Original Article

Development of a loop-mediated isothermal amplification assay for detecting *Porphyromonas endodontalis*

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Abstract

Context: Loop-mediated isothermal amplification (LAMP) may be used in the future to detect infecting microorganisms. LAMP assays exist for the endodontic pathogens *Enterococcus faecalis* and *Fusobacterium nucleatum*, but not yet for *Porphyromonas endodontalis*.

Aim: To develop a LAMP assay for detecting *P. endodontalis*.

Settings and Design: It was an *in vitro* benchtop study.

Subjects and Methods: The National Center for Biotechnology Information GenBank Basic Local Alignment Search Tool was used to identify a segment of the dipeptidyl peptidase 11 (DPP11) gene unique to *P. endodontalis*. A primer design tool was used to generate six primers required for developing the LAMP assay. WarmStart Colorimetric LAMP 2X Master Mix was used to evaluate the LAMP assay, using purified *P. endodontalis* DNA as a control.

Statistical Analysis Used: Statistical parameters for sensitivity and specificity.

Results: The assay was performed in triplicate on pure DNA from *P. endodontalis* and *P. gingivalis* and on the DNA that was extracted from *P. endodontalis*, *P. gingivalis*, *F. nucleatum*, and *E. faecalis* cells and diluted two-fold from 1/2 to 1/256. Assays for the diluted samples were performed in triplicate, and the contingency tables indicated the LAMP assay to be 82% sensitive and 90% specific for *P. endodontalis*.

Conclusions: LAMP assay could be a highly sensitive and specific chairside detection method for *P. endodontalis*.

Keywords: Chairside assay; loop-mediated isothermal amplification; *Porphyromonas endodontalis*

INTRODUCTION

Porphyromonas endodontalis, an asaccharolytic, black-pigmented, Gram-negative rod, is highly sensitive to oxygen, and hence, difficult to cultivate from clinical samples.[1] Therefore, nucleic acid amplification methods, namely polymerase chain reaction (PCR) and quantitative PCR (qPCR), are used to identify specific genes of *P. endodontalis* and similar endodontic organisms.[2,3]

Loop-mediated isothermal amplification (LAMP) can amplify a limited amount of DNA into a million copies in just 30 min and in very small volumes.[4] Here, we aimed to develop a LAMP assay for *P. endodontalis* using primers directed at a unique region of the dipeptidyl peptidase 11 (DPP11) gene.

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SUBJECTS AND METHODS

The study was approved by the university research committee. The National Center for Biotechnology Information GenBank Basic Local Alignment Search Tool (BLAST) and New England Biolabs (NEB, Ipswich, MA, USA) Primer Design tools were used. The novel DPP11 gene of *P. endodontalis*, GenBank AB610284.1, has only 57.9% similarity with the DPP11 sequence in *P. gingivalis* and lesser still with those of the dipeptidyl peptidase (DPP) family of genes.[5] A BLAST sequence similarity search was used to identify a segment of the gene (bases 1501–1800) dissimilar to the sequences in *P. gingivalis* DPP11. The NEB Primer Design tool was used to generate the following primers in this segment for both q-PCR and LAMP assays: F3, 5'-GCT CTT CTG ACT CGC TAT GC-3'; B3, 5'-CGA CAG ATG CTG CAA AAC G-3'; FIP, 5'-GGC AGA TCC GTA CTC GGC AAT GAG CGA ATC CCT GCT GAG A-3'; BIP, 5'-TCT ATG CCT CCC GTG AGC GT GCG CAA GAG ACG ATC TCG3'; LF, 5'TCA CGT ATA GAA ATG GGT TGC T3'; LB,5'-TTC GAG GAG TTT ATG AAA AAT CCC G-3'. Primers were constructed by Sigma Chemical Co (St. Louis, MO, USA). Bacterial cells and DNA were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

WarmStart Colorimetric LAMP DNA detection kits were purchased from New England Biolabs. The kits were supplied with the WarmStart LAMP 2X Master Mix, containing a strand-displacing DNA polymerase "Bst 2.0" in an optimized LAMP buffer solution designed for end-point visualization. A primer mix was formulated to contain 16 μM FIP, 16 μM BIP, 2 μM F3, 2 μM B3, 4 μM loop F, and 4 μM loop B. The standard assay (50 μL) was performed in a microcentrifuge tube for 30 min at 65°C using a water bath, as follows: WarmStart master mix (25 μL), primer mix (6.0 μL), dilutions of target DNA (i.e. control DNA or supernatants of cell extracts, 2 μL), and molecular grade water (18.5 μL). Positive reactions were identified visually using solutions that turned from pink to yellow.

