Oral Microbiology Immunology 2004: 19: 196–200
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Oral Microbiology and Immunology

TaqMan real-time polymerase chain reaction assay for the correlation of *Treponema denticola* numbers with the severity of periodontal disease

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Yoshida A, Kawada M, Suzuki N, Nakano Y, Oho T, Saito T, Yamashita Y. TaqMan real-time polymerase chain reaction assay for the correlation of Treponema denticola *numbers with the severity of periodontal disease.*
Oral Microbiol Immunol 2004: 19: 196–200. © Blackwell Munksgaard, 2004.

Treponema denticola has been implicated in periodontitis, and the presence of this organism in periodontal pockets has been investigated. However, qualitative analysis is insufficient for the clinical evaluation of periodontal treatments, and quantification of *T. denticola* populations is essential for monitoring therapeutic efficacy. Therefore, we developed a quantitative method for *T. denticola* that uses the TaqMan real-time polymerase chain reaction assay. Using this system, we evaluated the relative and absolute numbers of this organism in saliva and subgingival plaque. Furthermore, we analyzed the relationship between the numbers of *T. denticola* and pocket depth, and found a significant positive correlation ($P < 0.0001$) between these parameters. This report demonstrates the broad potential of real-time polymerase chain reaction applications in periodontology.

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

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Accepted for publication January 11, 2004

Periodontitis is an infectious disease caused by periodontal bacteria that bring about destructive changes leading to loss of bone and connective tissue attachment (21, 25). *Treponema denticola,* which is a helically shaped microorganism, has been implicated as a major pathogen in this disease (10). This spirochete is present at significantly elevated levels in plaque samples from deep-pocket sites of severe periodontitis patients (20). Recently, an association between this organism and oral malodor was strongly indicated (12, 17, 18).

Of the various methods used for the detection of bacteria, the polymerase chain reaction (PCR) method represents one of the most sensitive and rapid for determining the prevalence of periodontal bacteria (3, 13, 23, 26). However, conventional PCR methods detect the plateau phase of the reaction and are unable to quantify accurately the number of bacteria (11). Since qualitative analyses are unsuitable for the evaluation of treatments, quantitative analysis is essential for monitoring therapeutic efficacy in clinical trials.

The TaqMan assay, which is based on the 5′-3′ exonuclease activity of *Taq* polymerase, has been developed for the quantitative detection of DNA (8). Briefly, an oligonucleotide probe with a fluorescent dye reporter attached to the 5′-end and a quencher dye attached to the 3′-end are used in the assay. When the probe hybridizes to its target template, the reporter dye is cleaved by the 5′-nuclease activity of *Taq* polymerase and emits a fluorescent signal, since it is freed from suppression by the quencher dye (7).

Quantification of *T. denticola* in oral specimens using real-time PCR has been reported previously (2, 19). However, the relationship between the absolute/relative numbers of *T. denticola* and the progression of periodontitis remains unclear. This study was undertaken to clarify the relationship between the absolute/relative numbers of *T. denticola* and periodontal status, and to adapt the TaqMan method to the evaluation of periodontitis.

Material and methods

Bacterial strains and culture conditions

The *T. denticola* strains ATCC 35404 and ATCC 35405 were cultured in TYGVS

medium at 37°C under anaerobic conditions (9, 16).

Study subjects

Forty patients who visited the Department of Preventive Dentistry, Kyushu University Dental Hospital, were examined for the presence of *T. denticola* in the saliva using conventional PCR; 26 patients were positive. All of them were given a full-mouth periodontal examination. The pocket depths of all teeth were measured at six sites (distal, mid and mesial for both the buccal and lingual surfaces of each tooth) using a periodontal probe. After periodontal examination, nine *T. denticola*-positive patients with moderate to severe periodontitis having at least four teeth with $\geq$4 mm pocket depth (three males and six females, 28–72 years old; mean $\pm$ SD of age, 54.1 ± 26.1) 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 before the study. All of the subjects who participated in this study understood the nature of the research project and provided their informed consent. The experimental protocol was reviewed and approved by the Ethics Committee of Kyushu University Dental Hospital.

