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Rapid detection of *Actinobacillus actinomycetemcomitans* using a loop-mediated isothermal amplification method

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Introduction: *Actinobacillus actinomycetemcomitans* has been implicated in the etiology of aggressive periodontitis. In this study, we applied a novel nucleic acid amplification method, called loop-mediated isothermal amplification (LAMP), which amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions, allowing the rapid detection of *A. actinomycetemcomitans.*
Methods: We designed the primers for detecting *A. actinomycetemcomitans* and evaluated the specificity and sensitivity of the assay.
Results: The LAMP primers used in this study successfully amplified serotypes a–e of *A. actinomycetemcomitans*, while other oral bacteria were not amplified. By measuring the precipitation of magnesium pyrophosphate, we could quantify the chromosomal DNA of *A. actinomycetemcomitans*. The detection limits using the real-time turbidimetry analysis were 5.8×10^2–5.8×10^7 copies of *A. actinomycetemcomitans* template DNA per reaction tube. In addition, the LAMP assay was used for the rapid detection of *A. actinomycetemcomitans* in clinical specimens from eight individuals. The results with the LAMP method were similar to those using conventional polymerase chain reaction.
Conclusion: Our results suggest that the LAMP-based assay is very useful for the rapid detection of *A. actinomycetemcomitans*.

Key words: *Actinobacillus actinomycetemcomitans*; loop-mediated isothermal amplification; periodontitis; rapid detection

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Periodontitis is an inflammation of the tissues supporting the teeth (3). It is generally accepted that periodontitis is an infectious disease caused by oral bacteria (6). *Actinobacillus actinomycetemcomitans* is a non-motile, gram-negative, capnophilic, fermentative coccobacillus that has been implicated in the etiology of localized aggressive periodontitis (1, 12, 22, 23, 25, 27).

Genetic analyses of infectious diseases have been developed to obtain detailed genetic information on the virulence and antibiotic resistance of microbes. Molecular-based methods are often used to diagnose infectious disease. Of these, the polymerase chain reaction (PCR) is one of the most widely used techniques (24). PCR-based detection of bacteria is sensitive and specific (2). However, this detection method requires specialized equipment, such as thermal cyclers, and several operations.

Recently, Eiken Chemical (Tochigi, Japan) developed loop-mediated isothermal amplification (LAMP), which constitutes a novel nucleic acid amplification method (16, 19). The LAMP reaction requires a DNA polymerase with strand displacement activity and a set of four oligonucleotide primers that recognize six distinct regions (F1, F2, F3, B1, B2 and B3) on the target DNA: the forward inner primer (FIP), back inner primer (BIP), and two outer primers (F3 and B3) (19). The FIP consists of a sequence complementary to F1 and the F2 sense sequence. The BIP consists of the B1 sense sequence and a sequence complementary to B2. Although these four primers are sufficient for the amplification reaction, the LAMP reaction can be accelerated by using additional

primers called loop primers (18): LF and LB. LAMP is a novel approach for nucleic acid amplification that is both selective and rapid. The primary characteristic of the LAMP method is its ability to amplify nucleic acids under isothermal conditions at temperatures near 65°C (16, 19).

The LAMP method is also characterized by both high specificity and high amplification efficiency. As the LAMP method uses four primers that recognize six distinct target DNA sequences, the specificity is extremely high. This method is also extremely efficient because there is no time lost through changes in temperature, because the reaction is isothermal. Therefore, the LAMP assay could potentially constitute a valuable tool for the rapid diagnosis of infectious diseases, especially for various viral and microbial infections (5, 7, 8, 10, 13). Previously, we applied the LAMP method for the rapid diagnosis of adult periodontitis (26).

In this study, we developed and evaluated a LAMP method to help with the rapid diagnosis of aggressive periodontitis. This is the first report on a LAMP method for the rapid detection of *A. actinomycetemcomitans*.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. *A. actinomycetemcomitans* was grown anaerobically at 37°C in Todd–Hewiit broth (Difco Laboratories, Detroit, MI) supplemented with 1.0% yeast extract (Difco Laboratories) (THY broth) or on THY agar plates.

