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Rapid detection of the cariogenic pathogens *Streptococcus mutans* and *Streptococcus sobrinus* using loop-mediated isothermal amplification

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Introduction: *Streptococcus mutans* and *Streptococcus sobrinus* are associated with the development of dental caries in humans. In this study, we developed a rapid, sensitive method for detecting these major cariogenic pathogens using loop-mediated isothermal amplification (LAMP). The assay procedure is quite simple: the amplification is carried out in a single tube under isothermal conditions at 63°C, and the result can be obtained in less than 1 h.
Methods: Initially, a set of six primers was designed by targeting *S. mutans*-specific and *S. sobrinus*-specific regions, identified using the genomic subtractive hybridization technique. We evaluated the specificities and sensitivities of these assays. Furthermore, we detected and quantified these bacteria in saliva and carious dentin from eight children.
Results: The sensitivities of the *S. mutans*-specific and *S. sobrinus*-specific LAMP methods, examined using agarose gel electrophoresis, were each one cell for a 30-min reaction. The detection limits using real-time turbidimetry analysis were 1 to 10^7 cells (3.28×10^1 to 3.28×10^8 fg *S. mutans* template DNA) per reaction tube and 1 to 10^5 cells (2.72×10^3 to 2.72×10^8 fg *S. sobrinus* template DNA) per reaction tube. Using these assays, we detected and quantified these cariogenic bacteria for evaluation of the LAMP assay for clinical diagnosis.
Conclusions: Our results suggest that the LAMP-based assay in combination with subtractive hybridization is valuable for preparing species-specific primers for closely related species. Furthermore, the LAMP-based assay will be a useful tool for the rapid and sensitive prediction of dental caries.

Key words: dental caries; loop-mediated isothermal amplification; *Streptococcus mutans*; *Streptococcus sobrinus*; subtractive hybridization

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Mutans streptococci are strongly associated with dental caries (14, 15), and two members of this bacterial group, *Streptococcus mutans* and *Streptococcus sobrinus*, have been implicated as etiological agents of human dental caries (9, 15). One of the important virulence properties of these organisms is their ability to form biofilms known as dental plaque on tooth surfaces (9, 15). Several studies have shown that the prevalence of *S. sobrinus* is more closely associated with high caries activity than that of *S. mutans* (8, 10), and *S. sobrinus* also has a higher acidogenic capacity compared to *S. mutans* (5). Moreover, it has been reported that children harboring both *S. mutans* and *S. sobrinus* have a significantly higher incidence of dental caries than those with *S. mutans* alone (23). These results suggest the importance of discriminating between the

two streptococcal species for predicting and preventing dental caries.

Various methods have been developed for identifying *S. mutans* and *S. sobrinus* to prevent dental caries (4, 6, 13). Of these, the polymerase chain reaction (PCR) is one of the most specific and sensitive methods for diagnosing infectious disease, and many applications of PCR for detecting pathogenic microorganisms have been reported (3, 7, 27, 30). However, the PCR method has several disadvantages, such as the need for rapid thermal cycling, technical skill, and several manipulations.

Notomi et al. developed the loop-mediated isothermal amplification (LAMP) method (21), which is an auto-cycling strand displacement DNA synthesis carried out by a DNA polymerase with high strand displacement activity and a set of specific primers that recognize a total of six distinct sequences on the target DNA. Therefore, the LAMP method should amplify the target sequence with high selectivity (19). The LAMP reaction technique amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions at temperatures between 60 and 65°C. It takes less than 1 h to yield more than 500 μg/ml DNA (19). Furthermore, technique-specific instruments are not necessary. Therefore, LAMP can be a simple, valuable tool for the rapid diagnosis of infectious diseases and should be easily applicable in clinical laboratories.

In this study, we initially performed genomic subtractive hybridization to identify species-specific regions that distinguish *S. mutans* and *S. sobrinus*. This technique successfully identified genomic differences between these two organisms, and we used the LAMP method for the qualitative and quantitative detection of *S. mutans* and *S. sobrinus*. We then evaluated the specificities and sensitivities of these assays. In addition, we performed a LAMP-based assay using clinical oral specimens. This is the first report of the rapid detection of cariogenic bacteria using the LAMP technique.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. All the streptococcal strains were grown and maintained in brain–heart infusion broth (Difco Laboratories, Detroit, MI) or Mitis–Salivarius agar (Difco Laboratories) at 37°C under aerobic conditions with 5% CO_2.