Clinical examination and preparation of oral specimens

After a full-mouth periodontal examination, following the World Health Organization (WHO) criteria, the dentition of each subject was divided into six segments (24). The deepest periodontal pockets in each segment were selected for microbial sampling from each patient, to a maximum of six samples from each patient, with 53 samples collected in total. Clinical samples were obtained from the patients 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 (pH 8.0), 2 mM EDTA] and boiled at 100°C for 5 min, and the supernatant was used as the PCR template (22). Whole saliva was collected from patients by expectoration into ice-chilled tubes (15). For the extraction of chromosomal DNA from the saliva, 0.5 ml of whole saliva was mixed and centrifuged with the same amount of phosphate-buffered saline [NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 11.5 g/l, KH_2PO_4 0.2 g/l (pH 7.4)] at 12,000 ***g*** for 15 min, and 200 µl of the lysis solution was added to the precipitate. The precipitate was vortexed in the cell lysis buffer, and the bacterial chromosomal DNA was extracted by boiling for 10 min. After centrifugation, the supernatant was used for chromosomal DNA preparation.

Primers and probes for real-time PCR

T. denticola-specific and ubiquitous primer-probe sets were designed using the Primer Express ver. 1.5 software (Applied Biosystems, Foster City, CA), as described previously (27, 28). Briefly, a fluorescent probe was used to monitor PCR product formation continuously. This oligonucleotide probe was labeled at the 5′-end with a reporter dye (FAM: 6-carboxyfluorescein) and labeled at the 3′-end with a quencher dye (TAMRA: 6-carboxytetramethylrhodamine). The *T. denticola*-specific primers and probe were designed from the 16S rRNA gene of *T. denticola*. The specificities of the primers and probe were initially confirmed using the BLAST software on the National Center for Biotechnology Information Server (http://www.ncbi.nlm.nih.gov/), and subsequently confirmed by conventional PCR (Table 1) and dot blot analysis using digoxigenin-labeled probes (data not shown). The conventional PCR assays that were used to confirm the specificities and universality of the primers were performed as follows: 94°C for 2 min, followed by 25 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min. The primer-probe set comprised the forward primer (Td1394-F), 5′-AGAGCAAGCTCTCCCTTACCGT-3′, the reverse primer (Td1498-R), 5′-TAAGGGCGGCTTGAAATAATGA-3′ and the probe (Td1444T), 5′-FAM-CAGCGTTCGTTCTGAGCCAGGATCA-TAMRA-3′. The oligonucleotide primers and probes for broad-range detection of bacteria were complementary to highly conserved regions within the 16S rRNA gene, as previously described (6). The chosen set included forward primer (Uni152-F), 5′-CGCTAGTAATCGTGGATCAGAATG-3′; reverse primer (Uni220-R), 5′-TGTGACGGGCGGTGTGTA-3′; and the probe (Uni177T), 5′-FAM-CACGGTGAATACGTTCCCGGGC-TAMRA-3′ (27, 28).

TaqMan assay for *T. denticola* quantification

The amplification and detection of DNA by real-time PCR were performed with the ABI PRISM 7700 Sequence Detection

