RESEARCH ARTICLE

Rapid and simple detection of eight major periodontal pathogens by the loop-mediated isothermal amplification method

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Abstract

Loop-mediated isothermal amplification (LAMP) was applied to develop a rapid and simple detection system for eight periodontal pathogens: *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, *Campylobacter rectus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola* and *Tannerella forsythia*. Primers were designed from the 16S ribosomal RNA gene for each pathogen, and the LAMP amplified the targets specifically and efficiently under isothermal condition at 64 °C. To simplify the manipulation of LAMP examination, boiled cells and intact cells suspended in phosphate-buffered saline (PBS) were tested as templates besides extracted DNA template. The detection limits were 1–10 cells per tube using extracted DNA template. However, LAMP methods using boiled cells and intact cells required 10–100 and 100–1000 cells per tube, respectively. LAMPs for *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* were then applied to clinical plaque samples, and the method demonstrated equal or higher sensitivity compared with the conventional real-time PCR method. These findings suggest the usefulness of the LAMP method for the rapid and simple microbiological diagnosis of periodontitis, and the possibility of LAMP examination without the DNA extraction step.

Introduction

Recent advances in molecular biological techniques enabled one to detect periodontal pathogens without cultivation. PCR is the typical example and is now widely used for the microbiological diagnosis of periodontitis (Slots *et al*., 1995; Harper-Owen *et al*., 1999; Maeda *et al*., 2003). PCR is very sensitive and highly specific and, compared with cultural methods, can be performed in a relatively rapid and simple fashion. However, the method requires special reagents and apparatus (such as a thermalcycler) and is still time-consuming to perform at chair-side or bedside.

Loop-mediated isothermal amplification (LAMP), a novel DNA amplification method, was originally developed by Notomi *et al*. (2000). The method relies on auto-cycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment. The LAMP reaction can be conducted under isothermal conditions ranging from 60 to 65 °C. The specificity is attributable to four primers that recognize six distinct sequences (Notomi *et al*., 2000). Continuous amplification under isothermal conditions produces an extremely large amount of target DNA within 30–60 min. Therefore, the method demonstrates high sensitivity and enables simple visual (naked-eye) judgment of DNA amplification through a color change of the reaction mixture with SYBR green I (Iwamoto *et al*., 2003). Further, it has recently been reported that LAMP can be accelerated using additional primers, termed loop primers (Nagamine *et al*., 2002). Because the method requires only one type of enzyme and special apparatus is not needed, LAMP is promising in the rapid and simple detection of microorganisms (Enosawa *et al*., 2003; Iwamoto *et al*., 2003; Parida *et al*., 2004; Savan *et al*., 2004) and may be suitable for routine examinations at dental chair-side.

Some attempts have already been made to detect several periodontal pathogens by LAMP, demonstrating the utility

(Maeda *et al*., 2005; Yoshida *et al*., 2005). In this study, we expanded the application of the LAMP method to eight major periodontal pathogens – *Aggregatibacter (Actinobacillus) actinomycetemcomitans*, *Campylobacter rectus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola* and *Tannerella forsythia* – and evaluated their sensitivity and specificity. Further, intact cells and boiled cells were tested as templates besides the extracted DNA template to examine the influence on LAMP sensitivity.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study were as follows: *A. actinomycetemcomitans* Y4, *C. rectus* ATCC 33238, *E. corrodens* ATCC 23834, *F. nucleatum* ATCC 25586, *P. gingivalis* 381, *P. intermedia* ATCC 25611, *T. denticola* ATCC 35405, *T. forsythia* ATCC 43037. *Porphyromonas gingivalis*, *F. nucleatum* and *P. intermedia* were cultured in modified GAM broth (Nissui Seiyaku Inc., Tokyo, Japan) at 37 °C in an anaerobic box (Model ANX-1; Hirasawa Works, Tokyo, Japan) containing 80% N_2, 10% H_2 and 10% CO_2. *Aggregatibacter (Actinobacillus) actinomycetemcomitans*, *C. rectus* and *T. denticola* were cultivated according to the methods described previously (Kokeguchi *et al*., 1989, 1991; Miyamoto *et al*., 1991). Cultured *T. forsythia* was kindly provided by Dr T. Hino (Hiroshima University Graduate School of Biomedical Science). The cells were harvested by centrifugation at 10 000 ***g*** for 20 min at 4 °C, and cell numbers were determined using a Petroff–Hauser counting chamber and a phase-contrast microscope.