Table 1. Specificity of the LAMP primers

Strain	Amplification with the following LAMP primers	
	S. mutans	*S. sobrinus*
Oral Streptococci		
Mutans group (serotype[1])		
S. mutans UA159 (c)	+	–
S. mutans XC (c)	+	–
S. mutans MT703R (e)	+	–
S. mutans OMZ175 (f)	+	–
S. mutans NCTC10449 (c)	+	–
S. mutans Ingbritt (c)	+	–
S. mutans GS-5 (c)	+	–
S. sobrinus MT8145 (d)	–	+
S. sobrinus OU8 (g)	–	+
S. sobrinus OMZ176 (d)	–	+
S. sobrinus AHT-K (g)	–	+
S. downei Mfe28 (h)	–	–
S. downei S28 (h)	–	–
S. ratti BHT (b)	–	–
S. ratti FA1 (b)	–	–
S. cricentus HS1 (a)	–	–
S. cricentus E49 (a)	–	–
Mitis-sanguinis group		
S. mitis 903	–	–
S. sanguinis ATCC 10556	–	–
S. sanguinis OMZ9	–	–
S. gordonii DL1	–	–
S. oralis ATCC 10557	–	–
Salivarius group		
S. salivarius HT9R	–	–
S. salivarius HHT	–	–
Anginosus group		
S. anginosus FW73	–	–
S. milleri NCTC10707	–	–
Other bacteria		
Lactobacillus rhamnosus JCM1136	–	–
L. rhamnosus JCM1553	–	–
L. rhamnosus JCM1561	–	–
L. rhamnosus JCM1563	–	–
L. rhamnosus JCM8134	–	–
L. rhamnosus JCM8135	–	–
L. casei subsp. *casei* JCM8130	–	–
L. casei subsp. *casei* JCM8132	–	–
Porphyromonas gingivalis W83	–	–
P. gingivalis W50	–	–
P. gingivalis 381	–	–
Treponema denticola ATCC 35404	–	–
T. denticola ATCC 35405	–	–
T. medium ATCC 700293	–	–
T. vincenti ATCC 35580	–	–
T. pectinovorum ATCC 33768	–	–
T. socranskii subsp. *paredis* ATCC 35535	–	–
T. socranskii subsp. *socranskii* ATCC 35536	–	–
Actinobacillus actinomycetemcomitans Y4	–	–
A. actinomycetemcomitans ATCC 29522	–	–
A. actinomycetemcomitans NCTC9710	–	–
A. actinomycetemcomitans SUNYaB67	–	–
A. actinomycetemcomitans SUNYaB75	–	–
Actinomyces naeslundii JCM8350	–	–
A. naeslundii JCM8351	–	–
Prevotella intermedia ATCC 25611	–	–
P. melaninogenica ATCC 25845	–	–
P. loescheii ATCC 15930	–	–
P. denticola ATCC 33185	–	–
P. corporis ATCC 33547	–	–
P. bivia ATCC 29303	–	–
P. pallens ATCC 700821	–	–
P. veroralis ATCC 33779	–	–
P. oralis ATCC 33322	–	–
Fusobacterium nucleatum ATCC 10953	–	–
Tannerella forsythia ATCC 43037	–	–
Escherichia coli DH5α	–	–
Haemophilus aphrophilus NCTC5980	–	–

[1]All serotypes of *Streptococcus mutans* and *Streptococcus sobrinus* are stock culture collections of the Kyushu Dental College.

DNA extraction

Chromosomal DNA was isolated from the bacteria listed in Table 1 using a PureGene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) or an IsoQuick Nucleic Acid Extraction Kit (ORCA Research, Bothell, WA) in accordance with the manufacturers' instructions for gram-positive bacteria.

