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Short communication

Loop-mediated isothermal amplification method for the rapid detection of *Enterococcus faecalis* in infected root canals

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Introduction: *Enterococcus faecalis* is a major pathogen in the etiology of apical periodontitis after root canal treatment. A loop-mediated isothermal amplification method, which amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions using a set of four specially designed primers and a DNA polymerase with strand-displacement activity, was developed for the rapid detection of *E. faecalis* in clinical specimens from root canals.
Methods: Primers for detecting *E. faecalis* from the *azoA* gene were designed. The specificity of this assay was evaluated using various oral bacteria and the sensitivity was evaluated using serially diluted *E. faecalis* chromosomal DNA. In addition, loop-mediated isothermal amplification assays were applied to the rapid detection of *E. faecalis* from endodontic samples.
Results: The loop-mediated isothermal amplification products amplified with the primer set were specific for *E. faecalis*. To confirm the specificity of the amplicon, the amplified products were digested with the restriction endonuclease *Sau*3AI. The lower detection limit of the *E. faecalis* primer set without the loop primer set was 10 μg/tube for a 50-min loop-mediated isothermal amplification reaction. Using loop primers increased the detection sensitivity by several orders of magnitude. Furthermore, *E. faecalis* was detected with the loop-mediated isothermal amplification assay in four root canals from 18 individuals and the detection results were consistent with those of conventional polymerase chain reactions.
Conclusion: These results indicate that the loop-mediated isothermal amplification assay is very useful for rapid detection of *E. faecalis* and diagnosis of endodontic infection.

Key words: *Enterococcus faecalis*; loop-mediated isothermal amplification; rapid detection; root canal infection

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Apical periodontitis is an infectious disease that is caused predominantly by anaerobic bacteria, which generally survive in the apical portions of root-filled teeth (13, 20–22, 31). *Enterococcus faecalis*, which possesses various survival and virulence factors (30), is a major pathogen in the etiology of apical periodontitis after root canal treatment. Many studies have reported the existence of *E. faecalis* in root-filled teeth with apical periodontitis (7, 8, 13, 19, 20, 22, 24, 29, 31).

Genetic analyses have been developed to detect and identify the microorganisms associated with endodontic infections. The polymerase chain reaction is one of the most widely used techniques (5, 12, 24, 26, 28). Although polymerase chain reaction-based detection of bacteria is sensitive and specific, it requires specialized equipment, such as thermal cyclers, and several operations.

Recently, Eiken Chemical developed a novel nucleic acid amplification method called loop-mediated isothermal amplification (14, 16–18). The loop-mediated isothermal amplification reaction requires a DNA polymerase with strand-displacement activity and a set of four specially designed primers, called inner and outer primers. First, a stem-loop DNA structure, in which the sequences of the two ends of DNA are derived from the inner primer, is constructed (17, 18). Subsequently, self-primed DNA synthesis occurs rapidly at the 3′ end of the stem-loop DNA structure, and one inner primer hybridizes to the loop on the product in the loop-mediated isothermal amplification cycle and initiates strand displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem that is twice as long as the original. The final products are the stem-loop DNA of the target DNA. Loop-mediated isothermal amplification is a novel approach for nucleic acid amplification, with high specificity, selectivity and rapidity (27). The primary characteristic of the loop-mediated isothermal amplification method is its ability to amplify nucleic acids under isothermal conditions at temperatures between 60 and 65°C (17, 18). Importantly, this method does not require denaturation of the DNA template. The second characteristic of this method is its high specificity. The loop-mediated isothermal amplification reaction requires a DNA polymerase with strand-displacement activity and a set of four specially designed primers, to improve specificity. Furthermore, the amplification efficiency of the loop-mediated isothermal amplification method is extremely high because there is no time loss with thermal change, the reaction being isothermal (17, 18).

