Root canal microbiota of teeth with chronic apical periodontitis

I. N. Rôças

J. F. Siqueira Jr

1 Department of Endodontics, Estácio de Sá University, Rio de Janeiro, RJ, Brazil.

Running headline: Microbiota of dental root canals

* Corresponding author.

Mailing address:

José F. Siqueira Jr, DDS, MSc, PhD
Faculty of Dentistry, Estácio de Sá University
Rua Alfredo Baltazar da Silveira, 580/cobertura, Recreio
Rio de Janeiro, RJ
Brazil 22790-701
Tel: +55 21 8874-1022
FAX: +55 21 2199-2204
e-mail: jf_siqueira@yahoo.com; siqueira@estacio.br
Abstract

Samples from infected root canals of 43 teeth with chronic apical periodontitis were analyzed for the presence and relative levels of 83 oral bacterial species/phylotypes using a reverse-capture checkerboard hybridization assay. Associations between the most frequently detected taxa were also recorded. The most prevalent taxa were Olsenella uli (74%), Eikenella corrodens (63%), Porphyromonas endodontalis (56%), Peptostreptococcus anaerobius (54%), and Bacteroidetes oral clone X083 (51%). When prevalence was considered only for bacteria present at levels >10^5, Bacteroidetes clone X083 was the most frequent (37%), followed by Parvimonas micra (28%), E. corrodens (23%), and Tannerella forsythia (19%). The number of target taxa per canal was directly proportional to the size of apical periodontitis lesion, with lesions >10 mm in diameter harboring a mean number of approximately 20 taxa. Several positive associations were for the first time disclosed for the most prevalent taxa, and may have important ecological and pathogenic implications. In addition to strengthening the association of several cultivable named species with chronic apical periodontitis, the present findings using a large-scale analysis allowed inclusion of some newly named species and as-yet-uncultivated phylotypes in the set of candidate pathogens associated with this disease.

Key words: endodontic microbiology, apical periodontitis, 16S rRNA gene, polymerase chain reaction, checkerboard DNA-DNA hybridization.
Introduction

Chronic apical periodontitis is arguably one of the most common forms of biofilm-induced diseases that affect humans (9). The disease develops after dental pulp necrosis and infection as a result of caries, trauma or iatrogenic clinical procedures. The environmental conditions in the necrotic root canal are conducive to the establishment of a microbiota conspicuously dominated by anaerobic bacteria. Bacterial profiles of the endodontic microbiota vary from individual to individual (37), i.e., each individual harbors a unique microbiota in terms of species richness and abundance. This indicates that apical periodontitis has a heterogeneous etiology, where no single species can be considered as the main endodontic pathogen and multiple bacterial combinations can play a role in disease causation.

Early studies of the microbiota associated with apical periodontitis were conducted using broad-range culture methods. These were followed by a generation of studies employing molecular detection methods such as species-specific polymerase chain reaction (PCR) and the original checkerboard DNA-DNA hybridization assay to target cultivable bacteria previously isolated from infected canals or from other oral diseased sites. These methods allowed the inclusion of some culture-difficult species in the set of candidate endodontic pathogens. The adoption of 16S rRNA gene clone library analysis allowed an even more comprehensive broad-range investigation of bacterial communities in endodontic infections. By this technique, not only cultivable species but also as-yet-uncultivated and uncharacterized bacteria can be identified. Studies using the 16S rRNA gene clone library analysis have revealed that 40-55% of
the bacterial taxa found in primary endodontic infections have not been
cultivated and validly named (20, 30). However, technical difficulties and high
cost can make it difficult to analyze a large number of samples by this method.
Cataloguing bacterial species in the oral cavity by clone libraries provides 16S
rRNA gene sequence data that can be used to design oligonucleotide probes or
primers to target both cultivable and as-yet-uncultivated bacteria. Primers are
used in PCR assays, which are however restricted by the need to perform
several individual reactions to survey several samples for the presence of
several species/phylotypes. Probes can be used in molecular biology
techniques suitable for large-scale clinical studies, including the reverse-capture
checkerboard hybridization assay.

