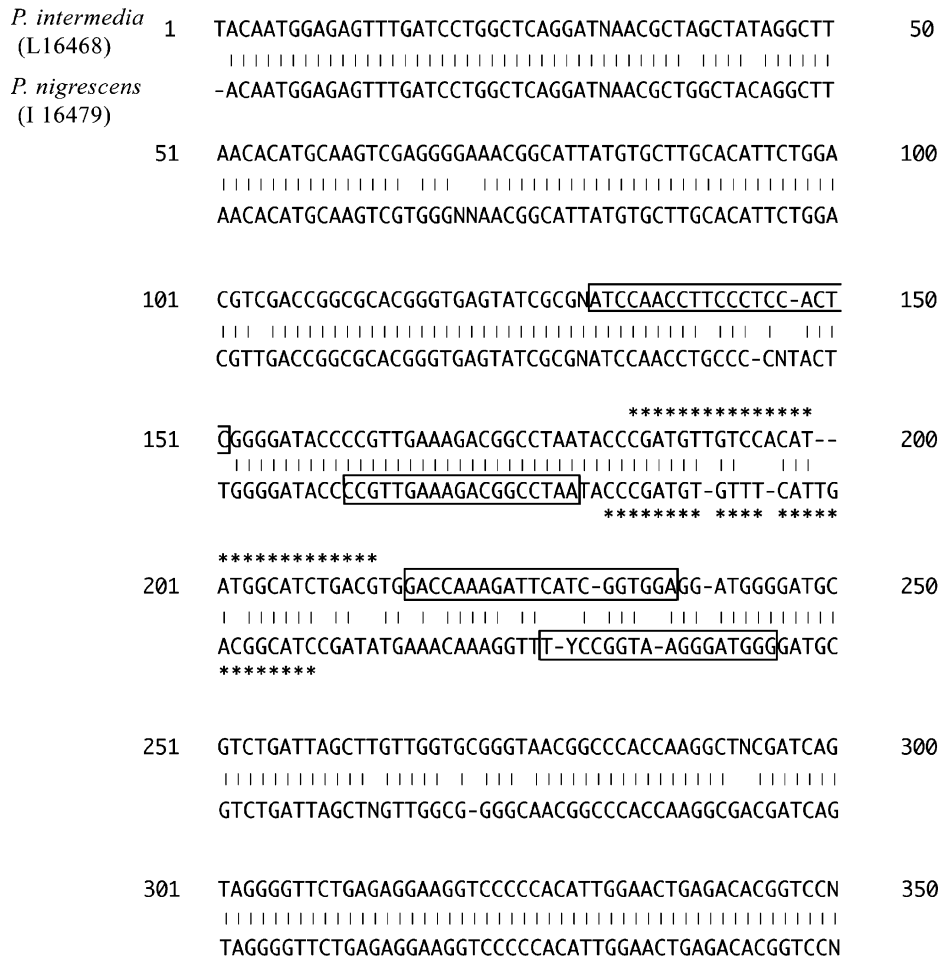

Fig. 1. Maximum matching analysis between the partial 16S ribosomal RNA gene sequences of *P. intermedia* and *P. nigrescens*. Maximum matching analysis between partial 16S rRNA genes of these two closely related species was performed using DNASIS-MAC version 3.7 (Hitachi Software Engineering, Tokyo, Japan), and variable regions were identified for designing the species-specific probe/primer sets. Dotted sequences indicate TaqMan hybridization probes, and boxed sequences show the forward and reverse primers. The probe/primer set for *P. intermedia* was designed according to the sequence of the complementary strand.

showed a credible error value (error = 0.0707) (Fig. 2). We confirmed the weight of the purified genomic DNA of *P. gingivalis*, which corresponded to 10^7 cells by the colony counting method and compared it to the genomic weight calculated by the hypothesis employed in this study. The former amount was approximately 15% less than the latter. Serially diluted genomic DNA solutions purified from each of the six pure bacterial species cultures, which were converted to number of cells, were employed for analyzing the standard curve used for quantitative detection with the newly designed probe/primer sets. The curve of each pathogen between logarithms of serially diluted genomic DNA and threshold PCR cycles was found to be linear over a wide range, corresponding to 10^2–10^7 cells (*P. gingivalis*, error = 0.0695; *A. actinomycetemcomitans*, error = 0.0288; *T. forsythia*, error = 0.0354; *T. denticola*, error = 0.0311; *P. intermedia*, error = 0.0898; and *P. nigrescens*, error = 0.0182) (Fig. 2).

