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Molecular analysis of the root canal microbiota associated with endodontic treatment failures

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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Introduction: The failure of endodontic treatment is usually caused by persistent/secondary intraradicular infections and *Enterococcus faecalis* has been considered to be the main pathogen involved. Nevertheless, the breadth of bacterial diversity involved with endodontic treatment failures remains to be consistently explored by culture-independent approaches.
Methods: This study determined the intraradicular microbiota of root-canal-treated teeth with post-treatment apical periodontitis using 16S ribosomal RNA gene clone library analysis.
Results: Bacteria were present in all cases, confirming the infectious etiology of post-treatment disease. Seventy-four bacterial taxa belonging to six phyla were found in the nine cases investigated. Of these, 55% were identified as as-yet-uncultivated phylotypes, which also made up a significant proportion of the microbiota in many cases. Twenty-five new phylotypes were identified. Most teeth harbored a mixed consortium, with a mean number of 10 taxa per case. Only 11 taxa were found in more than one case, revealing a high interindividual variability in the composition of the microbiota.
Conclusion: The current findings revealed new candidate endodontic pathogens, including as-yet-uncultivated bacteria and taxa other than *E. faecalis*, which may participate in the mixed infections associated with post-treatment apical periodontitis.

Key words: 16S ribosomal RNA gene clone library; apical periodontitis; endodontic treatment failure; root-canal-treated teeth

José F. Siqueira Jr, Faculty of Dentistry, Estácio de Sá University, Rua Baltazar da Silveira, 580/cobertura, Recreio, Rio de Janeiro, RJ 22790-701, Brazil
Tel.: + 55 21 8874 1022;
fax: + 55 21 2199 2204;
e-mail: jf_siqueira@yahoo.com; siqueira@estacio.br
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Endodontic treatment failure is usually characterized by the persistence or appearance of an apical periodontitis lesion (23). This post-treatment disease has been estimated to affect a significant proportion of the adult population even in developed countries, and when the costs of endodontic retreatment and restoration replacement are calculated, the cumulative economic burden may reach the order of billions of dollars (5).

Although extraradicular infections have been regarded as possible causes of post-treatment apical periodontitis (27), it has been well established that persistent or secondary intraradicular infections are the major etiological agents of endodontic treatment failures (12, 16, 18, 19, 24, 28). Culture studies have demonstrated that the microbiota of root-canal-treated teeth showing apical periodontitis mainly comprise one or two species; these are predominantly gram-positive bacteria with *Enterococcus faecalis* as the most prevalent taxon (12, 16, 28). However, culture-dependent approaches have some important drawbacks that can lead to underestimation of the actual breadth of bacterial diversity in diverse ecosystems (15). Limitations of the culturing procedures may be even more pronounced when one is investigating the microbiota of root-canal-treated teeth. To take a representative sample of treated canals is not an easy task because of the limitations imposed by the physical constraints of the root canal and the presence of filling material (24). Bacteria can be in low numbers in the canal or located in areas that are difficult to access with the paper points used for sampling. Therefore, a low number of bacterial cells may be sampled and if the sensitivity of the method used for identification is low, many bacteria can pass unnoticed. As a consequence, a very high sensitivity of the identification method can be of utmost importance. Because the polymerase chain reaction (PCR) is the

most sensitive method for bacterial identification (25), it can provide more reliable identification of the bacterial composition of the microbiota associated with endodontic treatment failures.

Recent culture-independent molecular biology studies have suggested that the microbiota in root-canal-treated teeth with apical periodontitis is more complex than previously shown by culture-dependent methods (20, 24). Bacteria have been detected in almost all treated canals associated with persistent disease and a higher mean number of taxa per case (four to six) has been observed (19, 24). While some molecular studies have confirmed that *E. faecalis* is the most prevalent species in failed cases (18, 24), other studies have questioned its role in treatment failures, either because it was absent (20) or present in a few cases and not as the dominant taxa (19), or because it has been as prevalent in treated cases with disease as in cases with no disease (6, 30). Taxa related to several genera, such as *Dialister*, *Eubacterium*, *Fusobacterium*, *Gemella*, *Mogibacterium*, *Peptostreptococcus*, *Prevotella*, *Propionibacterium*, *Selenomonas*, *Solobacterium*, *Streptococcus*, and *Veillonella*, and two as-yet-uncultivated bacteria, were detected in five root-canal-treated teeth by a study using clone library analysis (20). Denaturing gradient gel electrophoresis (DGGE) analysis of the bacterial communities in root-canal-treated teeth revealed that a mixed consortium occurred in most cases and the structure of the bacterial communities varied from individual to individual, suggesting that distinct bacterial combinations can play a role in treatment failures (19).