Genomic DNA from *P. endodontalis* (5 μg, from American Type Culture Collection [ATCC] strain #3540) and *P. gingivalis* (5 μg from ATCC strain #33277) were diluted two-fold from ½ to 1/256 in molecular grade water. The sensitivity and lower limit of detection of the LAMP assay were determined using diluted *P. endodontalis* DNA. Specificity of the newly developed LAMP assay for *P. endodontalis* was determined using *P. gingivalis* DNA.

To compare the sensitivity of the newly developed LAMP assay with that of qPCR, the target area between the forward and reverse outer primers was amplified by qPCR. Two-fold dilutions (1/2–1/256) of genomic DNA (starting at 10^4 copies/μL) from *P. endodontalis* and *P. gingivalis* were used to generate and compare the standard curves. The PCR mix included the following: 10 μL of each primer (F_3, B_3, 2 μM) was added to 20 μL of Chai Green Master Mix and 10 μL of quantitative whole genomic DNA from either *P. endodontalis* or *P. gingivalis*. Amplifications were performed in a single-channel Chai Open (qPCR) thermocycler (Chai Biotech, Santa Clara, CA, USA) as per the following program: initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 30 s, 62°C for 30 s, and at 72°C for 30 s. The terminal denaturation was performed at 72°C for 5 min. The PCR products were detected by monitoring the increase in fluorescence.

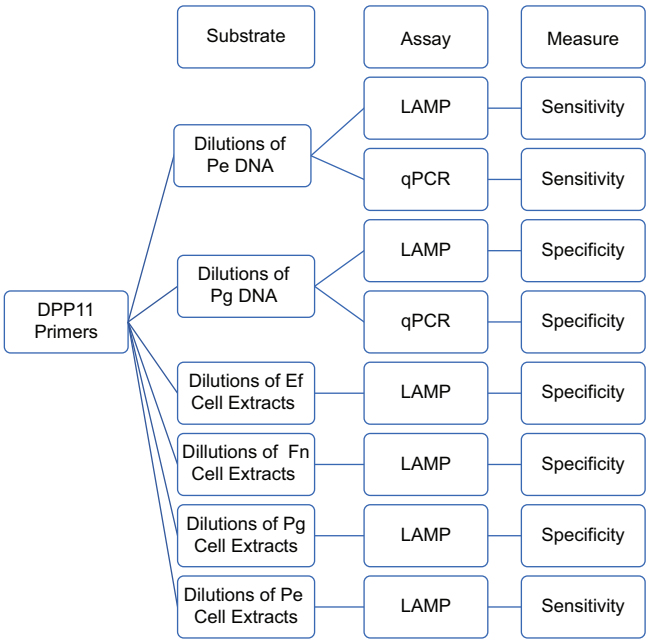
To simulate a clinical scenario, the newly developed LAMP assay was tested for sensitivity and specificity using DNA extracted from the cells of putative endodontic pathogens. *Enterococcus faecalis* cells (Ef, ATCC strain #19433) were inoculated in brain heart infusion (BHI) broth and allowed to grow for 12–24 h at 37°C. Likewise, *P. endodontalis* cells (Pe, ATCC strain #35406), *P. gingivalis* cells (Pg, ATCC strain # 33277), and *Fusobacterium nucleatum* cells (Fn, ATCC strain #10953) were inoculated in BHI broth (supplemented with 0.5 mg/mL hemin, 0.05 μg/ml menadione), and allowed to grow for 24–48 h at 37°C in an anaerobic chamber. Cells were removed from the culture medium by centrifugation at 6000 × g for 10 min at room temperature. The pelleted cells from 24-h to 48-h cultures were washed twice with Hank's balanced salt solution (HBSS), repelleted by centrifugation, dispersed in HBSS to achieve an optical density of 0.5 at 600 nm on a spectrophotometer, and then subjected to serial two-fold dilutions from ½ to 1/256. DNA was extracted from whole cells using the Instagene Matrix solution (Bio-Rad Laboratories). Each dilution of cells (1 mL) was added to 1 mL Instagene Matrix and boiled for 5 min. Cell debris settled by gravity and the supernatant containing the extracted DNA was used in the LAMP assays.

Statistical measures of sensitivity and specificity of the LAMP assay have been reported. The methods are summarized in Flowchart 1.

RESULTS

Since the genome of *P. endodontalis* is similar to that of *P. gingivalis*, the sensitivity and specificity of the new assay were measured using whole genomic DNA of the two organisms. All six primers were used to amplify the target region of DPP11 in genomic DNA of *P. endodontalis* and *P. gingivalis* by LAMP. The assay results, shown in Figure 1, indicated that only *P. endodontalis* DNA was detected and was positive even at 1/256 dilution (or 400 copies of DNA). Thus, in this simplified assay system, 100% specificity and sensitivity were obtained for *P. endodontalis* DNA.