Preparation of the LAMP template

InstaGene Matrix (Bio-Rad Lab, CA) was used for DNA extraction from cultivated strains and clinical dental plaque samples according to the manufacturer's instructions. Briefly, plaque samples and cultivated bacteria were suspended in 1 mL of phosphate-buffered saline [PBS (−)] (Gibco BRL, MD) and were pelleted and resuspended in 100 μL of InstaGene Matrix. The suspension was incubated at 56 °C for 30 min and then at 100 °C for 8 min. After the incubation, the suspension was centrifuged and 2 μL of the resulting supernatant was used as a template for LAMP and conventional real-time PCR (Maeda *et al*., 2003). To simplify the examination system, intact cells and boiled cells were tested as template besides the DNA extracted sample. Bacterial cells were suspended in 100 μL PBS (−), and 2 μL of the bacterial suspension was used for LAMP directly or after a boiling step for 5 min. To examine a detection limit of the LAMP method, templates were prepared from serial diluted cells of each periodontal pathogen.

Clinical samples

Sampling of subgingival plaque from periodontitis patients was performed at Okayama University Hospital of Medicine and Dentistry after obtaining informed consent. Samples were obtained from their periodontal pockets by inserting paper points (#45, Zipperer, Germany) as described previously (Nishimura *et al*., 1990), and DNA templates were prepared by InstaGene Matrix. The DNA samples were used for clinical microbiological diagnosis by conventional real-time PCR (Maeda *et al*., 2003). By real-time PCR, total bacterial counts, and numbers of *A. actinomycetemcomitans*, *P. intermeia* and *P. gingivalis* in the samples were quantified, and the remaining plaque DNA samples were stored at − 30 °C. Ten plaque DNA samples including $> 10^6$ of total bacterial counts and various numbers of the three species were selected from the sample stocks for the LAMP analysis.

Primers for LAMP

Primers for LAMP were designed to target the 16S rRNA gene. Primer Explorer version 2 (Fujitsu, Tokyo, Japan) was used at the Net Laboratory website (http://www.netlaboratory.com/) to nominate the candidate primers. A set of four primers, a forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3) (Notomi *et al*., 2000), containing species-specific sequences of the 16S rRNA gene, was selected from the candidates for each pathogen. Before the selection, species-specific regions on the 16S rRNA gene were identified by alignment of the published sequence data from the GenBank at the National Center for Biotechnology Information website. For the alignment, 16S rRNA gene sequences of *Prevotella nigrescens*, *Porphyromonas endodontalis* and *Escherichia coli* were also compared with their eight sequences in this study. One or two loop primers (LF, LB) were designed for each target and used to accelerate the reactions (Nagamine *et al*., 2002).

LAMP reaction

The LAMP reaction for each periodontal pathogen was carried out using a Loopamp DNA amplification kit (Eiken Chemical Co. Ltd, Tochigi, Japan) in a 25-μL volume. The reaction mixture contained 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3 primers, 2 μL of template, 1 μL of *Bst* DNA polymerase (8 U) and 12.5 μL of reaction mixture prepared in the kit. For acceleration of the LAMP reaction, 20 pmol of loop primer (LB or each of LF and LB) was added to the reaction mixture. The reaction mixture was incubated at 62, 64 or 66 °C for 60 min. After the incubation, the reaction was terminated by heating the reaction mixture at 80 °C for 2 min.

Detection of LAMP products

The LAMP product was detected by naked-eye inspection or agarose gel electrophoresis. For naked-eye detection, 1.0 μL of 10^{-1}-diluted SYBR Green I (Takara Bio Inc., Otsu, Japan) was added to the reaction mixture, and the color change was observed. For the electrophoretic analysis, 2 μL of reaction mixture was loaded on 2% agarose gel. The gel was stained with ethidium bromide (1 mg mL^{-1}) and assessed photographically under UV light (302 nm).