PCR amplification of conserved regions of the 16S ribosomal RNA (rRNA) gene followed by clone library construction has been widely used for unraveling the microbiota associated with different healthy and diseased sites in humans (15, 25). This approach allows the detection of almost every species in a sample, including as-yet-uncultivated and previously unknown bacteria. Identification by 16S rRNA gene sequencing is also more accurate than phenotype-based characterization (9). The purpose of the present study was to determine the bacterial diversity associated with root-canal-treated teeth with apical periodontitis lesions using 16S rRNA gene clone library analysis. Since approximately 40–55% of the taxa found in primary intraradicular infections are as-yet-uncultivated bacteria (13, 22), it seems likely that new candidate pathogens associated with endodontic failures can be revealed by this culture-independent approach.

Material and methods

Case description

Nine teeth were selected from patients (five female and four male; aged 30 to 80 years; mean age 48 years) who had been referred for root canal retreatment to the Department of Endodontics, Estácio de Sá University. All the root-canal-treated teeth were asymptomatic, showed radiographic evidence of apical periodontitis, and had endodontic therapy that had been completed more than 5 years earlier. All teeth were coronally restored and no direct exposure of the root canal filling material to the oral cavity was evident. Teeth showing frank exposure of the root filling material to the oral cavity were excluded from the study. The terminus of the root canal fillings ranged from 0 to 2 mm short of the radiographic apex, except for three cases (1R, 3 mm short; 3R, 4 mm short; and 2RH, 5 mm short). No case was overfilled. Selected teeth showed no significant gingival recession and absence of periodontal pockets deeper than 4 mm.

Sample collection and DNA isolation

Samples were taken in accordance with the guidelines of the Ethical Committee at Estácio de Sá University. After plaque removal and rubber dam application, the operative field was cleaned with 3% hydrogen peroxide and disinfected with a 2.5% sodium hypochlorite (NaOCl) solution; coronal restorations were then removed. Endodontic access was completed with a sterile high-speed carbide bur until the root canal filling was exposed. Two cases had a post, which was removed by ultrasonic vibration. After completion of the endodontic access, the tooth, clamp, and adjacent rubber dam were once again disinfected with 2.5% NaOCl. The NaOCl solution was then inactivated using sterile 5% sodium thiosulphate. Coronal guttapercha was removed using sterile Gates–Glidden burs and the apical material was retrieved using K-type and/or Hedström files. Root canal filling removal was always performed without the use of chemical solvents. Whenever possible, the retrieved material was transferred to cryotubes containing TE buffer (10 mM Tris–HCl, 1 mM ethylenediamine tetraacetic acid, pH 7.6) and also analyzed. Radiographs were taken to ensure that all filling material had been removed. A small amount of sterile saline solution was introduced into the root canal by syringe and the canal walls were then filed so that material could be obtained. Samples were initially collected by means of a #15 K-type file with the handle cut off. The file was introduced to a level approximately 1 mm short of the root 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 TE buffer and immediately frozen.

Samples were thawed to 37°C for 10 min and vortexed for 30 s. Afterwards, the microbial suspension was pelleted by centrifugation for 10 min at 5000 ***g***. The pellet was then resuspended in 180 µl buffer ATL supplied by QIAamp DNA Mini Kit (Qiagen, Valencia, CA) and 20 µl proteinase K (20 mg/ml) was added. Samples were incubated for 3 h at 56°C. Subsequently, total bacterial genomic DNA was isolated according to the protocol of the QIAamp DNA Mini Kit. The total bacterial DNA was eluted with 200 µl AE buffer (Qiagen) and stored at −20°C.

