Improved LAMP (loop-mediated isothermal amplification) and immunohistochemistry for detecting *Porphyromonas gingivalis* in periapical periodontitis

Taiichi Kitano

Nihon University Graduate School of Dentistry

Major in Oral pathology and Pathology

(Director : Prof. Masatake Asano)

Contents

An article published as in below and new unpublished data of LAMP to FFPE samples and of assessment of amplicons of PCR-LAMP to FFPE samples.

> Kitano T, Mikami Y, Iwase T, Asano M, Komiyama K (2016) Loop-mediated isothermal amplification combined with PCR and immunohistochemistry for detecting *Porphyromonas gingivalis* in periapical periodontitis. Journal of Oral Science 58, 163-169.

Abstract

Porphyromonas gingivalis is important in the development of marginal periodontitis. However, the precise role and localization of *P. gingivalis* in chronic periapical periodontitis remain unclear. Thus, methods that can detect *P. gingivalis* in formalin-fixed and paraffin-embedded (FFPE) tissue samples are needed. A technique combining loop-mediated isothermal amplification (LAMP) with PCR (PCR-LAMP) for detection of *P. gingivalis* was assesed, using 110 FFPE tissue samples of chronic apical periodontitis. PCR-LAMP specifically detected *P. gingivalis* with high sensitivity in FFPE tissue samples, and the sensitivity of the technique was higher than that of PCR or LAMP alone. The results of immunohistochemistry (IHC) confirmed the specificity of PCR-LAMP. IHC showed that *P. gingivalis* was localized in a granular layer of chronic apical periodontitis, a region that correlated with the localization of macrophages. This is the first study to describe the localization of *P. gingivalis* in human periapical periodontitis. In conclusion, PCR-LAMP was an effective tool for detecting *P. gingivalis* in periapical periodontitis. In addition, IHC results improve our understanding of the role of *P. gingivalis* in the progression of periapical periodontitis.

Introduction

Apical periodontitis can destroy tissues surrounding the tooth root apex and usually has a chronic course (1). Acute apical periodontitis can spread to other anatomical spaces, resulting in phlegmon and bacteriemia (2,3). Various bacteria species have been identified in periapical lesions, but the main pathogens involved in apical periodontitis development are unclear (4).

Porphyromonas gingivalis is a primary etiological agent in human marginal periodontitis and has many pathogenic factors, including exotoxin, endotoxin (lipopolysaccharide), fimbriae, hemagglutinins, and several enzymes, which induce a local chronic inflammatory host response and destroy periodontal supportive tissues (5,6). In addition, *P. gingivalis* was also identified in root canals, and each positive case correlates with clinical symptom severity, including gingival swelling (7,8). It is important to detect the bacterium in the root canal during endodontic treatment. Thus, the aim of this study was to develop a suitable method for detecting *P. gingivalis* in periapical periodontitis. Culture has been used to detect *P. gingivalis* in root canals during endodontic treatment (prevalence, 6.7%–15%) (9,10) but is poorly suited for clinical diagnosis because it requires time-consuming microbiological culture and laboratory confirmation and has a low detection rate. Molecular biological techniques including PCR have recently been developed, and these

methods have much higher detection rates for *P. gingivalis* (28%–43%) (11,12).

Loop-mediated isothermal amplification (LAMP) is an effective DNA amplification method. It relies on autocycling strand-displacement DNA synthesis using *B*st DNA polymerase and is performed under isothermal conditions. Its specificity depends on four primers recognizing six different sequences. An inner primer containing sequences of the sense and antisense strands of the target DNA starts reacting. The following strand-displacement DNA synthesis primed by an outer primer egresses a single-stranded DNA. This serves as a template for DNA synthesis primed by the second inner and outer primers, which hybridize to the other end of the target, thereby producing a stem–loop DNA structure. In subsequent LAMP cycling, one inner primer hybridizes to the loop on the product and starts strand-displacement DNA synthesis, the product of the original dumbbell-like DNA structure and a new dumbbell-like DNA structure with a stem twice as long (13). Continuous amplification under isothermal conditions yields a large quantity of target DNA within 30–60 min. Therefore, the method has high sensitivity and facilitates simple visual judgment of DNA amplification, through assessment of the turbidity of the reaction by-product magnesium pyrophosphate (13). Two more primers (loop primers) can be added to make the reaction shorter and more sensitive (14). Because *B*st DNA polymerase works under isothermal conditions, special devices such as thermal cyclers are unnecessary. LAMP is widely used in medical, biological, and agricultural research (15,16).

