Apical Root Canal Microbiota as Determined by Reverse-capture Checkerboard Analysis of Cryogenically Ground Root Samples from Teeth with Apical Periodontitis

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Abstract

Introduction: Bacteria located in the apical root canal system potentially participate in the pathogenesis of apical periodontitis. Detection and identification of apical bacteria can be compromised because of limitations in conventional sampling and identification procedures. This study identified several bacterial taxa in the apical and middle/coronal segments of primarily infected root canal system by using pulverized root segments and a culture-independent molecular method.

Methods: Seventeen extracted teeth with attached apical periodontitis lesions were sectioned to obtain 2 root fragments (apical and middle/coronal segments). Root fragments were cryogenically ground, and DNA was extracted from samples. After multiple displacement amplification, DNA from samples was used as template in a reverse-capture checkerboard hybridization assay targeting 28 bacterial taxa.

Results: Bacterial DNA was detected in all samples. The most prevalent taxa in the apical root canal system were Olsenella uli (76.5%), Prevotella baroniae (71%), Porphyromonas endodontalis (65%), Fusobacterium nucleatum (53%), and Tannerella forsythia (47%). O. uli, P. endodontalis, and Propionibacterium acnes were as frequently detected in apical samples as they were in middle/coronal samples. P. baroniae, T. forsythia, and F. nucleatum were found more frequently in the apical part of the canal as compared with matched coronal segments. Streptococcus species were more prevalent in middle/coronal samples.

Conclusions: Several candidate endodontic pathogens were very prevalent in the apical root canal system. The apical microbiota was usually complex and differed in species composition when compared with the microbiota of middle/coronal samples from the same tooth. (J Endod 2010;36: 1617–1621)

Key Words

Apical periodontitis, endodontic infection, polymerase chain reaction, reverse-capture checkerboard DNA-DNA hybridization, 16S rRNA gene

Apical periodontitis is caused by bacteria infecting the necrotic root canal system (1). The disease develops in the periradicular tissues adjacent to the portals of exit of bacteria and their products, ie, the apical, lateral, and furcal foramina. The term apical periodontitis refers to the fact that the disease most commonly develops around the root apex, where the apical foramen, which is the main communication between the root canal system and the periodontium, and the huge majority of lateral foramina are located. Therefore, bacteria occurring in the apical root canal are recognizably in a strategic position to participate in disease causation (2).

Virtually all studies evaluating the endodontic microbiota do not make any distinction of root canal segments. This is because in the clinical condition, samples are taken by using files or paper points introduced in the main canal up to its apical portion. Consequently, bacteria present in each sample are actually a pool from the entire root canal, including its coronal, middle, and apical segments. Specific analysis of the microbiota in the different segments might have to be carried out in teeth that are indicated for extraction.

Despite the benefits of culture-independent molecular biology techniques, these methods have several limitations. This is because bacteria are not always detectable in all samples (3–8). Introduction of culture-independent molecular biology techniques has sidestepped many of the limitations of culture and has consequently significantly increased the knowledge of the bacterial diversity in endodontic infections (9, 10). Several newly named species have emerged as candidate endodontic pathogens (10).

Although it is by and large recognized that bacteria in the apical canal are supposedly more important as to the etiology of apical periodontitis and persistence after treat—
ment (2, 11, 12), it is somewhat surprising how modest is the number of studies investigating the apical microbiota. As early as in 1894, Miller (13) emphasized the different morphology of the apical microbiota in comparison to the most coronal microbiota. Actually, the few studies available demonstrated that the apical microbiota differs significantly from that occurring in the more coronal aspects of the canal (intraradicular analysis) in terms of predominant morphotypes (14), bacterial community profile (5), and anaerobe:facultative ratio (15). Culture and molecular studies have identified many suspected endodontic pathogens in the apical canal, including black-pigmented anaerobic rods, Peptostreptococcus species, Pseudoramibacter alactolyticus, Treponema denticola, Fusobacterium nucleatum, Porphyromonas endodontalis, Filifactor alocis, and the uncultivated phylotype Bacteroidetes clone X083 (16–19). All studies of the human apical microbiota used extracted teeth, only 1 used ground tooth powder for the overall profiling of bacterial communities but with no identification (5), and 2 studies used molecular methods to detect samples taken by files and paper points (18, 19).

The present study was undertaken to identify several bacterial taxa in the apical and middle/coronal segments of the infected root canal system of teeth with apical periodontitis. For analysis, the study was carried out by using cryogenically ground samples from extracted teeth, which were screened for the presence of 28 bacterial taxa by using a culture-independent molecular biology technique.

