Exploring Bacterial Diversity of Endodontic Microbiota by Cloning and Sequencing 16S rRNA

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Abstract

Introduction: The characterization of microbial communities infecting the endodontic system in each clinical condition may help on the establishment of a correct prognosis and distinct strategies of treatment. The purpose of this study was to determine the bacterial diversity in primary endodontic infections by 16S ribosomal-RNA (rRNA) sequence analysis. **Methods:** Samples from root canals of untreated asymptomatic teeth (n = 12) exhibiting periapical lesions were obtained, 16S rRNA bacterial genomic libraries were constructed and sequenced, and bacterial diversity was estimated. **Results:** A total of 489 clones were analyzed (mean, 40.7 ± 8.0 clones per sample). Seventy phylotypes were identified of which six were novel phylotypes belonging to the family *Ruminococcaceae*. The mean number of taxa per canal was 10.0, ranging from 3 to 21 per sample; 65.7% of the cloned sequences represented phylotypes for which no cultivated isolates have been reported. The most prevalent taxa were *Atopobium rimae* (50.0%), *Dialister invisus*, *Prevotella oris*, *Pseudoramibacter alactolyticus*, and *Tannerella forsythia* (33.3%). **Conclusions:** Although several key species predominate in endodontic samples of asymptomatic cases with periapical lesions, the primary endodontic infection is characterized by a wide bacterial diversity, which is mostly represented by members of the phylum *Firmicutes* belonging to the class *Clostridia* followed by the phylum *Bacteroidetes*. *(J Endod 2011;37:922–926)*

Key Words

16S rRNA, bacterial diversity, clinical study, *in vivo*, metagenomic analysis, primary endodontic infection

Although the bacterial community in the oral cavity may comprise over 700 species or taxa (1), only a limited number of species find proper conditions to colonize the root canal system (2). Most data on the endodontic microbiota were obtained by culture approaches (3–5), but culture-dependent methods grossly underestimate the extent of bacterial diversity in polymicrobial infections (6). Furthermore, phenotypic profiles variations and methodological bias also limit the conclusions drawn from culture studies (7).

In the last decade, 16S rRNA analysis became a powerful phylogenetic framework for the identification of fastidious, slow-growing, or not-yet-cultivable bacteria, which makes a high proportion of the microorganisms in the oral cavity (8–10). Therefore, it is likely that currently unknown species are present in disease infections, emerging new concepts in the pathogenesis of several human infections and redirecting therapeutic strategies.

The assessment of the human microbioma by metagenomic approaches revealed that these techniques are sensitive and specific (6, 11) to evaluate the bacterial diversity of root canal infections (10, 12–15). These studies have shown that the prevalence of yet non-cultivable bacteria remarks over 50% of the microbiota in primary endodontic infections (10, 14), in persistent periradicular lesions (15), or in mixed endodontic infections (16). Previous data suggested that endodontic infections are characterized by a consortium of microorganisms, more restricted than observed in other sites of the oral cavity, evidencing a selection within this niche (16), which may lead to a specific microbiota able to induce periradicular inflammation.

Studies using cloned libraries analysis of endodontic microbiota usually evaluated a low number of samples, a maximum of five cases in primary infection (13). The major drawback of this method is that it usually detects only the most abundant organisms because of the relative expensive cost of the conventional sequencing approach (17, 18). A recently published study investigated the bacterial diversity of seven endodontic cases, symptomatic or not, including one of a treatment failure case, by 16S rRNA gene sequence analysis using cloned libraries sequenced by conventional Sanger capillary and deep coverage pyrosequencing (16). As expected, deep sequencing data revealed a larger number of taxa involved in endodontic infections, although the same phyla were more frequently found in both analysis, and most taxa detected by the latter technique were unique. All of these studies on bacterial diversity of endodontic infections revealed high intersubject variability, indicating the need for studies using very homogenous diagnosis criteria in a significant number of cases in order to improve our prognosis and lead to the development of rational treatment strategies. In order to provide data on the endodontic microbiota, this study intended to explore the bacterial diversity

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profiles of asymptomatic primary endodontic infection cases exhibiting periradicular lesions.

Materials and Methods

Patient Profiles

Patients aged 18 to 60 years of age were enrolled in this study after approval by the Institutional Ethical Committee for Human Subjects and written informed consent. Samples were taken from 12 single-rooted teeth with asymptomatic primary endodontic infection with radiographic evidence of periapical lesions. Patients who received antibiotic therapy in the last 3 months and/or exhibited periodontitis were excluded.

