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Detection of periodontal pathogen *Porphyromonas gingivalis* by loop-mediated isothermal amplification method

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Abstract

A method for nucleic acid amplification, loop-mediated isothermal amplification (LAMP) was employed to develop a rapid and simple detection system for periodontal pathogen, *Porphyromonas gingivalis*. A set of six primers was designed by targeting the 16S ribosomal RNA gene. By the detection system, target DNA was amplified and visualized on agarose gel within 30 min under isothermal condition at 64 °C with a detection limit of 20 cells of *P. gingivalis*. Without gel electrophoresis, the LAMP amplicon was directly visualized in the reaction tube by addition of SYBR Green I for a naked-eye inspection. The LAMP reaction was also assessed by white turbidity of magnesium pyrophosphate (a by-product of LAMP) in the tube. Detection limits of these naked-eye inspections were 20 cells and 200 cells, respectively. Although false-positive DNA amplification was observed from more than 10^7 cells of *Porphyromonas endodontalis*, no amplification was observed in other five related oral pathogens. Further, quantitative detection of *P. gingivalis* was accomplished by a real-time monitoring of the LAMP reaction using SYBR Green I with linearity over a range of 10^2–10^6 cells. The real-time LAMP was then applied to clinical samples of dental plaque and demonstrated almost identical results to the conventional real-time PCR with an advantage of rapidity. These findings indicate the potential usefulness of LAMP for detecting and quantifying *P. gingivalis*, especially in its rapidity and simplicity.

Keywords: Loop-mediated isothermal amplification; *Porphyromonas gingivalis*; Periodontitis

1. Introduction

Porphyromonas gingivalis, an oral black-pigmented anaerobic Gram-negative bacterium, has been frequently isolated from diseased lesions of periodontitis and strongly implicated as a key etiological agent of periodontal diseases [1–3]. For microbiological diagnosis of periodontal diseases, several techniques such as immunoassay, biochemical tests and hybridization using nucleotide probes have been developed [4]. However, since all these methods required bacterial culture, isolation and identification of anaerobic species such as *P. gingivalis*, have been laborious and time-consuming for routine clinical examinations. Recently advances of molecular biological techniques enabled culture-independent methods, such as polymerase chain reaction (PCR) [5–7]. Although PCR is very sensitive and highly specific, special regents and apparatus such as

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thermalcycler are needed. Therefore, this examination is centralized in highly sophisticated facilities and still time consuming.

Loop-mediated isothermal amplification (LAMP), a novel DNA amplification method has originally developed by Notomi et al. [8]. The method relies on autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment. The amplification products are stem-loop DNAs with several inverted repeats of the target and exhibit cauliflower-like structure with multiple loops. The LAMP reaction can be conducted under isothermal condition ranging from 60 to 65 °C. The specificity is attributable to a set of two specially designed inner and outer primers that recognize six distinct sequences. Continuous amplification under isothermal condition produces extremely large amount of target DNA as well as large amount of by-product, magnesium pyrophosphate, within 30–60 min. Therefore, the method demonstrates high sensitivity and enables simple visual (naked-eye) judgment of the reaction through a color change of mixture with SYBR Green I [9] or a white turbidity of magnesium pyrophosphate [10]. Quantitative detection of LAMP is possible by a real-time monitoring of the turbidity [10–12] or fluorescence of ethidium bromide [13]. Further, it has recently been reported that LAMP can be accelerated using additional primers, termed loop primers [13]. Therefore, the LAMP may be promising in rapid detection of microorganisms [9,11,12,14]. In addition, since the method requires only one type of enzyme and special apparatus is not needed, the method would be suitable for routine examinations at dental chair-side. In this study, we evaluated the potential of LAMP for the development of simple and rapid detecting system for periodontal pathogen *P. gingivalis*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study were as follows: *P. gingivalis* FDC 381, *P. gingivalis* ATCC 33277, *P. gingivalis* W83, *P. gingivalis* W50, *Porphyromonas endodontalis* ATCC 35406, *Prevotella intermedia* ATCC 25611, *Fusobacterium nucleatum* ATCC 25586, *Campylobacter rectus* ATCC 33238, *Actinobacillus actinomycetemcomitans* Y4 and *Escherichia coli* K12. *P. gingivalis*, *P. endodontalis*, *F. nucleatum*, and *P. intermedia* were cultured in the 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. *A. actinomycetemcomitans* and *C. rectus* were cultivated according to the methods described previously [15,16]. *E. coli* was cultured aerobically in Luria–Bertani medium. The cells were harvested by centrifugation at 10,000*g* for 20 min at 4 °C, and cell numbers were determined using Petroff–Hauser counting chamber and phase-contrast microscope.

