Uncultivated Phylotypes and Newly Named Species Associated with Primary and Persistent Endodontic Infections

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Endodontic infections have been traditionally studied by culture methods, but recent reports showing that over 50% of the oral microbiota is still uncultivable (B. J. Paster et al., J. Bacteriol. 183:3770–3783, 2001) raise the possibility that many endodontic pathogens remain unknown. This study intended to investigate the prevalence of several uncultivated oral phylotypes, as well as newly named species in primary or persistent endodontic infections associated with chronic periodontal and periapical diseases. Samples were taken from the root canals of 21 untreated teeth and 22 root-filled teeth, all of them with radiographic evidence of periapical bone destruction. Genomic DNA was isolated directly from each sample, and 16S rRNA gene-based nested or heminested PCR assays were used to determine the presence of 13 species or phylotypes of bacteria. Species-specific primers had already been validated in the literature or were developed by aligning closely related 16S rRNA gene sequences. Species specificity for each primer pair was confirmed by running PCRs against a panel of several oral bacteria and by sequencing DNA from representative positive samples. All species or phylotypes were detected in at least one case of primary infections. The most prevalent species or phylotypes found in primary infections were Dialister invisus (81%), Synergistes oral clone BA121 (33%), and Olsenella uli (33%). Of the target bacteria, only these three species were detected in persistent infections. Detection of uncultivated phylotypes and newly named species in infected root canals suggests that there are previously unrecognized bacteria that may play a role in the pathogenesis of periradicular diseases.

Periradicular diseases are arguably among the most common inflammatory diseases that affect humans (10). Overwhelming evidence indicates that microorganisms infecting the root canal of the teeth are the major causative agents of these diseases (27). Data from culture and molecular studies have demonstrated that the microbiota associated with primary endodontic infections is conspicuously dominated by anaerobic bacteria and that an infected root canal can harbor from 10 to 30 bacterial species (22, 33, 34, 37). On the other hand, the microbiota of persistent endodontic infections associated with treatment failure has been shown to be composed of fewer species, with dominance of facultative bacteria (32, 35). Even though over 300 bacterial species have already been isolated from or detected in infected root canals, no single species has been consistently found to be the major endodontic pathogen.

Application of molecular genetic methods to the analysis of the bacterial diversity in the oral cavity has revealed a still broader spectrum of extant bacteria than previously reported by cultivation approaches (23). Overall, over 700 different species belonging to 11 divisions (or phyla) of the domain Bacteria have been detected in the oral cavity of humans (17, 23, 24). About 50% of these bacteria are known only by 16S rRNA gene sequences (phylotypes) (23). This raises the interesting possibility that uncultivated and as-yet-uncharacterized species that have remained undetected in studies by traditional identification methods may make up a large fraction of the living oral microbiota and may participate in the etiology of oral diseases, including periodontal diseases.

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and difficulties in identifying these species by phenotype-based methods can be one of the reasons for this.

It has been revealed that the main putative endodontic pathogens are also the main periodontal pathogens, with few (if any) exceptions (27, 37). Several new species or phylotypes have been suggested to be involved with the pathogenesis of periodontal diseases (19, 23), but it is still uncertain whether most of these phylotypes are present in infected root canals associated with periradicular diseases. Therefore, the present investigation intended to use a devised nested or heminested PCR assay to survey samples from primary or persistent endodontic infections for the presence of some newly named bacterial species and uncultivated phylotypes that have been recently detected in association with periodontal diseases. Most of the target bacteria had never been previously found in endodontic infections. For the species already detected in endodontic infections, prevalence had not been previously inferred because of the small number of samples examined (11, 22).

**MATERIALS AND METHODS**

**Subjects.** Root canal samples collected for previous investigations (25, 29, 32) and stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) at −20°C were available for reanalysis in this study. Samples were taken from patients who had been seeking root canal treatment or retreatment at the Department of Endodontics, Estácio de Sá University, Rio de Janeiro RJ, Brazil. Only teeth from adult patients (ages ranging from 18 to 80 years), all of them having radiographic evidences of periradicular disease, were included in this study. Overall, 43 root canal samples were obtained and grouped as follows, according to the clinical diagnoses: (i) 21 cases of primary endodontic infections (untreated teeth) associated with asymptomatic chronic periroot lesions and (ii) 22 cases of persistent endodontic infections (root-filled teeth) associated with asymptomatic chronic periroot lesions, which had been selected for retreatment.