DNA techniques

Routine molecular biology techniques were performed as described by Sambrook et al. (21). Chromosomal DNA was isolated from the bacteria listed in Table 1 with an IsoQuick Nucleic Acid Extraction kit (ORCA Research, Bothell, WA) or a PureGene DNA Isolation kit (Gentra Systems, Minneapolis, MN).

Primer design for LAMP

The LAMP reaction was conducted according to the original reports by Nagamine et al. and Notomi et al. (17, 19). The oligonucleotide primers used in this study are listed in Table 2. The LAMP method requires a set of four specially designed primers (F3, B3, FIP and BIP), which recognize a total of six distinct sequences (F1, F2, F3, B1, B2 and B3) in the target DNA (Fig. 1). The two inner primers, FIP and BIP, contain two distinct sequences corresponding to the sense and antisense sequences of the DNA, one for priming in the first stage and the other for self-priming in later stages. The FIP consisted of the sequence of F1c and the sense sequence of F2 (5′-F1c-F2-3′). The BIP consisted of the sequences of B1 and B2c (5′-B1-B2c-3′). These four primers amplified the target DNA. We designed two additional loop primers: primer F, located between F1 and F2, and loop primer B, located between B1 and B2. The addition of two loop primers enhanced the specificity and reactivity (16). These two primers

Table 1. Bacterial strains used in this study

Actinobacillus actinomycetemcomitans ATCC 29522
Actinobacillus actinomycetemcomitans ATCC 29523
Actinobacillus actinomycetemcomitans ATCC 43718
Actinobacillus actinomycetemcomitans OMZ534
Actinobacillus actinomycetemcomitans SUNYaB67
Actinobacillus actinomycetemcomitans SUNYaB75
Actinobacillus actinomycetemcomitans NCTC9709
Actinobacillus actinomycetemcomitans NCTC9710
Actinobacillus actinomycetemcomitans TN-1
Actinobacillus actinomycetemcomitans Y4
Actinobacillus actinomycetemcomitans IDH781
Actinobacillus actinomycetemcomitans JP2
Actinobacillus actinomycetemcomitans ATCC 29524
Actinobacillus actinomycetemcomitans ATCC 43717
Porphyromonas gingivalis W50
Porphyromonas gingivalis 381
Porphyromonas gingivalis ATCC 33277
Treponema denticola ATCC 31211
Treponema medium ATCC 700293
Treponema vincentii ATCC 35580
Treponema socranskii subsp. *paredis* ATCC 35535
Treponema socranskii subsup. *socranskii* ATCC 35536
Treponema pectinovorum ATCC 33768
Prevotella intermedia ATCC 25611
Prevotella melaninogenica ATCC 25845
Prevotella nigrescens ATCC 25261
Prevotella loescheii ATCC 15930
Prevotella corporis ATCC 33547
Prevotella pallens ATCC 700821
Prevotella oralis ATCC 33322
Prevotella veroralis ATCC 33779
Prevotella loescheii ATCC 15930
Tannerella forsythia ATCC 43037
Fusobacterium nucleatum ATCC 10953
Haemophilus aphrophilus NCTC5980
Streptococcus mutans Xc
Streptococcus mutans OMZ175
Streptococcus mutans MT 703R
Streptococcus mutans MT8148
Streptococcus mutans UA159
Streptococcus sobrinus OU8
Streptococcus sobrinus OMZ176
Streptococcus sobrinus 6715
Streptococcus sobrinus AHT-K
Streptococcus sobrinus MT8145
Streptococcus mitis 903
Streptococcus sanguinis ATCC 10556
Streptococcus sanguinis OMZ9
Streptococcus sanguinis 556
Streptococcus gondonii DL1
Streptococcus oralis ATCC 10557
Streptococcus salivarius HT9R
Streptococcus cricentus HS1
Lactobacillus rhamnosus ATCC 7469
Lactobacillus rhamnosus ATCC 11443
Actinomyces viscosus ATCC 15988
Actinomyces naeslundii ATCC 12104
Actinomyces naeslundii ATCC 19039
Escherichia coli DH5α