Real-time PCR

Real-time PCR was performed for quantitative detection of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* as described previously (Maeda *et al.*, 2003). The GENEAMP^{R} 5700 SEQUENCE DETECTION SYSTEM (PE Applied Biosystems) was used for monitoring the fluorescence from dsDNA-binding SYBR Green I. The PCR mixture contained 2 × SYBR Green PCR Master Mix (PE Applied Biosystems), 20 pmol of forward and reverse primer, and 2 μL of extracted DNA. Both forward and reverse primers were designed from the species-specific regions of 16S rRNA gene (Maeda *et al.*, 2003). The thermocycling program was 40 cycles of 95 °C for 15 s and 60 °C for 1 min with an initial cycle of 95 °C for 10 min. The data were analyzed using the GENEAMP 5700 SDS software (PE Applied Biosystems).

Results and discussion

Primer design and specificity of LAMP

Primer sets for the LAMP of eight periodontal pathogens are listed in Table 1. The primer sets were designed to include species-specific sequences of the 16S rRNA gene. Locations of the LAMP primers in the 16S rRNA gene sequence were shown in supplementary Fig. S1. Because species-specific sequences are conveniently identified, the 16S rRNA gene is now widely used as a target for molecular microbiological diagnosis. By targeting the 16S rRNA gene of a multicopy gene, high sensitivity would be expected. On the other hand, the sequence similarity of 16S rRNA gene among the closely related species sometimes causes nonspecific amplification. However, because LAMP was a highly specific method that required six distinct sequences for the amplification, the 16S rRNA gene was used in the current study.

Before the serial experiments, optimum reaction temperature was determined using the designed primers. The reactions of LAMP for each periodontal pathogen were performed under isothermal conditions at 62, 64 or 66 °C for 60 min using the DNA-extracted template from 10^3 cells of each target. LAMP was performed successfully under the three different thermal conditions. The LAMP product amplified at 64 °C exhibited a slightly larger amount of DNA as compared with other conditions in all tested strains (data not shown). Therefore, the series of experiments were performed at 64 °C.

The specificity of LAMP was examined using an extracted DNA template prepared from a cell mixture of all tested species (10^3 cells of each species) or a cell mixture of seven species (10^3 cells of each species) without the target. The LAMP products were electrophoretically detected and are shown in Fig. 1. LAMP reactions with the designed primer set for each periodontal pathogen successfully amplified the DNA by using the DNA-extracted template including all tested species. The LAMP products appeared as a ladder-like pattern on the agarose gel because of its characteristic structure (Notomi *et al.*, 2000); however, the primer sets did not amplify the DNA with the extracted DNA template prepared from the cell mixture excluding the target (Fig. 1). These results demonstrated the high specificity of the designed primer sets of LAMP for each periodontal pathogen.

Sensitivity of LAMP

For sensitivity testing, LAMP reaction was performed for 30 or 60 min using serial diluted templates prepared by Insta-Gene Matrix. The LAMP amplicons were detected by both agarose gel electrophoresis and naked-eye inspection. The results of electrophoretic detection for the 60-min LAMP are shown in Fig. 2. The detection limits of LAMP for the tested periodontal pathogens were less than one cell per tube except for *E. corrodens*. DNA amplification was occasionally seen in the seven species when the amount of template DNA was genome equivalent in 0.5 cell (Aa, Cr, Pg, Td and Tf in Fig. 2). By targeting the 16S rRNA of multicopy gene, high sensitivity was obtained. Ten target cells were required as template for the detection of *E. corrodens*. The LAMP conditions for the tested species were the same other than the primers. The lower sensitivity of LAMP for *E. corrodens* may be due to the primer design, and a new designed primer set would be needed for higher sensitivity. However, because the current sensitivity was not critically inferior to other PCR-based molecular techniques (Slots *et al.*, 1995; Harper-Owen *et al.*, 1999; Lyons *et al.*, 2000; Sakamoto *et al.*, 2001; Maeda *et al.*, 2003), the method is possibly applicable to clinical examination.

