JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2003, p. 4438–4441 Vol. 41, No. 9
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.9.4438–4441.2003

Development of a 5′ Nuclease-Based Real-Time PCR Assay for Quantitative Detection of Cariogenic Dental Pathogens *Streptococcus mutans* and *Streptococcus sobrinus*†

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A 5′ nuclease TaqMan PCR assay was developed for the quantitative detection of the major cariogenic bacteria *Streptococcus mutans* and *Streptococcus sobrinus*. The absolute and relative numbers of bacteria were measured by this method. This assay will be useful for quantifying these organisms in oral specimens and for analyzing biofilm formation.

Dental caries is one of the most common infectious diseases afflicting humans (10). Although 200 to 300 bacterial species have been found associated with dental plaque, only *Streptococcus mutans* (serotype c, e, and f mutans streptococci) and *Streptococcus sobrinus* (serotype d and g mutans streptococci) have been consistently linked with the formation of human dental caries (10–12). Additionally, *S. mutans* and *S. sobrinus* are occasionally associated with nonoral infections, principally subacute bacterial endocarditis (7, 14, 19). Consequently, various methods have been developed to identify *S. mutans* and/or *S. sobrinus* (2, 3, 9). Several of these methods are PCR-based bacterial detection systems (15, 16). Most of the PCR-based diagnosis systems reported are qualitative analyses and are therefore unsuitable for accurate evaluation of caries susceptibility or caries activity. Quantitative analysis is essential for monitoring the cell number and/or ratio of cariogenic bacteria in oral specimens, such as dental plaque and saliva. Furthermore, monitoring the number of cariogenic bacteria in oral biofilm is required from the perspective of biofilm research.

A real-time PCR assay with the TaqMan system based on the 5′-3′ exonuclease activity of *Taq* polymerase has been developed for the quantitative detection of DNA copy number (8). Briefly, an oligonucleotide probe with a reporter fluorescent dye attached to its 5′ end and a quencher dye attached to its 3′ end is designed to hybridize to the target gene. During PCR amplification, the quencher dye of the probe is cleaved by the 5′ nuclease activity of *Taq* polymerase, resulting in the accumulation of reporter fluorescence. The release of the fluorescent dye during amplification allows for the rapid detection and quantification of DNA (6).

This report describes a method for the absolute and relative quantification of human cariogenic bacteria, including *S. mutans* and *S. sobrinus*, from oral specimens by using a TaqMan PCR assay. In spite of the importance of these organisms as cariogenic dental pathogens, quantitative detection of *S. mutans* and *S. sobrinus* by TaqMan PCR has not been reported.

TABLE 1. Strains and amplification results

Strain	Source or distribution	Amplification with primer:		
		S. mutans	*S. sobrinus*	Universal
Oral streptococci				
Mutans group (serotype)				
Streptococcus mutans	Human			
GS-5 (c)		+	−	+
MT8148 (c)		+	−	+
Xc (c)		+	−	+
MT703R (e)		+	−	+
OMZ175 (f)		+	−	+
Streptococcus sobrinus	Human			
MT8145 (d)		−	+	+
OMZ176 (d)		−	+	+
6715 (g)		−	+	+
OU8 (g)		−	+	+
Streptococcus downei	Monkey			
Mfe28 (h)		−	−	+
S28 (h)		−	−	+
Streptococcus ratti	Rat			
BHT (b)		−	−	+
FA1 (b)		−	−	+
Streptococcus cricetus	Hamster			
E49 (a)		−	−	+
HS1 (a)		−	−	+
Mitis-sanguinis group				
Streptococcus mitis 903	Human	−	−	+
Streptococcus sanguinis ATCC 10556	Human	−	−	+
Streptococcus gordonii DL1	Human	−	−	+
Streptococcus oralis ATCC 10557	Human	−	−	+
Salivarius group				
Streptococcus salivarius HT9R	Human	−	−	+
Anginosus group				
Streptococcus anginosus FW73	Human	−	−	+
Other bacteria				
Porphyromonas gingivalis ATCC 33277	Human	−	−	+
Actinibacillus actinomycetemcomitans Y4	Human	−	−	+
Treponema denticola	Human			
ATCC 35404		−	−	+
ATCC 35405		−	−	+
Bacteroides forsythus ATCC 43037	Human	−	−	+
Fusobacterium nucleatum ATCC 10953	Human	−	−	+
Prevotella intermedia ATCC 25611	Human	−	−	+
Haemophilus aphrophilus NCTC 5908	Human	−	−	+
Eikenella corrodens 1085	Human	−	−	+
Escherichia coli DH5α	GIBCO BRL	−	−	+

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† Respectfully dedicated to the memory of Toshihiko Koga, who passed away 14 October 2001.