Amplification of 16S rRNA genes

16S rRNA genes from DNA extracts were initially amplified using the universal primers 8f (AGA GTT TGA TYM TGG C) and 1492r (GYT ACC TTG TTA CGA CTT) (11). Most samples resulted in weak amplification so a hemi-nested PCR approach using primers 8f and 519r (GTR TTA CCG CGG CTG CTG) was carried out. Aliquots (5 µl) of the DNA extracts from clinical samples were used as target in the first PCR. Next, 2 µl of the resulting PCR product from each sample was used in the second round of amplification with primers 8f/519r.

All PCR amplifications were performed in 50 µl reaction mixture containing 1 µM concentration of each primer, 5 µl 10 × PCR buffer, 2 mM $MgCl_2$, 2 U *Tth* DNA polymerase, and 0.2 mM of each deoxyribonucleoside triphosphate (all reagents from Biotools, Madrid, Spain). Negative controls consisting of sterile ultrapure water instead of sample were included.

Preparations were amplified in a DNA thermocycler (Mastercycler personal; Eppendorff, Hamburg, Germany). The PCR temperature profile for the first reaction using primers 8f/1492r included an initial denaturation step at 95°C for 1 min, 26 cycles at 94°C for 45 s, 50°C for 45 s, and

Table 1. Bacterial taxa found in root-canal-treated teeth with apical periodontitis

	Clinical samples								
Bacterial taxa	1R	3R	4R	5R	1RH	2RH	3RH	4RH	5RH
Actinobacteria									
Actinomyces naeslundii							1 (3)		
Actinomyces radingae							1 (3)		
Actinomyces sp. oral clone 3RH-11[1]							1 (3)		
Actinomyces sp. oral clone 3RH-14[1]							1 (3)		
Actinomyces sp. oral clone 3RH-29[1]							1 (3)		
Actinomyces sp. oral clone 3RH-48[1]							1 (3)		
Atopobium rimae		1 (3)							
Bifidobacterium sp. oral clone 5RH-30[1]									1 (3)
Corynebacterium durum							6 (17)		
Corynebacterium sp. oral clone 3RH-4[1]							1 (3)		
Corynebacterium sp. oral clone 3RH-18[1]							2 (6)		
Corynebacterium sp. oral clone 3RH-41[1]							1 (3)		
Corynebacterium sp. oral clone 3RH-42[1]							1 (3)		
Olsenella genomosp. C1		1 (3)							
Olsenella uli									1 (3)
Propionibacterium sp. oral strain FMA5							1 (3)		
Bacteroidetes									
Bacteroidales oral clone 1R-7[1]	1 (14)								
Bacteroidetes oral clone AU126									1 (3)
Bacteroidetes oral clone X083	2 (29)	5 (16)				2 (5)	1 (3)		
Flavobacteriaceae genomosp. C1			1 (100)	3 (38)					
Porphyromonas gingivalis						3 (7)			
Prevotella baroniae		2 (6)				6 (14)			
Prevotella denticola									3 (10)
Prevotella nigrescens									5 (16)
Prevotella oris	1 (14)	2 (6)							1 (3)
Tannerella forsythia							1 (3)		
Tannerella sp. oral clone 3RH-44[1]							1 (3)		
Uncultured *Bacteroidetes* bacterium clone 1767	1 (14)								
Uncultured *Saprospiraceae* bacterium clone BR03BG08				1 (13)	3 (30)		2 (6)		
Firmicutes									
Clostridiales oral clone 5R-28[1]				1 (13)					
Clostridiales oral clone 5R-48[1]				1 (13)					
Dialister invisus	1 (14)								
Dialister sp. oral clone 9N-1						1 (2)			1 (3)
Enterococcus faecalis							2 (6)		2 (6)
Enterococcus sp. oral clone 5RH-27[1]									1 (3)
Enterococcus sp. oral clone 5RH-39[1]									1 (3)
Eubacteriaceae oral clone P2PB_46 P3						7 (16)			
Eubacterium sp. oral clone 3RH-1[1]							1 (3)		
Eubacterium sp. oral clone BP2-88							1 (3)		
Eubacterium sp. oral clone JS001						1 (2)			
Eubacterium yurii subsp. *yurii*							2 (6)		
Lachnospiraceae oral clone MCE9_31						2 (5)			
Peptostreptococcus stomatis							1 (3)		
Peptostreptococcus sp. oral clone FG014		1 (3)							1 (3)
Pseudoramibacter alactolyticus						11 (26)			
Selenomonas sp. oral clone 5RH-14[1]									1 (3)
Shuttleworthia satelles									2 (6)
Solobacterium sp. oral clone 5RH-44[1]									1 (3)
Solobacterium sp. oral clone K010						6 (14)			
Streptococcus constellatus									4 (13)
Streptococcus mutans							1 (3)	1 (5)	
Streptococcus oralis					1 (10)				
Streptococcus pyogenes					1 (10)				
Streptococcus sanguinis									1 (3)
Streptococcus sp. oral clone 5RH-38[1]									1 (3)
Veillonella sp. oral clone 1R-28[1]	1 (14)								
Veillonella sp. oral clone 4RH-12[1]								1 (5)	
Veillonella sp. oral clone BP1-85							1 (3)		
Fusobacteria									
Fusobacterium nucleatum						3 (7)			
Fusobacterium sp. oral clone BP2-79									1 (3)
Proteobacteria									
Brevundimonas diminuta					2 (20)				
Burkholderiales sp. oral clone 3RH-37[1]							1 (3)		
Campylobacter showae							2 (6)		
Dechlorospirillum sp. DB				1 (13)					