Compared with the 60-min LAMP, the sensitivity of the 30-min LAMP declined in all cases. Ten template cells were required for the detection of seven periodontal pathogens except for *E. corrodens*, and 100 cells were required for the detection *of E. corrodens* by the 30-min LAMP. The naked-eye inspection for each periodontal pathogen demonstrated equal detection limits to the electrophoretic analysis in both 30- and 60-min LAMP reactions (data not shown).

Table 1. LAMP primers for the periodontal pathogens

Target species	Primer	Sequence
A. actinomycetemcomitans	FIP	5′-CCCCACGCTTTCGCACATCATACCGAAGGCGAAGGCAG-3′
	BIP	5′-AGATACCCTGGTAGTCCACGCTTTCGGGCACCAGGGCTAAAC-3′
	F3	5′-TGCGTAGAGATGTGGAGGAA-3′
	B3	5′-GGCGGTCGATTTATCACGT-3′
	LB	5′-AAACGGTGTCGATTTGGGGAT-3′
C. rectus	FIP	5′-CGTCATAGCCTTGGTAGGCCGTAGGATGAGGCTATATCGTAT-3′
	BIP	5′-AGTCACACTGGAACTGAGACGGGTTTCCCCCATTGAGC-3′
	F3	5′-AGTCGGGAAAGTTTTCGGTG-3′
	B3	5′-AAAGTGTCATCCTCCACGC-3′
	LB	5′-ACGGTCCAGACTCCTACGG-3′
E. corrodens	FIP	5′-GGTAGGCCTTTACCCCACCAACCAAGACCTCGCGTTATTCGA-3′
	BIP	5′-ACGATCAGTAGCGGGTCTGAGAAATTCCCCACTGCTGCTC-3′
	F3	5′-CTAATACCGCATACGTCCTA-3′
	B3	5′-GTTGCCCCCATTGTCCAA-3′
	LB	5′-TGAGACACGGCCGCTCCTA-3′
F. nucleatum	FIP	5′-TCCAATATTCCCCACTGCTGCCGCCACAAGGGGACTGAGA-3′
	BIP	5′-TCCAGCAATTCTGTGTGCACGAGTACCGTCATTTTTTTCTTC-3′
	F3	5′-AGGCGATGATGGGTAGCC-3′
	B3	5′-AGCCGTCACTTCTTCTGTTG-3′
	LF	5′-TCCCGTAGGAGTAAGGGCC-3′
	LB	5′-TCGGAATGTAAAGTGCTTTCAGTTG-3′
P. gingivalis	FIP	5′-CACCACGAATTCCGCCTGCCTGAGCGCTCAACGTTCAGCC-3′
	BIP	5′-ATCACGAGGAACTCCGATTGCGCGCCTTCGTGCTTCAGTG-3′
	F3	5′-GGTAAGTCAGCGGTGAAACC-3′
	B3	5′-GCGTGGACTACCAGGGTAT-3′
	LB	5′-GCAGCTTGCCATACTGCGA-3′
P. intermedia	FIP	5′-CGTTACCCGCACCAACAAGCTAATATGGCATCTGACGTGGAC-3′
	BIP	5′-GCCCACCAAGGCTCGATCAGGGACCGTGTCTCAGTTCCA-3′
	F3	5′-ACGGCCTAATACCCGATGTT-3′
	B3	5′-CTGCCTCCCGTAGGAGTT-3′
	LF	5′-CATCCTCCACCGATGAATCTTTG-3′
	LB	5′-TAGGGGTTCTGAGAGGAAGGT-3′
T. denticola	FIP	5′-CATCCTGAAGCGGAGCCGTAGTACCGAATGTGCTCATTTAC-3′
	BIP	5′-GCTGGTTGGTGAGGTAAAGGCCATCTCAGTCCCAATGTGTCC-3′
	F3	5′-CCCTGAAGATGGGGATAGCT-3′
	B3	5′-TGCCTCCCGTAGGAGTTTG-3′
	LB	5′-CACCAAGGCAACGATGGGTAT-3′
T. forsythia	FIP	5′-CCATCCGCAACCAATAAATCTCTAATACCTCATAAAACAGG-3′
	BIP	5′-TAAGCCATCGATGGTTAGGGCGTGTCTCAGTACCAGTGTG3′
	F3	5′-GATAACCCGGCGAAAGTCG-3′
	B3	5′-TGCCTCCCGTAGGAGTCT-3′
	LB	5′-GTTCTGAGAGGAAGGTCCCC-3′