2.2. DNA extraction

Total bacterial DNAs were extracted from cultivated strains and clinical dental plaque samples by using InstaGene Matrix (Bio-Rad Lab. CA, USA) according to the instructions. Plaque samples and cultivated bacteria were suspended in 1 ml of phosphate-buffered saline [PBS (–)] (Gibco BRL, MD, USA), and were pelleted and resuspended in 100 μl of InstaGene Matrix. The suspension was incubated at 56 °C for 30 min and then 100 °C for 8 min. After the incubation, the suspension was centrifuged and 2 μl of the resulting supernatant was used for the detection of *P. gingivalis* by LAMP and the conventional real-time PCR [7]. To examine a detection limit, specificity, and a quantitative range of the LAMP, DNAs were prepared from various numbers of *P. gingivalis* as well as other oral pathogens.

2.3. 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 by inserting paper points (#45, Zipperer, Germany) into periodontal pockets as described previously [17], and DNAs were extracted from the plaques as described. The plaque DNA samples were used for clinical microbiological diagnosis by conventional real-time PCR as reported previously [7]. By this method, the number of *P. gingivalis* in plaque samples was quantified. The remaining plaque DNA samples were stored at −20 °C. Ten plaque DNA samples containing various numbers of *P. gingivalis* were selected randomly from the stocks for the analysis by LAMP.

2.4. Primers for LAMP

A set of six primers for LAMP was designed to target *P. gingivalis* 16S ribosomal RNA gene (16SrDNA). For the designing, Primer Explorer version 2 (Fujitsu, Tokyo, Japan) was used at the Net Laboratory website (http://www.venus.netlaboratory.com/partner/lamp/index.html). A forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3c) were used for a basic LAMP method, and additional two primers (loop primers, LFc and LB) were used for an accelerated LAMP. Further, to confirm the strict requirement of each primer, the LAMP was performed in the absence of one of the inner or outer primers. The primers' sequences and the locations were indicated

in Fig. 1. The F1c, B2c, B3c and LFc in the figure denoted complementary sequences. The FIP consisted of a sequence of F1c and a sense sequence of F2 (5′-F1c-F2-3′). The BIP consisted of a sequence of B1 and a B2c (5′-B1-B2c-3′).

2.5. LAMP reaction

The LAMP reaction for the detection of *P. gingivalis* was carried out using a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tochigi, Japan) in 25 μl volume. The reaction mixture contained 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3c primers, 2 μl of extracted DNA, 1 μl of *Bst* DNA polymerase and 12.5 μl of reaction mix prepared in the kit. For the acceleration of the LAMP reaction, 20 pmol of each LFc and LB was added to the reaction mixture. The reaction mixture was incubated at 60, 62, 64 or 66 °C for 30 or 60 min. After the incubation, the reaction was terminated by heating the reaction mixture at 80 °C for 2 min.

2.6. Detection of LAMP products

For naked-eye detection, 1.0 μl of 10^{-1} or 10^{-3}-diluted SYBR Green I (Takara Bio Inc., Otsu, Japan) was added to the reaction mixture, and the color change was observed. The inspection of the tube containing the 10^{-3}-diluted SYBR Green I was performed under UV light (302 nm wave length). The color change of 10^{-1}-diluted SYBR Green I was judged under natural light. Prior to the addition of SYBR Green I, white turbidity of the reaction mixture by magnesium pyrophosphate (by-product of LAMP) was also inspected.