All the teeth with primary endodontic infections showed various lesions and necrotic pulps. All the teeth with persistent infections had endodontic therapy completed ≥2 years earlier. Root-filled teeth were coronally restored and no direct exposure of the filling material to the oral cavity was evident. Terminals of the root canal fillings ranged from 0 to 4 mm short of the radiographic apex. All selected teeth showed no significant gingival recession and an absence of periodontal pockets of >4 mm deep.

**Sampling procedures and DNA extraction.** Root canal samples were taken from untreated or root-filled teeth under strict aseptic conditions as previously described (25, 29, 32). Endodontic files with the handle cut off and paper points used for sampling the canals were transferred to erytubes containing 1 ml of 5% dimethyl sulfoxide in trypticase-soy broth (Difco, Detroit, MI) and immediately used for sampling the canals were transferred to cryotubes containing 1 ml of 5% dimethyl sulfoxide in trypticase-soy broth (Difco, Detroit, MI) and immediately used for sampling the canals were transferred to cryotubes containing 1 ml of 5% dimethyl sulfoxide in trypticase-soy broth (Difco, Detroit, MI) and immediately used for sampling the canals were transferred to cryotubes containing 1 ml of 5% dimethyl sulfoxide in trypticase-soy broth (Difco, Detroit, MI) and immediately used for sampling. Primers sequences were as previously described or heminested PCR method devised to detect the target species/phylotypes in endodontic samples. In the first PCR, a practically full-length 16S rRNA gene fragment was amplified using a pair of universal 16S rRNA gene primers, which consisted of the forward universal primer 279 (9) and the reverse universal primer 1492r (38). Aliquots of 5 μl (each) of the supernatant from clinical samples were used as targets in the first PCR. PCR amplification was performed with 25 μl of the reaction mixture containing 0.2 μM concentration of reverse universal primers, 2.5 μl of 10X PCR buffer (Biotools, Madrid, Spain), 2 mM MgCl2, 1.25 U of Tth DNA polymerase (Biotools), and 0.2 mM each deoxyribonucleoside triphosphate (Biotools). PCRs were performed in 25-well microtiter plates. Negative controls consisting of sterile ultra-pure water instead of sample were included with each batch of samples analyzed.

Preparations were amplified in a DNA thermocycler (Masterecycler personal; Eppendorf, Hamburg, Germany). The PCR temperature profile for the universal reaction included an initial denaturation step at 97°C for 1 min; followed by 26 cycles, each of a denaturation step at 97°C for 45 s, primer annealing step at 55°C for 45 s, and an extension step at 72°C for 1 min; and a final step of 72°C for 4 min. PCR cycling conditions for the second round of amplification specific for D. invisus comprised an initial denaturation step of 95°C for 10 s; followed by 26 cycles, each of denaturation at 95°C for 4 s, primer annealing step at 55°C for 4.5 s, and an extension step at 72°C for 1 min; and a final step of 72°C for 4 min. For T7M oral clone B025, cycling conditions consisted of an initial denaturation step of 95°C for 10 min; followed by 28 cycles, each of denaturation at 95°C for 30 s, primer annealing at 65°C for 1 min, and an extension step at 72°C for 1 min; and a final extension step of 72°C for 3 min. For O. profusa and Synergistes oral clone B121, the temperature profile included an initial denaturation step at 95°C for 2 min, and a touchdown PCR was performed as follows: a denaturing temperature of each cycle was carried out at 95°C for 30 s. The annealing temperature was initially set at 68°C and was then lowered 0.5°C every other cycle until it reached 63°C. Seventeen additional cycles were carried out at 63°C. Primer annealing was performed using this scheme for 30 s, and primer extension was carried out at 72°C for 1 min. The final extension step was at 72°C for 5 min. The temperature profile for the other species/phylotypes encompassed an initial denaturation step at 95°C for 2 min, and touchdown PCR as follows: a denaturing temperature of each cycle at 95°C for 30 s and an annealing temperature initially set at 64°C and then lowered 0.5°C every other cycle until it reached 61°C. Twenty-one additional cycles were carried out at 61°C. Primer annealing was performed using this scheme for 30 s, and primer extension was carried out at 72°C for 1 min. The final extension step was at 72°C for 5 min. PCR amplifications were separated by electrophoresis in a 1.5% agarose gel, which was stained with 0.5 μg/ml ethidium bromide and viewed under UV transillumination. A 100 bp DNA ladder digest (Biotools) served as the molecular size standard.

