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Development of a 5′ Fluorogenic Nuclease-Based Real-Time PCR Assay for Quantitative Detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*†

Akihiro Yoshida, Nao Suzuki, Yoshio Nakano,* Takahiko Oho, Miki Kawada, and Toshihiko Koga

Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, Fukuoka 812-8582, Japan

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A 5′ nuclease TaqMan PCR was developed for the quantitative detection of the periodontopathic bacteria *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. The relative numbers of bacteria were measured by the comparative threshold cycle method. This simplified method is a way of obtaining the relative quantities of these organisms from specimens and of monitoring the effect of therapy.

Periodontitis is an inflammation of the supporting tissues of the teeth (4). It is generally accepted that periodontal diseases are infectious diseases caused by oral bacteria (5). *Actinobacillus actinomycetemcomitans* is a nonmotile, gram-negative, capnophilic, fermentative coccobacillus that has been implicated in the etiology of localized juvenile periodontitis (12, 16, 18), while *Porphyromonas gingivalis* is a gram-negative, black-pigmented anaerobe that is strongly implicated as a major pathogen in adult periodontitis (14, 19). Several PCR-based systems that use oral specimens for the detection of oral bacterial infections, especially periodontitis, have been reported (1, 2, 13, 15). Most previous diagnostic systems are qualitative and are therefore unsuitable for the evaluation of treatment, as quantitative analysis is essential for monitoring the effect of therapy in treatment trials.

The TaqMan assay based on the 5′-3′ exonuclease activity of *Taq* polymerase has been developed for quantitative detection of DNA (9). Briefly, an oligonucleotide probe that has a reporter fluorescent dye attached to its 5′ end and a quencher dye attached to its 3′ end is used for the assay. When the probe hybridizes to its target template, the reporter dye is cleaved by the 5′ nuclease activity of *Taq* polymerase and becomes capable of emitting a fluorescent signal, since it is no longer suppressed by the quencher dye (8).

This report describes a simple, rapid method for the relative quantification of major periodontopathic bacteria, including *A. actinomycetemcomitans* and *P. gingivalis*, in saliva and subgingival plaque. The method uses a TaqMan PCR assay and the comparative threshold cycle ($\Delta\Delta Ct$) method. This is the first reported TaqMan method developed for the detection of *A. actinomycetemcomitans*.

The bacterial strains used in this study are listed in Table 1. The strains of *A. actinomycetemcomitans* and *P. gingivalis* were cultured as described previously (15, 17). Subgingival plaque and saliva samples from patients with periodontitis were prepared as described previously (15).

The oligonucleotide primers and probes, designed by using Primer Express (version 1.5) software (Applied Biosystems, Foster City, Calif.), are listed in Table 2. The sequences of the universal primers and a probe for a broad range of bacteria are complementary to highly conserved regions within the 16S rRNA gene (7). The *A. actinomycetemcomitans*- and *P. gingivalis*-specific primers and probes were designed from the *lktA* (6) and 16S rRNA genes, respectively. The specificities of the primers and probes were confirmed by conventional PCR (Table 1) and dot blot analysis with digoxigenin-labeled probes (data not shown), respectively. Conventional PCR with universal primers amplified a DNA fragment of a similar size (68 bp) from all the strains listed in Table 1. The fluorescent probes were dually labeled with a reporter dye (6-carboxyfluorescein [FAM]) covalently attached at the 5′ end and a quencher dye (6-carboxytetramethylrhodamine [TAMRA]) covalently attached at the 3′ end. The primers used for real-time PCR were also used for conventional PCR (Table 2). The conventional PCR assays used to confirm the specificities and universalities of the primers were performed as follows: 94°C for 5 min, followed by 25 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min. For each real-time PCR, 20 μl of a mixture containing 1 μl of lysed cells, 1× TaqMan Universal PCR master mixture (Applied Biosystems), each sense and antisense primer at a concentration of 200 nM, and 250 nM TaqMan probe was placed in each well of a 96-well plate. Amplification and detection were performed with the ABI PRISM 7700 sequence detection system (Applied Biosystems) with the following cycle profile: 50°C for 2 min, 95°C for 10 min, and 60 cycles at 95°C for 15 s and 58°C for 1 min. Optimal AmpErase uracil-*N*-glycosylase enzyme activity requires a 2-min step at 50°C (10). *Ct* is defined as the cycle at which the fluorescence becomes detectable above the background fluorescence and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer-probe set by using the *Ct* values obtained from amplification of known quantities of DNA. To check the linearity of the detection system, solutions of lysed *A. actinomycetemcomitans* or *P. gingivalis* were amplified in successive 10-fold dilu-

* Corresponding author. Mailing address: Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81 92 642 6423. Fax: 81 92 642 6354. E-mail: yosh@dent.kyushu-u.ac.jp.