The loop-mediated isothermal amplification assay has proven to be a beneficial tool that facilitates genetic testing for the rapid diagnosis of infectious disease (3, 9, 10, 27, 32). In this study, we developed and evaluated a loop-mediated isothermal amplification method for the rapid detection of *E. faecalis*.

The following bacterial strains were used in this study: *E. faecalis* [ATCC19433 (JCM5803), ATCC19433 (JCM7783), ATCC29212], *Candida albicans* (ATCC18804), *Actinomyces viscosus* (ATCC15987, ATCC15988, ATCC43146), *Actinobacillus actinomycetemcomitans* (TN-1), *Streptococcus mutans* (Xc, GS-5), *Streptococcus sobrinus* (OMZ176), *Streptococcus mitis* (903), *Streptococcus sanguinis* (ATCC10556), *Streptococcus gordonii* (DL1), *Streptococcus oralis* (ATCC10557), *Streptococcus salivarius* (HT9R), *Porphyromonas gingivalis* (W83, W50, 381), *Treponema denticola* (ATCC35404, ATCC35405), *Fusobacterium nucleatum* (ATCC10953), *Tannerella forsythia* (ATCC43037), *Prevotella intermedia* (ATCC25611), *Prevotella melaninogenica* (ATCC25845) and *Prevotella nigrescens* (ATCC 25261). The *E. faecalis* strains were grown in Trypticase Soy Broth (Becton-Dickinson Co., Sparks, MD) at 37°C under anaerobic conditions.

Routine molecular biology techniques were performed as described elsewhere (25). Chromosomal DNA was isolated from the bacteria using an IsoQuick Nucleic Acid Extraction kit (ORCA Research, Bothell, WA) or a Pure Gene DNA Isolation kit (Gentra Systems, Minneapolis, MN).

The oligonucleotide primers used in this study are listed in Table 1. The loop-mediated isothermal amplification method requires a set of four specially designed primers [F3, B3, the forward inner primer, and the backward inner primer] that recognize a total of six distinct sequences (F1, F2, F3, B1, B2 and B3) in the target DNA (32). The two inner primers, the forward inner primer and backward inner primer, contain two distinct sequences that correspond to the sense and antisense sequences of the DNA, one for priming in the first stage and the other for self-priming in later stages. forward inner primer consists of complementary sequence F1 (F1c) and direct sequence F2 (F2). Backward inner primer consists of complementary sequence B1 (B1c) and direct sequence B2 (B2). The two outer primers, F3 and B3c (the sequence complementary to B3), are located outside the F2–B2 region. To increase amplification efficacy, two loop primers, the forward loop primer (LF) and backward loop primer (LB), were designed using PRIMER EXPLORER software, version 3.0 (Fujitsu, Tokyo, Japan), as shown in Fig. 1. The specific primers for *E. faecalis* were designed from the *azoA* gene, which encodes azoreductase (2). The specificities of the designed primers were initially confirmed using BLAST on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/) and then confirmed using loop-mediated isothermal amplification.

The loop-mediated isothermal amplification reaction was carried out in a 25-μl volume containing 1.6 μM each of forward inner primer and backward inner primer, 0.2 μM each of F3 and B3, 0.8 μM each of LF and LB, 1.4 mM each of deoxynucleoside triphosphate (dNTP), 0.8 M betaine (Sigma, St Louis, MO), 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.2% Tween-20, 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs, Beverly, MA), and 5 μl target DNA. The mixture was incubated at 66°C using a conventional heating block, and then heated to above 80°C for 2 min to terminate the reaction.