Subtractive hybridization

To design species-specific LAMP primers for *S. mutans* and *S. sobrinus*, genomic subtractive hybridization was performed as previously described (1, 20, 24, 28). Briefly, chromosomal DNA from *S. mutans* UA159 was completely digested with *Sau*3AI and purified using a PCR purification kit (Qiagen GmbH, Hilden, Germany). The *Sau*3AI-digested DNA fragments of *S. mutans* were ligated with the oligonucleotide adapters RBam12 and RBam24 (Table 2) for 18 h at 11°C and purified using a PCR purification kit. To prepare the driver DNA, chromosomal DNA from *S. sobrinus* OMZ176 was digested with *Hin*dIII and *Eco*RI. After digestion, the DNA was precipitated with ethanol–sodium acetate (final concentration: 0.3 M) and dissolved in distilled water. The first subtractive hybridization was performed in 10 μl of a reaction mixture containing 2 μg of the driver DNA from *S. sobrinus*, 20 ng of the R-adaptor-linked *Sau*3AI fragments from *S. mutans*, 10 mM EPPS (3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid), and 1 mM EDTA. The amount was determined according to the subtractive hybridization protocol (24) with brief modifications. The DNA mixture was denatured at 100°C for 2 min and then placed at 55°C. After adding 2 μl of 5 M NaCl, the mixture was left to hybridize at 55°C for 24 h. A 2-μl aliquot of the reaction mixture was diluted to 40 μl with a PCR mixture containing 100 pmol RBam24, 0.25 mM of each deoxynucleoside triphosphate, 5 U *ExTaq*, and 1x *ExTaq* buffer (Takara Bio, Shiga, Japan) to fill in the ends corresponding to the RBam24 adaptor. After denaturation for 5 min at 94°C, the mixtures were amplified by PCR for 30 cycles of 1 min at 70°C, 3 min at 72°C, and 1 min at 94°C, followed by 1 min at 94°C and 10 min at 72°C. The PCR products were purified using a PCR purification kit. The RBam24 adaptor was removed from the PCR products by digestion with *Sau*3AI, and the DNA fragments were purified using the PCR purification kit. In a 40-μl volume, 2 nmol of the second adaptors, JBam12 and JBam24 (Table 2), were ligated for 18 h at 11°C and purified using a PCR purification kit. The second-round subtractive hybridization was performed with 2 ng DNA from the first round PCR products and 2 μg of the driver DNA from *S. sobrinus*, prepared as described above. The second-round PCR products were digested with *Sau*3AI, cloned into *Bam*HI-digested pBluescript II SK$^+$ (Stratagene, La Jolla, CA), and then used to transform *Escherichia coli* DH5α (Invitrogen, San Diego, CA) by heat shock. The nucleotide sequences of the inserts were determined using an ABI PRISM 3100 or 3700 (Applied Biosystems, Foster City, CA).

To identify an *S. sobrinus*-specific region, the same procedures were performed for *S. sobrinus* OMZ176 as the tester and *S. mutans* UA159 as the driver.

Table 2. Oligonucleotides used in this study

Adaptor, primer	Sequence[1]	Amplicon size (bp)	Source or reference
Adaptors			
RBam12	5′-GATCCTCGGTGA-3′		24
RBam24	5′-AGCACTCTCCAGCCTCTCACCGAG-3′		
JBam12	5′-GATCCGTTCATG-3′		
JBam24	5′-ACCGACGTCGACTATCCATGAACG-3′		
LAMP primers			
Streptococcus mutans			
F3	5′-CGGGGCAAATTCCTGGTT-3′	218	This study
B3	5′-AGCAATGTGCATGGAGCC-3′		
FIP	5′-GCCCACGGTGGGTACTGGTA -CAGGTGAAGAAGCCGTAGC-3′ (F1c-F2)		
BIP	5′-GGTCATTGTGTGGCCAAGGGT -CTTGCCAAGACCTGTCTCC-3′ (B1-B2c)		
LF	5′-CCGTTAAATTGGCACAGACTCC-3′		
LB	5′-GGTGACCTTAAGGGCATGATGG-3′		
Streptococcus sobrinus			
F3	5′-GGGAGGCTCAAAGGAACT-3′	214	This study
B3	5′-GATGATTTGCTCATCATAGTCTG-3′		
FIP	5′-GGTAGCAAAGGTTAAATAGCCCAT -CGCTATTTTTACTGCTACAGC-3′ (F1c-F2)		
BIP	5′-TGCTTCTCTCTCTTATCAGTATCG -GTCTTTATGACCAGTTGTCGA-3′ (B1-B2c)		
LF	5′-TCCTACGGCAATGCCAATG-3′		
LB	5′-TTGGTCAACACACTAGAACCCG-3′		
Real-time PCR			33
Primers			
Smut3368-F	5′-GCCTACAGCTCAGAGATGCTATTCT-3′	114	
Smut3481-R	5′-GCCATACACCACTCATGAATTGA-3′		
Ssob287-F	5′-TTCAAAGCCAAGACCAAGCTAGT-3′	88	
Ssob374-R	5′-CCAGCCTGAGATTCAGCTTGT-3′		
Fluorescent probes			
Smut3423T	5′-FAM-TGGAAATGACGGTCGCCGTTATGAA -TAMRA-3′		
Ssob298T	5′-FAM-CCTGCTCCAGCGACAAAGGCAGC -TAMRA-3′		

[1]F1c, sequence complementary to F1; B2c, sequence complementary to B2.

Analysis of clones and the primer design for LAMP

The homologies of the sequences from the DNA fragments isolated using subtractive hybridization were analyzed using BLAST on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/). The oligonucleotide primers for the LAMP reaction were designed from the DNA fragments specific for *S. mutans* UA159 or *S. sobrinus* OMZ176. The primers were designed using PRIMER EXPLORER Version 2.0 software (Fujitsu, Tokyo, Japan; Fig. 1 and Table 2).