Table 1. Strains and amplification results

Strain	Source	Amplification with the following primers	
		Td[d]	Universal
Treponema denticola			
ATCC 35404	ATCC[a]	+	+
ATCC 35405	ATCC	+	+
Actinobacillus actinomycetemcomitans			
Y4	Socransky[b]	−	+
JP2	KU[c]	−	+
Porphyromonas gingivalis			
W83	KU	−	+
ATCC 33277	KU	−	+
Tannerella forsythensis ATCC 43037	ATCC	−	+
Fusobacterium nucleatum ATCC 10953	KU	−	+
Prevotella intermedia ATCC 25611	ATCC	−	+
Haemophilus aphrophilus NCTC 5908	KU	−	+
Eikenella corrodens 1085	KU	−	+
Streptococcus anginosus FW73	KU	−	+
Streptococcus sobrinus 6715	KU	−	+
Streptococcus gordonii DL1	KU	−	+
Streptococcus mutans Xc	KU	−	+
Streptococcus salivarius HT9R	KU	−	+
Escherichia coli DH5α	Gibco BRL	−	+

[a]ATCC, American Type Culture Collection, Manassas, VA.
[b]S. S. Socransky, Forsyth Dental Center, MA.
[c]KU, the culture collection in Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, Fukuoka, Japan.
[d]Td, *T. denticola.*

System (Applied Biosystems). For each real-time PCR, 20 μl of a mixture that contained 1 μl lyzed cells, 1× qPCR Mastermix (Eurogentec S.A., Seraing, Belgium), 200 nM of each sense and antisense primer, and 250 nM TaqMan probe was placed in each well of a 96-well MicroAmp Optical Reaction Plate with Optical Caps (Applied Biosystems). The following DNA amplification conditions were used: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, followed by 60 cycles of 95°C for 15 s and 58°C for 1 min. The critical threshold cycle (*Ct*) was defined as the first cycle in which the fluorescence was detectable above the background, and this value was inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer-probe set using the *Ct* values for the amplification of known quantities of DNA extracted from samples that contained 1.6 to 1.6×10^9 *T. denticola*. Observations of *T. denticola* bacteria were made under dark-field microscopy. For the analysis, the cell cultures were centrifuged at 1,500 ***g*** for 20 min, washed twice with phosphate-buffered saline, and resuspended in phosphate-buffered saline. The bacterial cell counts were performed by phase-contrast microscopy with a Petroff-Hausser bacterial counter (Hausser and Son, Philadelphia, PA). To determine the linearity and detection limit of the assay, solutions of lyzed *T. denticola* were amplified in serial 10-fold dilutions in a series of real-time PCRs, which allowed the calculation of the correlation coefficient from the standard curve of the *Ct* values. For the relative quantification, the numbers of *T. denticola* DNA copies were normalized to those of the 16S rRNA gene using the simplified comparative threshold cycle (ΔΔ*Ct*) method, as described previously (28). Briefly, the fold-difference (*N*) in the number of *T. denticola*-specific 16S rRNA gene copies relative to the number of 16S rRNA gene copies was determined as follows:

$$N = 2^{\Delta Ct} = 2^{(Ct\ T.\ denticola - Ct\ 16S\ rRNA)}$$

The Δ*Ct* values for the sample and 16S rRNA were determined by subtracting the average *Ct* value for the target gene from the average *Ct* value for the 16S rRNA gene.

Statistical analysis

Logarithmic transformation was performed for the numbers of *T. denticola* that were detected from patients to improve normality, and these values were compared with the parameter of periodontal pocket depth by linear regression analysis. Statistical significance was judged as when the P-value was < 0.05.

Results

Specificity and sensitivity of the TaqMan assay for *T. denticola*

Initially, we confirmed the specificities of the *T. denticola*-specific primer pairs by BLAST analysis (data not shown). In addition, the specificities of the primers were confirmed by conventional PCR using the bacterial species listed in Table 1. The *T. denticola* primers amplified the chromosomal DNA of *T. denticola*, but not the chromosomal DNAs of the other bacteria listed in Table 1. The sensitivity of the real-time PCR assay for *T. denticola* was measured with chromosomal DNA from *T. denticola*. The TaqMan PCR assay with 10-fold serial dilutions of *T. denticola* was able to detect bacterial DNA mixtures in which the quantity of DNA was above the linear range of DNA from 1.6 and 1.6×10^6 *T. denticola* cells per reaction mixture, with *Ct* values ranging from 15.8 to 34.5 (Fig. 1A,B).

