Susceptibility of As-yet-uncultivated and Difficult-to-culture Bacteria to Chemomechanical Procedures

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Abstract

Introduction: A significant portion of the bacteria taking part of the microbiome associated with apical periodontitis still remain to be cultivated and phenotypically characterized. This molecular study evaluated the prevalence of selected as-yet-uncultivated and difficult-to-culture bacterial taxa in infected root canals and their susceptibility to chemomechanical procedures. **Methods:** Root canals of single-rooted teeth with apical periodontitis were prepared using rotary nickel-titanium instruments and 2.5% sodium hypochlorite as the irrigant. DNA extracts from samples taken before (S1) and after (S2) chemomechanical preparation were surveyed for the presence of 7 as-yet-uncultivated phylotypes and 1 difficult-to-culture species using end-point polymerase chain reaction. Samples were also subjected to quantitative analysis of total bacteria and levels of the 2 most prevalent taxa. **Results:** *Bacteroidaceae* sp. HOT-272 (24%) and *Fretibacterium fastidiosum* (20%) were the most prevalent taxa in S1. Their mean counts in S1 were 8.25×10^3 and 2.13×10^3 rRNA gene copies, corresponding to 0.18% and 0.55% of the total bacteria. Chemomechanical debridement promoted a highly statistically significant reduction in total bacterial counts ($P < .001$), but 64% of the canals were still positive for bacterial presence. Of the target taxa, only *Bacteroidaceae* sp. HOT-272 and *F. fastidiosum* were detected in S2 (each one in 1 sample). The reduction in counts of both taxa was also highly significant ($P < .001$). **Conclusions:** Findings confirmed that several as-yet-uncultivated and difficult-to-grow bacterial taxa can participate in the microbiome associated with apical periodontitis. Two of them were found in relatively high prevalence but rarely as a dominant species. Chemomechanical procedures were highly effective in completely eliminating these taxa or at least substantially reducing their numbers. *(J Endod 2014;40:33–37)*

Key Words

Apical periodontitis, bacterial reduction, endodontic treatment, molecular microbiology, uncultivated bacteria

Studies of the human oral microbiome have shown that about 40%–60% of the bacterial species inhabiting the oral cavity still remain to be grown in the laboratory (1). The Human Oral Microbiome Database lists over 200 oral taxa as still unnamed and uncultivated (2). As-yet-uncultivated bacteria can be either bacteria that are relatively easy to cultivate on ordinary media but for some reason have not yet been cultivated or bacteria that are truly culture resistant (1). For a species to be validly named, strains have to be cultivated and phenotypically characterized. In addition, culture of a given species is crucial for the inference of ecologic and pathogenic behavior as well as the determination of antibiotic susceptibility. In this article, we refer to as-yet-uncultivated phylotypes as those that have not yet been grown in artificial media and are known only by a 16S rRNA gene signature and difficult-to-culture species as those that have already been characterized and formally named but that cannot be grown using ordinary media and conditions.

Cross-sectional culture-independent molecular studies have suggested an association of many uncultivated phylotypes with diseased conditions (1). The association is even stronger when the same phylotype is found in significantly higher prevalence in diseased sites than in healthy sites (3, 4). In endodontic infections, because healthy pulp sites are normally sterile, there is no way to compare conditions of health and disease. One may assume that the mere presence of a given species in significant abundance in the previously sterile root canal environment may suggest a pathogenic, or at least an ecologic, role in the mixed bacterial community (5).

Molecular microbiology analyses of different types of endodontic infections have revealed that a significant proportion of the microbiome consists of as-yet-uncultivated and difficult-to-culture bacteria (6–8). For instance, Sakamoto et al (6) reported that uncultivated phylotypes accounted for approximately 55% of the taxa (richness) and more than 38% of the clones sequenced (abundance) in samples taken from root canals of teeth with apical periodontitis. In pus aspirates from acute apical abscesses, uncultivated phylotypes have been shown to represent approximately 24%–46% of the taxa found (6, 9) and 6% to more than 30% of the clones sequenced (6, 10). Uncultivated phylotypes from several bacterial groups have been identified, including representatives of the genera *Atopobium*, *Oribacterium*, *Megasphaera*, and *Desulfobulbus* and the phyla *Bacteroidetes*, *Synergistetes*, and TM7 (6–8, 11–13). Some of them have been detected in relatively high frequencies, including *Bacteroidaceae* sp. HOT-272 (synonym, *Bacteroidetes* oral clone X083) (6, 14–18) and *Synergistetes* oral clones W090 and W028 (13, 14, 19, 20), which

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have been recently successfully cultivated and formally named as *Fretibacterium fastidiosum* (21).

There is only limited information available from intervention studies evaluating whether the prevalence of these uncultivated phylotypes and difficult-to-culture species significantly decreases after endodontic treatment procedures. For inferences to be made about the role of these new taxa in endodontic infections and their susceptibility to treatment, there is a need for using quantification techniques. This study was undertaken to evaluate the prevalence of selected as-yet-uncultivated and culture-resistant bacterial taxa in root canals of teeth before and after chemomechanical procedures. Quantification using real-time PCR (qPCR) was further performed for the most prevalent taxa, which were also detected in some postinstrumentation samples. This investigation is part of the ongoing efforts of our laboratory to identify prominent as-yet-uncultivated bacteria associated with apical periodontitis and to evaluate their susceptibility to antimicrobial treatment procedures.