LAMP reaction

The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical, Tochigi, Japan). A reaction mixture (25 μl) containing 1.6 μM of each inner primer (FIP and BIP), 0.2 μM of each outer primer (F3 and B3), 0.8 μM of each loop primer (LF and LB), 2× reaction mix (12.5 μl), *Bst* DNA polymerase (1 μl), and 1 μl target DNA was incubated at 63°C for *S. mutans* and *S. sobrinus* using a conventional heating block, and was heated at over 95°C for 2 min to terminate the reaction.

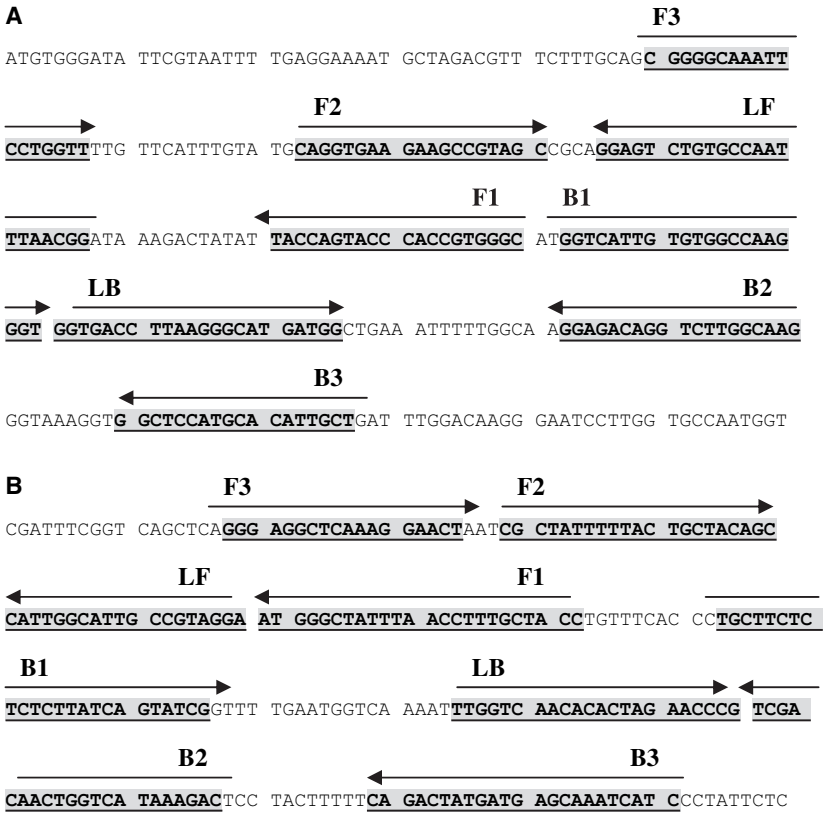

Fig. 1. Locations and names of the target sequences used as primers for *Streptococcus mutans* LAMP (A) and *Streptococcus sobrinus* LAMP (B). Target sequences were identified by subtractive hybridization using *S. mutans* UA159 and *S. sobrinus* OMZ176.

Detection of the LAMP products

The LAMP products were visually detectable upon the addition of 1 µl of original SYBR Green I (Molecular Probes, Eugene, OR) diluted 1 in 10 into the control solution and amplification products, respectively. The solution turned green in the presence of a LAMP amplicon, while it remained orange with no amplicon. The LAMP products were then electrophoresed on 1.8% agarose gels and stained with ethidium bromide. To confirm the structure, the amplified products were digested with the appropriate restriction enzymes (*Hae*III for *S. mutans* and *Mse*I for *S. sobrinus*) and electrophoresed on 1.8% agarose gels (Fig. 2).

The amplification of the gene was confirmed by real-time monitoring of the increase in the turbidity, which results from the white precipitate of magnesium pyrophosphate, using a Loopamp real-time turbidimeter (LA-200; Teramecs, Kyoto, Japan), while sequentially measuring the absorbance of the reaction mixture at 650 nm (17, 18). The turbidity was calculated using the following equation:

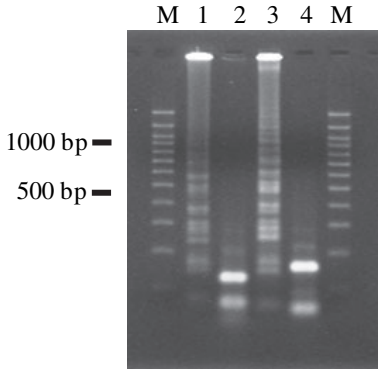

Fig. 2. Restriction analysis of the LAMP products. Lanes: M, 100-base-pair DNA ladder used as a size marker; lane 1, *Streptococcus mutans*-LAMP assay amplification; lane 2, *Hae*III digestion of the *Streptococcus mutans*-LAMP assay products; lane 3, *S. sobrinus*-LAMP assay amplification; lane 4, *Mse*I digestion of the *S. sobrinus*-LAMP assay products. *S. mutans* UA159 and *S. sobrinus* OMZ176 were used for these experiments.