**Materials and Methods**

**Specimen Collection and Processing**

DNA extracts from samples collected for a previous investigation (5) were stored and available for reanalysis in this study. The examined material consisted of 17 extracted teeth randomly collected from 14 patients in the Oral Surgery Clinic, Estácio de Sa University, Rio de Janeiro. All teeth had necrotic pulps, radiographic evidence of apical periodontitis and extensive caries lesions, and were extracted for prosthetic reasons or by request of the patient. The study protocol was approved by the Ethics Committee of the Estácio de Sa University, and informed consent was obtained from the patients.

Specimen collection and processing were as described previously (5). Briefly, each tooth was profusely rinsed with sterile saline solution immediately after extraction, 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 thiosulfate. These solutions were scrubbed onto the root surfaces by using sterile cotton applicators. After disinfection, the external root surfaces were sampled by using a sterile #80 paper point dampened with TE buffer (10 mMol/L Tris-HCl, 1 mMol/L ethylenediaminetetraacetic acid [EDTA], 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 (5).

Next, the teeth were sectioned perpendicularly to their long axis at 2 points: first, at the enamel-cementum junction, discarding the crown, and further at 4–6 mm from the apex to generate a fragment representing the apical third of the root and another representing the middle/coronal thirds. Apical and middle/coronal third segments were transferred to 15 mL Falcon (BD Biosciences, Mississauga, ON, Canada) tubes sterilized by gamma irradiation and then immediately stored at –20°C.

A 6750 freezer mill (Speex, Metuchen, NJ) operated at the liquid nitrogen temperature was used to cryogenically grind each tooth fragment as described previously (5). After grinding, root powder samples were stored at –20°C.

**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. To maximize DNA extraction from gram-positive bacteria, a step of preincubation with lysozyme for 30 minutes was added. DNA from a panel of several oral bacterial species was also prepared to serve as controls (20).

**Multiple Displacement Amplification**

DNA extracts from clinical samples were subjected to whole genome amplification by using the Illustra GenomiPhi V2 DNA Amplification kit (GE Healthcare, Piscataway, NJ), following the manufacturer’s instructions. In brief, 1 μL of DNA template was added to 9 μL of sample buffer containing random hexamer primers, denatured at 95°C for 3 minutes in a thermocycler, and then cooled to 4°C. An aliquot of 1 μL of enzyme mix containing the phi29 DNA polymerase and additional random hexamers was mixed with 9 μL reaction buffer containing deoxyribonucleoside triphosphates. This mixture was added to the denatured sample to a final volume of 20 μL and then incubated at 30°C for 1.5 hours. Afterwards, the enzyme was inactivated by 10-minute incubation at 65°C, and the amplified material was stored at –20°C. This multiple displacement amplification (MDA) step was used to achieve a better performance of the subsequent broad-range PCR assay, particularly for samples with low number of bacteria.

**Broad-range PCR for Checkerboard Analysis**

DNA from clinical samples amplified by MDA was used as template in a 16S rRNA gene-based PCR protocol. A practically full-length 16S rRNA gene fragment was amplified by using universal primers 8f and 1492r. The forward primer was labeled at the 5 end with digoxigenin.

PCR amplifications were performed in 50 μL of reaction mixture containing 2 μL of DNA extract (MDA products), 1 μmol/L concentration of each primer, 5 μL of 10 × PCR buffer (Fermentas, Burlington, Canada), 3 mMol/L MgCl2, 2 U of Tag DNA polymerase (Fermentas), and 0.2 mMol/L of each deoxyribonucleoside triphosphates (Biotools, Madrid, Spain). One negative control was included for every 5 samples analyzed.

Preparations were amplified in a DNA thermocycler (Mastercycler Personal; Eppendorff, Hamburg, Germany). The PCR temperature profile included an initial denaturation step at 95°C/1 min, 35 cycles at 94°C/45 s, 50°C/45 s, and 72°C/1.5 min, and a final step at 72°C/20 min. PCR amplicons were separated by electrophoresis in a 1.5% agarose gel, stained with GelRed (Biotium, Hayward, CA), and viewed under ultraviolet transillumination.

**Reverse-capture Checkerboard Assay**

The reverse-capture checkerboard assay was performed as described previously (21, 22). Labeled PCR products (40 μL) were used in a reverse-capture checkerboard assay to determine the presence of 28 bacterial taxa. Probes were based on 16S rRNA gene sequences of the target bacteria and were described and validated previously (21, 23). In addition to the 28 taxon-specific probes, 2 universal probes were included in the assay to serve as controls. Two lanes in the membrane contained standards at the concentration of 105 and 104 cells, which were treated the same way as the clinical samples.

