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Bacterial reduction and persistence after endodontic treatment procedures

M. Sakamoto[1], J. F. Siqueira Jr[2], I. N. Rôças[2], Y. Benno[1]

[1]Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama, Japan, [2]Department of Endodontics, Estácio de Sá University, Rio de Janeiro, RJ, Brazil

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Bacteria that persist after endodontic disinfection procedures may lead to treatment failure. Over 50% of the bacteria found in endodontic infections are as-yet-uncultivated so investigations of bacteria that endure treatment procedures should include techniques that side-step cultivation. This culture-independent study evaluated the bacterial reduction promoted by intracanal disinfection procedures and identified the taxa persisting after treatment. Samples taken from the infected canals of teeth with apical periodontitis before treatment (S1), after instrumentation using NaOCl as irrigant (S2) and after interappointment medication with a calcium hydroxide paste (S3) were subjected to 16S rRNA gene clone library and real-time polymerase chain reaction analyses. The S2 and S3 samples from five of the 15 canals showed negative results. In the other cases, instrumentation and instrumentation/medication promoted a significant reduction (99.67% and 99.85%, respectively) in the number of bacteria when compared to S1 samples. Forty-three distinct bacterial taxa were identified, of which 24 (56%) were as-yet-uncultivated phylotypes. Nineteen of these 43 taxa (including eight as-yet-uncultivated phylotypes) were disclosed in post-treatment samples, with streptococci being the most prevalent taxa. Findings demonstrated that culture-independent methods provide a detailed insight into the effects of intracanal disinfection protocols, helping to define more effective strategies to deal with endodontic bacteria, including as-yet-uncultivated phylotypes.

Key words: antimicrobial treatment; apical periodontitis; endodontic microbiology; real-time polymerase chain reaction; 16S rRNA gene clone library

José F. Siqueira Jr, Avenida Almte Ary Parreiras 311/1001, Icaraí, Niterói, RJ, Brazil 24230-322
Tel.: +55 21 2503 7289 (ext. 249);
fax: +55 21 2503 7289 (ext. 223);
e-mail: jf_siqueira@yahoo.com
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Because apical periodontitis lesions have an infectious etiology, the success of the endodontic treatment depends on how effective the clinician is in eradicating bacteria from the root canal system (24). During the treatment of infected root canals, two steps assume special relevance with regard to bacterial elimination – the chemomechanical preparation and the interappointment medication. The former is of paramount importance for root canal disinfection, because instruments and irrigants act primarily on the main canal, which is the most voluminous area of the system and consequently harbors the largest number of bacterial cells. Sodium hypochlorite (NaOCl) remains the most popular irrigant because of its potent antimicrobial and tissue-dissolving abilities (33). Nevertheless, studies have revealed that the chemomechanical preparation *per se* is not sufficient to predictably render root canals bacteria-free, with about 40–50% of the prepared canals still containing cultivable bacteria (2, 3, 23). To overcome the limitations of chemomechanical procedures in disinfecting the entire root canal system, the use of an interappointment medication has been advocated (23, 32). Calcium hydroxide has been the most used intracanal medication, and associations with other medicaments, such as camphorated paramonochlorophenol (CMCP) or chlorhexidine, have shown superior antimicrobial efficacy (28, 30).

Most clinical trials evaluating the antibacterial effectiveness of intracanal procedures and the bacterial species persisting after treatment have been based on traditional culture-dependent methods (3, 14, 23, 32, 31). However, results from these studies may have been influenced by significant limitations of culture procedures, including low sensitivity, inability to detect fastidious and as-yet-uncultivated

bacteria, and misidentification of species with aberrant phenotypic behavior (27). Culture-independent molecular biology methods can overcome most of the limitations of culture approaches and have revealed that 40–55% of the endodontic microbiota is made up of as-yet-uncultivated phylotypes (15, 22). Therefore, molecular methods have the potential to provide more reliable results with regard to bacterial elimination by intracanal disinfection procedures. The present study aimed to evaluate the efficacy of an antibacterial endodontic protocol through 16S rRNA gene clone library and quantitative real-time polymerase chain reaction (qPCR) analyses.