Quantitative detection of *T. denticola* in oral specimens

We tested the application of the real-time PCR method for the detection of *T. denticola* in saliva and subgingival plaque samples from the right maxillary first molar of nine patients. The absolute numbers of *T. denticola* were determined using the standard curves (Fig. 1B), and the bacterial percentages were calculated by the simplified ΔΔ*Ct* method. As shown in Table 2, the bacterial numbers ranged from 0 to 8.61×10^4 cells per mixture in the subgingival plaque, and from 0 to 1.34×10^5 cells per mixture in the saliva. The percentages

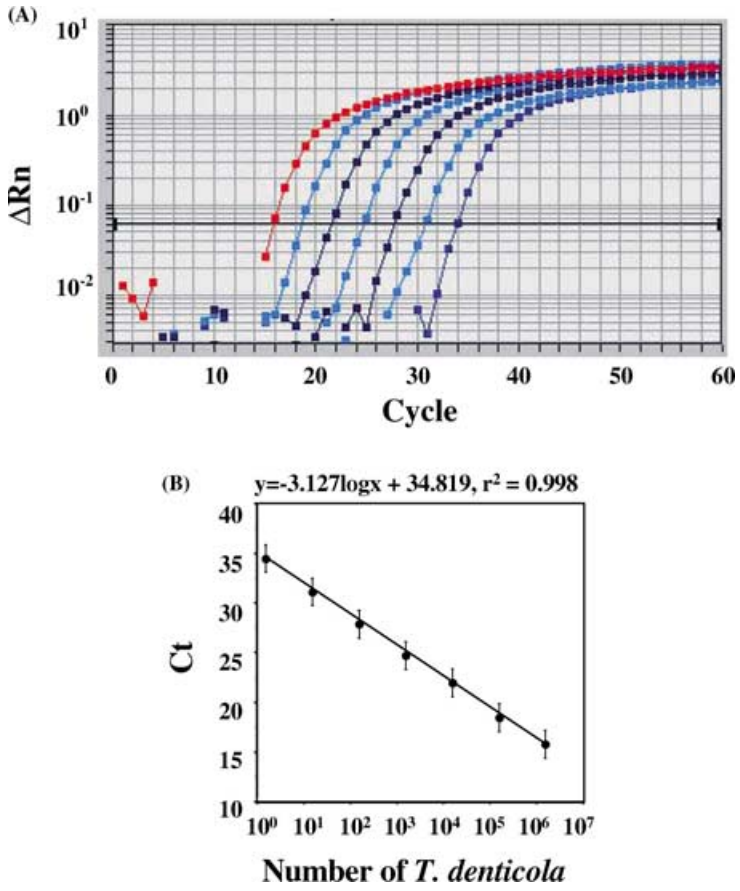

Fig. 1. The dilution curve (A) and standard calibration curve (B) for the *T. denticola* real-time PCR assay. The dilution curve was generated from amplification plots using serially diluted cell lysates (A). The threshold cycle was measured and plotted against the $\log_{10}$ of the dilution. The standard calibration curve was generated from the known bacterial colony forming units (CFU) of the cell lysates (B). The threshold cycle was measured and plotted against the $\log_{10}$ of the copy number. Each point represents the average of triplicate PCRs. The error bars indicate the SD ($n = 3$) of the data.

