Total and Specific Bacterial Levels in the Apical Root Canal System of Teeth with Post-treatment Apical Periodontitis

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Abstract

Introduction: Most studies of the microbiota in root canal–treated teeth focused only on the main canal, not distinguishing regions nor incorporating the intricate anatomy in the analysis. Moreover, most of them provided only prevalence data. This study was designed to evaluate the total bacterial counts and the presence, levels, and relative abundance of candidate endodontic pathogens exclusively in the apical root canal system associated with post-treatment apical periodontitis. **Methods:** Apical root specimens obtained during periradicular surgery of 27 adequately treated teeth with persistent apical periodontitis were cryogenically ground. DNA was extracted from the powder, and real-time polymerase chain reaction was used to quantify the total bacteria and 7 bacterial taxa. **Results:** Samples from 21 teeth were positive for bacteria. *Streptococcus* species were the most prevalent (76%) followed by members of the *Actinobacteria* phylum (52%) and *Pseudoramibacter alactolyticus* (19%). The mean total bacterial load in the apical root segments was 5.7×10^4 cell equivalents per root apex (or 2.1×10^4/100 mg root powder). Streptococci comprised from 0.02%–99.9% of the total bacterial counts, Actinobacteria from 0.02%–84.7%, and *P. alactolyticus* from 67.9%–99%. Although *Enterococcus faecalis* was found in only 3 (14%) cases, it was dominant in 2. **Conclusions:** *Streptococcus* species, members of the *Actinobacteria* phylum, and *P. alactolyticus* were the most prevalent taxa in the apical canal system and dominated the bacterial populations in many cases of post-treatment apical periodontitis. *(J Endod 2015;41:1037–1042)*

Key Words

16S ribosomal RNA gene, post-treatment apical periodontitis, quantitative real-time polymerase chain reaction, root canal–treated teeth

Post-treatment apical periodontitis is an inflammatory disease associated with root canal–treated teeth and is mostly caused by persistent or secondary intraradicular infection (1). Culture studies have identified some candidate pathogens (2, 3), but molecular microbiology methods have significantly expanded the number of species involved with post-treatment apical periodontitis (4–6). Because *Enterococcus faecalis* has been frequently detected in many culture (2, 3) and molecular studies (4, 7, 8), this species has reached the status of the most important species associated with endodontic treatment failure. Nevertheless, evidence mounted over the last decade indicates that *E. faecalis* may not be the main pathogen in post-treatment apical periodontitis (6, 7, 9). Several other bacterial taxa, including streptococci, members of the phyla *Actinobacteria*, and some anaerobic bacteria, usually composing a mixed community, have also been commonly found in association with this disease (4–6). In addition, the large majority of studies dealing with the microbiota of root canal–treated teeth with apical periodontitis were restricted to prevalence analyses. The pathogenic and ecologic relevance of a given species in the disease process also relates to its absolute counts and relative abundance in relation to the total bacterial community.

Post-treatment apical periodontitis is usually caused by bacteria that remained unaffected by endodontic antimicrobial procedures (10). Persistent bacteria are located on untouched root canal walls or in areas such as lateral canals, apical ramifications, dentinal tubules, and isthmuses (11–13). In most of these areas, bacteria are inaccessible to instruments and substances used in endodontic treatment. Likewise, conventional microbiological sampling procedures using paper points cannot reach those areas, and, consequently, the most important bacterial species involved with endodontic treatment failure may not be collected and identified. Because important endodontic pathogens can pass unnoticed when using conventional sampling, a different approach has been recommended (14). This consists of crushing the root in a cryogenic mill and using molecular methods to analyze the DNA extracted from the powder. So far, no study has used this technology to evaluate the bacteriologic conditions in root canal–treated teeth.

Morphologic studies have shown that most bacteria causing post-treatment apical periodontitis are located in the apical root canal system (11, 15, 16). Therefore, selective analysis of the apical microbiota is required for the identification of the taxa directly involved with this disease. Studies using conventional sampling with paper points cannot identify the bacterial taxa present exclusively in the apical root canal.

Therefore, the technical limitations of conventional sampling procedures rule out the ability to selectively identify bacteria in apical canal as well as in areas other than the main root canal. Grinding apical root segments of extracted teeth has been proposed to provide a more representative sample for a selective and more accurate analysis of the

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apical endodontic microbiota (14, 17, 18). For this analysis to be performed, extracted teeth or apical root fragments obtained by root-end resection have to be used.

The present study evaluated the total bacterial counts and the presence, levels, and relative abundance of several candidate endodontic pathogens in the apical root canal of adequately treated teeth with apical periodontitis. Root specimens obtained by root-end resection during periradicular surgery were pulverized in a cryogenic mill and subjected to quantitative real-time polymerase chain reaction (qPCR).