Specificity of species-specific probe/primer sets

The newly designed species-specific probe/primer sets showed no cross-reactivity with any other species when used with the DNA samples of the reference strains and clinical samples of bacterial profiles from our previous study (data not shown) (2). In addition, there was no cross-reactivity of the probe/primer set for *A. actinomycetemcomitans* with *H. influenzae* (data not shown). Further, the differential identification and quantification of *P. intermedia* and *P. nigrescens* were confirmed. The determined amounts of *P. intermedia* DNA were accurately quantified by real-time PCR, even in the presence of the same amount of *P. nigrescens* genomic DNA (Fig. 3a). The quantitative detection of *P. nigrescens* genomic DNA was not affected by *P. intermedia* DNA (Fig. 3b).

Crude genomic DNA samples were extracted from 10 subgingival plaque (P-pocket) and 10 tongue surface debris (P-tongue) specimens taken from patients with advanced periodontitis, as well as from 10 subgingival plaque (H-pocket) and 10 tongue surface debris (H-tongue) specimens from periodontal healthy individuals, and analyzed (Table 3). The identification and quantification of theoretical cell numbers of periodontal bacteria were estimated by real-time PCR, and their specific reactivity with the targeted species was again demonstrated by a conventional PCR assay.

Comparison of proportion of periodontal bacteria in clinical specimens

The prevalence and amount of the targeted species harbored by the subjects were compared using a real-time PCR assay