Materials and methods

Subjects

Patients presenting to the endodontic clinic at Estácio de Sá University for evaluation and treatment of apical periodontitis were considered for this study. Eighteen single-rooted teeth showing clinical and radiographic evidence of asymptomatic apical periodontitis lesions were included. All teeth had enough crown structure for adequate isolation with a rubber dam, and showed an absence of periodontal pockets deeper than 4 mm. The study protocol was institutionally approved and informed consent was obtained from the patients.

Endodontic treatment and sampling procedures

A rubber dam and an aseptic technique were used throughout the endodontic treatment. The protocol for disinfection of the working field has been described elsewhere (20). Briefly, each tooth was cleansed with pumice and, after rubber dam isolation, the operative field was sterilized with 3% hydrogen peroxide and 2.5% NaOCl solution. Complete access preparations were made and the operative field was again swabbed with 2.5% NaOCl. NaOCl solution was then inactivated by sterile 5% sodium thiosulphate. A bacteriological sample of the disinfected tooth surface was obtained with sterile paper points, which were transferred to fluid thioglycolate medium. For the tooth to be included in the study, samples taken from the working field after surface disinfection had to be uniformly negative. Following this criterion, three teeth were excluded.

The first root canal sample (S1) was taken as described previously (22). Briefly, a K-type file size no. 15 with the handle cut off was introduced to a level approximately 1 mm short of the tooth apex, based on diagnostic radiographs, and a gentle filing motion was applied. Afterwards, two sequential paper points were placed to the same level and used to soak up the fluid in the canal. The cut file and the two paper points were transferred aseptically to cryotubes containing 1 ml TE buffer [10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6] and immediately frozen.

All root canals were prepared by the alternated rotary motions (ARM) technique (29), using hand Nitiflex files (Maillefer, Ballaigues, Switzerland) and copious irrigation with 2.5% NaOCl. The canals were instrumented to at least a size 35 at the working length (which was 1 mm short of the tooth apex as determined by radiographs). Small files (no. 15 or no. 20) were taken to the patency length following each file size used in the apical preparation. After instrumentation was completed, the canal was irrigated with 17% EDTA and then with 2.5% NaOCl. Each canal was dried using sterile paper points and flushed with 2 ml 5% sodium thiosulfate to inactivate the NaOCl solution. Subsequently, the canal walls were gently filed and a post-instrumentation sample (S2) was taken from the canal as described above.

A calcium hydroxide/CMCP/glycerin paste was placed over the entire length of the prepared canal using lentulo spiral fillers. This paste was prepared by mixing equal volumes of glycerin and CMCP and then gradually adding calcium hydroxide powder until a creamy consistency was achieved. The paste was packed at the level of the canal entrance and a radiograph was taken to check for adequate placement (homogeneous filling throughout the entire extent of the prepared canal). The access cavity was then temporized with temporary cement to a thickness of at least 4 mm (Coltosol, Coltène/Whaledent Inc., Cuyahoga Falls, OH).

The second appointment was scheduled for 1 week later. At this time, the tooth was isolated with a rubber dam, the operative field was disinfected as previously, and a control bacteriological sample was obtained from the operating field. The temporary filling was removed and the calcium hydroxide paste was rinsed out of the canal using 2.5% NaOCl and a K-file, followed by inactivation with 5% sodium thiosulphate. The root canal walls were filed lightly to remove loose calcium hydroxide remnants, and a post-medication sample (S3) was taken from the canals. Canals were then filled with gutta-percha and sealer and the tooth was temporarily filled with ionomer cement before a permanent restoration was positioned.

qPCR analysis

DNA extraction procedures were as outlined earlier (22). Samples from all cases were initially screened for the presence of bacteria by a qualitative end-point PCR assay using universal 16S rRNA gene primers (1). Cases that showed positive results in post-treatment samples were selected for qPCR analysis.