In the present study, PCR and LAMP (PCR-LAMP) were combined in an attempt to develop a highly sensitive method for identification of *P. gingivalis* in formalin-fixed paraffin embedded (FFPE) chronic apical periodontitis tissues. To confirm the specificity of PCR-LAMP, immunohistochemistry (IHC) with anti-*P. gingivalis* specific antibody was performed because this technique is able to visualize the target antigen directly.

Materials and Methods

Sample collection

A total of 110 FFPE chronic apical periodontitis tissues were obtained from the archives of the Department of Pathology at Nihon University School of Dentistry (60 males and 50 females; age range, 6–87 years; average age, 45.2 years). Disease diagnosis was confirmed by two pathologists (T.K. and K.K.). This study was approved by the Ethics Committee of Nihon University School of Dentistry (EP2013-8).

Clinical findings

Patient clinical findings were obtained from clinical records. Symptoms of pain, pus drainage, fistula, and swelling related to active levels of periapical lesions were analyzed in relation to chronic apical periodontitis.

Standard bacterial strains

Genomic DNAs of standard *Streptococcus salivarius* HHT, *Staphylococcus aureus* 209P, *Enterococcus faecalis* JCM 5803, *Aggregatibacter actinomycetemcomitans* Y4, *Fusobacterium nucleatum* ATCC 25586, *Porphyromonas endodontalis* JCM 8526, and *P.*

gingivalis ATCC 33277 were prepared. All samples were kindly provided by Dr. Muneaki Tamura (Nihon University School of Dentistry).

Genomic DNA extraction and quality test

Ten-micrometer-thick serial sections were sliced from each FFPE tissue block and processed for genomic DNA (gDNA) extraction by using the ReliaPrep. FFPE gDNA Miniprep System (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Sections in 1.5-mL centrifuge tubes were deparaffinized by xylene and hydrophilized by 100% alcohol. Two hundred microliters each of lysis buffer and proteinase K were added to the tubes, which were then incubated overnight at 56°C. After heating at 80°C for 1 h, 10 μL of RNase was added to each tube, and the tubes were further incubated at room temperature for 5 min. Two hundred twenty microliters of BL buffer and 240 μL of 100% ethanol were added, and the sample in each reaction tube was transferred to a binding column, centrifuged at 10,000 × g, and washed twice with a wash solution. DNAs were eluted by 30 μL each of the elution buffer provided, and the eluted DNAs were stored at −20°C. DNA concentration and purity were measured by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the DNA concentration was adjusted to 60 ng/μL using nuclease-free water.

Design of LAMP primers

The LAMP primer set for *P. gingivalis* detection was designed by Primer Explorer v4 software (Fujitsu, Tokyo, Japan), using the target sequence of the *P. gingivalis* 16S rRNA coding region (Genbank AB035459). Because adequate sequences for the LB primer could not be generated with the software, we did not use this primer in the present study. The LAMP primers are listed in Table 1.

LAMP

LAMP reaction was performed using the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd, Tokyo, Japan) according to the manufacturer's instructions. Briefly, 6.25 μL of reaction buffer provided in the kit, 20 pmol each of FIP and BIP, 2.5 pmol each of F3 and B3 primers, 10 pmol of LF primer, and 1 μL of *B*st DNA polymerase were mixed in 0.2-mL tubes (total 11.5 μL/tube). After 1 μL of the DNA sample was added to each tube, the LAMP reaction was performed at 62°C, and the turbidity of each tube was monitored using the Loopamp real-time turbidimeter (LA-200; TERAMECS Co. Ltd., Kyoto, Japan). The threshold turbidity (i.e., the minimum turbidity for a positive result) was defined as 0.1, in accordance with the manufacturer's protocol (17).