The amplified products were subjected to agarose gel electrophoresis. The loop-mediated isothermal amplification products were digested with the appropriate restriction enzymes (*Sau*3AI for *E. faecalis* amplicons) and electrophoresed in 2% agarose gels. The sensitivities of the loop-mediated isothermal amplification assays were confirmed using serially diluted chromosomal DNA. Furthermore, loop-mediated isothermal amplification amplicons were detected in the reaction mixture directly with the naked eye on adding 1.0 μl 1 : 10-diluted original

Table 1. Oligonucleotide primers for loop-mediated isothermal amplification

Primer	Type	Sequence[1]	Amplicon size (bp)	Target	Source (strain)
Enterococcus faecalis					
Ef-F3	F3	5′-GCCGGAAATCGATGAAGA-3′	220	*azoA*	ATCC 19433
Ef-B3	B3	5′-TCCAGCAACGTTGATTGT-3′			
Ef-FIP	FIP	5′-CACTTTTTGTTGTTGGTTTTCGCTTTATTATCTGCTTGGGGTGC-3′			
Ef-BIP	BIP	5′-ATCTGCAGACAAAGTAGTAATTGCTCCAAGCTTTTAAGCGTGTC-3′			
Ef-LF	LF	5′-AAATGCTGCGCCAGCTCG-3′			
Ef-LB	LB	5′-TCCAATGTGGAACTTAAACGTACC-3′			

[1]Accession number: AY422207 for *Enterococcus faecalis azoA* gene. FIP, forward inner primer; BIP, backward inner primer.

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  1 ATGTCAAAAT TATTAGTTGT TAAAGCACAT CCATTAACAA AAGAAGAATC  50
 51 ACGCTCAGTT CGTGCGTTAG AAACATTTTT AGCTTCATAC CGTGAAACAA 100
101 ATCCATCAGA CGAAATCGAA ATTTTAGATG TTTATGCACC AGAAACAAAC 150
      F3                    F2
151 ATGCCGGAAA TCGATGAAGA ATTATTATCT GCTTGGGGTG CACTTCGAGC 200
          LF                                  F1
201 TGGCGCAGCA TTTGAAACAT TAAGCGAAAA CCAACAACAA AAAGTGGCTC 250
                                  B1
251 GTTTTAATGA ATTAACTGAT CAATTTTTAT CTGCAGACAA AGTAGTAATT 300
      LB                                        B2
301 GCTAATCCAA TGTGGAACTT AAACGTACCG ACACGCTTAA AAGCTTGGGT 350
                  B3
351 AGATACAATC AACGTTGCTG GAAAAACATT CCAATATACT GCAGAAGGAC 400
401 CAAAACCTCT AACAAGTGGT AAAAAAGCCT TACACATCCA ATCAAATGGC 450
451 GGCTTCTACG AAGGAAAAGA TTTTGCTTCT CAATACATTA AAGCGATTCT 500
501 AAACTTTATT GGCGTTGATC AAGTCGACGG ATTATTCATC GAAGGAATCG 550
551 ATCATTTCCC TGATCGCGCG GAAGAACTTT TAAATACTGC CATGACCAAA 600
601 GCAACTGAAT ACGGTAAAAC ATTCTAA
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Fig. 1. Nucleotide sequences of *Enterococcus faecalis azoA* gene used for loop-mediated isothermal amplification (LAMP) primer. The sequences used for LAMP primers are shown in bold type.

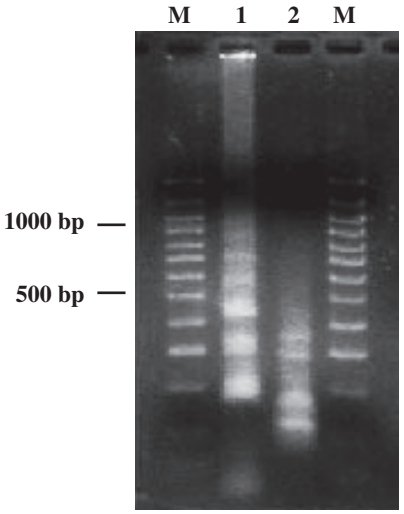

Fig. 2. Restriction analysis of loop-mediated isothermal amplification (LAMP) products. Lane M, 100-base-pair DNA ladder (Promega); lane 1, amplified products of *Enterococcus faecalis azoA*; lane 2, *E. faecalis azoA* digested with *Sau*3AI.