‡ Deceased.

TABLE 2. Oligonucleotide primers and probes

Designation	Sequence[a]	Amplicon size (bp)	Target	Source or reference
Primers				
Smut3368-F	5′-GCCTACAGCTCAGAGATGCTATTCT-3′	114	*gtfB*	UA159
Smut3481-R	5′-GCCATACACCACTCATGAATTGA-3′			
Ssob287-F	5′-TTCAAAGCCAAGACCAAGCTAGT-3′	88	*gtfT*	OMZ176
Ssob374-R	5′-CCAGCCTGAGATTCAGCTTGT-3′			
Uni152-F	5′-CGCTAGTAATCGTGGATCAGAATG-3′	69	16S rRNA	21
Uni220-R	5′-TGTGACGGGCGGTGTGTA-3′			
Fluorescent probes				
Smut3423T	5′-FAM-TGGAAATGACGGTCGCCGTTATGAA-TAMRA-3′		*gtfB*	UA159
Ssob298T	5′-FAM-CCTGCTCCAGCGACAAAGGCAGC-TAMRA-3′		*gtfT*	OMZ176
Uni177T	5′-FAM-CACGGTGAATACGTTCCCGGGC-TAMRA-3′		16S rRNA	21

[a] Accession numbers are M17361 for the *S. mutans gtfB* gene and D13928 for the *S. sobrinus gtfT* gene.

This is the first investigation of quantitative detection of *S. mutans* and *S. sobrinus* by using a TaqMan assay.

The bacterial strains used in this study are listed in Table 1. The *S. mutans* and *S. sobrinus* strains were cultured as described previously (15). Genomic DNA was isolated and purified using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.) in accordance with the manufacturer's instructions for gram-positive bacteria. Human saliva was prepared as described previously (15). Briefly, 500 μl of stimulated whole saliva and the same amount of phosphate-buffered saline (0.12 M NaCl, 0.01 M Na_2HPO_4, 5 mM KH_2PO_4 [pH 7.5]) were mixed and centrifuged at 12,000 × *g* for 10 min; 500 μl of cell lysis buffer (1.0% Triton X-100, 20 mM Tris-HCl, 2 mM EDTA [pH 8.0]) (18) was added to the precipitate, which was then incubated with 20 U of mutanolysin/ml and 0.2 mg of lysozyme/ml at 37°C for 2 h. The precipitate was vortexed, and the chromosomal DNA from the bacteria was extracted by boiling the precipitate at 100°C for 10 min. Plaque samples were collected from the buccal side of the upper first molar. One milligram (wet weight) of plaque was washed with phosphate-buffered saline two times. The precipitate was suspended in 100 μl of cell lysis solution and incubated with 20 U of mutanolysin/ml and 0.2 mg of lysozyme/ml at 37°C for 2 h. The lysate was boiled at 100°C for 10 min, and the chromosomal DNA was extracted.

Oligonucleotide primers and probes, designed using Primer Express 1.5 software (Applied Biosystems, Foster City, Calif.), are listed in Table 2. The universal primers and a probe for a broad range of bacteria were designed as previously described (4, 21). The *S. mutans*- and *S. sobrinus*-specific primers and probes were designed from the *gtfB* (17) and *gtfT* (5) genes, respectively. The specificities of the primers and probes were initially confirmed by BLAST with the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih

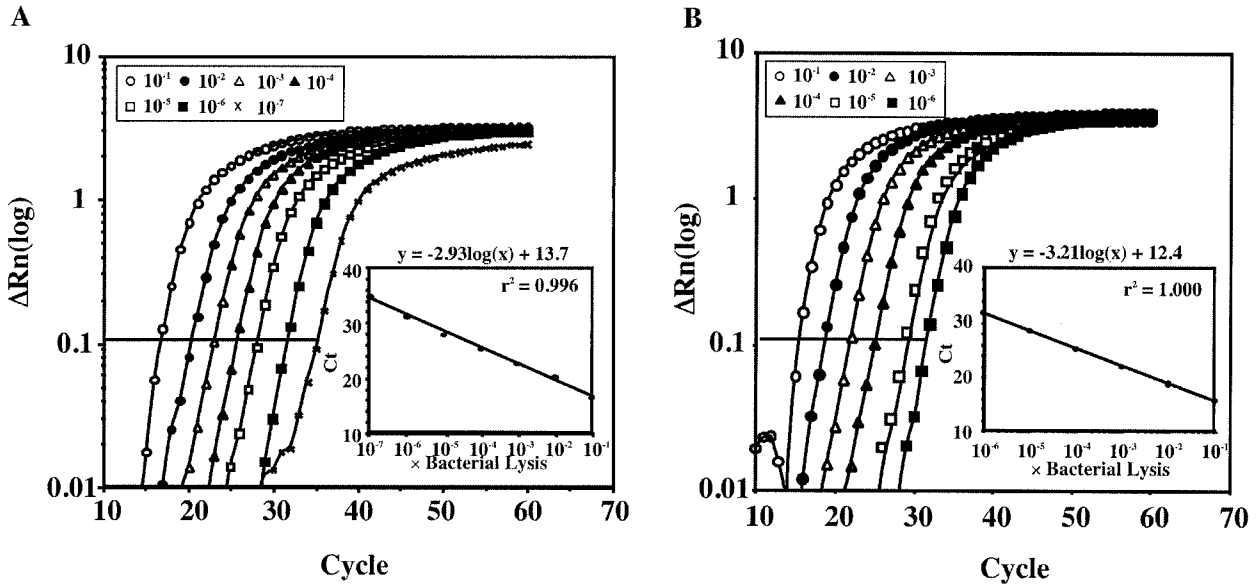

FIG. 1. Amplification plots of chromosomal DNA from lysed cells. Serial dilutions of chromosomal DNA were from *S. mutans* (A) or *S. sobrinus* (B). The log-transformed relative fluorescence [ΔRn(log)] was monitored as the increase in reporter dye intensity relative to the passive internal reference dye. The threshold fluorescence, or level at which the threshold cycle was determined, is shown. The standard curves were generated from the amplification plots in the small panels (correlation coefficient = 0.996 for *S. mutans* and 1.00 for *S. sobrinus*). *Ct* is the cycle number at which the threshold fluorescence was reached.

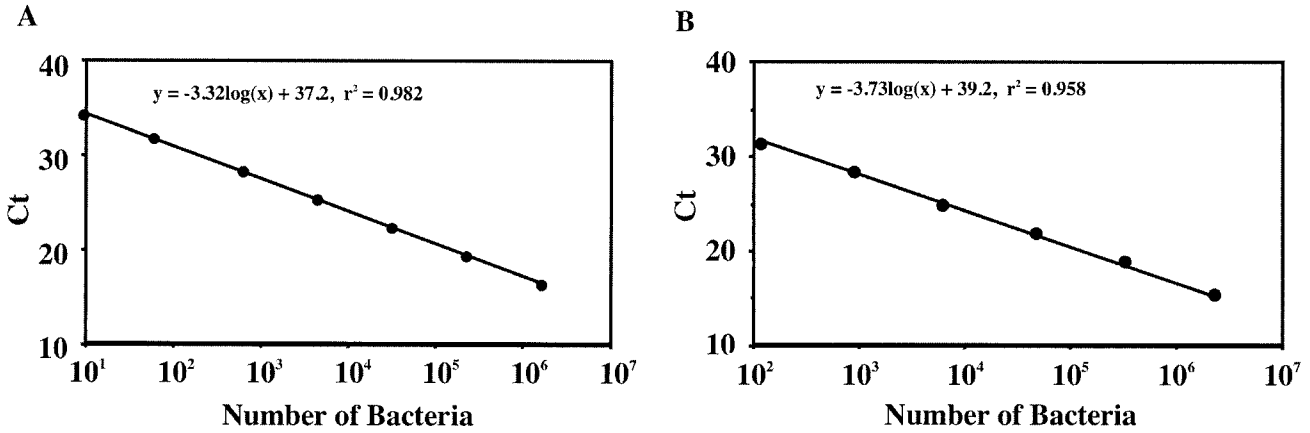

FIG. 2. Standard curves generated by known numbers of *S. mutans* (A) and *S. sobrinus* (B); linearity is shown for these organisms. The correlation coefficients are 0.982 for *S. mutans* and 0.958 for *S. sobrinus*. *Ct* is the cycle number at which the threshold fluorescence was reached.