Highly sensitive PCR-LAMP

To improve the sensitivity of LAMP, PCR was performed before the LAMP reaction (PCR-LAMP), a method similar to nested PCR. This method has advantages over nested PCR because it allows researchers to skip the time-consuming secondary PCR and because amplicons can be identified easily by simply measuring turbidity. The F3 and B3 primers for the LAMP reaction (Table 1) were used for the first PCR. In theory, the product sequence from the first PCR contains the LAMP target sequence. PCR was conducted in 5 μL of reaction mixture (Emeraldamp, TAKARA Bio, Kusatsu, Japan) containing 1 μL of DNA template. The temperature profile was initial denaturation for 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 2 min at 48°C, and 30 s at 72°C; final extension continued at 72°C for 7 min. PCR products were 100-fold diluted by nuclease-free water and were used as templates for secondary LAMP. The LAMP reaction was performed in the manner described above.

Electrophoresis and restriction enzyme assay

The LAMP amplicon exhibits a characteristic ladder pattern in electrophoresis (13). Thus, 5 μL of each PCR-LAMP product was applied to 2% agarose gel electrophoresis followed by ethidium bromide staining. The ladder bands were visualized by UV light. To further confirm the amplicon, the products were incubated with 2 μL of restriction enzyme (*Acc* II, TAKARA Bio) for 15 min and then electrophoresed on 2% agarose gels. When two separate bands (163 bp and 109 bp) were observed after incubation with the restriction

enzyme, the amplicon in the products was considered to be correct (13).

IHC

Serial sections of FFPE tissues were sliced into 4-μm sections and processed for IHC. Briefly, the specimen was incubated for 5 min with 3% hydrogen peroxide, to block endogenous peroxidase. The specimen was also incubated for 5 min with 5% BSA/TBST, to block nonspecific binding. Then, a specific anti-*P. gingivalis* antibody raised by rabbit (1:2000, kindly provided by Prof. Yoshimitsu Abiko, Nihon University School of Dentistry at Matsudo) or anti-CD68 antibody (1:50, Dako Japan, Tokyo, Japan) was applied to the specimen for 60 min at room temperature. After the first antibody incubation, the slide was subsequently incubated with the Envision System as the secondary antibody (Dako Japan, Tokyo, Japan). Before each step, the slide was rinsed three times with phosphate buffered saline. The locations bound by secondary antibodies were visualized using the DAB system. The slide was then counterstained with hematoxylin and examined with an optical microscope.

Results

LAMP and highly sensitive PCR-LAMP

To evaluate LAMP sensitivity, DNA extracted from the standard *P. gingivalis* strain (ATCC 33277) was used as the positive control and deionized water as the negative control. Figure 1a shows that *P. gingivalis* DNA was detectable after 15–20 min in LAMP. The negative control was undetectable even when incubation time was extended to 60 min (Fig. 1a). In addition, LAMP did not detect DNA from oral bacteria other than *P. gingivalis* (Fig. 1a). We next examined the sensitivity of LAMP by using serial dilution of *P. gingivalis* DNA. The detection limit of LAMP was 50 fg/tubes, an amount that corresponds to only 21 copies per tube of *P. gingivalis* genomic DNA (Fig. 1b). However LAMP was unable to detect *P. gingivalis* consistently from FFPE samples. (Fig. 1c) The amount of DNA in FFPE samples may have been insufficient for LAMP. Because LAMP was unable to detect *P. gingivalis* in FFPE tissues, we developed a more sensitive method—PCR-LAMP. DNA extracted from standard *P. gingivalis* was first processed by PCR, and the PCR product was subsequently subjected to LAMP reaction. This resulted in amplification of *P. gingivalis*, as indicated by the real-time turbidimeter, which was similar to LAMP (Fig. 1c). By serial dilution of the template DNA from 5 ag/μL to 0.5 ng/μL the assessment of detection limit of PCR-LAMP was performed. The PCR-LAMP detection limit was 5 fg/μL. This value corresponds to two

copies of *P. gingivalis* per tube (Fig. 1d).