SYBR Green I (Molecular Probes, Eugene, OR) to the mixture and observing the solution color (32). The solution turned green in the presence of a loop-mediated isothermal amplification amplicon, while it remained orange with no amplification. Otherwise, the turbidity derived from the white magnesium pyrophosphate precipitate in the mixture was detected with the naked eye (14).

To apply the loop-mediated isothermal amplification assay to clinical specimens, 18 samples were taken from 18 patients with teeth that had previously been root-filled for at least 4 years and all of which showed radiographic evidence of apical periodontitis. No tooth showed signs of associated swelling or purulent exudate. The patients were aged 10–72 years (mean 35.9 years), six were female and 12 were male.

Endodontic samples were taken from infected root canals, as previously reported (6, 7). The teeth were individually isolated from the oral cavity with a previously disinfected rubber dam. After isolating the tooth, the plaque was removed and the tooth, surrounding dam and clamp were cleaned with 30% hydrogen peroxide, then swabbed with 5% potassium iodide. In multi-rooted teeth, the largest canal or the canal related to a periapical radiolucency was selected. A sterile paperpoint moistened in sterile saline was placed in the root canal, and left for 10 s. The paperpoint was transferred into 200 μl cell lysis buffer. The cells were boiled at 100°C for 5 min, and the supernatant served as the template (32).

The polymerase chain reaction technique was performed as previously described (12). The chromosomal DNA from endodontic samples was amplified using the polymerase chain reaction primers CAA GGC ATC CAC CGT and GAA GTC GTA ACA AGG targeted against the 16S/23S rDNA intergenic region. The polymerase chain reaction temperature conditions included an initial step of 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final step of 72°C for 10 min.

The loop-mediated isothermal amplification reaction for *E. faecalis* was performed at 66°C for 60 min and the results were checked by electrophoresis on 2% agarose gels. A successful loop-mediated isothermal amplification reaction with species-specific primers produces many bands of different sizes (10, 19). A characteristic ladder of multiple bands is seen in Fig. 2.

The loop-mediated isothermal amplification products amplified with the primer set F3, B3, forward inner primer, backward inner primer, LF and LB demonstrated specificity for *E. faecalis*, and were negative for the DNA of other oral bacteria species. To confirm the specificity of the amplicon, the amplified products were digested with the restriction endonuclease *Sau*3AI, and subjected to agarose gel electrophoresis (Fig. 2).

The sensitivity of this assay for *E. faecalis* was evaluated using a serial dilution of *E. faecalis* chromosomal DNA. The *E. faecalis* primer set without the loop primer set had a lower detection limit of 10 μg/tube for a 50-min reaction (Table 2). The lower detection limit of the *E. faecalis* primer set with the pair of loop primers was 100 fg/tube for a 50-min reaction (Table 2).

Root canal samples from 18 individuals were subjected to the *E. faecalis* loop-mediated isothermal amplification assay. *E. faecalis* was detected in four root canals; these results were comparable to the results of polymerase chain reaction-based detection. We confirmed the inhibitory effects of the oral specimens on loop-mediated isothermal amplification. The presence of loop-mediated isothermal amplification inhibitors was assessed using lysates spiked with *E. faecalis*-negative root canal specimens and the effects were negligible (data not shown).