$$\text{Turbidity} = \ln \frac{(\text{Intensity of the incident light})}{(\text{Intensity of the transmitted light})}$$

Real-time PCR

A TaqMan-based real-time PCR was performed for quantitative detection of *S. mutans* and *S. sobrinus* as described previously (33). The oligonucleotide primers and probes used in this study are listed in Table 2. The amplification and detection of DNA using real-time PCR were performed with the LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany). For each real-time PCR, 20 µl mixture containing 5 µl template DNA, 5× LightCycler TaqMan Master Mix (Roche Diagnostics GmbH), each sense and antisense primer at a concentration of 500 nM, and 200 nM TaqMan probe was placed in each capillary. The following DNA amplification conditions were used: one cycle at 95°C for 10 min; 45 cycles at 95°C for 10 s, 58°C for 30 s, and 72°C for 1 s; and one cycle at 40°C for 30 s.

Preparation of saliva and carious dentin samples

Saliva and carious dentin samples were obtained from children who attended the Kyushu Dental College Hospital in Kitakyushu, Japan. The carious dentin samples were collected from vital carious teeth. All parents of the study participants understood the nature of the research project and provided informed consent. Each sample was weighed individually, suspended in 350 µl cell suspension solution, and incubated with 20 U mutanolysin/ml and 0.2 mg lysozyme/ml at 37°C for 1 h. The lysate was homogenized, and the chromosomal DNA was extracted using an Easy DNA kit (Invitrogen) according to the manufacturer's instructions. Finally, the concentrated DNA was dissolved in 10 µl Tris–EDTA (TE, pH 8.0) per 1 mg carious dentin. In addition, 350 µl resting saliva was incubated with 20 U mutanolysin/ml and 0.2 mg lysozyme/ml at 37°C for 1 h, and the chromosomal DNA was extracted using an Easy DNA kit (Invitrogen) according to the manufacturer's instructions.

Results

Subtractive hybridization

Thirty-three DNA fragments for *S. mutans* and 15 DNA fragments for *S. sobrinus* were obtained using the subtractive hybridization technique. The nucleotide sequences of the isolated clones were determined and subjected to a homology

Table 3. Sequences obtained by subtractive hybridization

Clone	Fragment size (bp)	Region containing ORF[1]	Predicted protein size (aa)[2]	Best match in database (homologue)	*E* value[3]
Sm3–15	834	2–833	276	Putative pyruvate dehydrogenase, TPP-dependent E1 component alpha-subunit	5e-140
Ss6	318	79–317	79	Hypothetical protein	9e-04

[1]The regions corresponding to the predicted open reading frames (ORFs) were searched using the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).
[2]The number of amino acids (aa) in the predicted protein is shown.
[3]Highest scoring match in GenBank with the blastx algorithm.

search. The fragment sizes ranged from 110 to 834 base pairs for *S. mutans* and 192 to 386 base pairs for *S. sobrinus*. The *S. mutans*-specific and *S. sobrinus*-specific primers were designed from DNA fragments Sm3-15 and Ss6, respectively (Table 3).

Specificity of the LAMP assay

The specificities of the designed primers were initially confirmed using BLASTN (2), as previously described, and the specificities of the LAMP assay for detecting *S. mutans* and *S. sobrinus* were examined by carrying out reactions with DNA samples of various oral bacteria (Table 1). A successful LAMP reaction with the *S. mutans*-specific and *S. sobrinus*-specific primers produced several bands of different sizes on agarose gel electrophoresis. When the sample tube did not contain target DNA, no amplification was observed. To confirm that the products were amplified from the target region, we digested the products with a restriction enzyme: *Hae*III for *S. mutans* and *Mse*I for *S. sobrinus*. Each amplicon digested with restriction endonuclease was subjected to agarose gel electrophoresis (Fig. 2). *Hae*III recognizes the nucleotide sequences in the LAMP products of *S. mutans* producing 92- and 126-base pair fragments and *Mse*I recognizes the nucleotide sequences in the LAMP products of *S. sobrinus* producing 71- and 143-base pair fragments. As shown in Fig. 2, the sizes of the fragments generated were in good agreement with the predicted sizes. Furthermore, DNA sequences of the LAMP products were investigated and confirmed the specificities of the LAMP products.