Electrophoresis and restriction enzyme assay

The products of LAMP and PCR-LAMP were visible by electrophoresis using 2% agarose gel and exhibited a ladder band that correlated with *P. gingivalis* DNA amplification. LAMP amplicons exhibited the usual ladder pattern in electrophoresis (13). To further confirm the structure of these LAMP and PCR-LAMP products, they were subjected to digestion with the restriction enzyme *Acc* II. Fragments were analyzed by 2% agarose gel electrophoresis, and the respective band sizes were 163 bp and 109 bp, for both the LAMP and PCR-LAMP products (Fig. 1e) (13).

PCR-LAMP analysis of FFPE samples

PCR-LAMP was used to analyze genomic DNA extracted from 110 FFPE samples; *P. gingivalis* was detected in 67 samples (60.9%) (Fig. 2; Table 2). In contrast, PCR and LAMP alone failed to detect *P. gingivalis* (0%) (Table 2).

Confirmation of PCR-LAMP products from FFPE samples

To confirm PCR-LAMP products amplified from FFPE samples, the final products were digested using a restriction enzyme (*AccII*). The resultants were electrophoresed and

compared to the ladder bands of non-digested PCR-LAMP amplicon. All the non-digested samples except for negative control exhibited typical ladder bands of LAMP reaction (left panel of Fig. 3). In contrast, all digested samples showed only two bands whose sizes have accordance with the calculated sizes (163 bp and 109 bp, right panel of Fig. 3). These electrophoretic patterns were the similar to those of Fig. 1-f.

IHC

Next IHC was performed by using anti-*P. gingivalis* antibody to confirm the presence of *P. gingivalis* in the 67 FFPE samples in which *P. gingivalis* was detected by PCR-LAMP. An essential histopathological feature of the examined periapical lesions is that they are composed of a granuloma that has a small cystic mass with or without epithelium lining. The granulation layer contains various capillaries and varying amounts of inflammatory cell infiltrate, including macrophages. Thus, we also performed IHC with the anti-CD68 antibody, a marker of macrophages. *P. gingivalis* immunopositivity was observed in 14 of the 67 FFPE specimens (Fig. 4; Table 2).

P. gingivalis organisms appeared as tiny immunopositive particles in the granular layer and were mostly present around and in the cytoplasm of macrophages (Fig. 3). *P. gingivalis* immunopositivity was not observed in the FFPE specimens in which no *P. gingivalis* had been detected by PCR-LAMP (Table 2).

Discussion

Chronic apical periodontitis lesions are caused by the action of virulence factors of bacteria and by inflammatory cytokines and chemokines after local pathogen stimulation in root canals (18). Bacterial persistence in clinically asymptomatic periapical lesions is a major cause of prolonged endodontic treatment (19). *P. gingivalis* is a known cause of marginal periodontitis but is also found in root canals (8,9), which suggests that it is a cause of periapical periodontitis. However, the presence and localization of *P. gingivalis* in periapical lesions of chronic apical periodontitis have not been reported. A highly sensitive method for *P. gingivalis* detection was developed. First, a LAMP primer set specific for *P. gingivalis* DNA was designed, and performed a LAMP reaction. The primer set specifically detected *P. gingivalis*, and the detection limit was approximately 21 bacterial copies. However, LAMP did not detect *P. gingivalis* in FFPE samples. During FFPE preparation, the samples were soaked in formaldehyde. This treatment forms crosslinks between proteins and nucleic acids, and one report noted that the quality of DNAs extracted from FFPE samples was not suitable for DNA detection methods such as PCR (20). Thus, using the idea of nested PCR, a highly sensitive method that combined LAMP and PCR was developed to produce a much more sensitive technique for detecting *P. gingivalis* in FFPE samples. (21). PCR-LAMP accurately detected *P. gingivalis* and had a detection limit superior to that of LAMP alone. PCR-LAMP