Table 1. (continued)

Bacterial taxa	Clinical samples								
	1R	3R	4R	5R	1RH	2RH	3RH	4RH	5RH
Escherichia sp. oral clone 3RH-30[1]							1 (3)		
Mesorhizobium amorphae					1 (10)				
Paracoccus sp. oral clone 5RH-43[1]									1 (3)
Petrobacter succinimandens				1 (13)					
Pseudomonas aeruginosa					1 (10)			16 (80)	
Pseudomonas putida								1 (5)	
Pseudomonas sp. oral clone 4RH-18[1]								1 (5)	
Stenotrophomonas maltophilia					1 (10)				
Terrahaemophilus aromaticivorans									1 (3)
Synergistes									
Synergistes sp. oral clone BA121		20 (63)				1 (2)			
Total number of taxa	6	7	1	6	7	11	26	5	20
Total number of clones	7	32	1	8	10	43	36	20	31

Data presented as number of clones (%).
[1]Novel phylotypes identified in this study.

72°C for 1.5 min, and a final step at 72°C for 15 min. PCR cycling conditions for the second round of amplification using primers 8f/519r included an initial denaturation step at 95°C for 5 min, 28 cycles at 94°C for 30 s, 55°C for 1 min, 72°C for 1.5 min, and final extension at 72°C for 20 min. Amplicons were separated by electrophoresis in a 1.5% agarose gel, stained with 0.5 μg/ml ethidium bromide, and viewed under ultraviolet transillumination (BTS 20M; Uvitec, Cambridge, UK).

Clone library analysis

16S rRNA gene clone library analysis was performed as described previously (22). PCR products were purified using an UltraClean PCR Clean-up DNA purification kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Purified amplicon was 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 recombinants and used as templates for sequencing. Forty-eight clones (white colonies) were randomly selected from each sample, except when fewer white colonies were available (in which case all were analyzed). Clones not showing the inserts were excluded and the final number of clones sequenced per sample is shown in Table 1. Sequencing was conducted using primers 8f and 519r, a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All sequences were checked by the CHIMERA CHECK program of the Ribosomal Database Project-II (RDP-II) (4) to eliminate chimeras which could result from the amplification of an accidental mixture of bacterial genes – no chimeric sequences were detected. Nucleotide sequences were analyzed by a BLASTN search (2) for the nearest matches. Database sequences with the highest similarities and score-bits to our sequences were chosen as their identification. The criterion to define a novel phylotype was set at sequences that differed from the closest GenBank entry by more than 2% (8). The sequences were aligned with the CLUSTAL W program (29) and corrected by manual inspection. A neighbor-joining phylogenetic tree was constructed from the alignment using the MOLECULAR EVOLUTIONARY GENETICS ANALYSIS package (MEGA version 2.1) (10). Distances were calculated with the Jukes–Cantor algorithm and robustness of the phylogeny was tested by bootstrap analysis with 500 iterations.