The present study was undertaken to evaluate the presence and relative
levels of 83 bacterial taxa in necrotic root canals of teeth with chronic apical
periodontitis by using a reverse-capture checkerboard hybridization assay.
Target taxa for investigation included cultivable species previously linked to
endodontic infections as well as newly characterized species and as-yet-
uncultivated phylotypes that have been recently detected in clone libraries from
periodontal (19, 22) and endodontic infections (20, 30). Some of them have
never been previously found in infected root canals and others have never been
tested against a large number of samples. Associations between the most
frequently detected taxa were also calculated.
Material and methods

Subjects and sample collection

Ethical approval for the study was granted by the Ethics Committee of the Estácio de Sá University, Rio de Janeiro, Brazil. Root canal samples were taken from 43 patients presenting to the endodontic clinic at Estácio de Sá University for evaluation and treatment of apical periodontitis. Only single-rooted teeth from adult patients older than 22 years, all of them having necrotic pulps and radiographic evidence of apical periodontitis lesion, were included in this study. The size of each lesion was calculated by taking the average of the lesions’ largest dimension and the extent in the direction perpendicular to the largest dimension. All cases were asymptomatic at the time of treatment, 7 of which had associated sinus tract. Selected teeth showed an absence of periodontal pockets deeper than 4 mm.

Samples were taken from the necrotic root canals under strict aseptic conditions and after a two-step disinfection protocol of the operative field with 2.5% NaOCl as previously described (37). Endodontic files with the handle cut off and paper points used for sampling the canals were transferred to cryotubes containing TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and immediately frozen at –20°C. Further, samples were brought to room temperature and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. DNA from a panel of several oral bacterial species was also prepared to serve as controls (39).
Oligonucleotide probes

Six taxon-specific probes and two universal probes were described in previous studies (3, 5). The other probes used in this study were designed as follows: 16S rRNA gene sequences of each of the target bacteria were retrieved from the GenBank and aligned with the sequences of their nearest neighbors in the phylogenetic tree. Potential probes with a melting temperature of approximately 51-52°C were designed from these areas. BLAST-based algorithm (1) was then used to verify their uniqueness. Probes were synthesized with multiple thymidines at the 5' end and were tested against purified DNA from the panel of oral species. No cross-reactions were observed for the probes used in this study, except for a TM7-group specific probe (5' – CCC GTC AAT TCC TTT ATG TTT TA – 3'), which was excluded from the panel. Probe sequences are depicted in appendix 1 (supplemental material).

Checkerboard hybridization assay

The PCR-based, reverse-capture checkerboard assay was carried out as described by Paster et al. (21) with some modifications. Initially, whole-genomic DNA extracts from clinical samples were used as templates in a 16S rRNA gene-based PCR protocol consisting of two steps. First, a practically full-length 16S rRNA gene fragment was amplified from 5 µl of the DNA extracts using universal primers 8f and 1492r. Next, 1 µl of the resulting PCR product from each sample was used to run two sets of partial 16S rRNA gene amplification, one using primers digoxigenin-8f and 519r and the other using primers digoxigenin-515f and 1492r. Primers 8f and 1492r were as described by Paster
et al. (21), while primers 519r and 515f were modified from the sequences reported by Hutter et al. (16) to accommodate a broader range of oral species/phylotypes. Therefore, two different fragments, which together encompass the nearly full-length 16S rRNA gene, were obtained for each sample. This two-step hemi-nested approach was used to achieve a better performance of PCR, particularly for samples with low number of bacteria (25). Since three different checkerboard runs had to be performed for each sample (only 30 probes fit on each checkerboard), the first PCR products were used as templates for three subsequent sets of labeled hemi-nested amplification with the two primer pairs. Using the same PCR product for the subsequent labeled reactions reduced the variation in the samples (8).