detection required only two or more copies of *P. gingivalis*, whereas LAMP alone required more than 21 copies. To confirm the effectiveness of PCR-LAMP for *P. gingivalis* detection in FFPE samples, it to analyze 110 clinical samples were used. *P. gingivalis* was detected in 60.9% of these FFPE samples. This rate is much higher than those reported in previous studies of culture methods (detection rate 6.7%–15%) and PCR (detection rate 28%–43%) (10-12). Then, to examine the accurate amplification by PCR-LAMP from the FFPE samples, the final products of PCR-LAMP were analyzed. The results supported the accuracy of PCR-LAMP. Furthermore, to confirm the specificity of PCR-LAMP, IHC was performed using the same 110 FFPE samples. The results of IHC showed that *P. gingivalis* was present only in the FFPE samples in which *P. gingivalis* was detected by PCR-LAMP. Taken together, these findings indicate that PCR-LAMP is a highly sensitive, highly specific detection system that requires only a small amount of DNA. The advantages of PCR-LAMP are that it is highly sensitive, faster, and cheaper in detecting specific culpable species. It thus has considerable potential in DNA diagnosis of intractable disease. For example, acute bacterial infection is a cause of Henoch-Schönlein purpura (22). The incidences of renal and gastrointestinal complications are higher for Henoch-Schönlein purpura patients with periapical lesions than for those without periapical lesions, and the former often undergo tooth extraction, which markedly improves their condition (23). However, some of these patients exhibit a transient flare-up after tooth extraction (24). It may be possible to use PCR-LAMP to evaluate disease

activity and pathogenesis in Henoch-Schönlein purpura patients.

Our IHC results confirm the findings of PCR-LAMP and indicate that *P. gingivalis* was present in the granular layer of chronic apical periodontitis, as particles in and around macrophages, but not in the periphery. Only a few histopathological studies have used clinical samples of pathogenic bacteria, despite the importance of such bacteria in periapical lesion development. To our knowledge, this is the first report of *P. gingivalis* localization in human periapical periodontitis.

In conclusion, PCR-LAMP was an effective tool for detecting small amounts of species-specific DNA. PCR-LAMP will enable extensive studies of bacterial presence in various types of clinical samples. In addition, the present IHC results increase our understanding of the role of *P. gingivalis* in the progression of periapical periodontitis.

Conclusions

PCR-LAMP was a strong tool in detecting small levels of species specific DNA. PCR-LAMP will enable extensive studies on bacteria presence in many kinds of clinical sample. In addition, our results of IHC may contribute to understand the role of *P. gingivalis* on the progression of periapical periodontitis.

Acknowledgements

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Tables

Table 1. Primers used for LAMP

Name	Sequence
F3	5’-TAGGGGAACTGAGAGGTT-3’
B3	5’-CGATGCTTATTCTTACGGTAC-3’
FIP	5’-ACCAATATTCCTCACTGCTG-CCCACACTGGTACTGAGAC-3’
BIP	5’-GAAGGAAGACTGTCCTAAGGATT-TGCAATACTCGTATCGCC-3’
LF	5’-CTCCCGTAGGAGTCTGGTCC-3’