Results

All cases were positive for the presence of bacteria. However, some cases still displayed a low number of bacteria irrespective of the hemi-nested PCR protocol used. Overall, 74 different taxa were identified from 188 clones sequenced (Fig. 1). Forty-one taxa (55%) were identified as as-yet-uncultivated phylotypes, i.e. species that are known only by 16S rRNA gene sequences. Twenty-five of these phylotypes were novel in that they have never been previously detected in other sites. Sequences for the novel phylotypes were deposited in the GenBank database under accession numbers AB308084 to AB308108. Six bacterial phyla were represented in this study. Twenty-nine taxa (39%) belonged to the *Firmicutes* (18 of which were uncultivated phylotypes). The other phyla represented in this study were *Actinobacteria* (16 taxa, 10 uncultivated phylotypes), *Bacteroidetes* (13 taxa, seven uncultivated phylotypes), *Proteobacteria* (13 taxa, four uncultivated phylotypes), *Fusobacteria* (two taxa, one uncultivated phylotype), and *Synergistes* (one uncultivated phylotype) (Table 1).

A mean of 10 taxa were detected per treated root canal, ranging from 1 to 26. Only 11 taxa were found in more than one case. They included *Bacteroidetes* oral clone X083 (four cases), uncultured *Saprospiraceae* bacterium clone BR03BG08 (three cases), *Prevotella oris* (three cases), *Prevotella baroniae* (two cases), *Pseudomonas aeruginosa* (two cases), *Streptococcus mutans* (two cases), *Synergistes* sp. oral clone BA121 (two cases), *Dialister* sp. oral clone 9N-1 (two cases), *E. faecalis* (two cases), *Flavobacteriaceae* genomosp. C1 (two cases), and *Peptostreptococcus* sp. oral clone FG014 (two cases). A high interindividual variability in the composition of the microbiota was clearly evident.

The most dominant taxon also varied from sample to sample: *Bacteroidetes* oral clone X083 (sample 1R), *Synergistes* sp. oral clone BA121 (3R), *Flavobacteriaceae* genomosp. C1 (4R as the only taxon detected and 5R), *Saprospiraceae* clone BR03BG08 (1RH), *Pseudoramibacter alactolyticus* (2RH), *Corynebacterium durum* (3RH), *P. aeruginosa* (4RH), and *Prevotella nigrescens* (5RH). As-yet-uncultivated phylotypes made up a significant fraction of the microbiota in root-canal-treated teeth because they represented about 50% of the total number of clones sequenced.

Discussion

The 16S rRNA gene clone library analysis revealed a broader diversity of bacterial