.gov/) and then confirmed by conventional PCR (Table 1) and dot blot analysis with digoxigenin-labeled probes (data not shown), respectively. Several strains of *S. mutans* and *S. sobrinus* of all serotypes served as positive controls, and the other bacteria served as negative controls for the bacterium-specific primers and probes (Table 1). Conventional PCR assays used to confirm the specificity and universality 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 Mix (Applied Biosystems), 200 nM (each) sense and antisense primer, and 250 nM TaqMan probe was placed in each well of a 96-well plate. Amplification and detection were performed using 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 of 95°C for 15 s and 58°C for 1 min. The critical threshold cycle (*Ct*) is defined as the cycle at which the fluorescence becomes detectable above background and is inversely proportional to the logarithm of the initial number of template molecules. The standard curves for each organism were plotted for each primer-probe set by using *Ct* values obtained from the amplification of genomic DNA extracted from samples containing 1.7×10^0 to 1.7×10^9 CFU of *S.*

TABLE 3. Number of *S. mutans* and *S. sobrinus* cells detected in oral specimens[a]

Specimen type and patient no.	*S. mutans*		*S. sobrinus*	
	No. of cells[b]	% of bacteria[c]	No. of cells[b]	% of bacteria[c]
Saliva				
1	$6.33 \times 10^1 \pm 0.09 \times 10^1$	$1.33 \times 10^{-1} \pm 0.04 \times 10^{-1}$	3.66 ± 0.40[d]	$2.57 \times 10^{-2} \pm 0.38 \times 10^{-2}$
2	$7.41 \times 10^1 \pm 0.76 \times 10^1$	$2.50 \times 10^{-2} \pm 0.67 \times 10^{-2}$	$4.43 \times 10^1 \pm 1.84 \times 10^1$[d]	$5.00 \times 10^{-3} \pm 1.41 \times 10^{-3}$
3	$1.12 \times 10^2 \pm 0.11 \times 10^2$	$6.22 \times 10^{-2} \pm 0.57 \times 10^{-2}$	ND[e]	ND
4	$4.43 \times 10^1 \pm 0.24 \times 10^1$	$1.93 \times 10^{-2} \pm 0.11 \times 10^{-2}$	$5.83 \times 10^1 \pm 0.51 \times 10^1$[d]	$1.33 \times 10^{-2} \pm 0.10 \times 10^{-2}$
5	$1.26 \times 10^2 \pm 0.12 \times 10^2$	$8.62 \times 10^{-2} \pm 0.09 \times 10^{-2}$	$4.57 \times 10^2 \pm 0.13 \times 10^2$	$1.88 \times 10^{-1} \pm 0.37 \times 10^{-1}$
6	$1.97 \times 10^3 \pm 0.03 \times 10^3$	1.46 ± 0.02	ND	ND
7	$1.34 \times 10^2 \pm 0.04 \times 10^2$	5.57 ± 0.01	2.87 ± 0.11[d]	$3.89 \times 10^{-2} \pm 0.14 \times 10^{-2}$
8	$2.15 \times 10^1 \pm 0.02 \times 10^1$	$4.60 \times 10^{-3} \pm 0.01 \times 10^{-3}$	$1.53 \times 10^2 \pm 0.16 \times 10^2$	$1.49 \times 10^{-2} \pm 0.13 \times 10^{-2}$
9	$5.05 \times 10^2 \pm 0.14 \times 10^2$	$1.03 \times 10^{-1} \pm 0.04 \times 10^{-1}$	$8.76 \times 10^2 \pm 0.41 \times 10^2$	$7.40 \times 10^{-2} \pm 0.72 \times 10^{-2}$
10	5.55 ± 0.11[d]	$1.60 \times 10^{-3} \pm 0.00 \times 10^0$	ND	ND
Plaque				
1	ND	ND	ND	ND
2	ND	ND	ND	ND
3	$3.76 \times 10^1 \pm 1.26 \times 10^1$	$2.70 \times 10^{-3} \pm 0.70 \times 10^{-3}$	ND	ND
4	$2.44 \times 10^1 \pm 0.06 \times 10^1$	$6.37 \times 10^{-3} \pm 0.86 \times 10^{-3}$	$1.64 \times 10^1 \pm 0.30 \times 10^1$[d]	$1.15 \times 10^{-3} \pm 0.07 \times 10^{-3}$
5	$3.38 \times 10^2 \pm 0.04 \times 10^2$	$2.95 \times 10^{-2} \pm 0.04 \times 10^{-2}$	$5.17 \times 10^2 \pm 0.05 \times 10^2$	$2.21 \times 10^{-2} \pm 0.30 \times 10^{-2}$
6	$3.44 \times 10^4 \pm 0.06 \times 10^4$	5.41 ± 0.50	ND	ND
7	$3.13 \times 10^1 \pm 0.12 \times 10^1$	$1.05 \times 10^{-3} \pm 0.07 \times 10^{-3}$	$2.55 \times 10^2 \pm 0.02 \times 10^2$	$1.03 \times 10^{-2} \pm 0.03 \times 10^{-2}$
8	$2.92 \times 10^1 \pm 0.12 \times 10^1$	$9.00 \times 10^{-4} \pm 2.83 \times 10^{-4}$	$2.39 \times 10^2 \pm 0.11 \times 10^2$	$5.40 \times 10^{-3} \pm 0.14 \times 10^{-3}$
9	$4.82 \times 10^4 \pm 0.34 \times 10^4$	7.16 ± 0.84	$4.76 \times 10^4 \pm 0.19 \times 10^4$	6.95 ± 0.28
10	4.25 ± 0.19[d]	$6.00 \times 10^{-4} \pm 0.00 \times 10^0$	ND	ND