Table 2. Number of *T. denticola* in oral specimens[a]

Patient no.	Conventional PCR Td[b] detection	Real-time PCR Cell number	% of Td
Saliva			
1	+	$8.77 \times 10^4 \pm 0.11 \times 10^4$	1.80 ± 0.17
2	+	$6.49 \times 10^4 \pm 0.12 \times 10^4$	$2.53 \times 10^{-1} \pm 1.00 \times 10^{-1}$
3	+	$1.29 \times 10^5 \pm 0.10 \times 10^5$	$4.10 \times 10^{-2} \pm 0.85 \times 10^{-2}$
4	+	$1.34 \times 10^5 \pm 0.11 \times 10^5$	$3.23 \times 10^{-1} \pm 0.35 \times 10^{-1}$
5	+	$6.21 \times 10^4 \pm 1.16 \times 10^4$	$9.00 \times 10^{-1} \pm 0.71 \times 10^{-1}$
6	+	$1.47 \times 10^4 \pm 0.12 \times 10^4$	1.02 ± 0.08
7	+	$1.05 \times 10^5 \pm 0.00$	1.07 ± 0.45
8	+	$1.08 \times 10^5 \pm 0.13 \times 10^5$	6.83 ± 1.92
9	+	$2.33 \times 10^3 \pm 0.11 \times 10^3$	1.00 ± 0.18
Subgingival plaque			
1	−	ND[c]	ND
2	+	$8.61 \times 10^4 \pm 1.40 \times 10^4$	9.77 ± 4.35
3	+	$5.59 \times 10^2 \pm 2.16 \times 10^2$	$5.40 \times 10^{-2} \pm 3.87 \times 10^{-2}$
4	−	ND	ND
5	+	$5.03 \times 10^4 \pm 0.44 \times 10^4$	2.70 ± 1.84
6	+	$1.40 \times 10^4 \pm 0.13 \times 10^4$	$2.63 \times 10^{-1} \pm 6.11 \times 10^{-2}$
7	+	$7.33 \times 10^2 \pm 0.12 \times 10^2$	$1.53 \times 10^{-2} \pm 2.52 \times 10^{-3}$
8	+	$8.17 \times 10^4 \pm 0.15 \times 10^4$	2.30 ± 0.69
9	+	$4.67 \times 10^4 \pm 0.01 \times 10^4$	1.90 ± 1.22

[a]Data are expressed as means ± SD ($n = 3$).
[b]Td, *T. denticola*.
[c]ND, not detected.

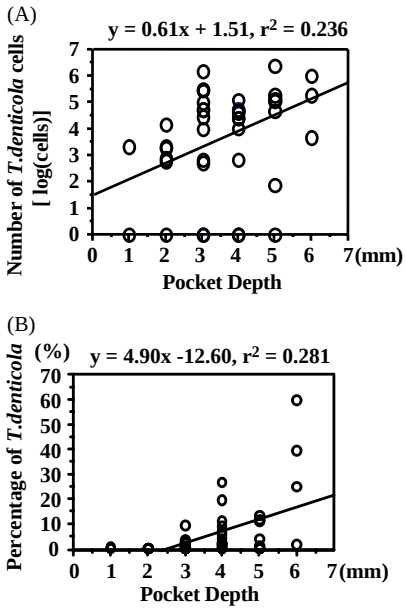

Fig. 2. The relationship between the number of *T. denticola* bacteria and pocket depth. (A) Correlation between the log-transformed absolute numbers of *T. denticola* and pocket depth for 53 periodontal pockets. (B) Correlation between the percentages of *T. denticola* and pocket depth for 53 periodontal pockets.

of these organisms ranged from 0 to 9.77% in the subgingival plaque and from $4 \times 10 \times 10^{-2}$ to 6.83% in the saliva.

Relationship between the number of *T. denticola* and pocket depth

In this study, the number of *T. denticola* cells in 53 samples from nine patients before periodontal treatment were analyzed using the TaqMan assay, and related to periodontal pocket depth. The relationship between the log-transformed number of *T. denticola* cells and pocket depth is shown in Fig. 2(A). Using simple regression analysis, the log-transformed number of *T. denticola* cells was linearly correlated with pocket depth ($y = 0.610x + 1.510$; $r^2 = 0.236$; $P < 0.0001$), as shown in Fig. 2(A). Simple regression analysis of the percentage of *T. denticola* bacteria in relation to pocket depth also showed a positive correlation ($y = 4.900x - 12.601$; $r^2 = 0.281$; $P < 0.0001$), as shown in Fig. 2(B).