The reverse-capture checkerboard assay was performed by using the Minislot-30 and Miniblotter-45 system (Immunetics, Cambridge, MA). First, 100 pmol of probe in TE buffer (10 mMol/L Tris HCl, 1 mMol/L EDTA, pH 8.0) was introduced into the horizontal wells of the Minislot apparatus and cross-linked to the Hybond-N+ nylon membrane (AmershamPharmaica Biotech, Buckinghamshire,
After hybridization, the membrane was washed and blocked in a buffer with casein. The membrane was sequentially incubated in antidigoxigenin antibody conjugated with alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany) and ultrasensitive chemiluminescent substrate CDP Star (Roche Molecular Biochemicals). Finally, a square of x-ray film was exposed to the membrane in a cassette for 20 minutes to detect the hybrids. Prevalence of the target species/phylotypes was recorded as the percentage of cases examined.

Results

Sterility control samples taken from the external root surface after disinfection yielded no PCR product. Negative PCR controls showed the predicted results. All apical and middle/coronal samples were positive for the presence of bacteria as determined by PCR with universal 16S rRNA gene primers. All samples were also positive for bacteria in the reverse-capture checkerboard assay as revealed by the 2 universal probes. The results revealed that 27 and 20 taxon-specific probes tested reactive with 1 or more apical and middle/coronal root canal samples, respectively. All samples were positive for at least 1 taxon-specific probe. The number of target bacterial taxa detected in the apical samples ranged from 1–25 (mean, 8 taxa), whereas in middle/coronal samples the number of taxa ranged from 1–18 (mean, 5 taxa).

The most prevalent taxa detected in the apical root canal system were Olsenella uli (13/17, 76.5%), Prevotella baronii (12/17, 71%), Porphyromonas endodontalis (11/17, 65%), Fusobacterium nucleatum (9/17, 53%), and Tannerella forsythia (8/17, 47%) (Fig. 1). Of the most prevalent species/phylotypes detected in this study, O. uli, P. endodontalis, and Propionibacterium acnes were as frequently detected in the apical segment as they were at the more coronal aspects of the root canal system. However, whereas both O. uli and P. endodontalis were often concomitantly present in both samples from the same tooth, P. acnes was either present in the apical sample or in the middle/coronal sample, with only the samples from 1 tooth sharing this species. Other species such as P. baronii, T. forsythia, and F. nucleatum were found more in the apical part of the canal as compared with the matched coronal segments. Streptococcus species were more prevalent in middle/coronal samples than in matched apical samples. Overall, several target species/phylotypes were more frequently detected in the apical segment. Some of the least prevalent species were found exclusively in apical samples, including Actinomyces israelii, Eubacterium sibiricum, Campylobacter rectus, Prevotella nigrescens, and Treponema soorenskii.

Samples from the apical canal were compared for similarities (shared target taxa) with their matched samples from the middle/ coronal segment. The median and mean of shared bacterial taxa between apical and middle/coronal segments of the same tooth were 27% and 41%, respectively, ranging from 0% (ie, the taxa found in the apical sample were different from those found in the middle/coronal sample of the same tooth) to 100% (ie, the taxa found in the apical sample were the same as those found in the middle/coronal sample of the same tooth).

Discussion

Only 2 studies have been published that use molecular methods to identify bacteria located in the apical root canal. One study (19) used a conventional species-specific PCR assay directed to 10 bacterial species, whereas the other (18) used the same reverse-capture checkerboard approach as used in the present study to detect 28 target species/phylotypes. The main difference between those studies and the present one was that a cryogenic grinding approach was used to pulverize root samples so the bacterial taxa entrapped in the intricate anatomy of the root canal system could be incorporated in the present analysis. Furthermore, samples from the coronal aspects of the canal were included in the analysis for sake of comparison between the microbiota present in apical and middle/coronal parts of the canal system.

The cryogenic grinding approach has been commonly used in forensic studies to recover DNA from tooth and bone for molecular analysis (24, 25). By incorporating this technique, the present protocol is expected to provide a more representative sample from the entire root canal system, including not only the main root canal but also irregularities, dentinal tubules, and ramifications. Therefore, it provides a more accurate picture of the apical root canal microbiota. However, the main disadvantage of this approach refers to the fact that it can only be applied to teeth with indication for forensic studies to recover DNA from tooth and bone for molecular analysis. The negative PCR results obtained for control samples from the root surface reveal that our disinfection protocol succeeded in rendering the root surfaces free of detectable bacterial DNA contaminants before analysis.