The qPCR assay was carried out using the LightCycler system (Roche Diagnostics, Mannheim, Germany) and the dsDNA-binding dye SYBR Green I. The total number of bacteria in samples was determined with *Porphyromonas gingivalis* JCM 8525 cells as a standard using universal primers TotalF (5′-TCC TAC GGG AGG CAG CAG T-3′) and TotalR (5′-GGA CTA CCA GGG TAT CTA ATC CTG TT-3′) (16). The qPCR amplification protocol has been described elsewhere (21). Briefly, amplification was performed in a 20-µl final volume containing 2 µl template DNA, 2 µl LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics), 0.5 µM of each primer and 3 mM $MgCl_2$. The protocol included an initial denaturation step at 95°C for 10 min, followed by 45 cycles of heating at 20°C/s to 95°C with a 0-s hold, cooling at 20°C/s to 60°C with a 5-s hold, heating at 20°C/s to 72°C with an 18-s hold and heating at 20°C/s to 84°C with a 1-s hold. Fluorescent products were detected at the last step of each cycle. After amplification, a melting curve was obtained by heating at 20°C/s to 95°C, cooling at 20°C/s to 70°C and heating slowly at 0.1°C/s to 95°C with fluorescence collection at 0.1°C intervals. Melting peaks were used to determine the specificity of the qPCR. Data were analysed using the LightCycler analysis software. Data obtained from samples S1, S2 and S3 were analysed statistically for differences using the Student's *t*-test comparing pairs of groups with the significance level established at 5% ($P < 0.05$).

16S rRNA gene clone library analysis

Nine samples (three cases) were randomly selected for 16S rRNA gene clone library analysis, which was performed as described previously (22). The primers used for the PCR amplification of 16S rRNA

gene sequences were 27f and 1492r (12). Because the S2 and S3 samples resulted in weak amplification, 2.5 μl of the first reaction was used as template for reamplification using the same PCR conditions. PCR products were purified, ligated into the plasmid vector pCR® 2.1, and then transformed into One Shot® INVαF′ competent cells using the Original TA Cloning Kit (Invitrogen, San Diego, CA). Plasmid DNAs were prepared using the TempliPhi DNA Amplification Kit (Amersham Biosciences, Piscataway, NJ) from randomly selected recombinants and used as templates for sequencing. Sequencing was conducted using the 27f and 519f primers (12), a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). All sequences were checked by the CHIMERA CHECK program of the Ribosomal Database Project-II (RDP-II) (7), and compared with similar sequences of the reference microorganisms by FASTA search (17). Database sequences with the highest similarities and scorebits to our sequences were chosen as their identification. The criterion to define a novel phylotype was set as sequences that differ from the closest GenBank entry by more than 2%.

Results

qPCR analysis

All initial samples (S1) were positive for the presence of bacteria as shown by the qualitative end-point PCR assay. Five samples showed negative PCR results for both S2 and S3 samples and were excluded from qPCR analysis. The other 10 samples showed positive results for S2 and S3 treatments and were subjected to a qPCR reaction for determination of bacterial reduction after treatment. The mean number of 16S rRNA gene copies in these 10 initial samples (S1) was 1.72×10^7 (range 3.97×10^4 to 6.47×10^7). After instrumentation (S2), the mean values of the number of 16S rRNA gene copies decreased to 5.75×10^4 (range 8.62×10^3 to 3.55×10^5), while after intracanal medication (S3) the mean number reached 2.53×10^4 (range 7.45×10^3 to 4.53×10^4). S2 and S3 samples showed a mean reduction of 99.67% (range 46.55–99.94%) and 99.85% (range 65.54–99.98%), respectively, in the number of bacteria when compared to S1 samples. S3 samples showed an overall percentage decrease of 56% in the number of bacteria when compared to S2. The number of 16S rRNA gene copies in S2 and S3 was significantly reduced in comparison to S1 ($P = 0.02$). There was no significant difference when comparing S2 and S3 ($P = 0.35$). Data from the qPCR analysis are depicted in Table 1.