**Sequencing.** To confirm the specificity of the primers, randomly selected representative PCR products for each target species or phylotypes were purified with a PCR purification system (Wizard PCR Prep; Promega, Madison, WI) and then sequenced directly on the ABI 377 automated DNA sequencer using dye terminator chemistry (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom). Sequence data and chromatograms were inspected and edited by using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (14). Sequences were then analyzed using the BLAST algorithm (1).
RESULTS

After the first round of amplification using universal bacterial primers, all samples yielded an amplicon of approximately 1,500 bp. This indicated that bacteria were present in all cases examined, demonstrating the suitability of the DNA for PCR analysis and indicating the absence of inhibitors in the reaction mixture. Negative controls using sterile ultrapure water instead of sample yielded no amplicon.

The sequences of primers specific for each species or phylotype are shown in Table 1. The specificity of each primer was tested against a panel of representative oral bacteria. The use of each primer set resulted in no PCR product of the expected size from nontargeted species. Primers specific for *O. uli*, *O. profusa*, *A. parvulum*, and *P. pallens* resulted in one band of the expected size when respective reference DNA was used. To confirm the specificity of each species- or phylotype-specific PCR primer, representative PCR products of the expected size obtained from clinical samples were sequenced and compared to the original sequences in GenBank database. The primer sequence for *D. invisus* was originally designed to target *Dialister* oral clone GBA27 (19) but it also annealed without any mismatches to the sequences of *D. invisus* and *Dialister* oral clones FY011 and BS095. However, sequences of the eight randomly selected representative PCR products from clinical samples all showed higher levels of similarity to *D. invisus* (99.4 to 100%). Nonetheless, even though our discussion hereafter relies on this species, the possibility exists that some of the nonsequenced PCR products could have been from other *Dialister* phylotypes. For *O. uli*, the levels of similarity among the sequences were 98.7 to 100%. Two amplicons generated by the *O. uli* primer yielded sequences of a new *Olsenella* phylotype, EI15. For *O. profusa*, the levels of similarity among the sequences were 99.1 to 100%. For *P. pallens*, the levels of similarity among the sequences were 99.8 to 100%. For *A. parvulum*, the level of similarity between the sequences was 99.3%. For *Synergistes* clones BH017/D084 (formerly *Deferribacteres* BH017/D084), the levels of similarity among the sequences were 98.6 to 100%. All sequences showed higher similarities to clone BH017. For *Synergistes* BA121, the levels of similarity among the sequences were 99.3 to 100%.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer pairs (5’–3’)</th>
<th>Base position (amplicon size in bp)</th>
<th>Reference or source</th>
</tr>
</thead>
</table>
| *Actinobaculum* oral clone EL030 | AGA GTT TGA TCC TGG CTC AG<sup>a</sup>  
CGC AGA ATC CGT GGA AAG A  
8–27  
825–843 (844) | 19                                                  |                                   |
| *Atopobium parvulum*            | AGA GTT TGA TCC TGG CTC AG  
TGC GGC ACG GAA GAA ATA CTC CCC  
8–27  
784–807 (827) | 19                                                  |                                   |
| *Synergistes* oral clones BH017/D084 | AGA GTT TGA TCC TGG CTC AG  
CGT CAA TGT TTC CAT CTC CTA C  
8–27  
968–989 (988) | 19                                                  |                                   |
| *Synergistes* oral clone W090   | AGA GTT TGA TCC TGG CTC AG  
GAA AGT ACG TCG TCG CCC TTT CAG  
8–27  
954–977 (997) | 19                                                  |                                   |
| *Desulfobulbus* oral clone R004 | AGA GTT TGA TCC TGG CTC AG  
GAA GCC ACC ACC CAC TTT CAT GGG  
8–27  
993–1016 (1,035) | 19                                                  |                                   |
| *Dialister invisus*             | CAG AAA TGC GGA GTT CTT CTT CG  
CCC GGG AAC GTA TTC ACC G<sup>b</sup>  
1007–1029  
1369–1387 (381) | 19                                                  |                                   |
| *Synergistes* oral clone BA121  | AGA GTT TGA TCC TGG CTC AG  
TGC GAA AGG GTC GAT CCG C<sup>a</sup>  
8–27  
982–1000 (999) | This study                                          |                                   |
| *Synergistes* oral clone E3_33  | AGA GTT TGA TCC TGG CTC AG  
ACA CTT GTA CTT CTC CAT ACA C  
8–27  
959–980 (1,000) | This study                                          |                                   |
| *Olsenella profusa*             | AGA GTT TGA TCC TGG CTC AG  
TGC GGC ACC ACC CAC TTT CAT GGG  
8–27  
788–809 (829) | This study                                          |                                   |
| *Olsenella uli*                 | AGA GTT TGA TCC TGG CTC AG  
TGC GGC ACC ACC CAC TTT CAT GGG  
8–27  
788–809 (829) | This study                                          |                                   |
| *Prevotella pallens*            | TGT GCG TTA TTG CAT GTA TCG TAT  
CCC CGA AGG GCA TAT TTA TTA CTC C  
442–465  
982–1003 (562) | This study                                          |                                   |
| *TM7* oral clone I025            | CCC TGC AGT GAG GGA TAA GA  
GTT TTC ATC GCT CGC TAA CTT G  
135–154  
590–611 (477) | 4                                                    |                                   |
| Universal 16S rRNA gene         | AGA GTT TGA TCC TGG CTC AG  
ACG GCT ACC TTG TTA CGA CTT  
8–27  
1492–1512 (1,505) | 38                                                   |                                   |