All PCR amplifications were performed in a 50 µl of reaction mixture containing 1 µM concentration of each primer, 5 µl of 10× PCR buffer, 3 mM MgCl₂, 2 U of Tth DNA polymerase and 0.2 mM of each deoxyribonucleoside triphosphate (all reagents from Biotools, Madrid, Spain). Negative controls consisting of sterile ultrapure water instead of sample were included with each batch of samples analyzed.

Temperature profile for the first PCR reaction using primers 8f/1492r was: 95°C/1min, 26 cycles at 94°C/45s, 50°C/45s, and 72°C/1.5min, and 72°C/15min. Cycling conditions for the second round of amplification using primers digoxigenin-8f/519r or digoxigenin-515f/1492r included: 95°C/5min, 28 cycles at 94°C/30s, 55°C/1min, 72°C/1.5min, and 72°C/20min. Amplicons were separated by electrophoresis in agarose gels and viewed under ultraviolet transillumination.
Labeled PCR products obtained with primers 8f/519r and 515f/1492r were mixed using equal proportions of each (45 µl) and used in the checkerboard assay to determine the presence and levels of 83 bacterial taxa. 16S rRNA gene probes were synthesized with multiple thymidines at the 5’ end. Two lanes in each membrane contained standards at 10^5 and 10^6 cells, which were treated the same way as the clinical samples. Probes were randomly distributed along three different membranes. Each membrane shared the two universal probes, which served as controls. A total of 1350 hybridizations can be performed simultaneously using a single membrane. Overall, 3569 hybridizations were carried out in this study, excluding the universal probes.

The checkerboard assay was performed using the Minislot-30 and Miniblotter-45 system (Immunetics, Cambridge, MA) (3, 8, 21, 25). First, 100 pmol of probe in Tris-EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) were introduced into the horizontal wells of the Minislot apparatus, and crosslinked to the Hybond- N+ nylon membrane (AmershamPharmacia Biotech, Buckinghamshire, England) by ultraviolet irradiation using a Stratalinker 1800 (Stratagene, La Jolla, CA) on autocrosslink position. The polythymidine tails are preferentially crosslinked to the nylon, leaving the specific probe available for hybridization (21). The membrane was then prehybridized at 55°C for one hour. Subsequently, 90 µl of the labeled PCR products with 50 µl of 55°C preheated hybridization solution was denatured at 95°C/5 min and loaded on the membrane using the Miniblotter apparatus. Hybridization was carried out at 54°C for two hours.
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 ultra-sensitive chemiluminescent substrate CDP Star (Roche Molecular Biochemicals). Finally, a square of X-ray film was exposed to the membrane in a cassette for 20 min in order to detect the hybrids.

**Data analysis**

Prevalence of the target species/phylotypes was recorded as the percentage of cases examined. The obtained chemiluminescent signals were also evaluated using ImageJ (http://rsb.info.nih.gov/ij/) and converted to counts by comparison with standards at known concentrations run on each membrane. Because of the recognized difficulties in inferring absolute counts for PCR-amplified samples and because estimates had to be made for counting as-yet-uncultivated phylotypes, counts were transformed into semi-quantitative data and categorized as follows: a level below detection (or absence); a level <10^5 bacteria; a level =10^5 bacteria; a level >10^5-<10^6 bacteria; a level =10^6 bacteria; and a level >10^6 bacteria. A heavy infection was considered for levels >10^5.

Relative risk (RR) with 95% confidence interval was used to examine pairs of the most prevalent taxa for associations. RR calculations were based on the detection of the target taxa in each subject, irrespective of their proportional recovery in samples. Moreover, data on presence/absence of taxa found at least in 5 samples were used for calculation of Euclidean distances, percent disagreement or Pearson coefficient, and then subjected to cluster
analysis by Ward’s method or unweighted pair group method using arithmetic averages (UPGMA) to determine bacterial associations in complexes.