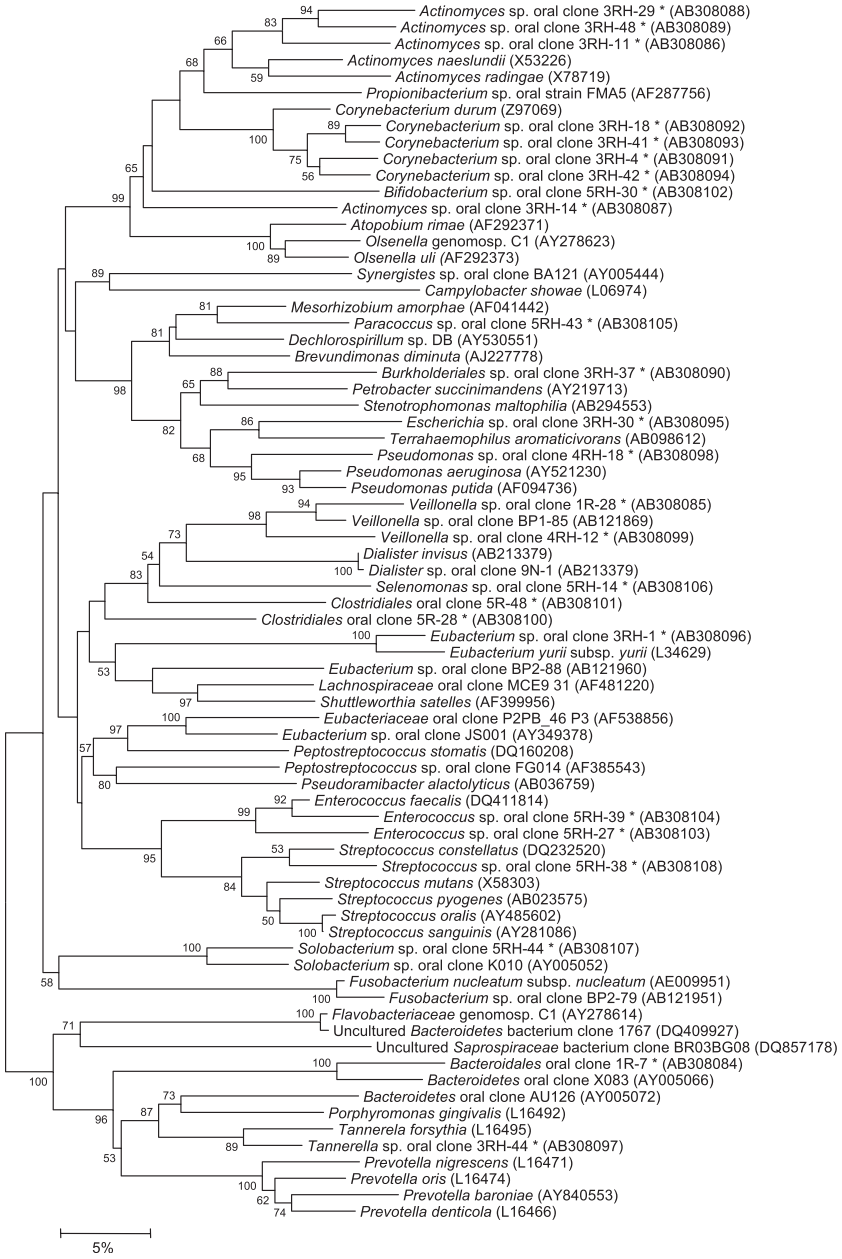

Fig. 1. Phylogenetic tree of bacterial species and phylotypes identified in samples from root-canal-treated teeth with apical periodontitis. Sequences were aligned with CLUSTAL W, and distances were calculated using the Jukes–Cantor algorithm. Bootstrap values (based on 500 replicates) are represented at each node when >50%. The scale bar represents a 5% difference in nucleotide sequences. Asterisks indicated novel phylotypes identified in this study.

taxa in root-canal-treated teeth than heretofore reported. Of the eight bacterial phyla previously recognized to have members composing the primary endodontic microbiota (13, 21, 22, 26), six were represented in this study. Most of the inferred species belonged to the *Firmicutes* phylum, which is in consonance with studies of primary infections (untreated canals) (13, 21, 22). However, relatively more members of the *Actinobacteria* phylum were disclosed as compared to primary infections. Representatives of *Spirochaetes* and TM7 phyla were not detected and this suggests that the root canal environment modified by treatment is no longer conducive to the establishment of the members of these phyla.

More than one-half of the taxa detected have not yet been cultivated and phenotypically characterized. Among them, there were 25 novel phylotypes. Our findings are in line with other molecular studies that have demonstrated that 40% to 60% of the species detected in the oral cavity (1, 8, 14), including primary endodontic infections (13, 22), remain to be cultivated. Collectively, as-yet-uncultivated phylotypes were also present in high proportions, corresponding to about one-half of the clones sequenced. Many phylotypes are suspected to play a role in different oral disease conditions, including caries (3), periodontal diseases (8, 14), and halitosis (7). Therefore, there is no evidence to suggest that the as-yet-uncultivated segment of the microbiota is less important than the cultivable segment in the etiology of post-treatment apical periodontitis.