Table 1. Bacterial load and per cent reduction determined for root canal samples of 10 teeth with apical periodontitis lesions

Case	Initial samples (S1)	Post-instrumentation samples (S2)	Post-medication samples (S3)
1T	6.47×10^7	3.55×10^5 (99.45%)	4.53×10^4 (99.93%)
2T	3.97×10^4	2.12×10^4 (46.55%)	7.45×10^3 (81.26%)
4T	1.61×10^6	3.22×10^4 (97.99%)	2.31×10^4 (98.56%)
5T	5.78×10^6	2.03×10^4 (99.65%)	3.28×10^4 (99.43%)
6T	1.53×10^7	1.45×10^4 (99.91%)	2.54×10^4 (99.83%)
7T	1.35×10^7	4.69×10^4 (99.65%)	2.18×10^4 (99.84%)
8T	2.14×10^5	2.89×10^4 (86.50%)	4.38×10^4 (79.53%)
9T	3.82×10^7	2.76×10^4 (99.93%)	7.71×10^3 (99.98%)
10T	4.23×10^4	8.62×10^3 (79.61%)	1.46×10^4 (65.54%)
11T	3.28×10^7	1.95×10^4 (99.94%)	3.09×10^4 (99.91%)
Mean	1.72×10^7	5.75×10^4 (99.67%)	2.53×10^4 (99.85%)

Samples were taken before treatment, after instrumentation with 2.5% NaOCl as irrigant, and after 1 week of calcium hydroxide intracanal medication.

16S rRNA gene clone library analysis

Clone libraries were constructed for S1, S2 and S3 samples from three cases. Forty-three different taxa were identified from the 191 clones sequenced, of which 24 taxa (56%) were identified as phylotypes, i.e. species that have not yet been cultivated and are known only by their 16S rRNA gene sequences. One phylotype was novel in that it has never been previously detected in other sites (*Solobacterium* oral clone 6Ta-2, deposited in the GenBank database under the accession number AB256031). Twenty-seven taxa (63%) belonged to the *Firmicutes* (14 of which were uncultivated phylotypes). The other phyla represented in this study were *Bacteroidetes* (six taxa, three uncultivated phylotypes), *Actinobacteria* (four taxa, three uncultivated phylotypes), *Proteobacteria* (three taxa, two uncultivated phylotypes), *Fusobacteria* (two taxa, one uncultivated phylotype) and *Synergistes* (one uncultivated phylotype).

Bacterial taxa found in samples examined by clone library analysis are displayed in Table 2. Means of 11 taxa were detected in S1 samples, four taxa in S2 samples and five taxa in S3 samples. The most dominant taxa in S1 samples were the novel phylotype *Solobacterium* oral clone 6Ta-2 (31% of the clones in sample 6T), *Bacteroides*-like sp. oral clone X083 (37% in sample 7T) and *Pseudoramibacter alactolyticus* (26% in sample 11T). Nineteen taxa (eight uncultivated phylotypes) were identified in post-treatment samples. No taxon detected in post-treatment samples was found to be dominant in the initial samples. *Streptococcus* species were detected in all post-treatment samples and were also the most dominant taxon in these samples, except for sample S2 from case 7T, in which *Solobacterium* sp. oral clone K010 corresponded to 56% of the clones sequenced.

Discussion

Diligent antimicrobial treatment can occasionally fail to promote total eradication of bacteria from root canals, with consequent selection of the most resistant segment of the microbiota. Gram-negative bacteria, which are common members of primary intraradicular infections, are usually eliminated following treatment, though studies have reported that some anaerobic rods, such as *Fusobacterium nucleatum* and *Prevotella* species, are among the most common species found in post-instrumentation samples (3, 18, 31). The present study also revealed the occurrence of some gram-negative bacteria in post-treatment samples but our findings, by and large, are in agreement with most culture-dependent studies, which reported that gram-positive bacteria are predominant in both post-instrumentation and post-medication samples (3, 5, 4, 6, 10, 18). Streptococci, especially *Streptococcus mitis*, were found in all post-treatment samples, confirming that these bacteria may represent a treatment problem because of persistence (5).