Results

All sample extracts were positive for PCR amplification using the broad-range 16S rRNA gene primers, indicating that bacteria were present in all examined samples, and that significant inhibitors of the PCR reaction were not present. Negative controls yielded no amplicons.

The results of the reverse-capture checkerboard analysis revealed that 72 of the 83 oligonucleotide probes tested were reactive with one or more clinical samples. All the 43 samples were positive for at least two taxon-specific probes. The number of taxa per infected canal ranged from 2 to 45 (mean, 14.3; median, 12). Root canals of teeth with lesions <5 mm in diameter (n=14) harbored a mean number of 11.7 taxa. Canals of teeth with lesions having 5 to <10 mm in diameter (n=15) showed a mean number of 16 taxa, while the canals associated with lesions larger than 10 mm (n=14) presented a mean number of 19.9 taxa. Three canals harbored more than 40 taxa and they all were associated with lesions ≥10 mm in diameter. Canals of teeth with sinus tracts exhibited a mean number of 16.7 taxa.

Taxa detected more frequently included Olsenella uli (32/43 cases-74%); Eikenella corrodens (27/43-63%); Porphyromonas endodontalis (24/43-56%); Peptostreptococcus anaerobius (23/43-54%); Bacteroidetes oral clone X083 (22/43-51%); Tannerella forsythia (22/43-51%); Dialister invisus (19/43-44%);
and *Fusobacterium nucleatum* (19/43-44%). Prevalence values for the 83 taxa are depicted in Figure 1.

The mean number of bacterial taxa present at high counts (>10⁵) in infected canals ranged from 0 to 10 (mean: 2.6, median: 2). Thirty-five cases showed at least one target taxon at levels above 10⁵. Of the 36 taxa detected at levels >10⁵, the most frequent were *Bacteroidetes* oral clone X083 (16/43-37%); *Parvimonas micra* (12/43-28%); *E. corrodens* (10/43-23%); *T. forsythia* (8/43-19%); *P. anaerobius* (5/43-12%); *D. invisus* (5/43-12%); and *Synergistes* oral clone BA121 (5/43-12%). Figure 2 depicts the prevalence of bacterial taxa encountered at levels >10⁵.

Several pairs of bacterial taxa were positively associated (RR>1). Strong positive associations occurred between *O. uli* and *E. sulci; Bacteroidetes* clone X083 and *P. micra, P. baroniae*, or *P. anaerobius*; and *T. forsythia* and *E. sulci* or *P. micra, P. micra* and *P. baroniae* were positively related to all the other taxa, except *F. nucleatum*. *T. forsythia* was the only species that showed positive associations with all the other most prevalent taxa. Other positive associations are shown in Table 1.

Cluster analysis using different distance measures and linkage techniques revealed 4 clusters, with only minor differences among the different methods. The Ward’s method with Euclidean distances was selected to display the results (Figure 3). A major cluster composed almost exclusively by the most prevalent taxa stood out against the other three. Several of the positive associations observed in RR calculations were confirmed in this broader analysis.
Discussion

Although the original checkerboard approach has already been used in endodontic microbiology research (4, 11, 32, 38, 39, 42), this is the first study to use the reverse-capture checkerboard assay to investigate the presence and relative levels of a large panel of target taxa in infected root canals of teeth with chronic apical periodontitis. This approach has important advantages over the original checkerboard method. One advantage relates to PCR amplification of samples previously to hybridization, which increases the method's sensitivity when compared to the original assay, even though recent introduction of multiple-displacement amplification may have overcome the problem of low sensitivity of the original checkerboard (4). In fact, the most significant advantages are related to the use of oligonucleotide probes instead of whole genomic probes. Oligonucleotide probes display higher specificity, since all the probe sequences in a panel can be designed to have similar $T_m$ values. This allows for a stringent hybridization temperature to be used for all probes in an individual membrane (21). Moreover, mismatches are not tolerated due to the considerable reduction of bond strength between short probes and the target (17). Oligonucleotide probes still have the advantage that they can be designed to detect both cultivable and as-yet-uncultivated bacteria, while in the original checkerboard method with whole genomic probes only cultivable species are targeted.