The most prevalent taxa included as-yet-uncultivated phylotypes and cultivable species, most of which had never been previously found in root-canal-treated teeth. Six of the 11 taxa found in more than one case were as-yet-uncultivated phylotypes. Except for *Synergistes* sp. oral clone BA121 (26), none of the others had been detected in treated canals. *Bacteroidetes* oral clone X083, the most frequent taxa in the current study, has been found in untreated canals of teeth with chronic apical periodontitis (22). Phylotypes were also present as the most dominant taxa in many cases. High occurrence of as-yet-uncultivated phylotypes, sometimes as a significant proportion of the microbiota, raises the interesting possibility that they are previously unrecognized bacteria that may be involved with endodontic treatment failures.

E. faecalis was found in only two of the nine cases, and in none of them was it the most dominant taxon. These findings are in agreement with our previous study using PCR-DGGE (19) and argue against the assumption that this is the most important species involved in endodontic treatment failures. *E. faecalis* certainly may be involved with the etiology of post-treatment disease in some cases, particularly given its many features that allow it to survive in treated canals (17, 30), but it is usually present in a mixed culture and not as a single species as found by culture studies.

Previous findings from a study using PCR-DGGE revealed mixed infections in most treatment failure cases, with high interindividual variability in the bacterial community profiles (19). This is corroborated by the current results. Bacterial profiles differed considerably among individuals, with most taxa being found in only one case. Even considering the small sample size, these results contest previous culture findings and point to a

heterogeneous etiology of post-treatment apical periodontitis lesions.

Overall, a mean number of 10 taxa were present in root-canal-treated teeth. This figure is far higher when compared to previous culture-dependent studies (12, 16, 28). This difference is likely to be related to the highest sensitivity of PCR and the ability to detect as-yet-uncultivated bacteria. Indeed, these advantages of molecular methods also help to explain why bacteria were detected in almost all treated cases with post-treatment disease in this and other studies (18, 19, 24), while culture studies have found bacteria in 44% to 85% of the cases (12, 16, 28).

The profile of the microbiota associated with root-canal-treated teeth was by and large different from that of primary infections and this is most probably a result of the different environmental conditions in treated and untreated canals. While primary infections occur in an environment where the necrotic root canal affords enough space and nutrients for many oral bacteria to survive and flourish, the treated canal comprises a rather harsh environment where space and nutrients are restricted. Only the bacteria able to adapt to these barren conditions can establish in the canal and participate in the etiology of post-treatment disease. These bacteria can be remainders of the primary infection that persisted after intracanal treatment measures (persistent infection) or they may have entered the canal during or after professional intervention (secondary infection). The occurrence of taxa uncommonly found in the oral cavity, such as *Pseudomonas* species/phylotypes and several other members of the *Proteobacteria* phylum, indicates that these bacteria may have been introduced to the canal during treatment, possibly after a breach in the aseptic chain. On the other hand, the presence of oral taxa that are commonly found in untreated canals suggests that they are bacteria previously present in primary infections, which have endured the treatment procedures. However, given the cross-sectional nature of this study, one cannot preclude the possibility of these bacteria being introduced to the canal by leakage of saliva at the time of treatment or even after root canal filling.

In conclusion, 16S rRNA gene clone library analysis of the root canal microbiota associated with endodontic treatment failures demonstrated that the bacterial diversity is greater than so far described and a significant proportion of the microbiota is represented by as-yet-uncultivated bacteria. Bacterial taxa other than *E. faecalis* and including as-yet-uncultivated bacteria may be involved in the etiology of post-treatment disease, usually in mixed infections. Although the subject population was small, the data reported here bring important additional information about the etiology of post-treatment apical periodontitis. Several new candidate pathogens were disclosed, most of which had not been previously found in association with endodontic treatment failures. Knowledge of the bacterial diversity in persistent/secondary endodontic infections can direct further studies towards a comprehensive large-scale clinical survey to link particular species/phylotypes with post-treatment disease.

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