Influence of the template preparation procedure

For further application of the LAMP method to clinical diagnosis, we attempted to simplify template preparation. Besides the extracted DNA template by InstaGene Matrix, intact cells and boiled cells were tested as templates to examine the influence of crude templates on sensitivity. The LAMP reaction was performed for 60 min and the amplicon was detected by agarose gel electrophoresis. The seven tested species except for *E. corrodens* demonstrated the same results; that the detection limits for boiled cells and intact cells were 10 and 100 cells per tube, respectively. The representative result of *A. actinomycetemcomitans* is shown in Fig. 3. For the detection of *E. corrodens*, 10 times more cells were required compared with other species. The results suggested the possibility of the direct use of the plaque sample for the LAMP assay. Because intact bacteria required 100 times the cell number for DNA amplification as compared with extracted DNA template, 1% of bacterial cells were estimated to be injured during the manipulation and incubation step at 64 °C.

The sensitivity of LAMP for the crude templates (10–100 cells per tube) was higher than that of culture

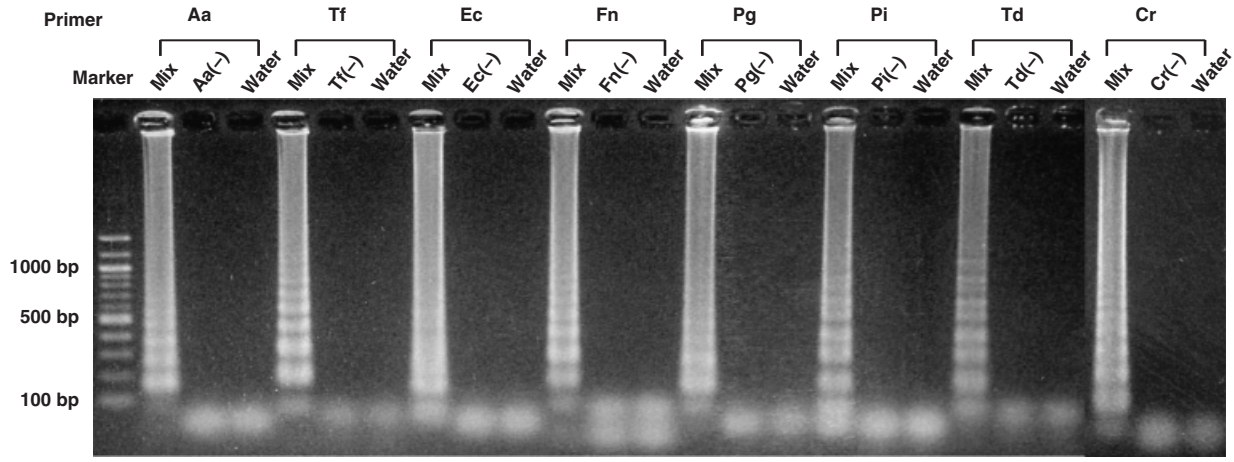

Fig. 1. Specificity of LAMP. The LAMP primer set was designed for each periodontal pathogen and the specificity was tested. Abbreviations Aa, Tf, Ec, Fn, Pg, Pi, Td and Cr represent *Aggregatibacter* (*Actinobacillus) actinomycetemcomitans*, *Tannerella forsythia*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola* and *Campylobacter rectus*, respectively. The LAMP product was electrophoretically detected on 2% agarose gel. DNA amplification was observed in all LAMP reactions for each periodontal pathogen with the DNA-extracted template including 10^3 cells of all tested species (lane Mix). No amplicon was seen when template DNA was prepared from the mixture of seven species (10^3 cells of each species) excluding the target species [lane (-) with the abbreviation of excluded species]. No amplicon was seen in the negative control of the water template.

