Bacterial Community Profiling of Cryogenically Ground Samples from the Apical and Coronal Root Segments of Teeth with Apical Periodontitis

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Abstract

Bacteria located at the apical part of infected root canals are arguably directly involved in the pathogenesis of apical periodontitis. This study was conducted to profile and further compare the bacterial communities established at the apical and middle/coronal segments of infected root canals. Extracted teeth with attached apical periodontitis lesions were sectioned so as to obtain two root fragments representing the apical third and the coronal two thirds. Root fragments were subjected to a cryogenic grinding approach. DNA was extracted from root powder samples and used as a template for bacterial community profiling using a 16S ribosomal RNA gene-based seminested polymerase chain reaction/denaturing gradient gel electrophoresis approach. The mean number of bands in apical samples from teeth with primary infections was 28, ranging from 18 to 48, whereas in the middle/coronal samples, it was also 28, ranging from 19 to 36. Findings showed that the profile of bacterial community colonizing the apical third of infected root canals is as diverse as that occurring at the middle/coronal thirds. A high variability was observed for both interindividual (samples from the same region but from different patients) and intraindividual (samples from different regions of the same tooth) comparisons. The methodology used to prepare and analyze samples was highly effective in disclosing a previously unanticipated broad diversity of endodontic bacterial communities, especially at the apical part of infected root canals. *(J Endod 2009;35:486–492)*

Key Words

Apical periodontitis, denaturing gradient gel electrophoresis, endodontic infection, polymerase chain reaction, 16S ribosomal RNA gene

Microbial diversity in infected root canals has been widely explored by culture and molecular technology (1). The latter has significantly expanded the list of putative endodontic pathogens to include several culture-difficult species and as-yet-uncultivated phylotypes previously undetected by culture-based approaches (2). Culture-independent molecular methods have revealed that primarily infected root canals can harbor about 10 to 30 bacterial species/phylotypes (2), numbers much higher than previously shown by culture studies (3).

As for human endogenous infections, there is a current trend to move away from the concept that a single pathogen causes a disease toward a more holistic concept that the community is indeed the unit of pathogenicity (4). In this context, the recognition of community profiles involved with some type of disease may represent an important step toward a better understanding of the pathogenesis of the disease in addition to setting the grounds for the establishment of more effective therapeutic protocols. There are several molecular methods for microbial community profiling analyses (5), and one of the greatest advantages of molecular methods in this regard refers to the ability of providing profiles that also include as-yet-uncultivated bacteria (6). The denaturing gradient gel electrophoresis (DGGE) is a culture-independent technique proposed by Muyzer et al (7) that has been widely used for fingerprinting of microbial communities in diverse environments (6). DGGE was introduced in endodontic microbiology research to study bacterial endodontic communities by Siqueira et al in 2004 (8). Since then, several other studies have profiled endodontic communities in different clinical conditions and from different geographic locations (4, 8–12). In addition to confirming that the different types of endodontic infections are polymicrobial (8, 10), the main findings from DGGE studies indicate that even the infection associated with treated teeth is usually mixed (12); some underrepresented uncultivated bacteria, such as members of the *Synergistes* phylum, may be commonly found in infected root canals (9, 11); bacterial communities follow a pattern according to the clinical condition (chronic apical periodontitis, acute apical abscesses, and treated teeth) (8, 12); and there is a great interindividual variability in endodontic communities associated with the same clinical disease, being still more pronounced when individuals from different geographic locations are analyzed (4, 8, 10, 12).

Bacteria in the apical part of infected root canals are in a strategic position to inflict damage to the host. So far, only a few studies have investigated the microbiota present in this critical area. These studies show that the apical microbiota is conspicuously dominated by anaerobic bacteria (13–16). When the apical microbiota is compared with that occurring in the more coronal aspects of the canal, there appear to be some inter-

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esting differences in the predominant morphotypes, the number of cells, and the anaerobe:facultative ratio (14, 17, 18). To date, no molecular study has profiled the microbiota at the apical third of infected canals and compared the structure of the microbiota with that occurring at the coronal parts of the canal.

An important limitation of most studies evaluating the endodontic microbiota refers to sampling procedures. Paper points are mostly used, but it is widely acknowledged that only bacteria occurring in or very close to the main root canal are usually sampled (19). Thus, bacteria located in other regions of the entire system including ramifications, dentinal tubules, isthmuses, irregularities, and even in some untouched areas of the main canal can pass unnoticed by the identification method.

The present study was undertaken to disclose and further compare the bacterial community profiles of the microbiota established at the apical and middle/coronal segments of infected root canals. For this, segments of extracted teeth with apical periodontitis were cryogenically ground and extracted DNA subjected to DGGE fingerprinting analysis.