Material and Methods

Case Selection

Twenty-eight single-rooted and single-canal teeth from 23 patients attending the endodontic clinic at the Department of Endodontics, Estácio de Sá University, Rio de Janeiro, Brazil, for the treatment of apical periodontitis were included in this study. All teeth presented with intact pulp chamber walls, necrotic pulp as determined by pulp sensitivity tests, and clinical and radiographic evidence of asymptomatic apical periodontitis. The following conditions were excluded from the study: teeth with gross carious lesions or root/crown fracture, teeth subjected to any previous endodontic procedures, presence of symptoms, antibiotic therapy within the previous 3 months, and patients with periodontal disease. The study protocol was approved by the Ethics Committee of the Estácio de Sá University, and informed written consent was obtained from all participants.

Sample Taking and Treatment Procedures

Root canal samples were taken following strict aseptic measures, which included rubber dam isolation and a 2-step disinfection protocol of the operative field with the sequential use of 6% hydrogen peroxide and 2.5% sodium hypochlorite (NaOCl). This disinfection protocol was applied before and after completing the access preparation. NaOCl was inactivated with 5% sodium thiosulfate, and sterility control samples were taken from the cavosurface angle of the access cavity. For inclusion of the tooth in the study, sterility control samples had to be negative for end-point PCR using universal 16S rRNA gene-based primers. Accordingly, 3 teeth had to be excluded.

A microbiologic sample was taken from the root canals immediately before instrumentation (S1 sample). Sterile 5% sodium thiosulfate was placed in the pulp chamber without overflowing, and a small instrument was used to carry the solution into the canal. A small hand instrument was placed in the canal approximately 1 mm short of the radiographic root apex and operated in a gentle circumferential filing motion to emulsify the root canal contents into the sodium thiosulfate solution. Sterile paper points were consecutively placed in the canal at the same level to soak up the fluid. Each paper point was left in the canal for about 1 minute, transferred to cryotubes containing Tris-EDTA buffer (10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH = 7.8), and immediately frozen at −20°C.

Root canal preparation was performed using the BioRaCe series of instruments (FKG Dentaire, La Chaux-de-Fonds, Switzerland). Apical preparation was done at the working length established 1 mm short of the foramen as determined by an electronic apex locator. Preparation size ranged from the BR5 (size 40, 0.04 taper) to the BR7 (size 60, 0.02 taper) instrument depending on the root anatomy and initial canal diameter. Patency of the apical foramen was frequently checked with a small instrument throughout the procedures. The irrigating solution (2.5% NaOCl) was delivered using NaviTip needles (Ultradent, South Jordan, UT) inserted up to 4 mm short of the WL. Two milliliters of NaOCl solution were used after each instrument size. Root canal preparation procedures were completed in a single appointment.

After the apical preparation was finished, the smear layer was removed by rinsing the canal with 5 mL 17% EDTA followed by 5 mL 2.5% NaOCl. The canal was dried using sterile paper points and flushed with 1 mL 5% sodium thiosulfate for 1 minute for NaOCl inactivation. In sequence, a postinstrumentation sample (S2) was taken from the canals as described for S1.

Qualitative Endpoint PCR

Clinical samples were thawed at room temperature, and DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Aliquots of 5 μL DNA extracts were used as templates in a 16S rRNA gene-based PCR method for the detection of 7 as-yet-uncultivated bacterial phylotypes and 1 difficult-to-culture species in endodontic samples. Oligonucleotide primers are listed in Table 1 and were as described previously (11, 12, 14, 15). Endpoint PCR amplification was performed in 50 μL reaction mixture containing 1 μmol/L concentration of each primer, 5 μL 10× PCR buffer (Fermentas, Ontario, Canada), 3 mmol/L $MgCl_2$, 1.25 U Taq DNA polymerase (Fermentas), and 0.2 mmol/L each deoxyribonucleoside triphosphate (Invitrogen Life Technologies, Carlsbad, CA). Negative controls consisting of sterile ultrapure water instead of a sample were included with each batch of samples analyzed. For positive controls, samples from previous studies (14, 16) that were positive for the target taxa using another molecular technique were PCR-amplified using the same primers and protocol described herein, and PCR products were sequenced to confirm specificity of the method. These PCR products were diluted and used as positive controls.

Temperature profiles for *Bacteroidaceae* sp. HOT-272, *F. fastidiosum*, TM7 sp. HOT-356, and *Desulfobulbus* sp. HOT-041 were as described previously (11, 12, 15). Cycling profiles for *Oribacterium* sp. HOT-102, *Atopobium* sp. HOT-416, *Megasphaera* sp. HOT-123, and TM7 sp. HOT-346 encompassed an initial denaturation step at 95°C for 2 minutes and touchdown PCR as follows: a denaturing temperature of each cycle at 95°C for 30 seconds and an annealing temperature initially set at 60°C and then lowered 0.5°C every other cycle until it reached 55°C. Thirty-six additional cycles were performed at 55°C. Primer annealing was performed using this scheme for 30 seconds, and primer extension was performed at 72°C for 1 minute. The final extension step was at 72°C for 5 minutes. PCR amplicons were separated by electrophoresis in a 1.5% agarose gel, which was stained with GelRed (Biotium, Hayward, CA) and visualized under ultraviolet illumination. The presence of amplicons of the expected size for each primer pair was considered as a positive result (Table 1). A 100−base pair DNA ladder (New England BioLabs, Beverly, MA) served as the molecular size standard. PCR amplicons generated from positive clinical samples were sequenced to confirm identification.

qPCR Analysis

The 2 most prevalent taxa were the only ones to be present in S2 samples and then were subjected to qPCR analysis. The 16S rRNA gene-based qPCR was performed with Power SYBR Green PCR Master