Table 2. Oligonucleotide primers for loop-mediated isothermal amplification

Primer	Sequence (5′ → 3′)	Amplicon size (bp)	Target	Source (strain)
F3	CCTTTGAAATGGCGGAGGAA	221	*dam*	HK1651
B3	GGCGCCTTGGTAGATTTCG			
FIP	TGCCGGCGTAATTGGTGAAGTT-CCGTAATTTACTGCGACCCG			
B2P	CACCAGCGTGATCTCGCCAA-TGGTTGGAGATCAGCACTTG			
LF	TTGTGAAAGCGGGGCGTAGG			
LB	CACACCATGGAACAACGCAACA			

were designed using PRIMER EXPLORER software, version 3.0 (Fujitsu, Tokyo, Japan), as shown in Fig. 2 and Table 2. The primers for *A. actinomycetemcomitans* were designed from the *dam* genes, which encode DNA adenine methyltransferase (4). The specificities of the designed primers were initially confirmed using blast on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/) and then confirmed by LAMP for the chromosomal DNA of various oral bacteria (Table 1).

LAMP

For the LAMP reaction, a LAMP mixture was made in a 25-μl reaction volume containing 1.6 μM (each) of FIP and BIP, 0.2 μM (each) of F3 and B3, 0.8 μM (each) of loop F and loop B, 1.4 mM of each deoxynucleoside triphosphate, 0.8 M betaine, 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.2% Tween-20, 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs, Beverly, MA), and 5 μl of target DNA. The mixture was incubated at 67°C using a conventional heating block and was heated to over 80°C for 2 min to terminate the reaction.

Detection of the LAMP products

LAMP amplicons in the reaction mixture were detected by the naked eye on addition of 1.0 μl of 1/10-diluted original SYBR Green I (Molecular Probes, Eugene, OR) to the mixture and observation of the solution color. The solution turned green in the presence of a LAMP amplicon, while it remained orange with no amplification. Alternatively, the turbidity derived from the white precipitate of magnesium pyrophosphate in the mixture was detected visually (14). For electrophoretic analysis, 2 μl of reaction mixture was loaded on a 2% agarose gel, which was stained with ethidium bromide (1 mg/ml) and assessed photographically under ultraviolet light (302 nm). To confirm the structure of the LAMP product, the amplicon was digested with *Sau*3AI (Takara Bio, Shiga, Japan) and was subjected to electrophoresis. The specificity of LAMP products was confirmed by DNA sequencing.

Clinical samples

Human subgingival plaque was prepared as previously described (24). Briefly, subgingival plaque samples were obtained by inserting a sterile endodontic paperpoint into the subgingival site for 10 s. The paperpoint was transferred into 200 μl phosphate-buffered saline (0.12 M NaCl, 0.01 M Na_2HPO_4, 5 mM KH_2PO_4, pH 7.5) and centrifuged at 12,000 ***g*** for 5 min. The cells were resuspended in 100 μl cell lysis buffer (1.0% Triton X-100, 20 mM Tris–HCl, 2 mM EDTA, pH 8.0) (24) and boiled at 100°C for 5 min; the supernatant served as the template (24). Human saliva was prepared as described previously (20). Briefly, 500 μl of stimulated whole saliva and the same amount of phosphate-buffered saline were mixed and centrifuged at 12,000 ***g*** for 10 min; 500 μl cell lysis buffer were added to the precipitate, which was then incubated with 20 U mutanolysin/ml and 0.2 mg lysozyme/ml at 37°C for 2 h. The precipitate was spun and the chromosomal DNA from the bacteria was extracted by boiling the precipitate at 100°C for 10 min. One milligram (wet weight) of tongue coat was collected and washed with phosphate-buffered saline twice. The precipitate was suspended in 100 μl cell lysis solution and incubated with 20 U mutanolysin/ml and 0.2 mg lysozyme/ml at 37°C for 2 h. The lysate was boiled at 100°C for 10 min, and the chromosomal DNA was extracted.