Figure 1. Stacked bar chart of frequency of detection of bacterial species/phylotypes in apical and middle/coronal root canal samples of teeth with apical periodontitis. Total length of each bar stack indicates overall prevalence of bacterial species/phylotypes in diseased teeth, with no distinction of region. Different colors within each bar indicate prevalence of bacterial taxa specifically in apical samples, middle/coronal samples, or both.
The availability of sample material from endodontic infections is not always optimum for a comprehensive analysis to be performed. This can be because of the recognized limitations of root canal sampling techniques with files and/or paper points. In the specific case of this study, the small root fragments might have represented a small amount of DNA available for the checkboard identification analysis, especially if one considers that only a small aliquot of DNA extract can be used in PCR. To circumvent this limitation, the present study made use of nonspecific whole genomic amplification by means of MDA to generate large quantity of assay ready DNA, as previously recommended by Brito et al (26) for the conventional checkboard technique. MDA is a technique for nonspecific whole genome amplification by using phi29 bacteriophage DNA polymerase enzyme and random hexamer primers in an isothermal reaction (27). MDA has been shown to provide uniform amplification of the whole genome with extensive coverage, generate extremely long DNA products compared with previous whole genome amplification methods, and result in high DNA yields even starting from very low template amounts, with reduced amplification bias (27, 28). It has been regarded as an important pre-PCR procedure for the detection of low copy number sequences (29, 30). In addition, MDA provides enough amplified DNA to perform multiple analyses of the same sample.

A large intraindividual variability was demonstrated in this study, in which the mean or median numbers of bacterial taxa shared by the apical and middle/coronal samples from the same tooth were low. Only in 2 teeth were all detected taxa found in both samples, with a 100% match. Intraindividual variability means that the composition of the apical microbiota is different from the middle/coronal microbiota. Such differences were also observed for the same samples when using the PCR–denaturing gradient gel electrophoresis (DGGE) open-ended approach (5).

The occurrence of distinct species colonizing the different regions of the canal can be explained by ecological conditions. Ecological determinants are mostly represented by the physicochemical factors and type of nutrient availability, which are expected to vary according to the different root canal regions. Specifically, the apical canal presents a lower oxygen tension and larger availability of proteins and glycoproteins that are highly conducive to the establishment of anaerobic bacterial species, most of which are asaccharolytic and/or proteolytic, such as P. endodontalis, F. nucleatum, T. forsythia, and Treponema denticola (31, 32). However, some saccharolytic anaerobic species, such as O. uli and P. baroniae, can also be very prevalent, and these species are expected to derive nutrients from the carbohydrate portion of glycoproteins and/or food webs established with other members of the apical microbiota. Because this study was restricted to presence/absence (prevalence) data, further studies evaluating abundance of species in the communities are required to refine such ecological inferences.

Most of the taxa targeted in this study were anaerobic species, which might help explain the higher prevalence of several of them at the apical root canal segment as compared with samples from the coronal aspects of the same canals. Some taxa were as prevalent in the apical canal as they were at the coronal samples. The only group that was more frequently detected in the middle/coronal segments was the streptococci, which are predominantly facultative and saccharolytic bacteria and might be favored by the ecological conditions in coronal parts of the root canal system.

The mean number of target taxa was higher in the apical segment as compared with the middle/coronal segments of the canal. However, because this was a closed-ended molecular identification analysis with specific probes, the possibility exists that taxa other than those targeted in this study might have been present. In fact, a previous open-ended broad-range PCR-DGGE analysis of these very same samples demonstrated that the mean number of species/phylotypes per sample was virtually the same, although the composition was different. These findings demonstrate that the apical microbiota can be as diverse as in the middle/coronal segments of the canal. The high diversity of the apical microbiota should not be faced as a surprise. Bacteria in the apical canal compose the advance front of infection and thereby reside near an inflamed tissue area. Inflammatory exudate seeps into the apical canal and stagnates, creating a fluid phase that provides bacteria with nutrients in the form of glycoproteins and proteins. This represents optimal conditions for establishment and growth of a highly diverse microbiota.

The most prevalent species in the present study have been consistently detected in previous studies by using samples from main root canals of teeth with primary infections (with no distinction of location) or from abscess aspirates (21, 22, 33, 34). Specifically, when compared with previous results from a checkboard study of teeth with chronic apical periodontitis (21), a clinical condition similar to the present study, O. uli, P. baroniae, P. endodontalis, Streptococcus species, F. nucleatum, and T. forsythia were also among the most prevalent taxa. However, in a previous study with samples taken from the apical root canal with files and paper points, these species were either present in lower prevalence or even absent (18). Such differences are very likely to be related to 2 factors, pre-PCR MDA approach to increase the amount of DNA available for analysis and use of pulverized root segments, which allowed inclusion of bacteria present not only in the main canal but also in other locations of the root canal system.

In conclusion, several candidate endodontic pathogens were detected in the apical root canal system. These species fulfill an important requirement for endodontic pathogens, ie, “the microorganism must be spatially located in the root canal system in such a way that it or its virulence factors can gain access to the periapical tissues” (35). The apical microbiota was usually complex and differed in composition when compared with matched middle/coronal microbiota from the same teeth.

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