With the recent findings showing as-yet-uncultivated bacteria as constituents of a significant proportion of the endodontic microbiota (15, 22), studies on the effects of intracanal antimicrobial procedures should also rely on the detection of these bacteria. For the present study, we used two culture-independent methods, which have the potential to offer more detailed insights into complex bacterial communities (26). Clone library analysis showed that as-yet-uncultivated phylotypes corresponded to 56% of the detected taxa, which

Table 2. Bacterial taxa detected during treatment of three infected root canals associated with apical periodontitis. Data are based on 16S rRNA gene sequencing analysis

	Persisting taxa	
Taxa only in initial samples (S1)	Post-instrumentation (S2)	Post-medication (S3)
Bacteroides-like sp. oral clone X083 (3)	*Streptococcus mitis* (3)	*Streptococcus mitis* (3)
Pseudoramibacter alactolyticus (3)	*Acinetobacter junii* (1)	*Streptococcus sanguinis* (2)
Dialister sp. oral clone 9N-1 (2)	Flavobacteriaceae genomosp. C1 (1)	*Fusobacterium nucleatum* (1)
Fusobacterium nucleatum (2)	*Prevotella* sp. oral clone GU027 (1)	*Neisseria* sp. oral clone BP2-72 (1)
Mogibacterium sp. oral clone BP1-36 (2)	*Solobacterium* sp. oral clone K010 (1)	*Prevotella shahii* (1)
Atopobium sp. oral clone C019 (1)	*Staphylococcus aureus* (1)	*Propionibacterium acnes* (1)
Dialister invisus (1)	*Streptococcus parasanguinis* (1)	*Rothia* sp. oral clone BP1-65 (1)
Eubacteriaceae oral clone MCE10_174 (1)	*Streptococcus salivarius* (1)	*Rothia* sp. oral clone BP1-71 (1)
Eubacteriaceae oral clone P2PB_46 P3 (1)	*Streptococcus* sp. oral clone ASCF07 (1)	*Streptococcus cristatus* (1)
Eubacterium infirmum (1)		*Streptococcus salivarius* (1)
Eubacterium minutum (1)		*Veillonella parvula* (1)
Eubacterium sp. oral clone BP1-93 (1)		Uncultured *Lautropia* sp. clone 2.15 (1)
Eubacterium sp. oral clone JS001 (1)		
Filifactor alocis (1)		
Fusobacterium sp. oral clone CZ006 (1)		
Lachnospiraceae oral clone MCE9_173 (1)		
Lachnospiraceae oral clone MCE9_31 (1)		
Megasphaera sp. oral clone BB166 (1)		
Peptoniphilus lacrimalis (1)		
Peptostreptococcus sp. oral clone BP1-72 (1)		
Peptostreptococcus sp. oral clone CK035 (1)		
Porphyromonas gingivalis (1)		
Prevotella baroniae (1)		
Solobacterium sp. oral clone 6Ta-2 (1)		
Solobacterium sp. oral clone K010 (1)		
Synergistes sp. oral clone BA121 (1)		

Number of cases in which the taxon was found is indicated in parentheses.

is in agreement with our previous findings (22). Eight of the 19 taxa (42%) found in post-treatment samples were uncultivated phylotypes, suggesting that they are previously uncharacterized bacteria that may participate in persistent endodontic infections.

Samples were also quantitatively analysed to assess bacterial reduction after treatment. The greatest advantage of using qPCR assays for evaluation of antibacterial treatment protocols is that as-yet-uncultivated bacteria are also included in the analysis. Because different taxa have different numbers of 16S rRNA operons per genome (ranging from one to 14) (8, 19, 35), it is not feasible to infer a one-to-one relationship between the number of 16S rRNA gene copies detected by qPCR and the number of bacterial cells present in the sample. Because the determination of the precise bacterial cell number in a mixed consortium is difficult to achieve, the bacterial load calculated herein is referred to as 'rRNA gene copy numbers' (11).