To compare the sensitivity of the newly developed LAMP assay with that of qPCR, the forward and reverse outer primers were used to amplify the target region of DPP11



Flowchart 1: Experimental protocol for use of DPP11 primers

in genomic DNA of *P. endodontalis* and *P. gingivalis* by qPCR. The number of cycles for fluorescence (CT threshold level) to appear was proportional to *P. endodontalis* DNA concentration (3200–400 copies) and was sensitive to 400 copies of DNA (data not shown). *P. gingivalis* was detected only by scattered fluorescence after 27–35 cycles. qPCR allowed the quantitation of DNA extracted from diluted *P. endodontalis* cells (1/256 dilution corresponds to 400 copies of DNA).

To simulate a clinical scenario of assaying an infected pulp, DNA was extracted from serial dilutions of *P. endodontalis*, *P. gingivalis*, *F. nucleatum,* and *E. faecalis* cells. LAMP assays of the DNA indicated only *P. endodontalis* to be positive, with negative reactions for all dilutions of the other cells. Figure 2 shows the LAMP results of DNA extracted from cells of *P. endodontalis* and *P. gingivalis*. A false-negative result was noted at 1/32 dilution *of P. endodontalis*. A contingency table [Table 1] comparing the results of triplicate experiments for the four organisms indicated the LAMP assay to be 90% specific for *P. endodontalis* and 82% sensitive to a 1/256 dilution of cells.

DISCUSSION

Since cultivation might fail to detect important pathogens, molecular methods are used to identify the presence of specific bacteria in the ecological niche of infected root canals. Among the techniques used are whole genomic DNA probes, checkerboard DNA hybridization, and PCR-based assays.[6] PCR is considered the gold standard for detecting DNA and other nucleic acids.[7] PCR is often used in oral research to measure the effect of an endodontic sealer or medicament on endodontic microflora.[8,9] Likewise, PCR is

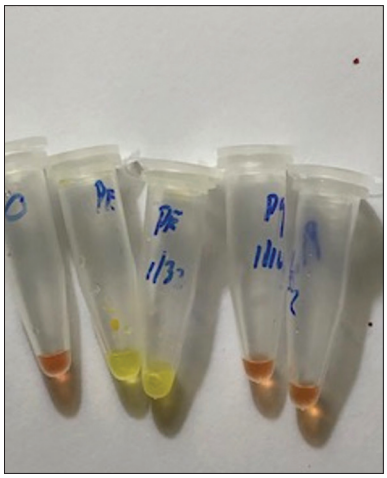


Figure 1: Loop-mediated isothermal amplification assay comparing *Porphyromonas endodontalis* (Pe) DNA with *Porphyromonas gingivalis* (Pg) DNA. From left: negative control, Pe DNA 6000 copies, Pe DNA 3000 copies; negative control, Pg DNA 6000 copies, Pg DNA 3000 copies

used is used to identify novel strains of bacteria associated with dental caries.[10,11] However, DNA amplification by PCR requires many cycles of heating and cooling to generate enough DNA for detection. In addition, it requires expensive equipment.

Like PCR, LAMP reproduces a specific genomic region from a small amount of starting material. However, LAMP does not use thermal cycling and the amplification occurs at a one temperature (usually between 60 and 65°C).

LAMP uses a special DNA polymerase ("Bst") that reads double-stranded DNA without requiring a rise in temperature to open the double helix ("strand displacement" activity). To copy the double-stranded DNA, LAMP uses spatially designed primers that form stem-like "loop" structures. Thus, DNA copying occurs at multiple locations, creating a multitude of DNA in a short period of time. Unlike PCR, which typically uses only one pair of primers, LAMP uses two or three sets of primers. Unlike PCR, which produces billions of identical copies of DNA, the final products of LAMP are stem-loop DNAs of various lengths and broccoli-like structures with multiple loops, adding up to an abundance of DNA.[12] Our data indicated that the LAMP assay was 90% specific for DNA extracted from *P. endodontalis* cells and negative for *P. gingivalis*, *F. nucleatum*, and *E. faecalis*.

In addition to its specificity, LAMP is well-known for its high sensitivity, similar to real-time qPCR. Our LAMP assay detected *P. endodontalis* with 80% sensitivity at very low concentrations, similar to qPCR (400 copies of genomic DNA), and a 1/256 dilution of whole cells. With color-based visualization of positive tests, LAMP is simpler than qPCR. It is a fast and simple diagnostic tool for the rapid detection and identification *of P. endodontalis*.