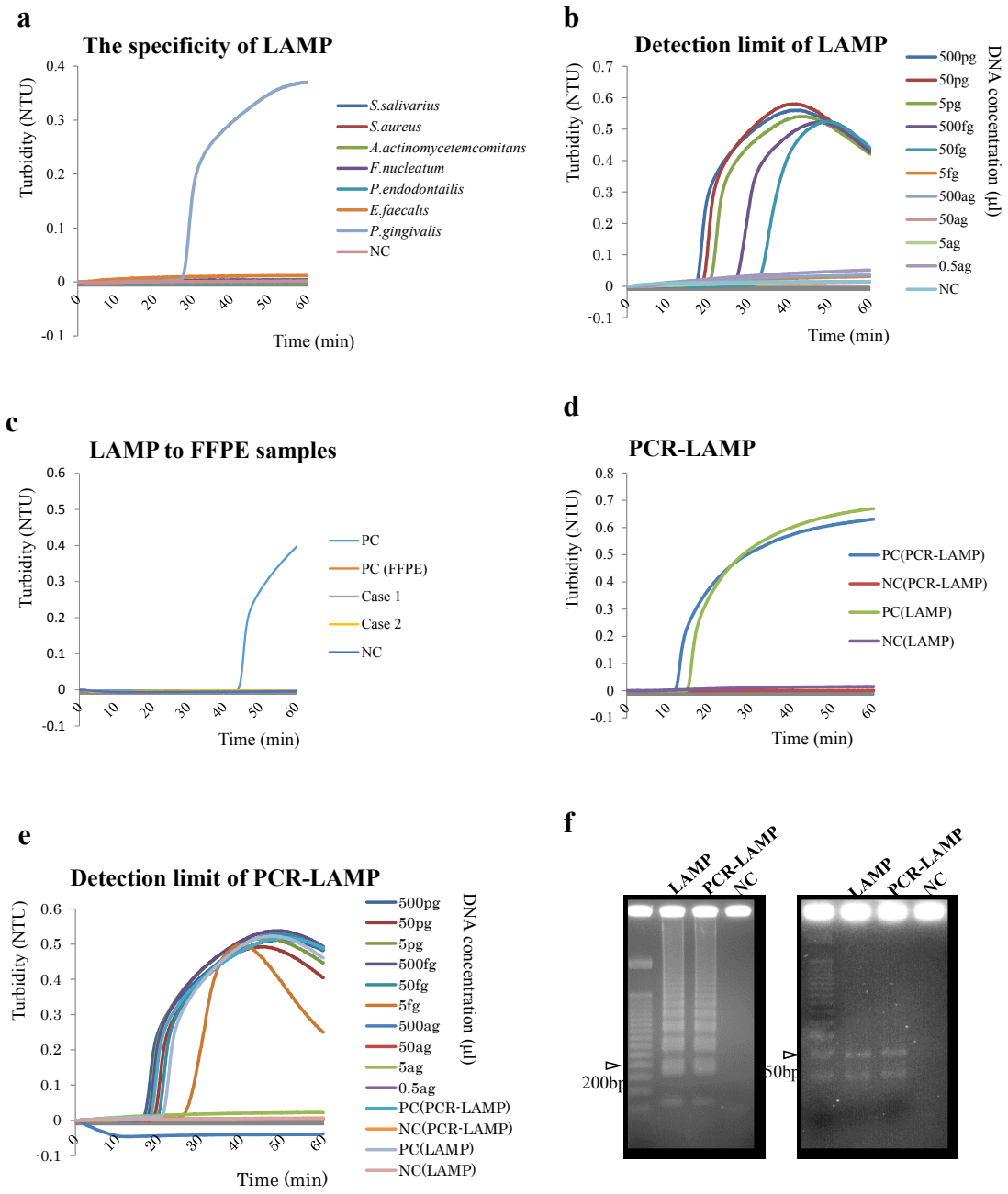
Table 2. Results of PG-DNA detection by PCR-LAMP and IHC

Method	Positive case	Positive ratio (%)
PCR-LAMP	67	60.9
LAMP	0	0
PCR	0	0
IHC	14	12.7

Total 110 cases were analyzed by each method. PCR and LAMP were 0%

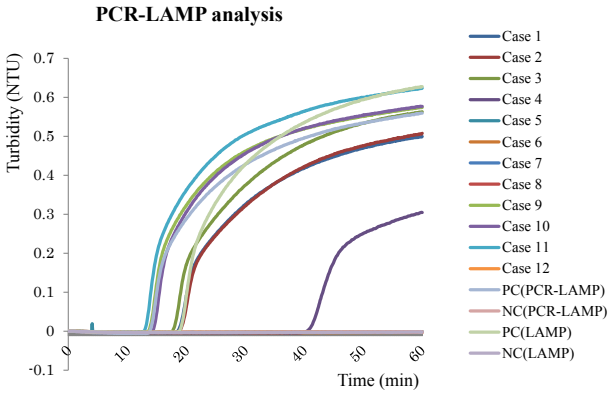
Figures

Fig 1. LAMP and PCR-LAMP



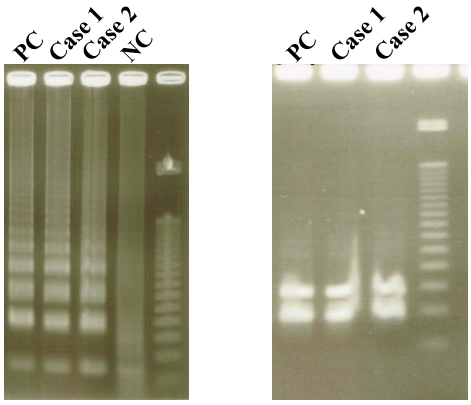
a) LAMP amplified only *P. gingivalis* DNA and did not amplify DNA of other bacteria species. b) The detection limit of LAMP was 50 fg/μL (21 copies/tube). c) LAMP alone failed to detect *P. gingivalis* genomic DNA from the FFPE samples. d) The detection limit of PCR-LAMP was 5 fg/μL (2 copies/tube). e) The products of PCR-LAMP and LAMP exhibited ladder pattern on electrophoresis (14). The NC yielded no result. After digestion by restriction enzyme (*Acc* II), both products converged to two bands on electrophoresis.