Results

Specificity of LAMP for *A. actinomycetemcomitans*

The LAMP assay successfully amplified the 262-bp target sequence of the *A. actinomycetemcomitans dam* gene at 67°C in 60 min. The product was evident on agarose gel electrophoresis as a ladder-like pattern on the gel, which is characteristic of the LAMP reaction and indicates the production of stem-loop DNA with inverted repeats of the target sequence. The specificity of the amplification was confirmed by restriction endonuclease digestion with *Sau*3AI for the *A. actinomycetemcomitans* amplicon (Fig. 3) and DNA sequencing (data not shown). Furthermore, the specificity of this assay for *A. actinomycetemcomitans* was confirmed using various oral bacteria (Table 1). This assay successfully amplified all of the serotypes (a–e) of *A. actinomycetemcomitans* strains, but no other oral bacteria. LAMP amplified an extremely large amount of target DNA and produced magnesium pyrophosphate as a by-product; the magnesium pyrophosphate production was confirmed as white turbidity (data not shown). The LAMP mixture with *A. actinomycetemcomitans* genomic DNA appeared green on the addition of SYBR Green I, whereas the original orange color did not change in the negative control tube (data not shown).

Sensitivity of LAMP for *A. actinomycetemcomitans*

To ascertain the detection limit of the LAMP assay for *A. actinomycetemcomitans*, serial 10-fold dilutions of genomic DNA were tested. The lower detection limit for the LAMP assay was 580 copies for a 20-min reaction (with the loop primers) (Table 3). No amplification was apparent when the sample tube did not contain target DNA (negative control).

Quantitative LAMP assay

Figure 4 shows the results of real-time turbidity measurements in LAMP reaction solutions containing 5.8×10^2–5.8×10^7 copies of *A. actinomycetemcomitans* template DNA per reaction tube. An increase in the quantity of the initial template DNA shortened the threshold time. A plot of the amplification time required to exceed a turbidity level of 0.1 (threshold time) vs.