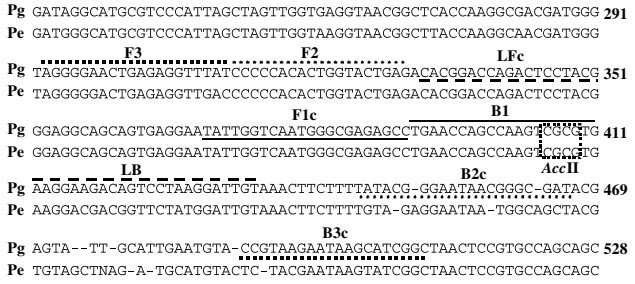

Fig. 1. Target sequence of 16SrDNA and primers for the LAMP. The nucleotide sequence of 16SrDNA of *P. gingivalis* (Accession No. L16492) was used for designing the primers and was shown with the sequence of *P. endodontalis* (L16491). A forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3c) were used for a basic LAMP method, and additional two loop primers (LFc and LB) were used for an accelerated LAMP. The F1c, B2c, B3c and LFc denoted complementary sequences. The FIP consisted of a sequence of F1c and F2 (5′-F1c-F2-3′). The BIP consisted of a sequence of B1 and B2c (5′-B1-B2c-3′). The numbers at the right side indicate the base position of the 16SrDNA. The restriction enzyme *Acc*II was used for digestion of the LAMP product, and the recognition site was surrounded by box.

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). To confirm a structure of the LAMP product, the amplicon was digested with *Acc*II (Takara Bio Inc.) and was subjected to the electrophoresis.

For quantitative detection, real-time monitoring of LAMP was performed using GeneAmp® 5700 Sequence Detection System (PE Applied Biosystems, Foster, CA, USA). SYBR Green I was used as a source of fluorescence. Original solution of SYBR Green I was diluted (3×10^{-3}), and 2 μl of the diluted SYBR Green I was added to the LAMP reaction mixture. All amplifications and detections were carried out in MicroAmp optical 96-well reaction plate with optical caps (PE Applied Biosystems). Accumulation of LAMP products was detected by monitoring the increase in fluorescence of dsDNA-binding SYBR Green at every 30 s for 40 min under isothermal condition at 64 °C. The data of real-time LAMP were analyzed using the GeneAmp® 5700 SDS software (PE Applied Biosystems).

2.7. Real-time PCR

Real-time PCR was performed for quantitative detection of *P. gingivalis* as described previously [7]. GeneAmp® 5700 Sequence Detection System (PE Applied Biosystems) was used for monitoring the fluorescence from dsDNA-binding SYBR Green. 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 (5′-cttgacttcagtggcggcag-3′) and reverse primer (5′-agggaagacggttttcacca-3′) was designed from the species-specific regions of 16SrDNA. 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 was analyzed using the GeneAmp® 5700 SDS software (PE Applied Biosystems).

3. Results and discussion

3.1. Primer design and specificity of LAMP

The primers of LAMP for the detection of *P. gingivalis* were designed to target the 16SrDNA. The 16SrDNA sequence of *P. endodontalis*, a same genus *Porphyromonas* of oral pathogen, was referred to the design. Candidate sequences for the primers were nominated by the Primer Explorer software (Fujitsu) and were compared to the corresponding sequences of *P. endodontalis*. A set of primers, which was the most specific to *P. gingivalis*, was selected in the current study (Fig. 1). In the primer set, F3, B2c, B3c and LB contained the specific

sequences of *P. gingivalis* comparing to *P. endodontalis*. By using the primer set, specificity, sensitivity, and quantitativity of the LAMP for *P. gingivalis* was investigated.

A successful LAMP reaction was seen with the complete set of primers and template DNA from four strains of *P. gingivalis* (Fig. 2(a)). The LAMP products appeared as a ladder-like pattern on the agarose gel due to its characteristic structure [8]. To confirm the specific amplification, the product was digested with *Acc*II, which recognize the sequence in B1. Consequently, many bands at different size were concentrated and fragmented to approximately 65- and 130-bp length in good agreement with the predicted size (Fig. 2(a)). The results demonstrate that the LAMP specifically amplified the target sequence in the form of stem-loop DNAs with various stem length and cauliflower-like structures with multiple loops. No difference was seen among the strains tested (data not shown).

Prior to the serial experiments, optimum reaction temperature was determined. The reactions of LAMP for *P. gingivalis* were performed under isothermal condition at 60, 62, 64 or 66 °C using extracted DNA from four strains of *P. gingivalis* (10^2 cells) prepared in this study. Although significant difference was not observed, the LAMP product amplified at 64 °C exhibited slightly larger amount of DNA as compared to others (data not shown). The series of experiments were performed at 64 °C hereafter. Since no difference was seen among the strains of *P. gingivalis* in any experiments, the results of FDC 381 strain were representatively shown in the figures.