<sup>a</sup> Universal 16S rRNA gene forward primer (base position relative to *E. coli* 16S rRNA gene).

<sup>b</sup> Universal 16S rRNA gene reverse primer (base position relative to *E. coli* 16S rRNA gene).
E3_33, the level of similarity between the sequences was 99.7%. For Synergistes clone W090 (formerly Deferribacteres W090), the levels of similarity among the sequences were 98.4 to 99.2%. For Desulfobulbus R004, the levels of similarity among the sequences of the expected size were 98 to 100%. A smaller PCR product (about 175-bp long) was also found in many specimens amplified with the Desulfobulbus R004 primer and showed 100% similarity to the D. invisus sequence. For Actinobaculum EL030, the levels of similarity among the sequences were 98 to 99.1%. For TM7 oral clone I025, the levels of similarity among the sequences were 98.7 to 100%.

All target species/phylotypes were found in at least one sample from primary endodontic infections (untreated teeth). Only the three most prevalent species in primary infections were detected in persistent endodontic infections (root-filled teeth), but at lower frequencies. Prevalence data for the target species/phylotypes in primary or persistent endodontic infections are represented in Table 2.

### DISCUSSION

In this study, we investigated the occurrence of some named species and uncultivated phylotypes in endodontic infections. Named species comprised newly proposed species or species that have undergone recent taxonomic reclassification (7, 8, 18). The target species are usually difficult to cultivate in artificial media and/or difficult to identify by phenotypic features (7, 8, 18), and this may have resulted in underestimation of their presence in endodontic infections. For instance, Dialister species have been consistently detected in endodontic infections only after the advent of molecular genetic approaches (22, 30). Dialister pneumosintes has been found at high prevalence in root canals from teeth associated with different forms of periradicular diseases (30). The newly named D. invisus is an anaerobic gram-negative coccobacillus closely related to Dialister pneumosintes, with 93% sequence similarity between the two taxa (8). Munson et al. (22) first isolated this species from infected root canals in a study using cultivation and 16S rRNA gene sequencing. This was the only species detected in all five cases analyzed in their study (22). In the present investigation, D. invisus was detected in 81% of the primarily infected root canals. This considerably high prevalence lends additional credence to the assumption that this species can be an important endodontic pathogen. Although in lower prevalence values, D. invisus was also detected in cases of failed endodontic treatment (14% of the teeth). This suggests that this species can also be involved with persistent endodontic infections.

Although Olsenella and Atopobium species have been recently isolated/detected in endodontic infections (5, 11, 22), no study had consistently investigated their prevalence in these infections. Cultural and molecular analyses of samples taken from five infected root canals revealed the occurrence of O. uli in three cases, A. parvulum in two cases, and O. profusa in one case (22). O. uli has been found to predominate over other gram-positive rods in root canal samples taken after chemomechanical preparation and intracanal medication, suggesting that this species can resist intracanal disinfection measures and thereby may be involved in persistent infections (5). Herein, O. uli was present in one-third of the samples from primary infections and in one root-filled tooth. O. profusa and A. parvulum were detected in 9.5% and 5% of the untreated teeth, respectively, but in no retreatment case. These findings revealed that Olsenella species, particularly O. uli, are common members of the microbiota associated with primary endodontic infections.

Prevotella species have been frequently found in different types of endodontic infections (2, 13, 34). The most commonly isolated/detected species include Prevotella intermedia, Prevotella nigrescens, and P. tannerae (2, 39), but others, including nonpigmented species, have also been found (13). In this study, we devised a pair of PCR primers to identify P. pallens, a black-pigmented Prevotella species, directly in clinical samples and detected this species in 9.5% of the primarily infected canals. This is the first report of the occurrence of P. pallens in endodontic infections.