Sampling Procedures

Teeth were isolated with a rubber dam, and caries/coronal restorations were removed with sterile high-speed burs. The operative field and pulp chamber were disinfected with 30% hydrogen peroxide followed by 2.5% sodium hypochlorite (NaOCl) and 5% sodium thiosulfate solution. Samples were obtained by sterile #15 paper points, which were inserted into the root canal (approximately 1 mm short the tooth apex) and transferred to 100 μL Tris EDTA (TE) buffer (50 mmol/L Tris/HCl, pH = 7.6; 1 mmol/L EDTA, pH = 8.0) and stored at −80°C.

Polymerase Chain Reaction Amplification

DNA was extracted by the addition of Tween 20 (0.5%) and proteinase K (0.2 mg/mL) solutions to the samples, followed by a 2-hour incubation at 55°C. Proteinase K was inactivated at 95°C for 5 minutes (19).

Five microliters of template DNA were used in 50-μL amplification reactions consisting of 1 U Platinum *Taq* polymerase (Invitrogen, São Paulo, Brazil), 5 μL buffer (10×), 3.0 mmol/L $MgCl_2$, 4.0 μL dNTP solutions (25 μmol/L each), and 1.0 μL each of 16S rRNA bacterial primer (0.5 μmol/L) (D88 [5′-AGAGTTTGATYMTGGCTCAG-3′] and E94 [5′-GAAGGAGGTGWTCCARCCGCA-3′]) (20). Genomic DNA of *Enterococcus faecalis* (ATCC 29212) and water were used as positive and negative controls, respectively. Polymerase chain reaction (PCR) was performed in a DNA thermocycler (MJ Research, Waltham, MA) adjusted to an initial denaturation step at 94°C for 4 minutes followed by 30 cycles at 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 90 seconds, and a final step at 72°C for 15 minutes. Three independent PCR reactions were performed for each sample. The 1,500-bp fragments were revealed after electrophoresis in 1% agarose gel and purified by using QIAquick Gel Extraction (Qiagen, North Rhine-Westphalia, Germany).

16S rRNA Cloning

The 16S rRNA gene products were ligated into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and inserted into electrocompetent DH5α *Escherichia coli* (2.5 kV). Transformed cells were selected in Luria–Bertani agar plates supplemented with ampicillin (100 μg/mL), suspended in 10 mmol/L Tris, and used as a template to determine the correct sizes of inserts by PCR. The cycling conditions followed an initial denaturation at 94°C for 10 minutes and included 30 cycles at 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 90 seconds followed by a final extension at 72°C for 10 minutes. The PCR products (approximately 1,500 bp) were submitted to electrophoresis in 1% agarose gel and purified with GTX PCR DNA Purification kit (GE Healthcare, Buckinghamshire, UK) before sequencing.

16S rRNA Sequencing

Fifty purified PCR products per sample were sequenced with an ABI prism cycle-sequencing kit (Big-Dye Terminator Cycle Sequencing kit) using primer (5′-TKACCGCGGCTGCTG-3) (20) in an ABI Prism 3100 genetic analyzer (Applied Biosystems–Hitachi, Foster City, CA).

Data Analysis

Sequences with more than four ambiguous characters were discarded. Sequences of approximately 500 bases were obtained, and the identity/approximate phylogenetic position was obtained by comparison with 16S rRNA gene sequences of the GenBank database through the Basic Local Alignment Search Tool (BLAST) algorithm considering a 98% similarity level. Clone sequences with low-scoring homologies (<97% similarity) into the GenBank database were sequenced by using M13 primer pairs in order to recover total gene lengths (1,500 bp).

Bionumerics software (Applied Maths, Saint-Martens-Latem, Belgium) was used for data entry, editing, sequence alignment, structure comparison, secondary structure comparison, similarity matrix generation, and phylogenetic tree construction. The similarity matrices were corrected for multiple base changes at a single position by the method of Jukes and Cantor (21). Phylogenetic trees were constructed by the Unweighted Pair Group Method with Arithmatic Mean (UPGMA) method (22).

Results

All samples were positive for bacteria. A total of 489 cloned 16S rRNA 500-bp fragments were appropriately sequenced (81.5% of the total number of sequences obtained); 40.6 ± 8.0 clones per sample were properly sequenced (range, 51–31) and used for the phylogenetic analysis. Six clones for which the sequences exhibited low-scoring homologies (<97% similarity) to sequences deposited in the GenBank were fully sequenced (1,500 bp of amplified 16S rRNA). The sequences of the six novel phylotypes were deposited in GenBank (HQ441585, HQ441588, HQ441583, HQ441590, HQ441586, and HQ441587).