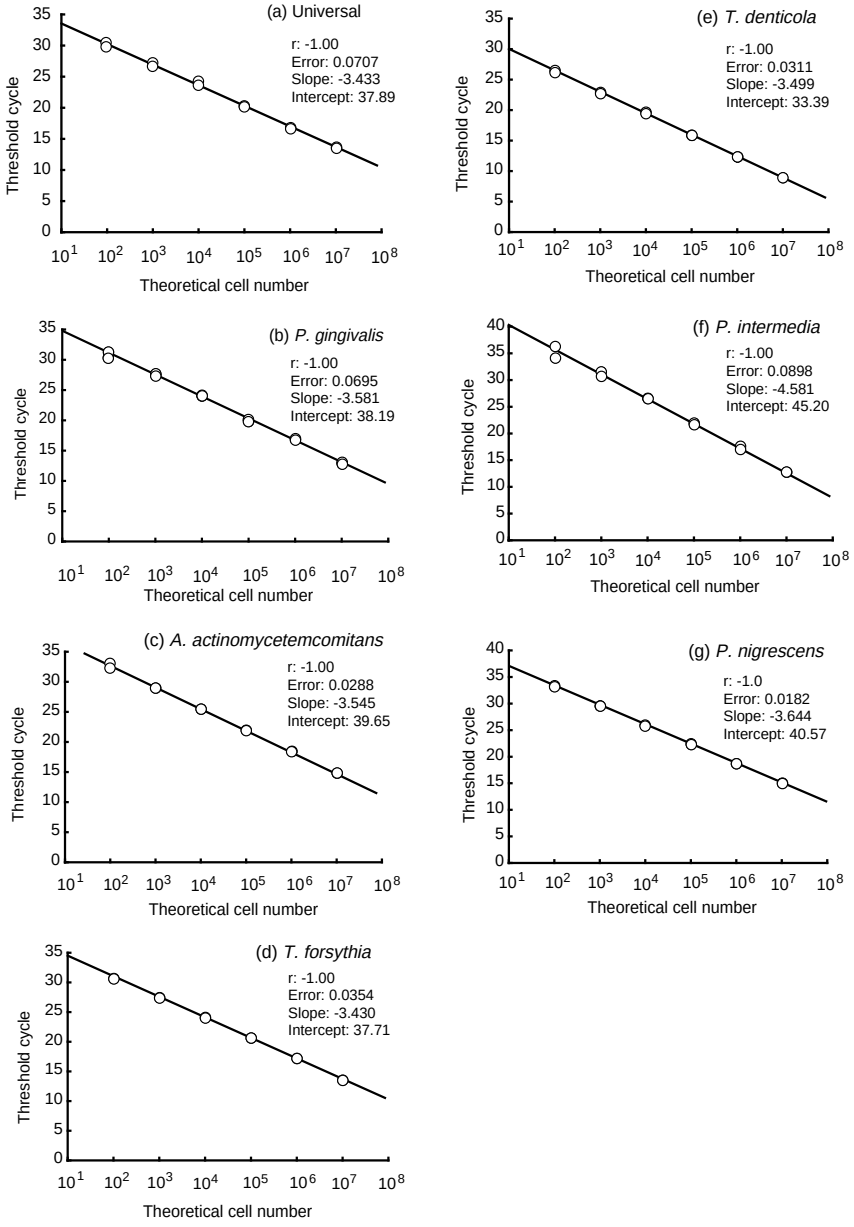

Fig. 2. Correlation between threshold cycle and number of cells. Standard curves from real-time PCR with the universal or species-specific probe/primer sets are shown. The threshold cycle is the cycle number when threshold fluorescence was reached. The theoretical cell number was calculated as described in Material and Methods. Reactions of the universal or species-specific probe/primer sets with genomic DNA of the targeted pathogen are shown. a) Universal with *P. gingivalis* DNA. Species specific with (b) *P. gingivalis*, (c) *A. actinomycetemcomitans*, (d) *T. forsythia*, (e) *T. denticola*, (f) *P. intermedia*, and (g) *P. nigrescens*. Each reaction was performed in duplicate. By plotting the standard curve values, using LIGHTCYCLER software version 3.5.28, we generated the represented data. 'Slope' represents the overall reaction efficiency. PCR efficiency (E) was calculated by the following formula; $E = 10^{-1/\text{slope}}$. 'Error' (mean squared error) provided clues to tube to tube variations, e.g. pipetting errors. The standard curve was considered reliable when the error value was <0.2. 'Intercept' is the value of y-intercept and 'r' is the correlation coefficient.

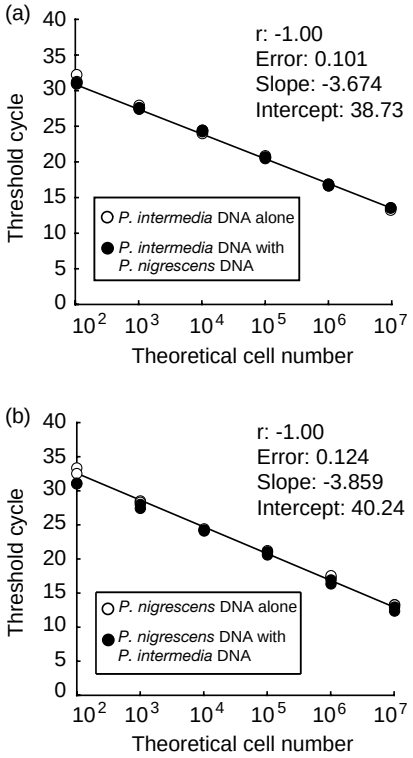

Fig. 3. Specific detection of *P. intermedia* and *P. nigrescens* using real-time PCR. We confirmed the differential identification and quantification of *P. intermedia* and *P. nigrescens*. Serial dilutions of genomic DNA from *P. intermedia* and *P. nigrescens,* corresponding to 10^7–10^2 cells (16.6 ng to 166 fg in *P. intermedia*, and 8.74 ng to 874 fg in *P. nigrescens*), were used as templates for real-time PCR. The reactions were performed with the targeted bacterial DNA in the presence or absence of another equivalent DNA template. Each assay was repeated twice. a) Standard curve from real-time PCR with *P. intermedia* specific probe/primer set. b) Standard curve from real-time PCR with *P. nigrescens* specific probe/primer set.

(Fig. 4). In the subgingival samples, all periodontal pathogens, except for *P. nigrescens*, were more prevalent in the diseased sites than in the healthy sites and in a significantly greater proportion in diseased pockets ($P < 0.05$). *P. nigrescens* was detected in greater proportions in the controls. In the tongue debris samples, greater proportions of *P. gingivalis* ($P = 0.0051$), *P. intermedia* ($P = 0.0342$), and *T. denticola* ($P = 0.0051$) were found in the patients than in the non-disease controls, but no significant differences were found in the proportions of *A. actinomycetemcomitans, T. forsythia*, and *P. nigrescens*. When we analyzed the presence or absence of the target species in samples from patients, the existing bacteria in the plaque samples were shown to be in the tongue debris taken from the same subject (Table 3; Fig. 4).