Discussion

It is necessary to establish the relationship between the levels of periodontopathic bacteria and periodontal status to obtain a better understanding of periodontitis. Quantification of the bacteria in the oral cavity is essential for the achievement of this goal. Previously, quantitative estimation of *T. denticola* at periodontal sites involved a biotin–avidin-enhanced ELISA procedure with a monoclonal antibody that was specific for serovariants of *T. denticola* (20). Although this method is very sensitive and specific for this organism, it is unsuitable for the determination of the percentages of specific bacteria. One of the advantages of the real-time PCR assay for the quantitative detection of bacteria is that it is able to detect both the absolute and relative numbers of bacteria. In evaluations of clinical treatments, monitoring of the relative numbers of bacteria is often required. Therefore, the quantification procedure should be matched to the clinical demands and/or situation. Armitage et al. previously defined the relationship between the percentage of subgingival spirochetes and the severity of periodontitis using dark-field microscopy (1). Dark-field microscopic analysis is suitable for the detection of total spirochete numbers, but is unsuitable for the identification of specific spirochete species in clinical specimens, due to similarities in shape. The aim of this investigation was to elucidate the relationship between pocket depth and the number of *T. denticola* bacteria in periodontal sites using an accurate, sensitive and specific method. Therefore, we developed the TaqMan method of quantification for *T. denticola*.

Initially, we established our own absolute quantification system and evaluated its reliability. The linear detection range of the real-time PCR assay was seven orders of magnitude above the background, ranging from 1.6 to 1.6×10^6 organisms per reaction mixture. Previously reported real-time PCR assays for the detection of bacteria have shown detection ranges of five to six orders of magnitude, and have shown similar levels of sensitivity (4, 5, 14).

Using this assay system, we calculated the relative and absolute numbers of *T. denticola* in clinical specimens. Variable numbers and percentages of *T. denticola* were detected in the saliva and subgingival plaque samples. In addition, this experiment revealed that the presence of polymerase inhibitors in saliva and subgingival plaque was negligible (data not shown). Our results indicate that the *T. denticola*-specific real-time PCR assay is applicable to the analysis of bacterial populations in saliva and subgingival plaque.

Linear regression analysis showed a significant positive correlation between the numbers of *T. denticola* bacteria and pocket depth, with a correlation coefficient of 0.496 ($P < 0.0001$). The slope of the regression line for the log-transformed number of *T. denticola* plotted against the pocket depth was 0.61. A significant positive correlation was also observed between the percentages of *T. denticola* and pocket depth. These results suggest that the number and percentage of *T. denticola* bacteria increase relative to increases in pocket depth. Our results are consistent with those of the previous ELISA-based investigation (20) and dark-field microscopy-based analysis (1). In addition, Armitage et al. (1) reported

positive correlations between the percentage of subgingival spirochetes and the following parameters: plaque index, gingival exudate, gingival index, bleeding tendency, connective tissue attachment loss and periodontal disease index. Taking into account these and our present results, we conclude that the number of *T. denticola* in subgingival plaque may be an indicator of disease progression.

The real-time PCR assay for periodontopathic bacteria is a sensitive and specific tool for monitoring bacterial numbers. This assay is able to distinguish between different spirochete species. Furthermore, in combination with measurements of other parameters, this method is a powerful tool for the clarification of periodontal disease.

Acknowledgments

We thank Dr Kazuyuki Ishihara and Kiyonobu Honma, Department of Microbiology, Tokyo Dental College, for useful suggestions regarding this research. This investigation was supported primarily by Research Fellowships from the Japan Society for the Promotion of Science for Young Scientists (N.S.), and by a research grant from the Nakatomi Foundation (A.Y.).

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