Seventy species/ phylotypes were identified in this study, most of which (n = 46) were present in only one sample. About 65.7% (n = 46) of the identified taxa were reported as-yet-uncultivable or as-yet-uncharacterized species. The mean number of bacterial taxa per canal was 10.0 (±5.5) ranging from 3 to 21. The phylogenetic tree, constructed based on 16S rRNA gene sequence comparison of 1,500 aligned bases (Jukes and Cantor method), is shown in Figure 1.

Most detected taxa belonged to the phylum *Firmicutes* (57.1% of the identified taxa). Within this phylum, *Clostriadiales* were found in 10 samples followed by *Selenomonadales* detected in 6 of 12 samples. Six novel phylotypes within *Firmicutes* were detected in patient #1 and classified as members of the family *Ruminococcaceae*.

The second most representative phylum was *Bacteroidetes* (Cytophaga-Flexibacter-Bacteroides phylum), accounting for 18.8% of the detected phylotypes and detected in 10 of the 12 studied samples. *Proteobacteria* (gammaproteobacteria, deltaproteobacteria, betaproteobacteria, and epsilonproteobacteria) represented 8.6% of the libraries. *Actinobacteria* (high G + C gram-positive bacteria) were detected in 8 of 12 samples and represented 7.14% of the cloned sequences. The least frequent phyla were *Synergistetes* (detected in 4.3% of the cloned libraries and in three samples), *Fusobacteria* (2.9%), and *Spirochaetes* (1.4%).

Interestingly, the microbiota of the only two cases (samples 7 and 8) in which *Clostridiales* and *Bacteroidetes* were not detected was the least diverse but included *Actinobacteria*. The most frequently detected species, *Atopobium rimae*, detected in 6 of the 12 studied samples (50.0%), is a member of the phylum *Actinobacteria*.