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TABLE 1. Strains and amplification results

Strain	Source	Amplification with the following primers[h]:		
		Aa	Pg	Universal
A. actinomycetemcomitans				
ATCC 29523	ATCC[a]	+	−	+
Y4	Socransky[b]	+	−	+
NCTC 9710	NCTC[c]	+	−	+
IDH 781	Asikainen[d]	+	−	+
IDH 1705	Asikainen	+	−	+
P. gingivalis				
W83	KU[e]	−	+	+
W50	KU	−	+	+
381	KU	−	+	+
ATCC 33277	KU	−	+	+
ATCC 49417	KU	−	+	+
Treponema denticola				
ATCC 35404	Ishihara[f]	−	−	+
ATCC 35405	Ishihara	−	−	+
Bacteroides forsythus ATCC 43037	Honma[g]	−	−	+
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, Boston, Mass.
[c] NCTC, National Collection of Type Cultures, London, England.
[d] S. Asikainen, University of Helsinki, Helsinki, Finland.
[e] KU, culture collection in the Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, Fukuoka, Japan.
[f] K. Ishihara, Tokyo Dental College, Chiba, Japan.
[g] K. Honma, Tokyo Dental College, Chiba, Japan.
[h] Aa and Pg, Aa-specific and Pg-specific primer pairs, respectively.

tions in a series of real-time PCRs so that a correlation coefficient could be calculated from the standard curve of *Ct* values. Detection and quantification were linear over the range of DNA concentrations examined. The quantity of DNA was linear over the range from 30 pg to 3 μg per reaction mixture for both species (Fig. 1A and 1B). The number of *A. actinomycetemcomitans* or *P. gingivalis* DNA copies was normalized to the number of 16S rRNA gene DNA copies by the method of Biéche et al. (3), with modifications. Briefly, we measured both the species-specific (*A. actinomycetemcomitans* and *P. gingivalis*) and the control-specific (16S rRNA gene) fluorescence for each specimen. In addition, we measured both types of fluorescence in four serial 10-fold dilutions of sample lysate. Then, we constructed standard curves for both the targets and the control for each sample. The results, expressed as the fold difference (*N*) in the number of target gene copies relative to the number of 16S rRNA gene copies, were determined as follows: $N = 2^{\Delta\Delta Ct} = 2^{(\Delta Ct\ \text{target} - \Delta Ct\ 16S\ \text{rRNA})}$, where $\Delta\Delta Ct$ is ΔCt target minus ΔCt 16S rRNA and ΔCt is the difference in threshold cycles for target and reference. The ΔCt 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. This study determined the numbers of *A. actinomycetemcomitans* and *P. gingivalis* bacteria in saliva and subgingival plaque samples from 10 patients with periodontitis (Table 3). Furthermore, conventional PCR was performed for comparison of the sensitivities of both the conventional and the real-time PCR analyses. The conventional PCR analysis was performed under the same conditions used for the real-time PCR, and the sensitivities were compared (Table 3). The real-time PCR analysis was more sensitive than the conventional PCR analysis in this assay.

One way to quantify bacteria is to use absolute quantification, which requires very precise sample collection. Another way is to use relative quantification by the $\Delta\Delta Ct$ method. From a clinical perspective, analysis of the percentage of specific bacteria in a region is often required to evaluate treatment. Lyons et al. (11) pointed out the importance of relative quantification rather than determination of the absolute number of a single species in a mixed sample.

The percentages of *A. actinomycetemcomitans* and *P. gingivalis* bacteria in each subgingival plaque sample varied by a few

TABLE 2. Oligonucleotide primers and probes

Designation	Sequence	Amplicon size (bp)	Target	Source or reference
Primers				
Aa1956-F	5′-CAGCATCTGCGATCCCTGTA-3′	147	*lktA*	JP2
Aa2102-R	5′-TCAGCCCTTTGTCTTTCCTAGGT-3′			
Pg1198-F	5′-TACCCATCGTCGCCTTGGT-3′	126	16S rRNA	W83
Pg1323-R	5′-CGGACTAAAACCGCATACACTTG-3′			
Uni152-F	5′-CGCTAGTAATCGTGGATCAGAATG-3′	69	16S rRNA	8
Uni220-R	5′-TGTGACGGGCGGTGTGTA-3′			
Fluorescent probes				
Aa2034T	5′-FAM-TCGAGTATTCCTCAAGCATTCTCGCACG-TAMRA-3′		*lktA*	JP2
Pg1238T	5′-FAM-GCTAATGGGACGCATGCCTATCTTACAGCT-TAMRA-3′		16S rRNA	W83
Uni177T	5′-FAM-CACGGTGAATACGTTCCCGGGC-TAMRA-3′		16S rRNA	8