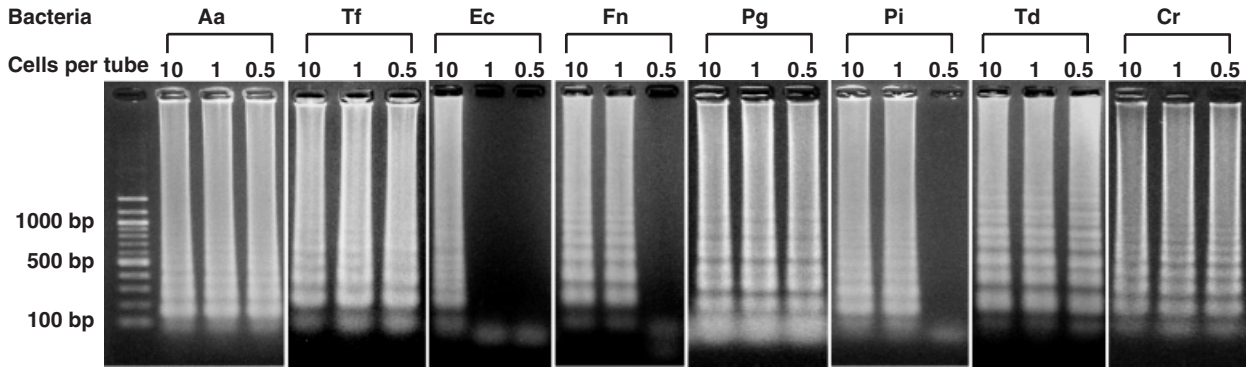

Fig. 2. Detection limit of LAMP. The DNA-extracted template was prepared from various cell numbers of each target, and LAMP was carried out at 64 °C for 60 min. The LAMP product was electrophoretically detected on 2% agarose gel. The target cell number equivalent to one reaction tube was shown on each lane. The detection limit of LAMP was less than one cell per tube except for *Eikenella corrodens*. The sensitivity tests were performed individually five times, and DNA amplification was occasionally seen when the amount of template DNA was equivalent to 0.5 cell. LAMP required 10 cells for the detection of *E. corrodens*. Abbreviations of the tested species correspond to those in Fig. 1.

methods (Lau *et al*., 2004; Boutaga *et al*., 2005), but was lower than the PCR method (Slots *et al*., 1995; Harper-Owen *et al*., 1999; Lyons *et al*., 2000; Sakamoto *et al*., 2001; Maeda *et al*., 2003). The relationship between the presence of periodontal pathogens and the clinical aspects of periodontitis have been demonstrated (Socransky *et al*., 1998; Tran & Rudney, 1999). Contrary to this, a few reports demonstrated the relationship between the quantities of periodontal pathogens and the clinical aspects (Socransky *et al*., 1991; Kawada *et al*., 2004). In addition, most periodontal pathogens are found in periodontally healthy subjects (Tran & Rudney, 1999) and the amounts of periodontal pathogens in periodontal pockets have been reported to vary a great deal (Socransky *et al*., 1991; Kawada *et al*., 2004). Therefore, the diagnostic cut-off of the quantities of periodontal pathogen cannot be defined so far. Eick & Pfister (2002) reported the cut-off of genome equivalents in 10^3–10^4 cells of periodontal pathogens using the microDent® kit (Hain Diagnostika, Germany) based on the PCR method. The utility of the kit was demonstrated in the report, but the definition of the cut-off seems to be unclear. In the current study, we cannot conclude that the crude templates are sufficient for the microbiological diagnosis of periodontitis. Simple DNA-purification methods using commercially