In the present study, several cultivable species were amongst the most frequently detected taxa, including *O. uli, E. corrodens, P. endodontalis* and *P. anaerobius*. *O. uli* was present in about three fourths of the samples, indicating
that this species is a very common member of the microbiota associated with chronic apical periodontitis. *O. uli* has only recently been recognized as a member of the endodontic microbial consortium of teeth with apical periodontitis (6, 20, 34). *E. corrodens* has been detected in endodontic infections mainly by molecular methods (28) and the frequency at which this species was found in this study is probably the highest ever reported for *E. corrodens* in endodontic infections. Even when presence at high levels was regarded, *E. corrodens* was one of the most prevalent taxon. *P. endodontalis* has been found in endodontic infections by culture (14), but its association with apical periodontitis has been strengthened by findings from molecular studies, whereby highest prevalence values have been reported (10, 12, 35). In this study, *P. endodontalis* was found in about one-half of the infected canals. In spite of being among the most prevalent taxa, both *O. uli* and *P. endodontalis* were usually found at low levels and consequently not as the dominant taxa in the mixed consortium. Although the low levels of infection may apparently put into question a pathogenic role for these taxa in chronic apical periodontitis, at least an ecological role in the mixed consortium can not be disregarded.

Other cultivable species frequently encountered in this study included *P. anaerobius*, *T. forsythia*, *F. nucleatum* and *P. micra*. *P. anaerobius* has been often reported to occur in infected root canals (20, 43). In the present study, this species was found in 23 cases, 5 of which as one of the dominant taxa. The important periodontal pathogen *T. forsythia* has been previously detected in endodontic infections only by molecular biology techniques (7, 33, 38), and the high prevalence observed in the present study, in many cases at high counts,
confirms the species association with apical periodontitis. *F. nucleatum* and *P. micra* have long been recognized as putative endodontic pathogens by studies using culture and molecular methods (10, 13, 38, 43).

Some newly named species, such as *P. baroniae*, *D. invisus* and *Peptostreptococcus stomatis*, were also found in somewhat high frequencies. Interestingly, *P. baroniae* was the most prevalent of the 7 *Prevotella* species targeted in this study. *P. baroniae* is likely synonym with *Prevotella clones PUS9.180 and E9_42-E4*, and has been recently linked to acute apical abscesses (18, 30). *D. invisus* was originally found in infected root canals of teeth with chronic apical periodontitis (20). Findings for this species are in line with several other recent studies that demonstrated *D. invisus* presence in association with both asymptomatic and symptomatic endodontic infections (24, 27, 29, 34). *P. stomatis* (or *Peptostreptococcus* clone CK035) is a newly named species that had been only previously detected in infected canals by studies using clone library analysis (29-31). This is the first study evaluating the occurrence of this species in a large number of root canal samples. *P. stomatis* was detected in about one fourth of the cases and in some of them as one of the most dominant taxa in the community.

*Treponema* species are examples of culture-difficult bacteria that have been identified in endodontic infections only by molecular methods (2, 26, 38). All ten cultivable and four as-yet-uncharacterized oral treponemes were targeted in this study. Of the 9 treponemes detected, *T. denticola* and *T. socransky* were the most prevalent species, which is in consonance with previous studies (2, 26). The other most frequently found *Treponema*
species/phylotypes included *Treponema* 6:G:G47 (16%), *T. putidum* (16%), *Treponema* II:10:D12 (14%) and *T. parvum* (12%). No *Treponema* species was found at levels >10^5.