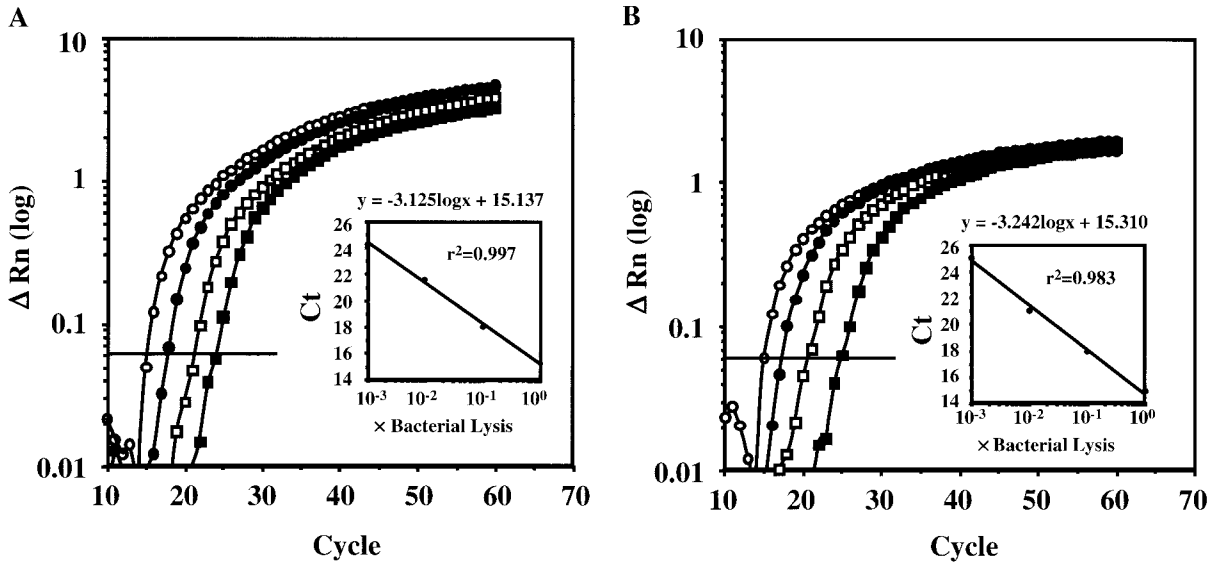

FIG. 1. Amplification of genomic DNA from lysed cells. Serial dilutions of genomic DNA were from *A. actinomycetemcomitans* (A) or *P. gingivalis* (B). The relative fluorescence (ΔRn) was monitored as the increase in the intensity of the reporter dye relative to the intensity of the passive internal reference dye. The threshold fluorescence, or the level at which the threshold cycle was determined, is shown. The standard curves were generated from the amplification plots in the insets (correlation coefficients, 0.997 for *A. actinomycetemcomitans* and 0.983 for *P. gingivalis*). *Ct* is the cycle number at which the threshold fluorescence is reached.

orders of magnitude (Table 3). Our results for *P. gingivalis* are consistent with those from a previous report (11). The proportion of *A. actinomycetemcomitans* bacteria ranged from 0 to 0.11%. Similar results were obtained with the saliva samples, in which the proportion of *P. gingivalis* bacteria ranged from 0 to 1.56% and that of *A. actinomycetemcomitans* bacteria ranged from 0 to 0.22%.

The simplified ΔΔ*Ct* method is accurate and useful for the relative quantification of periodontopathic bacteria. Further studies of this real-time PCR-based quantitative detection sys-

TABLE 3. Comparison of conventional PCR and real-time PCR

Sample and patient no.	Conventional PCR result		Real-time PCR result[c]	
	Aa[a]	Pg[b]	% of Aa	% of Pg
Saliva				
1	−	+	ND[d]	0.20 ± 0.02
2	+	−	0.19 ± 0.04	ND
3	+	+	0.22 ± 0.04	1.56 ± 0.06
4	+	−	0.20 ± 0.03	$5.11 \times 10^{-4} \pm 1.16 \times 10^{-4}$
5	−	+	ND	0.64 ± 0.02
6	−	+	ND	1.34 ± 0.13
7	+	+	0.11 ± 0.01	0.08 ± 0.00
8	−	+	ND	0.81 ± 0.06
9	−	−	ND	ND
10	−	−	ND	ND
Subgingival plaque				
1	−	+	ND	1.33 ± 0.20
2	−	−	$7.16 \times 10^{-4} \pm 0.47 \times 10^{-4}$	ND
3	−	−	$4.69 \times 10^{-3} \pm 0.45 \times 10^{-3}$	ND
4	+	−	0.11 ± 0.03	ND
5	−	+	ND	0.38 ± 0.04
6	−	+	ND	6.40 ± 0.43
7	+	+	0.02 ± 0.00	0.20 ± 0.02
8	−	+	ND	1.10 ± 0.16
9	−	−	ND	ND
10	−	−	ND	ND

[a] Aa, *A. actinomycetemcomitans*.
[b] Pg, *P. gingivalis*.
[c] Data are expressed as means ± standard deviation ($n = 3$).
[d] ND, not detected.

tem might be useful both for the evaluation of treatment and for elucidation of the etiologic role of unculturable oral bacteria in periodontitis.

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