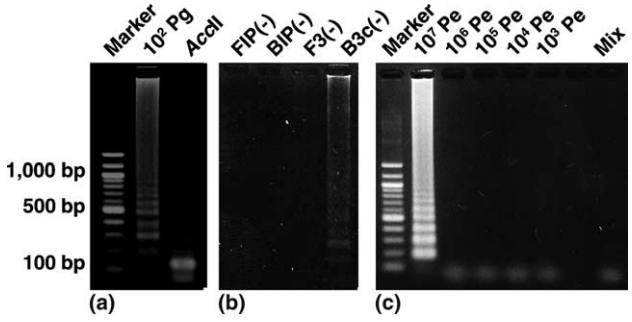

Fig. 2. (a) Electrophoretic pattern of the LAMP. The basic LAMP was carried out using extracted DNA from 10^2 cells of *P. gingivalis* (Pg), and the LAMP product was electrophoresed on 2% agarose gel. A digested sample of the LAMP product with *Acc*II was simultaneously electrophoresed. The accelerated LAMP demonstrated identical result (data not shown). No difference was seen among the four strains tested, and the result of FDC 381 strain was representatively shown. (b) Requirement of primers for the LAMP. The target sequence was tried to amplify in the absence of one of the inner (FIP and BIP) or outer primers (F3 and B3c). Template DNA was prepared from 10^7 cells of *P. gingivalis*. No amplification was seen in the absence of one of the inner primers. No significant amplification was seen in the absence of outer primers. (c) Specificity of the LAMP. Extracted DNAs from *P. endodontalis* (10^3–10^7) were used as templates for specificity testing of the accelerated LAMP. No amplicon was seen up to 10^6 cells. The amplicon was detected from 10^7 cells of *P. endodontalis*. In the last lane (Mix), reaction sample of the accelerated LAMP using the template DNA extracted from 10^7 cells mixture of *P. intermedia*, *F. nucleatum*, *C. rectus*, *A. actinomycetemcomitans*, and *E. coli* was loaded.

The specificity of the LAMP was examined by using related oral pathogens as templates. In addition, to examine the requirement of each primer, the LAMP was performed in the absence of one of the inner or outer primers. No amplicon was seen in the absence of FIP, BIP or F3 primer, and very slight amplicon was detected in the absence of B3c primer (Fig. 2(b)). Extracted DNA mixture from 10^7 cells of *P. intermedia*, *F. nucleatum*, *C. rectus*, *A. actinomycetemcomitans*, and *E. coli* was used as a template for the specificity testing of the LAMP, and no amplicon was observed (Fig. 2(c)). These results demonstrated that the each primer was required for the LAMP, and the reaction was specific to *P. gingivalis*. Even though a set of primers was designed in consideration of *P. endodontalis*, DNA was amplified from the extracted DNA of *P. endodontalis* in more than 10^7 cells (Fig. 2(c)). This may influence the results of clinical plaque samples. However, since the cell numbers more than 10^7 is extremely rare through the employed sampling method [7], the influences are considered to be small. By targeting the 16SrDNA of a multi-copy gene, high sensitivity would be expected. In addition, LAMP was a high specific method that required six distinct sequences for the amplification. Therefore, the 16SrDNA was employed for the first trial of LAMP, even though the 16SrDNA sequence was similar among genus *Porphyromonas*. Sufficient results were obtained in the current study, however, for more specific amplification, the target DNA may need to be changed. Species-specific genes responsible for the virulence or pathogenesis, such as fimbrilin (*fimA*) [18,19], would be candidates.

3.2. Sensitivity and quantitativity of LAMP

The LAMP amplicons were detected by agarose gel electrophoresis, naked-eye inspection, and real-time monitoring using GeneAmp® 5700 Sequence Detection System. The result of electrophoretic detection was shown in Fig. 3. The extracted DNA was prepared from the 200 cells of *P. gingivalis*, and serial 10-fold dilution of the extracted DNA was used for sensitivity testing. The accelerated LAMP using loop primers amplified the target within 30 min from the 10-fold dilution sample. The detection limit of the LAMP system was estimated to be 20 cells at the sampling stage before DNA extraction. Copy number of 16SrDNA in *P. gingivalis* is registered to be four on the website of TIGR (The Institute for Genome Research) database, and since 2 μl out of the 100 μl-DNA extracts was used for the LAMP, detection limit in one reaction tube was estimated to be near upon two copies. The basic LAMP required 60 min to detect the 200 cells of *P. gingivalis*. The