Sensitivity of the LAMP assay

To evaluate the detection limit of the LAMP assays, we determined the sensitivity of the LAMP assay by testing serial tenfold dilutions of a known concentration of DNA. The results were obtained using electrophoretic analysis. The detection limit of the *S. mutans* primer set with the loop primers was one cell (3.28×10^1 fg *S. mutans* template DNA) for a 30-min reaction. Similarly, the detection limit of the *S. sobrinus* primer set with the loop primers was one cell (2.72×10^3 fg *S. sobrinus* template DNA) for a 30-min reaction (Table 4).

LAMP analysis using real-time turbidimetry for a quantitative assay

Figure 3A shows the results of real-time turbidity measurements in LAMP reaction solutions containing 1 to 10^7 cells (3.28×10^1 to 3.28×10^8 fg *S. mutans* template DNA) per reaction tube, and Fig. 3B presents the results of real-time turbidity measurements in LAMP reaction solutions containing 1 to 10^5 cells (2.72×10^3 to 2.72×10^8 fg *S. sobrinus* template DNA) per reaction tube. As the quantity of the initial template DNA increased, the threshold time decreased. Plots of the amplification time required to exceed a turbidity of 0.1 (threshold time) vs. the log of the initial *S. mutans* (Fig. 4A) and *S. sobrinus* (Fig. 4B) template DNA concentrations demonstrated linear relationships with correlation coefficients of $r^2 = 0.9903$ and $r^2 = 0.9932$, respectively.

Table 4. Detection sensitivities of LAMP

	Results[1] for															
	S. mutans								*S. sobrinus*							
Time (min)	0	1	10	10^2	10^3	10^4	10^5	10^6	0	1	10	10^2	10^3	10^4	10^5	10^6
0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
15	−	−	−	−	±	+	+	+	−	−	−	−	−	±	±	±
30	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
60	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+

[1]Results were obtained by agarose gel electrophoresis. +, clearly visible; ±, visible but not clear; −, not visible.
Streptococcus mutans UA159 and *Streptococcus sobrinus* OMZ16 were used in this study.

Real-time PCR analysis for a quantitative assay

We developed a TaqMan real-time PCR assay to quantify *S. mutans* and *S. sobrinus*. The *S. mutans*-specific and *S. sobrinus*-specific primers and probes used for real-time PCR were designed from the *gtfB* and *gtfT* genes, respectively, by Yoshida et al. (33). Using these primers and probes, we developed a TaqMan PCR assay to quantify *S. mutans* and *S. sobrinus* on the LightCycler system. A standard curve was plotted for each primer–probe set using the threshold cycle (Ct) values obtained by amplifying successive 10-fold dilutions of a known concentration of DNA, and the correlations between Ct values and CFU counts were obtained ($y = -2.7129\log(x) + 32.102$, $r^2 = 0.986$ for *S. mutans*; $y = -3.153\log(x) + 28.121$, $r^2 = 0.994$ for *S. sobrinus*). The assay was capable of detecting the bacteria linearly over a range of 50 to 5×10^7 cells (1.64 to 1.64×10^5 pg *S. mutans* template DNA) per reaction mixture and 5 to 5×10^6 cells (1.36×10^1 to 1.36×10^7 pg *S. sobrinus* template DNA) per reaction mixture.

Quantitative detection of *S. mutans* and *S. sobrinus* from clinical specimens

We initially assessed the potential inhibitory effects of the oral specimens on LAMP and real-time PCR. We assessed the possible presence of LAMP and PCR inhibitors in saliva using lysates spiked with *S. mutans*-negative and *S. sobrinus*-negative saliva (1 µl per mixture) and carious dentin [ca. 1 µg (wet weight) per mixture] and observed negligible inhibition (data not shown). The real-time LAMP and the conventional real-time PCR were applied to saliva and carious dentin samples from eight children between 4 and 11 years of age. Comparing the real-time LAMP with conventional real-time PCR, the real-time LAMP was more sensitive than conventional real-time PCR. The number of *S. mutans* and *S. sobrinus* determined by real-time LAMP was almost the same or from 1 to 1000 times