Material and Methods

Sample Collection

The examined material consisted of 22 extracted teeth randomly collected from 18 patients in the Oral Surgery Clinic, Estácio de Sá University, Rio de Janeiro, Brazil. All teeth presented radiographic evidence of apical periodontitis and extensive caries lesions and were extracted for prosthetic reasons or by request of the patient. Eighteen teeth exhibited necrotic pulps (primary infection): three maxillary incisors, one mandibular incisor, two maxillary canines, four maxillary single-rooted premolars, two mandibular premolars, two maxillary molars (mesiobuccal roots), and four mandibular molars (mesial roots). The other four teeth had previous root canal treatment (secondary/persistent infection): one maxillary incisor, one mandibular incisor, one maxillary canine, and one maxillary premolar. All teeth showed no periodontal disease deeper than 4 mm, except for two mandibular molars from the same patient (samples 17 and 18). The four treated teeth and the two periodontally involved teeth were not discarded from the study in order to test the applicability of the experimental protocol used to investigate different clinical conditions. The study protocol was institutionally approved, and informed consent was obtained from the patients.

Immediately after extraction, each tooth was profusely rinsed with sterile saline solution, and a #15 sterile scalpel was used to remove all attached soft tissue, including the apical periodontitis lesion, from the root. The external root surfaces were cleaned with 3% hydrogen peroxide and disinfected with 2.5% sodium hypochlorite. The latter was inactivated by sterile 5% sodium thiosulphate. These solutions were scrubbed onto the root surfaces by using sterile cotton applicators. After disinfection, the external root surfaces were sampled by using a #80 sterile paper point dampened with TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, and pH 7.6). This sample served as sterility control and was assessed by means of polymerase chain reaction (PCR) with universal bacterial primers 968f and 1401r (see later). DNA extracted from a human saliva sample was used as positive controls.

Tooth Sectioning

The teeth were sectioned perpendicularly to their long axis at two points: first, at the enamel-cementum junction, discarding the crown, and further at 4 to 6 mm from the apex to generate a fragment representing the apical third of the root and another representing the coronal two thirds (Fig. 1). Each section was performed with the aid of sterile carborundum disks under conditions of strict asepsis. For one tooth, the coronal segment was lost so only the apical segment was available. Apical and middle/coronal-third segments were transferred to 15-mL Falcon tubes sterilized by gamma irradiation and then immediately stored at −20°C.

Cryogenic Grinding

A 6750 freezer mill (Spex, Metuchen, NJ) operated at the liquid nitrogen temperature was used to cryogenically grind each tooth fragment. Root fragments were transferred to individual cylindrical polycarbonate grinding vials. Frozen specimens were ground by a magnetically shuttling steel impact bar oscillating back and forth against two stationary stainless steel end plugs closing the vial (volume = 25 mL). The vial was immersed in liquid nitrogen during operation. The program used for cryogenic grinding included the following parameters: (1) precooling for 15 minutes (before grinding, when the vial was immersed in liquid nitrogen for sample freezing), (2) grinding for 1 minute (resulting from impact bar motion in each working cycle), and (3) recooling for 0.1 minute (a period between two working cycles when the oscillation of the impact bar was interrupted to avoid heating resulting from attrition). Two grinding cycles were performed per specimen. The impact bar frequency was set in 10, which is 20 actual impacts per second (one to each side). After grinding, root powder samples were stored at −20°C.

Stainless steel end plugs, the impact bar, and the polycarbonate vials were all disinfected after each use by the following protocol: brushing and washing with neutral detergent, rinsing with ethanol (70%), immersion in 2.5% sodium hypochlorite for 1 minute, rinsing with milliQ water, drying at 70°C, and sterilization under ultraviolet light for 15 minutes.

DNA Extraction

DNA was extracted from root powder samples by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) following the protocol recommended by the manufacturer for tissues. To maximize DNA extraction from gram-positive bacteria, a step of preincubation with lysozyme for 30 minutes was added according to the manufacturer's instructions.

Seminested PCR

DNA extracts were used as templates in a 16S rRNA gene-based nested PCR approach. One negative control consisting of sterile milliQ water instead of the sample was included for every five samples in all batches of samples analyzed. Aliquots of 3 μL of DNA extract from middle/coronal samples and 6 μL from apical samples were used in the first round of PCR amplification using universal bacterial primers 532f (5′-CGT GCC AGC AGC CGC GGT AA-3′) (20) and 1401r (5′-CGG TGT GTA CAA GAC CC-3′) (21). PCR amplification was performed in 50 μL of reaction mixture containing 0.2 μmol/L of each primer, 5 μL of 10× PCR buffer (Fermentas, Burlington, Canada), 2.5 mmol/L $MgCl_2$, 1.5 U *Taq* DNA polymerase (Fermentas), 0.2 mmol/L of each deoxyribonucleoside triphosphate (Biotools, Madrid, Spain), and milliQ water to complete the volume. Cycling parameters for this first round included an initial denaturation step at 94°C for 3 minutes, 30 cycles of a denaturation step at 94°C for 1 minute, a primer annealing step at 53°C for 1 minute, an extension step at 72°C for 2 minutes, followed by a final step of 72°C for 10 minutes for complete extension.

The PCR products generated in the first reaction (5 μL from middle/coronal samples and 8 μL from apical samples) were used in the second round of amplification, which was performed to increase the performance of the PCR in samples with low DNA concentration.