It is generally believed that the major cause of root canal failure is infection of

Table 2. Detection sensitivities of loop-mediated isothermal amplification

	Without loop primers Genomic DNA (fg/tube)[1]										With loop primers Genomic DNA (fg/tube)									
Primer set (min)	0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9
Enterococcus faecalis																				
0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
30	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+
40	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+
50	−	−	−	−	−	−	−	+	+	+	−	−	+	+	+	+	+	+	+	+
60	−	−	−	−	−	−	−	+	+	+	−	−	+	+	+	+	+	+	+	+

[1]+, clearly visible; −, not visible.

the apical portion of the root-filled tooth (7, 8, 11, 13, 19–22, 24, 31), and a polymerase chain reaction-based examination to check for infected root canals has been developed (24, 29). However, the polymerase chain reaction method has several intrinsic disadvantages (14, 17, 18), such as requiring expensive reaction equipment for rapid thermal cycling, as well as the time, labor and costs of the detection tests. By contrast, the loop-mediated isothermal amplification method needs only a water-bath at a constant temperature of 66°C or a conventional heating block. Other characteristics of the loop-mediated isothermal amplification method are its high specificity and high amplification efficiency, which enable the rapid detection of bacterial DNA (14, 17, 18). As the loop-mediated isothermal amplification method uses four primers that recognize six distinct regions on the template DNA, its specificity is extremely high. Recently, several studies have reported the application of loop-mediated isothermal amplification for the clinical diagnosis of infectious diseases (3, 4, 9, 10, 23, 32). In this investigation, we used the loop-mediated isothermal amplification method for the rapid detection of *E. faecalis*, which plays a major role in the etiology of persistent apical periodontitis lesions (30).

First, we evaluated the specificity of the loop-mediated isothermal amplification assay for this organism. We confirmed the specificity of the bacteria-specific primers using various oral bacterial DNA samples, because more than 700 bacterial species constitute the normal inhabitants of the oral cavity (1, 6). Furthermore, we evaluated the detection limits of these assays using serially diluted chromosomal DNA. The lower detection limit, with loop primers in a 50-min reaction, was 100 fg/tube for *E. faecalis*. Adding a set of loop primers to B3, F3, backward inner primer and forward inner primer accelerated the loop-mediated isothermal amplification reaction. These results accord with previous studies (15, 32).

We applied this assay to endodontic specimens from 18 individuals: four samples were positive and the same results were obtained using polymerase chain reaction-based detection. These results suggest that loop-mediated isothermal amplification-based detection can be applied to oral specimens. We are now comparing the relationship between the clinical endodontic condition and the existence of *E. faecalis*.

In conclusion, loop-mediated isothermal amplification was developed for the rapid and accurate detection of *E. faecalis*, one of the most frequently detected bacterial species in persistent endodontic infection. We showed that loop-mediated isothermal amplification is suitable for rapid screening of oral bacteria in infected root canals.

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References

1. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol 2005: **43**: 5721–5732.
2. Chen H, Wang RF, Cerniglia CE. Molecular cloning, overexpression, purification, and characterization of an aerobic FMN-dependent azoreductase from *Enterococcus faecalis*. Protein Expr Purif 2004: **34**: 302–310.
3. Endo S, Komori T, Ricci G et al. Detection of *gp43* of *Paracoccidioides brasiliensis* by the loop-mediated isothermal amplification (LAMP) method. FEMS Microbiol Lett 2004: **234**: 93–97.
4. Enosawa M, Kageyama S, Sawai K et al. Use of loop-mediated isothermal amplification of the IS900 sequence for rapid detection of cultured *Mycobacterium avium* subsp. *paratuberculosis*. J Clin Microbiol 2003: **41**: 4359–4365.
5. Fouad A, Barry FJ, Caimano M et al. PCR-based identification of bacteria associated with endodontic infections. J Clin Microbiol 2002: **40**: 3223–3231.
6. Gomes BP, Lilley JD, Drucker DB. Clinical significance of dental root canal microflora. J Dent 1996: **24**: 47–55.
7. Gomes BP, Pinheiro ET, Gade-Neto CR et al. Microbiological examination of infected dental root canals. Oral Microbiol Immunol 2004: **19**: 71–76.
8. Hancock HH, III, Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001: **91**: 579–586.
9. Hong TC, Mai QL, Cuong DV et al. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J Clin Microbiol 2004: **42**: 1956–1961.
10. Iwamoto T, Sonobe T, Hayashi K. Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J Clin Microbiol 2003: **41**: 2616–2622.
11. Mejare B. *Streptococcus faecalis* and *Streptococcus faecium* in infected dental root canals at filling and their susceptibility to azidocillin and some comparable antibiotics. Odontol Rev 1975: **26**: 193–204.
12. Molander A, Lundquist P, Papapanou PN, Dahlen G, Reit C. A protocol for polymerase chain reaction detection of *Enterococcus faecalis* and *Enterococcus faecium* from the root canal. Int Endod J 2002: **35**: 1–6.
13. Molander A, Reit C, Dahlen G, Kvist T. Microbiological status of root-filled teeth with apical periodontitis. Int Endod J 1998: **31**: 1–7.
14. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun 2001: **289**: 150–154.

15. Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 2002: **16**: 223–229.
16. Nagamine K, Kuzuhara Y, Notomi T. Isolation of single-stranded DNA from loop-mediated isothermal amplification products. Biochem Biophys Res Commun 2002: **290**: 1195–1198.
17. Nagamine K, Watanabe K, Ohtsuka K, Hase T, Notomi T. Loop-mediated isothermal amplification reaction using a nondenatured template. Clin Chem 2001: **47**: 1742–1743.
18. Notomi T, Okayama H, Masubuchi H et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000: **28**: E63.
19. Peciuliene V, Balciuniene I, Eriksen HM, Haapasalo M. Isolation of *Enterococcus faecalis* in previously root-filled canals in a Lithuanian population. J Endod 2000: **26**: 593–595.
20. Peciuliene V, Reynaud AH, Balciuniene I, Haapasalo M. Isolation of yeasts and enteric bacteria in root-filled teeth with chronic apical periodontitis. Int Endod 2001: **J34**: 429–434.
21. Pinheiro ET, Gomes BP, Ferraz CC, Sousa EL, Teixeira FB, Souza-Filho FJ. Microorganisms from canals of root-filled teeth with periapical lesions. Int Endod J 2003: **36**: 1–11.
22. Pinheiro ET, Gomes BP, Ferraz CC, Teixeira FB, Zaia AA, Souza Filho FJ. Evaluation of root canal microorganisms isolated from teeth with endodontic failure and their antimicrobial susceptibility. Oral Microbiol Immunol 2003: **18**: 100–103.
23. Poon LL, Leung CS, Tashiro M et al. Rapid detection of the severe acute respiratory syndrome (SARS) coronavirus by a loop-mediated isothermal amplification assay. Clin Chem 2004: **50**: 1050–1052.
24. Rocas IN, Siqueira JF, Jr, Santos KR. Association of *Enterococcus faecalis* with different forms of periradicular diseases. J Endod 2004: **30**: 315–320.
25. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. New York: Cold Spring Harbor Laboratory Press, 1989.
26. Sedgley C, Buck G, Appelbe O. Prevalence of *Enterococcus faecalis* at multiple oral sites in endodontic patients using culture and PCR. J Endod 2006: **32**: 104–109.
27. Seki M, Yamashita Y, Torigoe H, Tsuda H, Sato S, Maeno M. Loop-mediated isothermal amplification method targeting the *lytA* gene for detection of *Streptococcus pneumoniae*. J Clin Microbiol 2005: **43**: 1581–1586.
28. Siqueira JF, Jr, Rocas IN. PCR methodology as a valuable tool for identification of endodontic pathogens. J Dent 2003: **31**: 333–339.
29. Siqueira JF, Jr, Rocas IN. Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004: **97**: 85–94.
30. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. *Enterococcus faecalis*: its role in root canal treatment failure and current concepts in retreatment. J Endod 2006: **32**: 93–98.
31. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998: **85**: 86–93.
32. Yoshida A, Nagashima S, Ansai T et al. Loop-mediated isothermal amplification method for rapid detection of the periodontopathic bacteria *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. J Clin Microbiol 2005: **43**: 2418–2424.