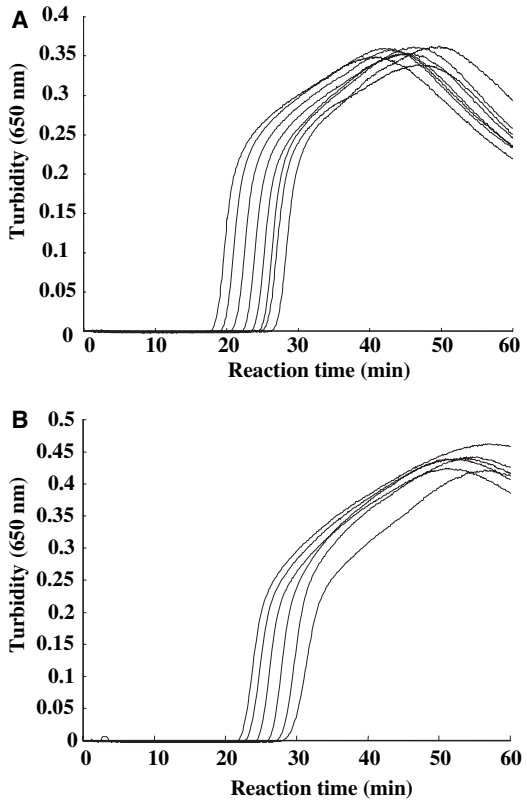

Fig. 3. Real-time monitoring of the *Streptococcus mutans* UA159 (A) and *Streptococcus sobrinus* OMZ176 (B) LAMP reactions. The curves, from left to right, represent the serial tenfold dilutions ranging in reverse from 1 to 10^7 cells (3.28×10^1 to 3.28×10^8 fg of *S. mutans* template DNA) per reaction tube and from 1 to 10^5 cells (2.72×10^3 to 2.72×10^8 fg of *S. sobrinus* template DNA) per reaction tube.

greater than determined by conventional real-time PCR (Table 5).

Discussion

Various methods have been used to detect *S. mutans* and *S. sobrinus*, including culture and PCR-based amplification methods (12, 22, 33). These techniques are specific and accurate, but most of them are technically demanding and time-consuming. Therefore, we focused on the LAMP method for rapidly detecting *S. mutans* and *S. sobrinus*. This is the first report to describe the application of the LAMP method to detect cariogenic bacteria.

The nucleotide sequences of *S. mutans* and *S. sobrinus* share high homologies, and regions of nucleotide sequence specificity between *S. mutans* and *S. sobrinus* are limited. We initially designed the primers for LAMP assays to detect these bacteria based on sequence information obtained from the BLAST database. However, because we could not identify regions specific to these organisms, we used the subtractive hybridization technique, which is often applied to identify unique DNA segments from bacteria and mammalian cells, to identify species-specific regions on chromosomal DNA. This technique provides preferential enrichment sequences that differ between two or more strains of bacteria isolated from patients with or without a particular disease (1, 25, 29, 31). We applied PCR-based subtractive hybridization to identify genetic elements unique to *S. mutans* strains associated with early childhood caries. Using this method, we chose the fragments Sm3–15 and Ss6 for designing *S. mutans*-specific and *S. sobrinus*-specific primers, respectively. The results suggested that the combination of the LAMP method and genomic subtractive hybridization was effective for designing species-specific LAMP primers to distinguish closely related species. We still cannot exclude the possibility of cross-reaction of the specific primers against other streptococci. However, the utilization of subtractive hybridization for designing the species-specific primers improved the specificities of the primers.

Determining the prevalence of *S. mutans* and *S. sobrinus* in the oral cavity is important because the detection and quantification of these bacteria are relevant for diagnosis and treatment planning. Straetemans et al. suggested that the delayed acquisition of these organisms reduces the incidence of caries in both the primary dentition and in the permanent

Table 5. Number of bacterial cells in oral specimens using by quantitative LAMP[a]

			Saliva[c]				Carious dentin[d]			
			Streptococcus mutans		*Streptococcus sobrinus*		*Streptococcus mutans*		*Streptococcus sobrinus*	
Patient no.	Age (yr)	Gender[b]	real-time LAMP	real-time PCR	real-time LAMP	real-time PCR	real-time LAMP	real-time PCR	real-time LAMP	real-time PCR
1	6	F	2.36×10^6	7.04×10^4	ND	ND	2.64×10^9	2.36×10^6	1.30×10^5	ND
2	10	M	2.90×10^3	ND[e]	2.87×10^1	ND	6.20×10^2	2.03×10^{1f}	8.93×10^1	ND
3	7	F	4.73×10^4	2.19×10^3	ND	ND	7.17×10^8	4.84×10^5	2.11×10^4	7.11×10^2
4	4	M	2.25×10^4	2.11×10^4	ND	ND	1.47×10^4	5.34×10^3	1.64×10^2	ND
5	11	F	2.90×10^3	1.06×10^{1f}	2.87×10^1	1.18×10^{1f}	6.02×10^2	8.68×10^{1f}	8.93×10^1	ND
6	10	M	2.40×10^3	2.93×10^{2f}	ND	ND	1.77×10^4	2.73×10^3	1.18×10^3	ND
7	10	M	5.06×10^3	1.65×10^4	3.34×10^1	ND	1.99×10^5	2.93×10^4	1.45×10^1	ND
8	7	F	2.40×10^3	ND	1.52×10^2	ND	8.99×10^2	8.18×10^{1f}	ND	ND

[a]A result from a 60-min reaction is shown.
[b]F, female; M, male.
[c]CFU per ml of saliva.
[d]CFU per mg of carious dentin.
[e]ND, not detected.
[f]Theoretical data.