Discussion

The bacterial 16S rRNA gene is a useful target for detection and quantification of bacteria in a variety of complex environmental and health-related situations, during which a multi-species population is sampled along with impurities, or where the bacteria are internalized within a matrix (1, 21, 23, 55–57). In theory, the variable regions of the 16S rRNA gene should provide a means for species-specific detection

Table 3. Analysis of bacterial distribution in subgingival plaque and tongue debris specimens by conventional and real-time PCR

	Universal	*P. gingivalis*		*A. actinomycetemcomitans*		*T. forsythia*		*P. intermedia*		*P. nigrescens*		*T. denticola*	
Sample no.*	Real-time PCR**	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR
P-pocket-1	2.86E+07	+	6.59E+02	+	8.18E+03	+	5.65E+04	+	1.22E+06	+	5.81E+04	+	8.53E+04
P-pocket-2	4.77E+07	+	8.04E+06	−	−	+	1.07E+06	+	1.94E+07	+	<100	+	3.87E+05
P-pocket-3	6.28E+07	+	1.15E+06	+	9.33E+04	+	4.15E+05	+	7.24E+06	+	3.31E+02	+	1.74E+05
P-pocket-4	1.99E+07	+	<100	+	2.69E+04	+	8.60E+04	+	5.79E+05	+	2.52E+05	+	7.74E+04
P-pocket-5	2.18E+07	+	3.94E+05	−	−	+	3.14E+05	+	2.93E+05	+	6.81E+04	+	1.05E+05
P-pocket-6	4.01E+06	+	2.66E+05	−	−	+	<100	+	2.36E+06	+	7.07E+04	+	2.42E+04
P-pocket-7	1.11E+07	+	2.60E+06	+	3.09E+05	+	<100	+	1.94E+06	+	<100	+	1.67E+05
P-pocket-8	6.67E+07	+	4.81E+05	−	−	+	3.44E+05	+	2.14E+07	+	1.06E+06	+	2.44E+05
P-pocket-9	1.88E+07	+	<100	−	−	+	1.16E+04	+	3.89E+05	+	5.76E+05	+	1.61E+04
P-pocket-10	2.29E+07	+	<100	+	1.90E+03	+	1.32E+04	+	3.15E+06	+	6.09E+05	+	3.73E+04
H-pocket-1	1.21E+06	−	−	−	−	+	<100	+	2.21E+04	+	2.01E+05	+	1.06E+04
H-pocket-2	1.04E+06	−	−	−	−	+	<100	−	−	+	3.33E+05	±	<100
H-pocket-3	2.52E+06	−	−	−	−	+	<100	−	<100	+	6.69E+03	−	−
H-pocket-4	7.39E+05	±	<100	−	−	+	2.33E+03	+	5.02E+03	+	<100	+	7.36E+03
H-pocket-5	5.88E+06	−	−	−	−	+	<100	−	−	±	<100	−	−
H-pocket-6	3.23E+06	+	1.96E+02	−	−	+	<100	+	7.14E+03	+	1.77E+02	±	<100
H-pocket-7	2.11E+06	−	−	−	−	−	−	±	<100	+	1.14E+04	−	−
H-pocket-8	4.19E+06	−	−	−	−	+	1.25E+03	+	1.61E+05	+	1.24E+05	+	3.15E+03
H-pocket-9	2.21E+05	+	1.14E+02	−	−	+	<100	−	−	+	2.52E+02	+	<100
H-pocket-10	2.63E+07	−	−	−	−	+	<100	−	−	±	<100	±	1.39E+03
P-tongue-1	1.35E+07	+	<100	+	<100	+	<100	±	4.89E+03	±	<100	±	9.36E+02
P-tongue-2	3.63E+07	+	2.79E+05	−	−	+	2.19E+05	+	8.02E+05	+	<100	+	7.10E+04
P-tongue-3	1.43E+08	+	<100	+	<100	+	2.18E+03	+	9.89E+05	+	<100	+	4.42E+04
P-tongue-4	3.47E+06	+	<100	+	<100	+	2.01E+02	±	2.74E+03	+	8.08E+03	±	1.16E+03
P-tongue-5	7.05E+06	+	1.41E+03	−	−	+	1.82E+03	+	2.74E+05	+	1.07E+05	+	1.18E+04
P-tongue-6	3.02E+07	+	<100	−	−	+	3.17E+03	+	4.78E+04	+	<100	±	6.02E+02
P-tongue-7	1.35E+08	+	<100	+	4.52E+04	+	<100	+	1.80E+05	+	<100	+	2.51E+04
P-tongue-8	1.61E+08	+	<100	+	<100	+	6.79E+04	+	2.92E+05	+	5.85E+04	+	1.10E+04
P-tongue-9	4.65E+07	+	2.30E+03	−	−	+	1.10E+05	+	1.18E+06	+	3.56E+05	+	3.24E+04
P-tongue-10	2.11E+08	+	<100	+	6.21E+03	+	4.97E+03	±	5.53E+04	+	2.10E+02	±	4.74E+03
H-tongue-1	8.83E+07	+	<100	+	<100	+	2.38E+05	+	2.97E+05	+	4.10E+02	+	2.91E+04
H-tongue-2	2.01E+08	+	<100	−	−	+	4.58E+06	±	4.27E+04	+	<100	+	1.52E+03
H-tongue-3	2.07E+06	−	−	−	−	+	9.56E+02	±	1.23E+03	+	<100	±	<100
H-tongue-4	1.01E+07	+	<100	−	−	+	8.45E+02	+	7.33E+04	±	<100	±	2.42E+03
H-tongue-5	3.47E+07	−	−	−	−	+	<100	±	6.70E+03	±	<100	±	6.64E+02
H-tongue-6	7.35E+05	+	<100	−	−	+	<100	±	9.28E+02	+	4.47E+03	±	<100
H-tongue-7	8.18E+06	−	−	−	−	+	<100	−	−	+	1.85E+04	±	<100
H-tongue-8	4.45E+06	−	−	−	−	+	1.33E+02	±	6.04E+03	+	3.51E+04	−	2.73E+02
H-tongue-9	6.67E+03	−	−	−	−	+	<100	−	−	−	−	±	<100
H-tongue-10	3.97E+07	−	−	−	−	−	−	−	−	−	−	−	−