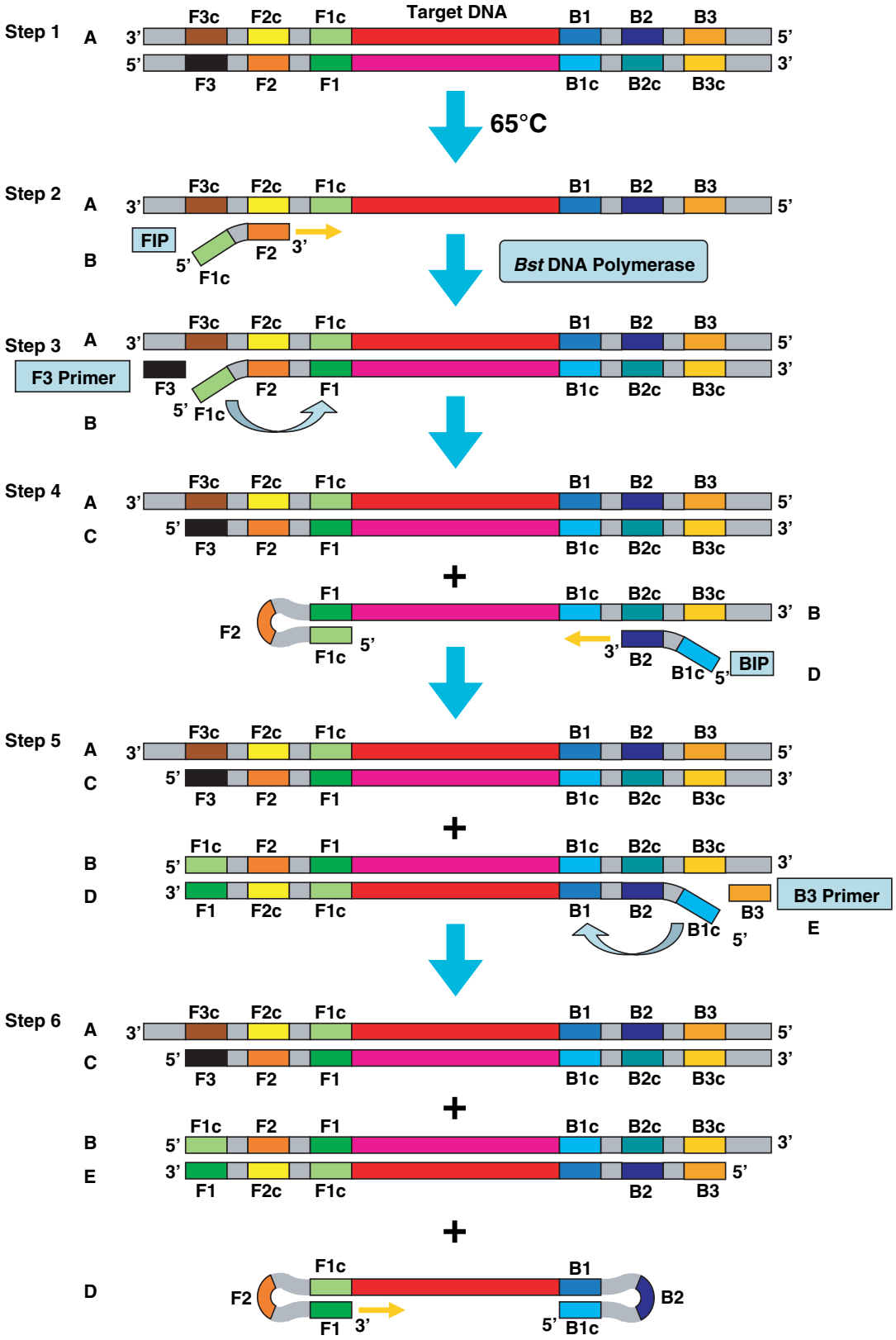

Fig. 1. The starting-material-producing steps of the loop-mediated isothermal amplification method. This figure shows the process starting from primer forward inner primer (FIP). The inner primer FIP hybridizes to F2c in the target DNA and initiates complementary strand synthesis (Step 2). The outer primer F3 slowly hybridizes to F3c in the target DNA and initiates strand displacement DNA synthesis, releasing an FIP-linked complementary strand, which can form a looped out structure at one end (Steps 3 and 4). This single-strand DNA serves as the template for back inner primer-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis, leading to the production of the dumb-bell form DNA (Steps 5 and 6).