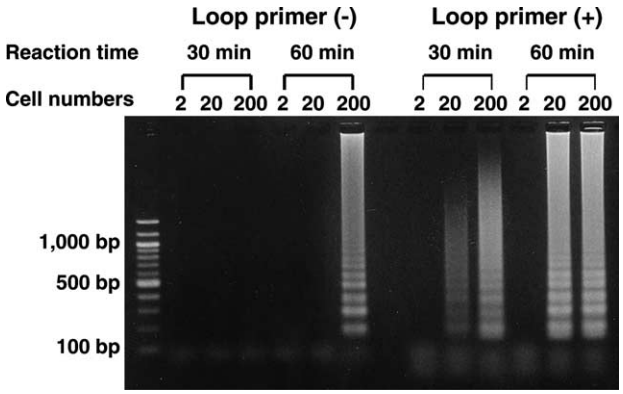

Fig. 3. Detection limit of the LAMP. Extracted DNA was prepared from various cell numbers (2, 20 and 200 cells) of *P. gingivalis* and was used as template for the basic [loop primer (−)] or the accelerated LAMP [loop primer (+)]. The reaction of LAMP was carried out at 64 °C for 30 or 60 min. The target DNA from 20 cells was amplified detectably on 2% agarose gel within 30 min by the accelerated LAMP.

loop primers prepared in this study dramatically accelerated the LAMP reaction and increased the sensitivity. The accelerated LAMP demonstrated an equivalent or higher sensitivity with the previously published PCR methods [5,6] or real-time PCR [7,20,21] in shorter time. Generally, the LAMP yields extremely large amount of DNA, and this enables naked-eye inspection [9,10]. The results of naked-eye inspections were shown in Fig. 4. The color of reaction mixture of successful LAMP exhibited green by the addition of SYBR Green I, whereas the original orange color did not change in the control tube. By using UV light, positive and negative reaction was more clearly distinguished. The naked-eye inspection with SYBR Green I demonstrated equivalent sensitivity to agarose gel electrophoresis both under UV and natural light (less than 20 cells). The sensitivity of inspection by white turbidity, a simpler eye judgment, was inferior to the electrophoresis. Two hundreds cells were required to definitely identified the reaction as positive. Although quantitative detection is difficult, the eye inspection was quite simple and rapid. Therefore it may facilitate the application of LAMP, especially in the place such as a dental chair-side.

For quantitative detection, real-time monitoring of the LAMP reaction was performed using loop primers. DNA extracts from 10^2 to 10^6 cells of *P. gingivalis* were subjected to the real-time LAMP. The increase in the fluorescent of SYBR Green I bound to the amplified double-strand DNA was scanned and measured by GeneAmp® 5700 Sequence Detection System at every 30 s. The scanning datum was shown in Fig. 5. The detection was linear over the rage examined, and all of the reaction reached plateau in 33 min. It has been reported that the real-time monitoring of LAMP reaction was achieved by measuring magnesium pyrophosphate [10–12] or fluorescence of ethidium bromide [13,22] with