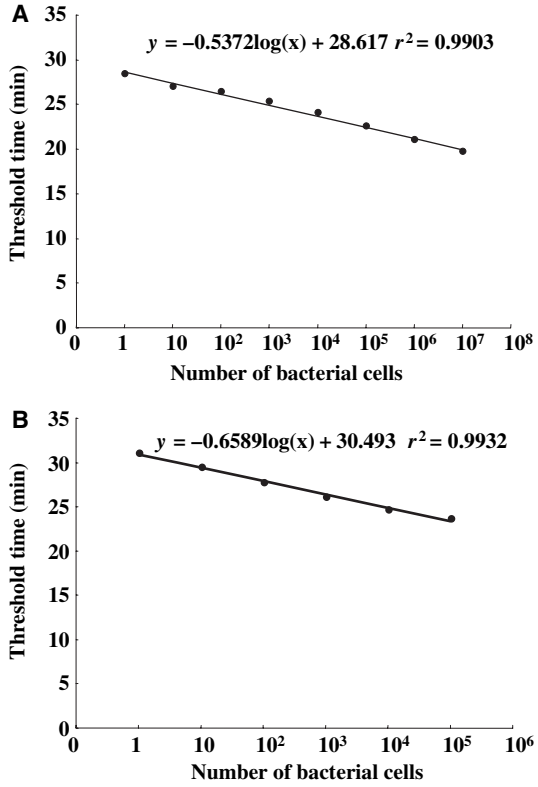

Fig. 4. Standard curves generated using known numbers of *Streptococcus mutans* UA159 (A) and *Streptococcus sobrinus* OMZ176 (B), showing linearity for these organisms. The correlation coefficients are 0.9903 for *S. mutans* and 0.9932 for *S. sobrinus*.

dentition at later ages (26). Therefore, it is critical to detect *S. mutans* and *S. sobrinus* during the early years for dental caries prediction and subsequent treatment. Furthermore, to evaluate caries susceptibility or caries activity accurately, quantitative analysis is essential for monitoring the cell number and the ratio of cariogenic bacteria in oral specimens.

In this study, we evaluated the detection limits of the LAMP assays using serially diluted chromosomal DNA. The detection limit with loop primers using electrophoretic analysis was one cell in a 30-min reaction for both *S. mutans* and *S. sobrinus*. The detection limits with loop primers using real-time turbidimetry analysis were 1 to 10^7 cells (3.28×10^1 to 3.28×10^8 fg *S. mutans* template DNA) per reaction tube and 1 to 10^5 cells (2.72×10^3 to 2.72×10^8 fg *S. sobrinus* template DNA) per reaction tube. The lower detection limits of the reported PCR-based detection systems for *S. mutans* and *S. sobrinus* were 25–100 cells (11, 12, 22). Furthermore, the lower detection limits of the TaqMan real-time PCR assays developed here were 50 cells (1.64 pg *S. mutans* template DNA) per reaction mixture and five cells (1.36×10^1 pg *S. sobrinus* template DNA) per reaction mixture. Compared to these reports, the LAMP-based detection system is more sensitive.

Using this assay system, we detected and quantified these cariogenic bacteria in saliva and carious dentin samples from eight children (Table 5). The numbers of these bacteria in each saliva and carious dentin sample varied by several orders of magnitude, and our results were consistent with previous reports (16, 33). The numbers of *S. mutans* and *S. sobrinus* determined by real-time LAMP were almost the same or from 1 to 10^3 times more than those determined by TaqMan-based real-time PCR. The differences in the detection methods might have caused the differences in the detection sensitivities. In this study, we confirmed that the LAMP-based quantitative detection system is applicable to oral specimens. However, further evaluation, especially for quantitative assays, is required.

As described above, the LAMP assay is an extremely rapid, highly sensitive, and specific method. In this study, we used a DNA extraction kit for more efficient extraction of DNA from clinical samples. However, simplification of the DNA extraction procedure from clinical specimens is essential to shorten the time required to perform this assay.

In conclusion, the LAMP assay developed in this study allows the rapid and sensitive identification of *S. mutans* and *S. sobrinus*. The results suggest that the assay would be useful for clinically diagnosing *S. mutans* and *S. sobrinus* infections and that it could contribute to diagnosing and predicting the development of human dental caries. Combined with the LAMP-based rapid detection system for periodontopathic bacteria (32), our novel detection system will facilitate the diagnosis of major oral infectious diseases. However, further improvements regarding these assays are needed.

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