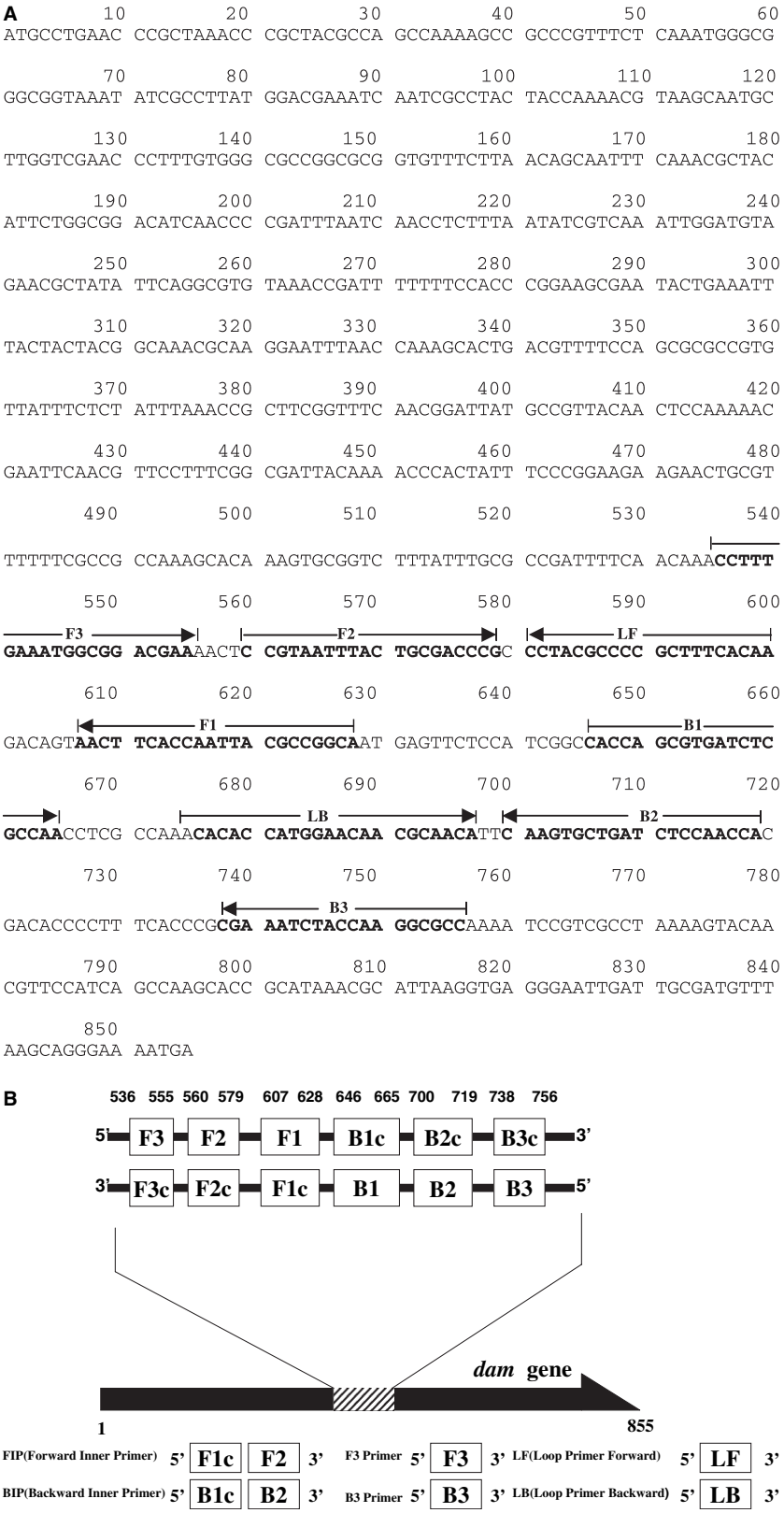

Fig. 2. (A) Nucleotide sequences of the *Actinobacillus actinomycetemcomitans dam* gene used for the loop-mediated isothermal amplification (LAMP) primers. The sequences used for the LAMP primers are shown in boldface, and are the consensus sequence among the *A. actinomycetemcomitans* strains. (B) Schematic representation of the primers used in this study.

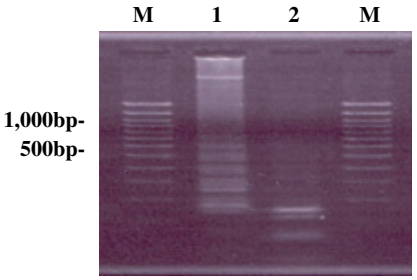

Fig. 3. Restriction analysis of the loop-mediated isothermal amplification products. Lane M, 100-bp DNA ladder (Promega, Madison, WI); lane 1, amplified products of *Actinobacillus actinomycetemcomitans dam*; lane 2, *A. actinomycetemcomitans dam* digested with *Sau*3AI.

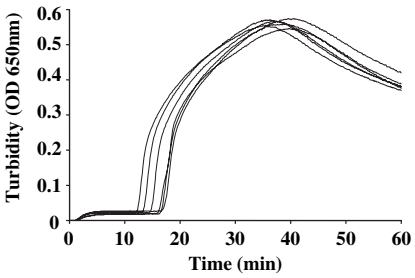

Fig. 4. The real-time sensitivity of *Actinobacillus actinomycetemcomitans* loop-mediated isothermal amplification, as monitored by measuring turbidity. The mean values of three independent experiments are plotted. After a 40-min reaction, the turbidity caused by the concentration of magnesium pyrophosphate decreased. Shown from left to right in the figure are the curves for decreasing concentrations (58,000,000–580) of bacteria. The detection limit was 580 copies.

the log of the initial template DNA showed a linear relationship, with a correlation coefficient of $r^2 = 0.9434$ (Fig. 5).