[a] Data are means ± standard deviations ($n = 3$).
[b] CFU per PCR mixture.
[c] Data were calculated by the modified ΔΔ*Ct* method.
[d] Theoretical data below the detection limits.
[e] ND, not detected.

mutans and 1.1×10^0 to 1.1×10^9 CFU of *S. sobrinus*. The numbers of CFU were determined by plating culture dilutions on tryptic soy agar (Difco Laboratories, Grand Island, N.Y.) plates.

To determine the linearity and detection limit of the assay, solutions of lysed *S. mutans* and *S. sobrinus* were amplified in successive 10-fold dilutions in a series of real-time PCRs (Fig. 1). Based on this approach, correlations were observed between the *Ct* and the CFU (Fig. 2). Detection and quantification were linear over a range from 1.7×10^1 to 1.7×10^7 CFU per reaction mixture for *S. mutans* and 1.1×10^1 to 1.1×10^6 CFU per reaction mixture for *S. sobrinus*. The presence of PCR inhibitors in dental plaque was assessed by the fluorescence levels for serial dilutions of lysed *S. mutans* and *S. sobrinus*. Lysates spiked with approximately 10 μg (wet weight) of *S. mutans*- and *S. sobrinus*-negative dental plaque in each mixture showed no inhibition (data not shown).

Using this assay, we determined the numbers of *S. mutans* and *S. sobrinus* bacteria in saliva and dental plaque from 10 individuals (Table 3). The numbers of these organisms in each saliva sample varied by several orders of magnitude, and our results for saliva samples are consistent with a previous report (20). In addition, the relative amounts of these organisms in oral specimens were calculated by the comparative *Ct* (ΔΔ*Ct*) method (21), with a simplification. Briefly, the results, expressed as the fold difference (*N*) between the number of target gene copies and the number of 16S rRNA gene copies, were determined as follows: $N = 2^{\Delta Ct} = 2^{(Ct\ \text{target}\ -\ Ct\ 16\text{S rRNA})}$. From a clinical perspective, it is often necessary to analyze the percentage of specific bacteria in a region. Lyons et al. (13) pointed out the importance of the relative quantification of bacteria in clinical specimens. The percentages of these organisms in saliva ranged from 0.0016 to 5.57% for *S. mutans* and from 0 to 0.19% for *S. sobrinus*. Note that the percentages of *S. mutans* and *S. sobrinus* normalized to the copy number of the 16S rRNA gene may be underestimated, because the copy number of the 16S rRNA gene is higher than that of *gtfB* or *gtfT*. Furthermore, we examined the absolute and relative numbers of these organisms in plaque samples on tooth surfaces (Table 3). This is the first report to quantify cariogenic bacteria from dental plaque samples by using a real-time PCR assay. The cell numbers ranged from 0 to 4.82×10^6 per mg (wet weight) of plaque for *S. mutans* and from 0 to 4.76×10^6 per mg (wet weight) for *S. sobrinus*. The percentages of these organisms in the plaque ranged from 0 to 7.16% (*S. mutans*) and from 0 to 6.95% (*S. sobrinus*). The range of cell numbers and percentages of these bacteria in dental plaque are similar to previous results with cell culture methods (1).

This investigation revealed that the TaqMan assay is accurate and useful for the absolute and relative quantification of cariogenic bacteria from oral specimens. Recently, dental plaque has been considered an oral biofilm, and monitoring the absolute or relative amount of cariogenic bacteria in oral biofilm is essential. Moreover, the TaqMan PCR-based quantification system is advantageous, since cell numbers can be monitored in the biofilm as it is. This assay system will be useful for clarifying how these bacteria behave in oral biofilm formation and will contribute to the development of biofilm research.

This investigation was supported by research fellowships from the Japan Society for the Promotion of Science for Young Scientists (to N.S.) and by a research grant from the Nakatomi Foundation (to A.Y.).

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