Because clone libraries from endodontic infections reveal that approximately one-half of the detected taxa still remain to be cultivated and characterized, a comprehensive analysis of the microbiota involved with different forms of apical periodontitis should include these bacteria (20, 30). An initial and essential step towards the recognition of as-yet-uncultivated bacteria as candidate endodontic pathogens is evaluating their prevalence in a large number of diseased cases to look for association. Ten of the 22 as-yet-uncultivated phylotypes targeted in this study were detected in at least 20% of the cases: *Bacteroidetes* clone X083, five *Synergistes* and two *Dialister* phylotypes, *Olsenella* genospecies C1, and *Tannerella* clone BU063. *Bacteroidetes* clone X083 occurred in one-half of the canals and was the most prevalent taxon at levels >10^5. These findings suggest that this phylotype can be a candidate endodontic pathogen that has been previously overshadowed by inherent limitations of culture methods. *Synergistes* bacteria have been only recently disclosed in endodontic infections by molecular biology techniques (20, 34), with clone BA121 being the most frequently detected phylotype (34). This was corroborated by the present findings. Other four as-yet-uncultivated *Synergistes* phylotypes were also often detected, indicating that these bacteria are also common members of endodontic infections and their occurrence had been underrated by culture studies. Overall, these association results indicate that several as-yet-uncultivated phylotypes should be included in the set of
candidate endodontic pathogens and efforts should be directed towards
development of culture media and strategies to cultivate and study some
important features of these bacteria, including pathogenicity and susceptibility to
antimicrobial agents.

Endodontic bacteria fall into 8 bacterial phyla, namely Firmicutes, Bacteroidetes, Spirochaetes, Fusobacteria, Actinobacteria, Proteobacteria, Synergistetes and TM7 (20, 29, 30, 34). The panel of taxa targeted in this study included representatives of all these phyla, except TM7. In fact, a TM7 group-specific probe had to be removed from the panel because of cross-reactivity to several other species (data not shown). Clone X112 from the Sulphur River 1 (SR1) phylum has been originally detected in subgingival samples from patients with marginal periodontitis (22) and was included in our panel. This clone was detected in two infected canals. This is the first report of a member of the phylum SR1 in infected canals. Although this finding expands the diversity of endodontic bacteria to include another phylum, the fact that clone X112 was detected in low prevalence and even so as very weak signals may indicate that it is not an important member of endodontic infections.

Culture studies have demonstrated that primary endodontic infections are characterized by a mixed consortium dominated by anaerobic bacteria and composed of a mean number of 2.6 to 5.4 taxa per canal (23, 36, 43, 44). Nonetheless, broad-range molecular analyses of the root canal microbiota of teeth with chronic apical periodontitis have revealed higher figures - 7 taxa in DGGE analysis (37), 11 taxa in T-RFLP analysis (30), 10-12 taxa in clone library analysis (29, 30) and 20 taxa in combined culturing and clone library
analyses (20). Therefore, data from culture studies tend to underestimate the number of bacterial taxa in infected canals, and this can be a result of difficulties or even impossibilities to cultivate a significant proportion of the endodontic microbiota. This was confirmed in the present study, where a mean number of about 14 taxa was present per canal. This value is almost the same as that reported by Brito et al. (4) when using the original checkerboard to detected 77 cultivable species in nonamplified samples from asymptomatic primary infections. Considering that only target species can be detected by the checkerboard technology, this figure might well be larger to include nontarget taxa and taxa at levels below the detection limits of the assay. The mean number of taxa per canal was clearly in direct proportion to the lesion size – small lesions (< 5mm) harbored 11.7 taxa, lesions from 5 to <10 mm harbored 16 taxa, and lesions >10 mm harbored about 20 species. These differences in species richness help explain the long-held concept that the endodontic treatment of teeth with large lesions have a lower success rate than teeth with small or no lesions (41).