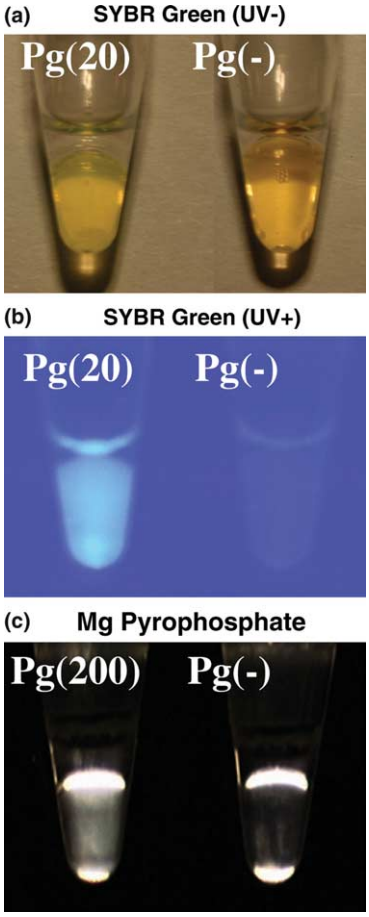

Fig. 4. Naked-eye inspection. Positive and negative reactions of the accelerated LAMP were distinguished by simple eye inspection. The original orange color of SYBR green I turned to be green in the positive reaction mixture (a), and the fluorescence of DNA-binding SYBR green I was visually detected under UV light (b). The detection limit of both naked-eye inspections with SYBR green I was less than 20 cells (Pg20). The positive reactions were shown with the negative control (Pg−). The LAMP reaction was also inspected through white turbidity of the tube caused by magnesium pyrophosphate, a by-product of LAMP (c). The detection limit of the white turbidity was less than 200 cells (Pg200).

equal level of sensitivity and quantitativity to the conventional real-time PCR [7,20,21]. By using the SYBR Green I, similar results were obtained in the current study. Although special apparatus, such as a turbidimeter or fluorescent detection system is required, the real-time LAMP will allow a quantitative detection of periodontal pathogens with an advantage of rapidity comparing to the conventional real-time PCR [7,20,21].

3.3. Examination of clinical dental plaque sample

The real-time LAMP and the conventional real-time PCR [7] were applied to the clinical dental plaque samples. The original 2 μl of extracted DNA sample and 2 μl of diluted sample (10^{-1}) were run in duplicate. The number of *P. gingivalis* examined by the both methods was

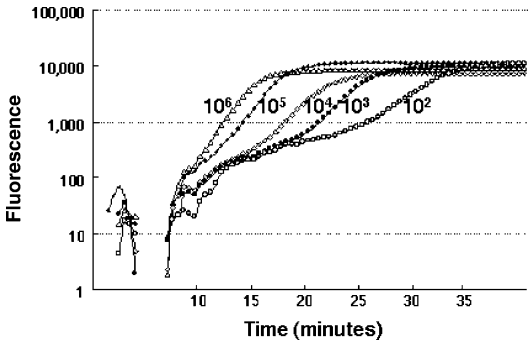

Fig. 5. Real-time monitoring of LAMP reaction. The accelerated LAMP was performed, and generated fluorescence intensity of DNA-binding SYBR Green I was monitored at every 30 s. Template DNAs were prepared from 10^2 to 10^6 cells of *P. gingivalis* and were used as standards for quantitative detection of *P. gingivalis* in clinical plaque samples.

Table 1
Cell numbers of *P. gingivalis* in plaque samples

Plaque sample	Real-time PCR	Real-time LAMP
1	Not detected[a]	Not detected[a]
2	Not detected[a]	Not detected[a]
3	165	202
4	388	480
5	1019	2609
6	2019	3206
7	23374	35277
8	24249	28371
9	190308	264144
10	404672	208534

[a] Less than 20 cells.

shown in Table 1. The number of *P. gingivalis* determined by the real-time LAMP was well corresponded to that analyzed by the real-time PCR. Although non-specific amplification was observed in *P. endodontalis* as described above, the influence was not practically seen in the current study. The set of primer seems to have sufficient specificity for the clinical examination. In addition, the results suggested that DNA contamination of other bacterial species did not influence the reaction of LAMP. On the other hand, slightly larger difference was seen in samples of 5 and 10 comparing to other samples because of unknown reason. Since the LAMP and real-time PCR are quite sensitive methods, small technical errors in experimental manipulations such as a pipetting might influence the results seriously.

Our final goal is to establish a simple and rapid examination system for periodontal pathogens in places such as private clinics or dental chair-side. In the current study, we employed the LAMP for the purpose. It demonstrated high efficacy and specificity, which was attributable to continuous amplification under isothermal condition with four primers. Comparing to the PCR or real-time PCR, only required device was water bath or heat block, and the most practically important benefit was the rapidity. Other than the LAMP, nucleic acid sequence-based amplification (NASBA) [23] and self-sustained sequence replication (3SR) [24] have been reported as isothermal amplification methods so far. Although the methods do not require thermal cycler, they are reported to be less specific, due mainly to low temperature (40 °C) for amplification and therefore requiring elaborate method for detection. The LAMP seems to have great advantage in its detection. Naked-eye inspection, electrophoresis analysis, and real-time monitoring of the reaction will be used according to the situation of the dental office.

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