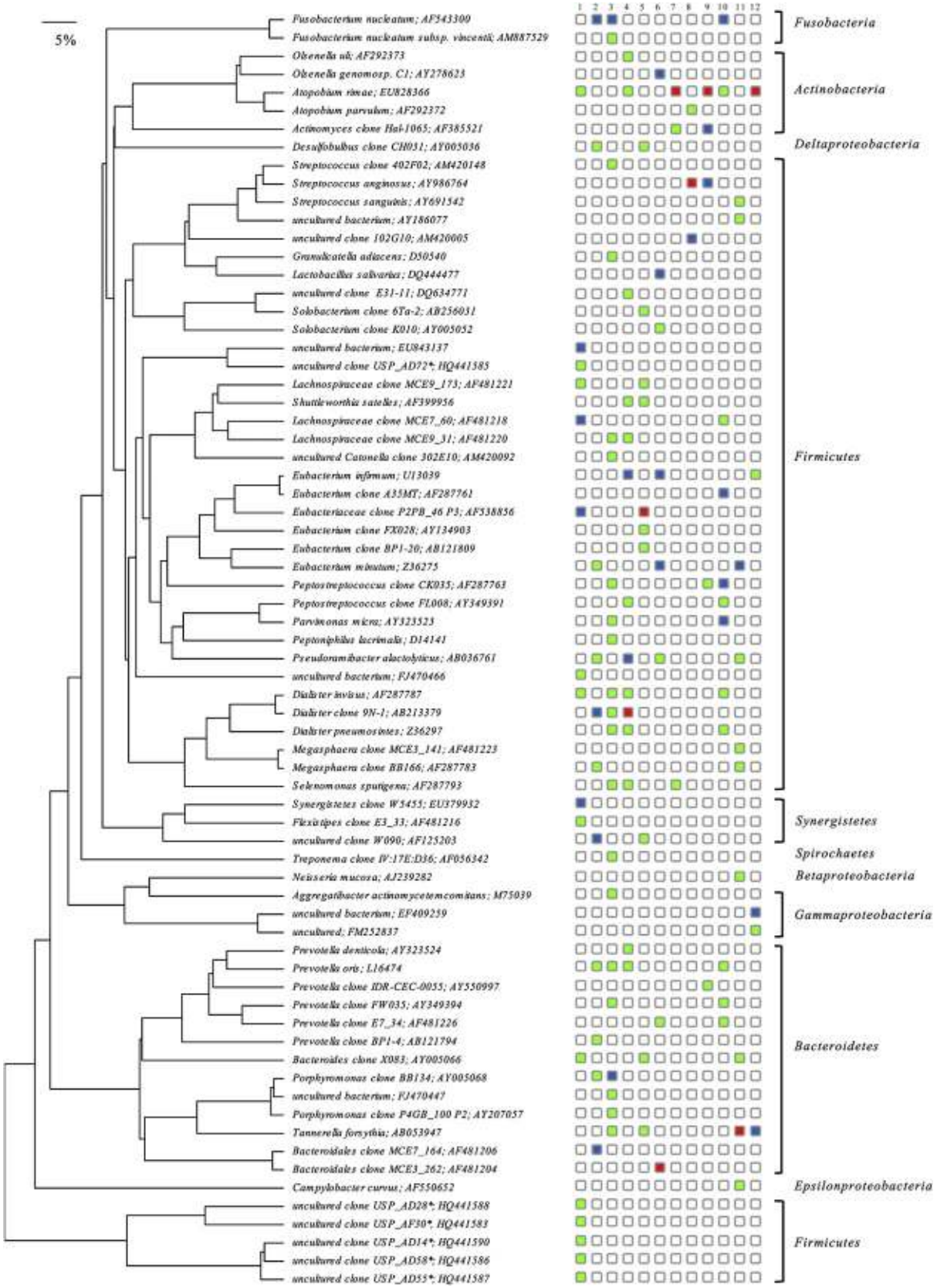

Figure 1. The phylogenetic tree of bacterial species/phylotypes identified in primary endodontic infection (n = 12). The scale bar represents 5% difference in nucleotide sequence. Accession numbers for 16S rRNA gene sequence are indicated for each strain according to GenBank. The column of boxes represents the bacterial profile of each subject, and proportion is indicated by the following colors: *white* (absence), *green* (<10%), *blue* (10%–25%), and *red* (>25%). The six novel phylotypes identified in this study are indicated by an *asterisk*.

Pseudoramibacter alactolyticus, *Prevotella oris*, *Tannerella forsythia*, and *Dialister invisus* were found in four samples each (33.3%%), whereas *Eubacterium infirmum*, *Eubacterium minutum*, *Peptostreptococcus* clone CK035, *Dialister* clone 9N-1, *Dialister pneumosintes*, *Selenomonas sputigena*, *Bacteroidetes* clone X083, and *Fusobacterium nucleatum* were each detected in three of the cases (25.0%).

Most of the species and phylotypes identified in this study represented less than 10% of the total microbiota in each sample. However, some species comprised more than 25% of the microbiota per sample. Furthermore, the highly prevalent specie, *Atopobium rimae,* was found in high proportion (over 25%) in three of the six samples that were positive for this specie.

Discussion

Primary endodontic microbiota is represented by a highly diverse bacterial community, as shown by the detection of 70 species/phylotypes by 16S rRNA libraries analysis. The use of cloned libraries sequencing analysis instead of high throughput sequencing methods may result in a lower assessment of the bacterial diversity (16). However, the currently used method enabled us to characterize the most abundant organisms, which are possibly more adapted to their niche and are arranged in biofilm communities (23).

The bacterial diversity observed in this study is about 43% higher than preceding studies (10, 14), possibly because of the larger number of evaluated samples (n = 12) and sequenced clones (n = 489). More than half (65.7%) of the detected taxa were yet-uncultivable or yet-uncharacterized species; this is similar to other data obtained by 16S rRNA analyses (8–10). The phylogenetic position of these not-yet-cultivated phylotypes were obtained, which may provide insight into their cultivation requirements (20) and metabolic outcomes.

On average, the microbiota of each root canal was characterized by 10 species/phylotypes, which is similar to other studies evaluating teeth with clinical and radiographic evidence of asymptomatic apical periodontitis (10, 14). Despite the use of very strict inclusion criteria (ie, the absence of periodontitis and single-rooted teeth with asymptomatic teeth with periapical lesions), most species/phylotypes were present in only one sample, confirming the large degree of intersubject variability (16, 24, 25).

Bacteria detected in the primary root–infected samples were distributed into the seven phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Synergistetes*, and *Spirochaetes*. These data are in accordance to data provided in the human microbiome study (26) in which, except for *Synergistetes*, these comprised 96% of the microbiota of the human oral cavity.