The necrotic root canal affords bacteria space and a moist, warm, nutritious and anaerobic environment, which is by and large protected from the host defenses. Even so, only a restricted assortment of oral bacteria is found in an individual infected canal. This indicates that selective pressures occur in the root canal system that favor the establishment of some species and inhibit others (43). One important ecological factor that helps to determine the composition of the root canal microbiota includes bacterial interactions. Positive bacterial interactions enhance the survival capacity of the interacting bacteria
and enable different species to coexist in habitats where neither could exist alone. Positive interactions can also result in enhanced pathogenicity due to additive or synergistic effects. In the present study, several positive associations were for the first time depicted for some taxa, particularly the newly named species and as-yet-uncultivated phylotypes. The most prevalent taxon *O. uli* was positively related to several other taxa and showed no negative associations. Some strong positive associations were observed for both *Bacteroidetes* clone X083 and *T. forsythia*, the latter being positively associated with all the other taxa tested.

This study also examined for the first time broader bacterial associations using cluster analysis and 4 major complexes were disclosed. One of these complexes involved 13 taxa, all of which very frequently detected. Cluster analyses of conventional checkerboard identifications have been used in periodontal microbiology research to evaluate bacterial associations in subgingival (40) and supragingival (15) plaque. However, direct comparisons between the endodontic complexes observed in the present study and the periodontal complexes cannot be performed because of the differences between the habitats and the inclusion of several new and as-yet-uncultivated taxa in the checkerboard panel used in this study. The factors dictating ecological interrelationships and the resulting pathogenic implications of the various combinations observed in the present study remain to be determined. Also, future research should address association of specific bacterial pairs or complexes with symptoms and other clinical conditions in endodontics.
The knowledge of the bacterial diversity involved with apical periodontitis has been substantially refined and redefined after about one decade of application of molecular biology methods to endodontic microbiology research. In addition to strengthening the association of several cultivable named species with chronic apical periodontitis, the present findings using a large-scale analysis allowed inclusion of some newly named species and as-yet-uncultivated phylotypes in the set of candidate pathogens associated with this disease.
Acknowledgements

This study was supported by grants 470417/2004-8 (INR), 300693/2005-2 (INR), and 304552/2006-2 (JFSJ), from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), a Brazilian Governmental Institution. The authors are grateful to Dr. Bruce Paster, for providing a detailed protocol of the checkerboard method and for his valuable advice, and to Mr. Marlei Gomes da Silva, for technical assistance.
References


FIGURE LEGENDs

**Figure 1.** Stacked bar chart of frequency of detection and levels of bacterial species/phylotypes in root canal samples of teeth with chronic apical periodontitis from 43 individuals. Total length of each bar stack indicates percentage of positive samples; *i.e.*, prevalence of bacterial species/phylotypes. Different colors within each bar indicate percentage of samples containing different levels of the species.

**Figure 2.** Stacked bar chart of frequency of detection of bacterial species/phylotypes in root canal samples at levels above $10^5$. Different colors within each bar indicate percentage of samples containing different levels of the species.

**Figure 3.** Dendrogram of a cluster analysis of 51 bacterial taxa present in primary root canal infections of teeth with chronic apical periodontitis using Euclides distance calculation and Ward’s method. The bacterial cluster on top (dark gray) is composed almost exclusively by the most prevalent taxa.
Figure 2
Figure 3
Table 1
Bacterial associations in infected root canal of teeth with chronic apical periodontitis as determined by relative risk calculation (95% confidence interval)