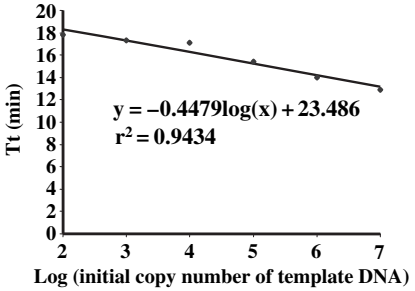

Fig. 5. The relationship between the threshold time (Tt) of each sample and the log of the amount of initial template DNA.

Rapid detection of *A. actinomycetemcomitans* from clinical specimens

Clinical cases with suspected periodontitis were submitted to our laboratory and were investigated both with LAMP and conventional PCR assays. No sample that was negative with the LAMP assay tested positive with PCR, and vice versa. We initially confirmed the inhibitory effects of the oral specimens on LAMP. The presence of LAMP inhibitors in subgingival plaque was assessed using lysates spiked with *A. actinomycetemcomitans*-negative saliva (1 µl per mixture) and dental plaque [ca 1 µl (wet weight) per mixture, mimicking subgingival plaque], and they inhibited the assay negligibly (data not shown). Therefore, we applied the assay to the rapid detection of *A. actinomycetemcomitans* in subgingival plaque from eight individuals (Table 4). As shown in Table 4, various detection patterns were observed.

Discussion

Recently, Eiken Chemical developed the LAMP technique, which is a novel nucleic acid amplification method (16, 19). The LAMP assay reported here is advantageous because of its rapid reaction, simple operation and easy detection. LAMP amplifies the target DNA without the temperature changes normally required for denaturing, annealing and extending DNA. LAMP operates under isothermal conditions at about 65°C for 1 h, making LAMP a simple, timesaving procedure, which allows the results to be obtained within 1 h of extracting the bacterial genome. No time is lost as a result of changes in temperature, as is the case with thermal cycling for PCR. Moreover, LAMP requires only simple reaction equipment; it can be performed using a regular laboratory bath or a heating block that provides a constant temperature of about 65°C. LAMP has been used to detect several viruses and bacteria (7, 8, 10, 11); recently, LAMP assays were developed for *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (26). There is a strong correlation between mixed infections by these organisms and adult periodontitis (9). In this study, we developed a LAMP method for the rapid detection of *A. actinomycetemcomitans*, which cannot be detected using an enzyme reaction, in comparison to other periodontal pathogens such as *P. gingivalis*, *T. forsythia* and *T. denticola*, which have been detected enzymatically (26). Therefore, we focused on a LAMP method for the rapid detection of *A. actinomycetemcomitans* and here report on its first application.

First, we evaluated the specificity of the LAMP assays toward this organism. The LAMP method was both highly specific and highly efficient. Since this method uses four primers that recognize six distinct sequences on the target DNA, its specificity is extremely high. Therefore, this is a potentially valuable tool for the rapid diagnosis of oral infectious diseases, in that over 500 species or phylotypes have been detected from the oral cavity. In this study, we initially examined the specificity of this assay using chromosomal DNA from various oral bacteria. The LAMP primers used in this study successfully amplified serotypes a–e of *A. actinomycetemcomitans*, while other oral bacteria were not amplified (Table 1). The LAMP method also has an extremely high amplification efficiency, due in part to its isothermal nature; there is no time lost arising from changes in temperature for enzyme function, and the inhibition reaction that occurs at later stages of amplification, a typical problem with PCR, is less likely to take place. In this study, we evaluated the detection limits of this assay using serially diluted chromosomal DNA. The lower detection limit with loop primers in a 60-min reaction was 5.8×10^2