*Crude genomic DNA samples were extracted from 10 subgingival plaque (P-pocket) and 10 tongue surface debris (P-tongue) specimens taken from patients with advanced periodontitis, as well as from 10 subgingival plaque (H-pocket) and 10 tongue surface debris (H-tongue) specimens from periodontal healthy individuals, and analyzed. f (+): detected, (−): not detected, (±): faintly detected.

and enumeration of complex bacterial populations by real-time PCR (24, 32, 33, 40, 55–57). Therefore, once species-specific TaqMan probe/primer sets are successfully designed for pathogens, a real-time PCR assay can be used as a reliable tool for rapid and highly sensitive enumeration. In the present study, multiple alignments of the 16S rRNA gene sequences of 100 oral bacterial species of interest were analyzed. The quantitative sensitivity and specificity of newly designed TaqMan probe/primer sets were verified using both reference strains and clinical specimens, for which the presence or absence of the target species had been established by conventional PCR.

The exact copy number of 16S rRNA operons within each cell of the numerous species of oral bacteria has not been clarified and doubling time varies among bacterial species; this represents the major limitation to the absolute determination of bacterial numbers by real-time PCR based on the 16S rRNA gene sequence (13, 25). However, it was previously reported that threshold cycles corresponding to the same amounts of genomic DNA of slow growing oral anaerobes were similar to each other (30, 40). Therefore, we utilized *P. gingivalis,* for which accurate genome size and weight are known, as the reference species to form a universal standard curve, which was then used to calculate the theoretical cell numbers of five other oral anaerobes.

The yield of the purified genomic DNA from pure cultured *P. gingivalis*, whose cell number had been calculated by colony counting, was considerably less than the theoretical weight, and the loss seemed to be unavoidable in the purification step (data not shown). Estimating cell numbers by colony counting might therefore not be suitable for a standard, though the present attempt to quantify periodontal bacteria was sufficiently reliable to compare bacterial proportions within various clinical samples. TaqMan probe/primer sets were employed for the identification and quantification of six periodontal bacteria harbored in subgingival plaque from diseased sites and on tongue surfaces of periodontitis patients by real-time PCR, and the results showed that all periodontal bacteria, except for *P. nigrescens*, were periodontitis-associated species (Table 3). These results demonstrate that such patients harbor greater amounts of periodontal pathogens in diseased subgingival sites. In addition, it was verified that the probe/