Materials and Methods

Case Description

Twenty-seven patients (20 women and 7 men aged 20–78 years, mean age = 49 years) who had been referred for periradicular surgery to a private practice from 1 of the authors (H.S.A.) were included in this study. Each patient contributed 1 root canal–treated tooth. All teeth were asymptomatic and presented with apical periodontitis lesions as determined radiographically and further confirmed by cone-beam computed tomographic (CBCT) imaging requested for surgery planning. Eleven cases were associated with sinus tracts. In addition to the presence of post-treatment apical periodontitis, the inclusion criteria were as follows: teeth endodontically treated at least more than 1 year previously; teeth with satisfactory coronal restorations and no direct exposure of the root canal filling material to the oral cavity; teeth with no significant gingival recession and absence of periodontal pockets deeper than 4 mm; and teeth exhibiting adequate root canal treatments on the basis of the apical terminus (from 0–1 mm short of the apex), homogeneous fillings (no voids), and tapered canal shape as determined both radiographically and by CBCT analysis. Approval for the study protocol was obtained from the Ethics Committee of the Estácio de Sá University.

Sample Collection

Specimens for molecular microbiologic analysis were obtained during periradicular surgery. For decontamination before surgery, the oral cavity was rinsed with 0.12% chlorhexidine for 2 minutes, and the area to be operated on was then swabbed with the same solution. An intrasulcular incision was used to reflect a full-thickness mucoperiosteal flap. After flap elevation, care was taken to avoid contamination of the surgical site with saliva. The apical periodontitis lesion was curetted and placed in 10% buffered formalin solution for histopathologic examination. Root-end resection was performed using a sterile Zekrya FG 28-mm bur (Maillefer, Ballaigues, Switzerland) under sterile saline irrigation. The apical root fragment (3- to 5-mm long) was then copiously rinsed with saline, and a #15 sterile scalpel was used to remove all attached soft tissue remnants from the root specimen. The apical root fragments were placed in sterile 15-mL Falcon tubes and then immediately frozen at −20°C. Surgery was completed by root-end preparation with ultrasonic tips and root-end filling with a bioceramic material. All surgical procedures were performed under magnification with an operating microscope.

Cryogenic Grinding

The apical root specimens were thawed and subjected to external disinfection and further cryogenic grinding as described previously (14). Briefly, the external root surfaces were cleaned with 3% hydrogen peroxide and disinfected with 2.5% sodium hypochlorite, which was then inactivated with 10% sodium thiosulfate. These substances were scrubbed along the entire extent of the root using sterile cotton applicators, except for 1 mm around the apical foramen. After disinfection, the external root surfaces were sampled by using a #80 sterile paper point dampened with Tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH = 7.6). This sample served as the sterility control and was assessed by means of a qPCR assay using universal bacterial primers (see later). These disinfecting and control sampling procedures were conducted under an operating microscope. A 6750 freezer mill (Spex, Metuchen, NJ) operated at the liquid nitrogen temperature was used to cryogenically grind each tooth fragment. After grinding, apical root powder samples were stored at −20°C.

DNA Extraction

Because the apical root fragments varied in sizes, root powder samples were weighed to permit analysis of data as the number of bacterial cells per 100 mg of root powder.

Next, the powder was suspended in TE buffer, and DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the protocol recommended by the manufacturer. The final volume of DNA from each clinical sample was 200 μL, and this was taken into account during calculations for quantification.

qPCR

For quantification of the levels of total bacteria and 7 bacterial groups or species, 16S ribosomal RNA gene-targeted qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7500 Real-time PCR instrument (Applied Biosystems) in a total reaction volume of 20 μL. Universal and group/species-specific primers were as described and validated previously (4, 19–25); their sequences and the respective annealing temperatures are shown in Table 1. Primers in a concentration of 0.5 μmol/L each and 2 μL of the DNA extract from each apical root specimen were added to the PCR master mix in MicroAmp Optical 96-well reaction plates. Plates were sealed, centrifuged, and then subjected to amplification. Cycling conditions for the qPCR included 95°C/10 min and 40 repeats of the following steps: 95°C/1 min, annealing for 1 minute (specific temperatures are shown in Table 1), and 72°C/1 min. At each cycle, the accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye (double-stranded DNA-binding SYBR Green). All measurements were performed in triplicate for samples and standards. In all experiments, triplicates of appropriate negative controls containing no template DNA were subjected to the same procedures to exclude or detect any possible contamination or carryover. After amplification, melting curve analysis of PCR products was performed to determine the specificity of the amplified products. Melting curve was obtained from 60°C–95°C, with continuous fluorescence measurements taken at every 1% increase in temperature. Data acquisition and analysis were performed using the ABI 7500 software v2.0.4 (Applied Biosystems).

Levels of bacterial groups or species were inferred for each sample based on obtained standard curves, which were constructed using DNA extracted from known concentrations of the following strains: *E. faecalis* American Type Culture Collection (ATCC) 29212, *Filifactor alocis* ATCC 35896, *Parvimonas micra* ATCC 33270, *Pseudoramibacter alactolyticus* C11b-d, *Streptococcus mutans* ATCC 25175 (for the *Streptococcus* genus primer), and strains of *Actinomyces israelii* (for the *Actinobacteria* phylum primer) and *Fusobacterium nucleatum* (for the *Fusobacterium* genus primer) isolated in a previous study (26). DNA isolated from pure cultures of these strains was quantified using a BioPhotometer (Eppendorf, Hamburg, Germany). Knowing the genome size of each species and the average molecular weight of 1 base pair (660 Da), the measured DNA value could then