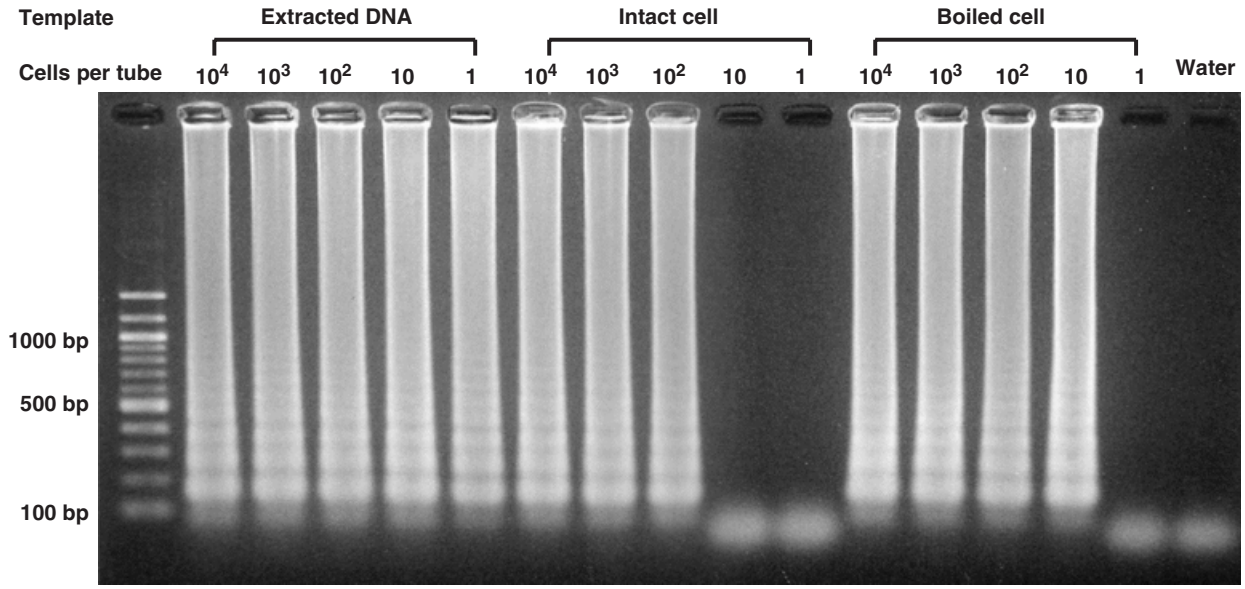

Fig. 3. Influence of the crude template on LAMP. Intact bacterial cells suspended in PBS (−) and boiled cells were tested as template besides the DNA-extracted template, and the influence on sensitivity was examined. Each LAMP was performed for 60 min, and the product was detected on 2% agarose gel. A representative result of *Aggregatibacter* (*Actinobacillus) actinomycetemcomitans* was demonstrated. The number on the gel denoted the target cell number equivalent to the amount of template in a reaction tube. The detection limit of LAMP using boiled cells and intact cells were 10 and 100 cells per tube, respectively.

available kits may give us a better result. However, it seems necessary to accumulate clinical data by quantitative, molecular-based microbiological methods before creating a guideline of microbiological diagnosis for periodontitis.

Examination of the clinical plaque sample

To evaluate the practicability, LAMP methods for *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* were applied to the clinical plaque samples. We have previously established a real-time PCR method for quantitative detection of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* (Maeda *et al*., 2003). The method is now applied to the clinical diagnosis of periodontitis in our hospital. DNA-extracted samples are being prepared from subgingival plaque, and the samples are stored at $-30\,^{\circ}C$ after the examination. From the sample stock, five samples containing more than one cell $2\,\mu L^{-1}$ (one cell per reaction tube) of the target and five samples containing less than one cell $2\,\mu L^{-1}$ (less than the quantitative range of real-time PCR) of the target were selected for each LAMP of the three periodontal pathogens. The LAMP reaction was performed for 60 min, and the LAMP product was detected by agarose gel electrophoresis. A representative result of *A. actinomycetemcomitans* is shown in Fig. 4. The LAMP amplicon was seen in all cases when the real-time PCR detected more than one cell $2\,\mu L^{-1}$ of the target. One of the five was positive in the LAMP for *A. actinomycetemcomitans* and *P. gingivalis*, and four of the five were positive in the detection of *P. intermedia* when the cell number in the template was less than one cell. Because the cell number was less than one cell $2\,\mu L^{-1}$, it was out of quantitative range of the real-time PCR, and the accurate cell number in the clinical sample was unknown. However, these results demonstrated equal or higher sensitivity of LAMP as compared with real-time PCR. In addition, sensitivity was at equal level with the LAMP using the extracted DNA templates from cultivated bacteria. This result implied that the contamination of bacterial species other than the target or the contamination of human cells during the sampling step is not so critical as to influence the sensitivity. Although the crude template without the DNA-extraction step was not tested for clinical samples, these findings may suggest that a relatively high sensitivity would be expected. In the current study, the LAMP for *C. rectus*, *E. corrodens*, *F. nucleatum*, *T. denticala* and *T. forsythia* was not applied to clinical samples, and the applicability remains to be elucidated.