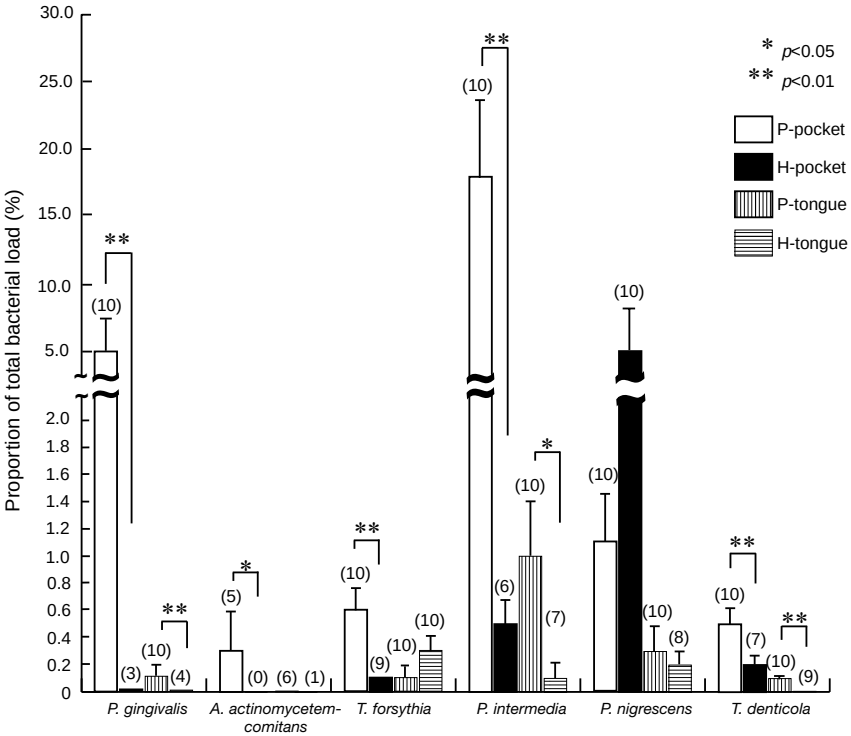

Fig. 4. Comparison of bacterial distribution in clinical specimens. The presence or absence and amount of targeted species harbored by the subjects were compared using real-time PCR. Crude genomic DNA from 10 subgingival plaque (P-pocket) and 10 tongue surface debris (P-tongue) samples taken from patients with advanced periodontitis, and 10 subgingival plaque (H-pocket) and 10 tongue surface debris (H-tongue) samples taken from periodontal healthy individuals were analyzed. Standard errors are shown as error bars. Number in parentheses: number of positive samples in which the targeted pathogen was present.

primer sets reacted accurately with crude DNA samples containing target bacteria.

In the past decade, systematic bacteriology has been reorganized based on gene analysis (1, 16, 27, 58). Further, intraspecies heterogeneity among strains of *P. intermedia* was investigated and a new species, designated as *P. nigrescens,* was proposed (14, 16, 46). Recently, a quantitative fluorescent *in situ* hybridization (FISH) assay for differential identification of *P. intermedia* and *P. nigrescens* was reported (19); however, a quantitative real-time PCR assay for these species that is more accurate and rapid than FISH has not been described. In the present study, we used a real-time PCR analysis method to identify *P. intermedia* and *P. nigrescens* organisms according to their different variable regions of 16S rRNA gene sequences. Our quantitative assay of clinical specimens showed that *P. intermedia* was related to periodontitis, whereas *P. nigrescens* displayed no obvious tendency in association with periodontal health status (Fig. 4). This contradictory occurrence was previously reported using sensitive detection methods, though they lacked quantitative ability (9, 17, 34), and the present investigation is the first to show quantitative findings in support of those previous reports.

It is known that bacterial species occurring in tooth and tongue samples are highly associated in individuals, and that most species are more frequently detected in tongue specimens as compared to those from subgingival sites (34, 39, 51, 52). The tongue dorsum is suggested to house an organized biofilm in which anaerobic bacteria may locate and thrive, from which anaerobic locations around the teeth are seeded. This hypothesis is consistent with the concept that mucosal surfaces serve as the initial colonization site and reservoir for oral sites (6, 15, 18, 28, 35). The present findings also indicate that tongue samples are advantageous for examining prevalence. In addition, halitosis is also suggested to be related to the microbial complex on the tongue (11, 38). The present real-time PCR method may assist further investigations to understand microbial roles in that condition.

In summary, the present real-time PCR method is suitable for the detection of six putative periodontal bacteria without cross-detection of genomic products from other species. Further, results of our quantitative analysis of clinical specimens suggest that the microbial population in the oral cavity is varied depending on periodontal health status and site of sample collection. The present findings may help future microbial studies of periodontal diseases as well as those of halitosis.

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