Fig 2. PCR-LAMP analysis of FFPE samples



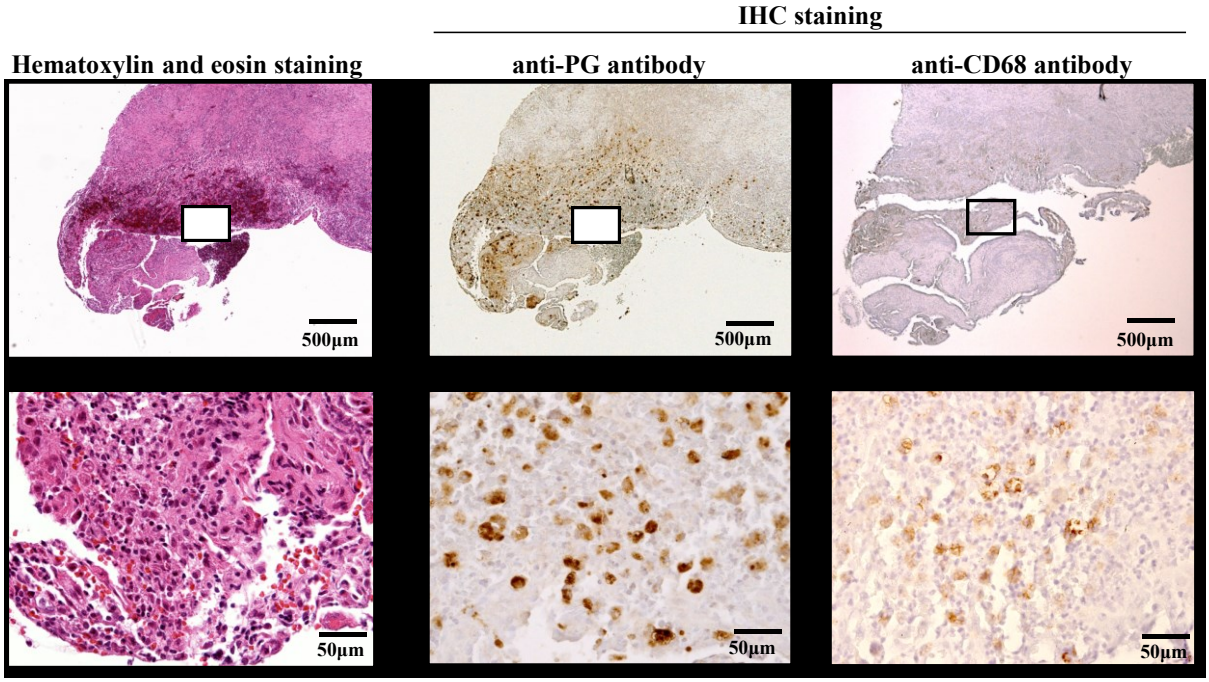
P. gingivalis-DNA could be detected in 67 cases with PCR-LAMP in total 110 cases. 12 *P. gingivalis*-positive cases were randomly selected and shown.

Fig, 3 Confirmation of the analysis of FFPE specimens



The two products of positive cases of PCR-LAMP to FFPE samples exhibited a ladder pattern on electrophoresis. After digestion by restriction enzyme (*Acc* II), products converged to two bands on electrophoresis.

Fig 4. Histopathological and immunohistochemical findings of chronic apical periodontitis



The left panels show hematoxylin and eosin staining. The middle panels show IHC staining with anti-*P. gingivalis* antibody. The right panels show IHC staining with anti-CD68 antibody. Anti-*P. gingivalis* (+) particles were observed in and around macrophages (CD68-positive cells) in chronic apical periodontitis. These cells were concentrated in the granular layer of chronic apical periodontitis tissue.