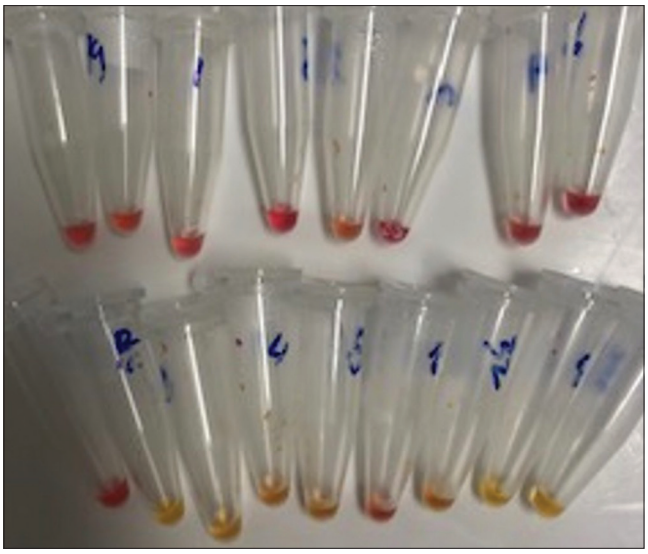


Figure 2: Loop-mediated isothermal amplification assay comparing cell extracts of *Porphyromonas endodontalis* (Pe) with those of *Porphyromonas gingivalis* (Pg). Top, Pg; from left: negative control and cell dilutions (½–1/128); bottom, Pe; from left: negative control and cell dilutions (½–1/256)

Table 1: Contingency table of loop-mediated isothermal amplification assay

Cell extract	+ LAMP assay	− LAMP assay
Porphyromonas endodontalis	19	4
Porphyromonas gingivalis	2	22
Fusobacterium nucleatum	1	23
Enterococcus faecalis	4	21

Sensitivity and specificity of the LAMP assay was measured using eight-fold dilutions of DNA from cell extracts. Cell extracts were diluted from ½ to 1/256 and experiments were run in triplicate, resulting in 24 assays for each organism. One experiment for *Porphyromonas endodontalis* and *Porphyromonas gingivalis* is shown in [Figure 2]. LAMP: Loop-mediated isothermal amplification

LAMP has been used to detect a number of periodontal pathogens.[13] Likewise, LAMP assays have been developed for the endodontic pathogens *E. facaelis* and *F. nucleatum*.[14,15] Traditionally, genetic identification of clinical bacterial isolates has been based on the 16S rRNA gene, which is approximately 1500 bp long and consists of nine (hyper) variable regions that are usually sufficient for species identification.[16] Indeed, *P. endodontalis* had previously been detected in infected root canals by 16S-gene-directed PCR using an amplicon of base positions 618–128.[17] However, we found cross-reactivity between *P. endodontalis* and *P. gingivalis* using the primers in qPCR (data not shown). In addition, Primer Explorer software did not reveal constructive primers for successful LAMP in this area that could differentiate between these species. Hence, we used primers constructed from the sequence of the DPP gene of *P. endodontalis* which has only 56% similarity with that of *P. gingivalis* and less similarity with the DPP family of genes.[5]

Besides its advantages, LAMP has limitations as well. Since endpoint colorimetric detection depends on a drop in pH, false positives and negatives can be generated if the pH is not strictly controlled. Nonspecific amplification could be another problem. Owing to the reaction conditions in LAMP (high concentration and nature of the LAMP primer sets), amplification can occur from the secondary structure of nontarget DNA. We found 10% and 18% false positives and false negatives, respectively, in the assays involving DNA extracted from cells. Similarly, owing to the more complex primer configurations, designing successful LAMP primer sets may be difficult. For any desired amplification of the target site, two or three sets of primers must be designed in a complex manner. In the present study, Primer Explorer software was used for optimal primer construction and reaction success. Another limitation was that the "Bst" polymerase is labile and sensitive to freeze-thaw cycles and storage at $>-20°C$.

In a recent editorial, Singh and Bolla stressed the importance of microbial evaluation in endodontics, with an emphasis on emerging technologies.[18] Methods of identification of pathogens in infected root canals continue to advance. Rather than relying on difficult cultivation techniques, high minimum threshold levels associated with whole chromosomal DNA probes, or expensive PCR technology, LAMP provides an innovative method for the rapid detection of microorganisms. Accordingly, the LAMP assay for *P. endodontalis* shows promising utility owing to its high sensitivity, pathogen specificity, and potential implementation at the point-of-care. However, laboratory testing cannot replicate the complex conditions of human pulp, and further testing using more complex samples from clinical specimens would be required.

CONCLUSIONS

LAMP can be used to detect the presence of *P. endodontalis* using a visual detection test that requires only a water bath at a single temperature and may be considered for point-of-care assays.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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