Because molecular methods are more sensitive and specific than culture and can detect as-yet-uncultivated bacteria, they can provide a more reliable and detailed insight into the effects of intracanal disinfection protocols. However, as with any other method, molecular technologies also have their limitations, which may have affected the results. Of particular interest, the ability to detect DNA from dead cells poses a major problem when one is investigating the immediate effectiveness of antibacterial treatment because DNA from cells that have recently died can still be detected (26). The results that some canals were negative for the presence of bacteria and that most of the other cases showed over 99% reduction in the number of rRNA gene copies might argue otherwise. The possibility exists that DNA from dead cells may have been destroyed by the effects of the substances used during treatment. NaOCl used throughout the treatments is known to kill bacteria and to degrade DNA, with the resultant fragments being undetectable by PCR (9, 13). Hydroxyl ions from calcium hydroxide also have oxidative damaging effects on DNA (25), and may have contributed to the degradation of free DNA from dead cells.

The antibacterial protocol used in this study encompasses large apical preparations with 2.5% NaOCl as irrigant, and application of calcium hydroxide/CMCP/glycerin medication for 1 week. Additional antibacterial effects are also expected after using 2.5% NaOCl irrigation to remove the calcium hydroxide paste from the canal. The whole protocol succeeded in rendering the root canals bacteria-free in five of 15 cases (33%). In most of the other cases, data obtained from the comparison between S1 and S2 or S3 revealed a significant reduction in the number of bacterial cells in the canals (>99%; $P < 0.05$). Although no significant difference was detected between samples S2 and S3, it is worth pointing out that post-medication samples showed an overall mean bacterial reduction of 56% in relation to post-instrumentation samples. If this difference assumes clinical relevance remains to be clarified in the light of culture-independent approaches.

It has been demonstrated by culture-dependent studies that cases showing no cultivable bacteria in the canal at the time of filling have a significantly better outcome (31, 34). Because of the low sensitivity of culture methods, a negative result does not imply that the canal has been rendered sterile, only that bacterial numbers have reached a threshold that is undetectable by culture and that may be compatible with periradicular tissue healing. Molecular methods can detect far fewer cells than culture and have the potential to demonstrate the actual effectiveness of a given antibacterial protocol and accurately establish the number of bacterial cells that characterize the threshold below which a satisfactory outcome can still be achieved. Further studies

should investigate the relationship between the outcome of the treatment and the levels of bacterial reduction achieved by different protocols as determined by culture-independent methods.