High sensitivity is one of the advantages of the LAMP method. However, the high sensitivity of LAMP sometimes causes a problem. We have recently established a LAMP method for the detection of *P. gingivalis* (Maeda *et al*., 2005). Using two loop primers, the method demonstrated high sensitivity with a detection limit of 1–2 copy genes per tube. We applied the method for routine clinical examinations and had the problem of nonspecific amplification in the negative control of the water template (unpublished data). It

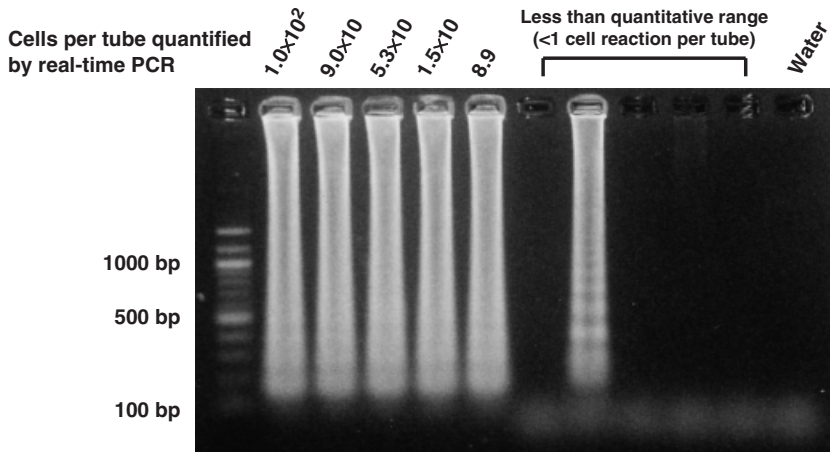

Fig. 4. Analysis of clinical plaque samples. The extracted DNA template was prepared from the subgingival plaque sample and was subjected to analysis by both real-time PCR and the LAMP method for *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia*. Before LAMP analysis, the target cell number in the reaction tube was quantified by real-time PCR, and an equal volume (2 μL) of the template was used for LAMP. Five samples containing numerous targets and five samples containing the targets under the quantitative detection limit of real-time PCR were selected for each LAMP. The LAMP products were detected from all samples that contained more targets than the quantitative detection limit of the real-time PCR. However, a positive result was obtained occasionally from samples under the quantitative range of the real-time PCR (one positive in the LAMP for *A. actinomycetemcomitans* and *P. gingivalis*, and four positives for *P. intermedia*). A representative result of *A. actinomycetemcomitans* was demonstrated.

is theoretically impossible to amplify DNA from water, and therefore a cause of the nonspecific amplification was considered to be contamination of *P. gingivalis* during the manipulations. Aerosol from the pipette might be the most probable cause. In the current study, a new primer set with only one loop primer was redesigned for *P. gingivalis*. Although the sensitivity declined a little, nonspecific amplification from the water sample has never been seen so far. Considering the high sensitivity of LAMP, scrupulous manipulations are required throughout the examinations.

The greatest advantage of the LAMP method is its simplicity and the rapidity attributed to continuous amplification under isothermal conditions within an hour (Notomi *et al*., 2000). As expected, each LAMP method for the eight periodontal pathogens was accomplished in an hour, and naked-eye inspection was possible with high specificity and sensitivity. Further, the trial of the crude template suggests the applicability of the LAMP method without the DNA-extraction step. Considering these findings, the LAMP methods established in the current study are supposed to be powerful tools for the microbiological diagnosis of periodontitis, especially in places such as private clinics, bedsides or dental chair-sides.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. Location of the LAMP primers.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-695X.2008.00417.x (This link will take you to the article abstract).

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