Firmicutes comprised the largest number of phylotypes, as observed also in culture-dependent (3) and independent studies (10, 13, 14, 27). Nineteen of the detected taxa within this phylum belonged to the class *Clostridia*, which was detected in 10 of 12 studied samples and includes the highly prevalent species/phylotypes detected in the libraries (ie, *Eubacterium infirmum*, *Eubacterium minutum*, *Peptostreptococcus* clone CK035, *Pseudoramibacter alactolyticus)*. In addition, the six novel identified phylotypes were also classified into *Firmicutes* in the *Clostridia* class and the *Ruminococcaceae* family. As far as we know, this is the first description of the detection of this family in the oral cavity. Members of this family, particularly of the genus *Ruminoccocus*, may be part of the resident microbiota in the gut of healthy subjects (28, 29), and their abundance seemed to be associated with dietary habits (30). The second most frequently seen class within this phylum was *Negativicutes*, family *Veillonellaceae,* detected in 7 of 12 samples and comprising the frequently detected species/phylotypes *Dialister invisus*, *Dialister* clone 9N-1, *Dialister pneumosintes*, and *Selenomonas sputigena* as well as the genus *Megasphaera*. On the other hand, *Bacilli*, which includes the family *Lactobacillaceae*, was detected in only five of the evaluated samples. This class includes the genus *Streptococcus*, which is highly prevalent in saliva and on the tongue surface (31, 32), suggesting that they may be less adapted to the endodontic niche than the preceding *Firmicutes* classes.

The second most abundant phylum was *Bacteroidetes,* which was detected in 10 of the 12 studied samples. This contrasts with data of a recent study in which *Bacteroidetes* was the most prevalent phylum in endodontic samples detected by pyrosequencing and conventional Sanger capillary sequencing (16). In the present study, the most prevalent species within this phylum were *Tannerella forsythia* and *Prevotella oris*, which were detected in 33% of the analyzed samples. *T. forsythia*, which is associated with periodontitis, has also been reported as a common organism in root canal infection. Previous data suggested an association between *T. forsythia* and pain of endodontic origin (33). However, despite its high prevalence (66%), relative levels of this species were not correlated to spontaneous pain by others (34), in agreement with the present finding of a high prevalence of *T. forsythia* in asymptomatic endodontic infections.

Although only four taxa were detected belonging to the phylum *Actinobacteria*, these organisms were found in 8 of 12 samples, including the two samples in which *Clostridiales* and the phylum *Bacteroidetes* were not detected, suggesting that this group may possibly be selected in the endodontic environment. Furthermore, the most prevalent species in this study, *Atopobium rimae* is an *Actinobacteria*. Previous studies reported that species of the genus *Atopobium* were detected in cases of systemic bacteremia (35, 36) and oral infectious disease, as periodontitis (37, 38) and persistent periradicular lesions (15, 39). The high prevalence of *A. rimae* in primary endodontic infections as shown here, which is associated with other data indicating a higher frequency of this species in periradicular soft tissues than in root ends (15), provided evidences to suggest this species as an important endodontic pathogen.

Bacteria belonging to the recently described phylum *Synergistetes* (40) are widespread in the environment as well as in animal samples. Members of this phylum were found in healthy patients, suggesting they could play a functional role in human microbiota, but may also act as opportunist pathogens (41). They were previously reported in endodontic infections, although in low prevalence (42, 43). The diversity and prevalence of *Synergistetes* seemed low when compared with other phylogenetic groups because only three phylotypes were detected in three samples. However, two *Synergistetes* taxa (clones W090 and W5455) were found in proportions ranging from 10% to 20%. These organisms were rarely cultivated, and a previous study reported the dependence of *Synergistetes* with *P. micra* for *in vitro* growth (44). Our data indicated that *Synergistetes* were found within a highly diverse microbiota, but *P. micra* was not detected concomitantly with this phylum in any of the endodontic samples.

Spirochaetes was the least prevalent phylum in this study, as previously shown in endodontic samples (10, 16). *Treponema* species/phylotypes may be part of the microbiota associated with asymptomatic and symptomatic endodontic infections (45), and they are usually represented by novel as-yet-uncultivated phylotypes, not only in endodontic sites but also in other niches of the oral cavity (26). *Spirochaetes* are abundant in subgingival samples of periodontitis subjects (26), and the studied subjects were free of periodontitis, suggesting the absence of cross-contamination from periodontal pockets. Thus, their low detection rate may indicate that this phylum may not be well adapted to the endodontic environment.

Our data pointed out that despite being highly diverse, the microbiota of primary endodontic infections is mostly represented by members of the phylum *Firmicutes* belonging to the classes *Clostridia* and *Negativicutes* and by members of the phylum *Bacteroidetes*. *Actinobacteria*, especially of the species *A. rimae*, were also highly prevalent, suggesting their fitness to the endodontic environment. The full knowledge on the bacterial agents infecting the root canal under different clinical conditions and their decrease/elimination after different treatment strategies may lead to the development of rational treatments based on the susceptibility of the colonizing microbiota.

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The authors deny any conflicts of interest related to this study.

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