<table>
<thead>
<tr>
<th>Taa</th>
<th>E. corrodens</th>
<th>P. endodontalis</th>
<th>P. anaerobius</th>
<th>Bacteroidetes X083</th>
<th>T. forsythia</th>
<th>D. invisus</th>
<th>F. nucleatum</th>
<th>P. micra</th>
<th>P. baroniae</th>
<th>E. sulci</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. uli</td>
<td>1.0</td>
<td>1.0</td>
<td>1.2</td>
<td>1.5</td>
<td>2.2</td>
<td>1.3</td>
<td>1.8</td>
<td>1.6</td>
<td>1.5</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>(0.7-1.8)</td>
<td>(0.6-2.1)</td>
<td>(0.7-2.7)</td>
<td>(0.8-3.8)</td>
<td>(0.9-6.3)</td>
<td>(0.6-3.3)</td>
<td>(0.8-5.4)</td>
<td>(0.7-4.8)</td>
<td>(0.6-4.5)</td>
<td>(1.2-30.0)</td>
</tr>
<tr>
<td>E. corrodens</td>
<td>-</td>
<td>1.0</td>
<td>2.8</td>
<td>1.0</td>
<td>1.6</td>
<td>1.0</td>
<td>1.0</td>
<td>1.4</td>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.6-1.8)</td>
<td>(1.3-6.8)</td>
<td>(0.6-2.0)</td>
<td>(0.8-3.3)</td>
<td>(0.5-2.1)</td>
<td>(0.5-2.1)</td>
<td>(0.7-3.4)</td>
<td>(0.6-3.2)</td>
<td>(0.8-4.7)</td>
</tr>
<tr>
<td>P. endodontalis</td>
<td>-</td>
<td>0.7</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
<td>1.1</td>
<td>1.1</td>
<td>1.9</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.4-1.3)</td>
<td>(0.8-2.6)</td>
<td>(0.7-2.8)</td>
<td>(0.6-2.2)</td>
<td></td>
<td></td>
<td>(0.9-4.5)</td>
<td>(1.0-6.3)</td>
<td>(0.5-2.3)</td>
</tr>
<tr>
<td>P. anaerobius</td>
<td>-</td>
<td>3.0</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>2.1</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.5-6.1)</td>
<td>(0.8-2.8)</td>
<td>(0.5-1.9)</td>
<td>(0.5-1.9)</td>
<td></td>
<td></td>
<td>(0.9-4.9)</td>
<td>(0.7-3.3)</td>
<td>(0.7-3.3)</td>
</tr>
<tr>
<td>Bacteroidetes X083</td>
<td>-</td>
<td>1.7</td>
<td>1.6</td>
<td>0.7</td>
<td>1.7</td>
<td>2.1</td>
<td>1.0</td>
<td>4.4</td>
<td>4.1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.9-3.1)</td>
<td>(0.8-3.3)</td>
<td>(0.4-1.4)</td>
<td>(1.7-13.0)</td>
<td>(1.6-12.3)</td>
<td></td>
<td>(0.4-2.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. forsythia</td>
<td>-</td>
<td>1.3</td>
<td>1.6</td>
<td>1.3</td>
<td>1.3</td>
<td>2.1</td>
<td>1.0</td>
<td>3.1</td>
<td>2.1</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.7-2.6)</td>
<td>(0.8-3.3)</td>
<td>(1.3-7.9)</td>
<td>(0.9-5.0)</td>
<td>(1.6-12.3)</td>
<td></td>
<td>(0.4-2.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. invisus</td>
<td>-</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>1.1</td>
<td>1.3</td>
<td>1.0</td>
<td>0.5</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.5-1.8)</td>
<td>(0.5-1.8)</td>
<td>(0.5-2.3)</td>
<td>(0.6-2.7)</td>
<td>(0.4-2.1)</td>
<td></td>
<td>(0.2-1.2)</td>
<td>(0.2-1.3)</td>
<td>(1.2-6.4)</td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>2.5</td>
<td>0.6</td>
<td>2.8</td>
<td>0.5</td>
<td>0.5</td>
<td>1.3</td>
<td>1.3</td>
<td>2.5</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1.2-5.4)</td>
<td>(1.2-5.4)</td>
<td>(0.7-3.2)</td>
<td>(1.2-5.4)</td>
<td>(0.7-3.2)</td>
<td>(0.6-2.7)</td>
<td></td>
<td>(1.2-6.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. micra</td>
<td>-</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td></td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.2-5.4)</td>
<td>(0.7-3.2)</td>
<td>(0.6-2.7)</td>
<td>(0.6-2.7)</td>
<td></td>
<td></td>
<td>(1.2-6.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. baroniae</td>
<td>-</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td></td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>E. sulci</td>
<td>-</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td></td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>