Table 3. Detection sensitivities of loop-mediated isothermal amplification

	Genomic DNA (CFU/tube)										
Time (min)	0	5.8	5.8×10^1	5.8×10^2	5.8×10^3	5.8×10^4	5.8×10^5	5.8×10^6	5.8×10^7	5.8×10^8	5.8×10^9
0	–	–	–	–	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–	–	–	±	±
20	–	+	+	+	+	+	+	+	+	+	+
30	–	+	+	+	+	+	+	+	+	+	+
40	–	+	+	+	+	+	+	+	+	+	+
50	–	+	+	+	+	+	+	+	+	+	+
60	–	+	+	+	+	+	+	+	+	+	+

Table 4. Patient characteristics and result of loop-mediated isothermal amplification (LAMP) detected in subgingival plaque samples

Patient no.	Age (year)	Gender	Pocket depth (probing region)	PCR			LAMP (CFU/tube)		
				Subgingival plaque	Saliva	Tongue coat	Subgingival plaque	Saliva	Tongue coat
1	66	F	2 mm (23)	+	−	+	+ $(3.35 \times 10^1 \pm 0.03 \times 10^1)^1$	−	+ $(2.20 \times 10^2 \pm 0.77 \times 10^2)^1$
2	67	F	3 mm (15)	+	−	+	+ $(1.01 \times 10^1 \pm 0.16 \times 10^1)^1$	−	+ $(3.39 \times 10^2 \pm 0.21 \times 10^2)^1$
3	35	F	3 mm (41)	+	−	−	+ $(4.20 \times 10^0 \pm 0.13 \times 10^0)^1$	−	−
4	58	M	2 mm (31)	+	−	−	+ $(5.67 \times 10^2 \pm 0.49 \times 10^2)^1$	−	−
5	51	F	3 mm (34)	+	+	−	+ $(2.86 \times 10^2 \pm 0.47 \times 10^2)^1$	+ $(1.03 \times 10^3 \pm 0.16 \times 10^3)^1$	−
6	43	F	3 mm (17)	+	+	−	+ $(2.20 \times 10^2 \pm 0.17 \times 10^2)^1$	+ $(1.00 \times 10^{-1} \pm 0.13 \times 10^{-1})^1$	−
7	24	M	4 mm (36)	±	−	−	+ $(2.57 \times 10^0 \pm 0.22 \times 10^0)^1$	−	−
8	41	F	5 mm (14)	+	−	−	+ $(1.90 \times 10^4 \pm 0.67 \times 10^4)^1$	−	−

+, clearly visible; ±, visible but not clear; −, not visible.
[1]Theoretical data below the detection limits.

colony-forming units/tube for *A. actinomycetemcomitans*. This sensitivity is consistent with previous studies (5).

To diagnose infectious disease, a quantitative analysis of bacteria is required. Quantitative detection using the LAMP technique is available by measuring the precipitation of magnesium pyrophosphate, a by-product of the LAMP reaction (15). As shown in Fig. 5, the standard curve obtained using the real-time turbidity measurements was linear. Therefore, the quantity of template DNA at an unknown concentration can be determined by comparing its threshold time with the threshold times of template DNA of known concentrations. To make the diagnosis of progressive periodontitis, quantification of *A. actinomycetemcomitans* is required. Therefore, using the real-time turbidity monitoring system, we estimated the number of *A. actinomycetemcomitans* from clinical samples and evaluated this system. Using this assay system, we examined oral specimens from eight *A. actinomycetemcomitans*-positive individuals and determined the clinical characteristics of the patients (Table 4). As shown in this table, we could detect *A. actinomycetemcomitans* from the oral samples without any inhibition (data not shown). Therefore, we confirmed that this assay system could be used for the clinical diagnosis of progressive periodontitis.

Our ultimate goal is to establish a simple, rapid examination system for periodontal pathogens that can be used in private clinics or at the dental chair-side. Combined with the previous LAMP-based assays for adult periodontitis, this detection system is a powerful tool for the diagnosis of periodontitis. In this paper, we reported a simple, sensitive, reliable, rapid diagnostic method for detecting *A. actinomycetemcomitans* based on genome amplification. Nevertheless, further research is needed on the quantification of bacterial DNA using LAMP. We plan to evaluate the reliability of the LAMP-based quantitative detection method by comparing it with other assay systems in a future study.

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