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References

1. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 1996: **11**: 266–273.
2. Bystrom A, Sundqvist G. Bacteriologic evaluation of the effect of 0.5 percent sodium hypochlorite in endodontic therapy. Oral Surg Oral Med Oral Pathol 1983: **55**: 307–312.
3. Bystrom A, Sundqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. Int Endod J 1985: **18**: 35–40.
4. Chavez de Paz LE, Dahlen G, Molander A, Moller A, Bergenholtz G. Bacteria recovered from teeth with apical periodontitis after antimicrobial endodontic treatment. Int Endod J 2003: **36**: 500–508.
5. Chavez de Paz L, Svensater G, Dahlen G, Bergenholtz G. Streptococci from root canals in teeth with apical periodontitis receiving endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005: **100**: 232–241.
6. Chu FC, Leung WK, Tsang PC, Chow TW, Samaranayake LP. Identification of cultivable microorganisms from root canals with apical periodontitis following two-visit endodontic treatment with antibiotics/steroid or calcium hydroxide dressings. J Endod 2006: **32**: 17–23.
7. Cole JR, Chai B, Marsh TL et al. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 2003: **31**: 442–443.
8. Farrelly V, Rainey FA, Stackebrandt E. Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl Environ Microbiol 1995: **61**: 2798–2801.
9. Fouad AF, Barry J. The effect of antibiotics and endodontic antimicrobials on the polymerase chain reaction. J Endod 2005: **31**: 510–513.
10. Gomes BP, Lilley JD, Drucker DB. Variations in the susceptibilities of components of the endodontic microflora to biomechanical procedures. Int Endod J 1996: **29**: 235–241.
11. Horz HP, Vianna ME, Gomes BP, Conrads G. Evaluation of universal probes and primer sets for assessing total bacterial load in clinical samples: general implications and practical use in endodontic antimicrobial therapy. J Clin Microbiol 2005: **43**: 5332–5337.
12. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, ed. Nucleic acid techniques in bacterial systematics. New York: John Wiley and Sons, 1991: 115–175.
13. McCarty SC, Atlas RM. Effect of amplicon size on PCR detection of bacteria exposed to chlorine. PCR Methods Appl 1993: **3**: 181–185.
14. McGurkin-Smith R, Trope M, Caplan D, Sigurdsson A. Reduction of intracanal bacteria using GT rotary instrumentation, 5.25% NaOCl, EDTA, and $Ca(OH)_2$. J Endod 2005: **31**: 359–363.
15. Munson MA, Pitt-Ford T, Chong B, Weightman A, Wade WG. Molecular and cultural analysis of the microflora associated with endodontic infections. J Dent Res 2002: **81**: 761–766.
16. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 2002: **148**: 257–266.
17. Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. Proc Natl Acad Sci U S A 1988: **85**: 2444–2448.
18. Peters LB, van Winkelhoff AJ, Buijs JF, Wesselink PR. Effects of instrumentation, irrigation and dressing with calcium hydroxide on infection in pulpless teeth with periapical bone lesions. Int Endod J 2002: **35**: 13–21.
19. Rainey FA, Ward-Rainey NL, Janssen PH, Hippe H, Stackebrandt E. *Clostridium paradoxum* DSM 7308T contains multiple 16S rRNA genes with heterogenous intervening sequences. Microbiology 1996: **142**: 2087–2095.
20. Rôças IN, Siqueira JF Jr, Santos KR. Association of *Enterococcus faecalis* with different forms of periradicular diseases. J Endod 2004: **30**: 315–320.
21. Sakamoto M, Huang Y, Ohnishi M, Umeda M, Ishikawa I, Benno Y. Changes in oral microbial profiles after periodontal treatment as determined by molecular analysis of 16S rRNA genes. J Med Microbiol 2004: **53**: 563–571.
22. Sakamoto M, Rôças IN, Siqueira JF, Benno Y. Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections. Oral Microbiol Immunol 2006: **21**: 112–122.
23. Shuping GB, Orstavik D, Sigurdsson A, Trope M. Reduction of intracanal bacteria using nickel-titanium rotary instrumentation and various medications. J Endod 2000: **26**: 751–755.
24. Siqueira JF Jr. Strategies to treat infected root canals. J Calif Dent Assoc 2001: **29**: 825–837.
25. Siqueira JF Jr, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J 1999: **32**: 361–369.
26. Siqueira JF Jr, Rôças IN. Exploiting molecular methods to explore endodontic infections: Part 1—current molecular technologies for microbiological diagnosis. J Endod 2005: **31**: 411–423.
27. Siqueira JF Jr, Rôças IN. Exploiting molecular methods to explore endodontic infections: Part 2—Redefining the endodontic microbiota. J Endod 2005: **31**: 488–498.
28. Siqueira JF Jr, de Uzeda M. Influence of different vehicles on the antibacterial effects of calcium hydroxide. J Endod 1998: **24**: 663–665.
29. Siqueira JF Jr, Rôças IN, Favieri A et al. Incidence of postoperative pain after intracanal procedures based on an antimicrobial strategy. J Endod 2002: **28**: 457–460.
30. Siren EK, Haapasalo MP, Waltimo TM, Orstavik D. In vitro antibacterial effect of calcium hydroxide combined with chlorhexidine or iodine potassium iodide on *Enterococcus faecalis*. Eur J Oral Sci 2004: **112**: 326–331.
31. Sjogren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J 1997: **30**: 297–306.
32. Sjogren U, Figdor D, Spangberg L, Sundqvist G. The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. Int Endod J 1991: **24**: 119–125.
33. Sundqvist G, Figdor D. Endodontic treatment of apical periodontitis. In: Orstavik D, Pitt Ford T, ed. Essential endodontology. Oxford: Blackwell Science Ltd, 1998: 242–277.
34. Waltimo T, Trope M, Haapasalo M, Orstavik D. Clinical efficacy of treatment procedures in endodontic infection control and one year follow-up of periapical healing. J Endod 2005: **31**: 863–866.
35. Ward BB. How many species of prokaryotes are there? Proc Natl Acad Sci USA 2002: **99**: 10234–10236.