In addition to newly named species, this study also focused the occurrence of as-yet-uncultivated bacteria in endodontic infections. All phylotypes targeted in this study were detected in at least one sample from primary endodontic infections; one phylotype was found in one case of persistent infection. Clones from the Synergistes division were found to be common members of the endodontic microbiota in untreated teeth. One-third of these samples harbored Synergistes oral clone BA121, which was first detected in one subject with refractory periodontitis (23). This phylotype was also detected in one root-filled tooth. Although firstly detected in endodontic samples (22), Synergistes clone E3_33 was detected in only one specimen. Clone BH017 (or D084) from the Synergistes group was also detected at a relatively high prevalence value, i.e., 29%, of the primary infections. Although clones BH017 and D084 were indistinguishable by our assay, all sequences from representative amplicons yielded higher similarities to the former. These two clones have also been among the most strongly associated with marginal periodontitis (19). Synergistes clone W090 was detected in 24% of the primarily infected root canals. This clone has been reported to occur in 73% of samples taken from subjects with periodontitis (19). Detection of these uncultivated Synergistes phylotypes at high prevalence in infected root
canals suggests that they can be previously unrecognized bacteria that play a role in the pathogenesis of periradicular lesions.

Other uncultivated phyotypes targeted in this study were also detected for the first time in primary endodontic infections. Desulfobulbus oral clone R004 and Actinobaculum oral clone EL030 were both detected in 14% of the samples. These phyotypes have been previously detected at high frequencies in subjects with marginal periodontitis (82% and 44%, respectively) (19). TM7 oral clone I025 was detected in 9.5% of the subjects with marginal periodontitis (82% and 44%, respectively) (19). TM7 oral clone I025 was detected in 9.5% of the samples. These findings expanding the list of phyla represented in endodontic infections to include TM7 bacteria. Candidate division TM7 is one of the several divisions described for the domain Bacteria, and because it has no cultivated representative, members can be identified only by molecular methods. Recent studies have demonstrated a high prevalence of TM7 bacteria, particularly clone I025, in association with periodontal diseases (4, 19). Based on our findings, it appears that clone I025 can be found in infected root canals, but it is not so prevalent in endodontic infections as it is in periodontal infections. Such a difference may be a result of different environmental conditions between the root canal and the gingival crevice, the latter being more favorable to the establishment of TM7 bacteria. However, differences may have been due to methodological reasons or geographical influence (3, 4).

As expected, samples from root-filled teeth yielded few positive results. Because of the restricted room for bacterial establishment and the low availability of nutrients within treated root canals, the number of bacterial species composing the microbiota associated with persistent endodontic infections is significantly lower than that occurring in primary endodontic infections (32, 35). Thus, only the bacterial species that are able to adapt to the bleak environmental conditions within filled root canals will succeed in causing persistent endodontic infections. E. faecalis has been shown to be the most frequently detected species in root-filled teeth, and 17 of the 22 cases examined herein had been positive for this facultative species in increased prevalence, molecular methods have also demonstrated to be more complex than previously anticipated. Since endodontic infections develop in a previously sterile environment, many bacteria, particularly clone I025, in association with periodontal diseases merits further elucidation.

The microbial etiology of periradicular diseases has been demonstrated to be more complex than previously anticipated by cultivation studies. In addition to detecting some cultivable species in increased prevalence, molecular methods have also expanded the list of putative endodontic pathogens by inclusion of some fastidious bacterial species or even uncultivated bacteria that had never been previously found in endodontic infections by cultivation procedures (27). As a consequence, a larger and ever-expanding number of species have been suspected to be involved with causation of periradicular diseases. Since endodontic infections develop in a previously sterile place which as such does not contain a normal microbiota, every bacterial species present in the mixed consortium has the potential to play a role in the infectious process. Although the PCR assay used in the present study does not allow quantitation, identification of the taxon present in endodontic samples and determination of their prevalence are still of extreme value to help unravel the bacterial diversity in infected root canals. This information will make it possible to use other techniques such as real-time PCR, DNA microarrays, or reverse-capture DNA-DNA hybridization assays to investigate the numbers of these taxa in root canal samples.

In conclusion, the present investigation has expanded the list of endodontic bacteria by including several newly named oral species, as well as novel phyotypes recently identified by 16S rRNA gene sequence analysis. These findings point to an urgent need for a steady and sustained effort to obtain these bacteria in culture so that their phenotypic traits, including virulence and susceptibility to antimicrobials, can be properly assessed.

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