Characterization of *Dialister* Species in Infected Root Canals

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Abstract

Members of the *Dialister* genus are asaccharolytic obligately anaerobic gram-negative coccobacilli that are culture-difficult or remain uncultivated. Their participation in endodontic infections has been only consistently demonstrated after advent of molecular biology approaches. This study was undertaken to characterize *Dialister* species in samples from primary endodontic infections using a devised 16S rRNA gene-based group-specific heminested PCR assay followed by sequencing of PCR products. Genomic DNA was isolated directly from clinical samples and used as template for PCR. Amplicons from positive specimens were sequenced and phylogenetically analyzed to determine species identity. Ten of 21 clinical samples yielded sequences with the highest percent similarities to oral *Dialister* species/phylotypes. Seven sequences were from *Dialister invisus*, and the other three sequences belonged to *Dialister pneumosintes*, *Dialister* oral clone BS095 and *Dialister* sp. clone IS013B24. Findings demonstrated that different *Dialister* species can take part in the microbiota associated with apical periodontitis lesions. *(J Endod 2006;32:1057–1061)*

Key Words

Apical periodontitis, *dialister*, endodontic infection, 16S rRNA gene sequencing

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0099-2399/$0 - see front matter

doi:10.1016/j.joen.2006.04.010

Dialister species are nonmotile, nonsporing, and asaccharolytic obligately anaerobic gram-negative coccobacilli. Members of this bacterial genus are either culture-difficult or remain to be cultivated and characterized, which may have resulted in underestimation of their role in human infections. Introduction of molecular biology methods for bacterial detection and identification has demonstrated that *Dialister* species are commonly found in the oral cavity, including in association with diseases such as marginal periodontitis (1), caries (2), halitosis (3), and apical periodontitis (4–6). Species of *Dialister* have also been detected in nonoral human sites, including bacterial vaginosis samples (7), Fallopian-tube specimens from women with salpingitis (8), urinary tract infections (9), intestinal tract (10), and brain abscess (11).

Isolation of *Dialister* species in endodontic infections has been only occasionally reported (12), conceivably because of difficulties in culturing and identifying these bacteria. Difficulties in isolation of *Dialister* species may be related to the fact that some species present strict anaerobic requirement, slow growth, are unreactive in commonly used biochemical tests, and may be even difficult to be distinguished from other species in primary culture (13, 14). Colonies grown on agar plates may require magnification to be visualized and growth in broth media is only slightly turbid at best (14, 15). In addition, many species may have specific growth requirements, which can account for their unculturability.

Two cultivable oral species of *Dialister* have been recognized and validly named – *Dialister pneumosintes* (16) and *Dialister invisus* (15). *D. pneumosintes* was firstly isolated by Olitsky and Gates in 1921, and originally named as *Bacterium pneumosintes* (17). It was then transferred to the genus *Dialister* (18), and further placed in the genus *Bacteroides* (19). In 1994, the genus *Dialister* was revived to accommodate this species (16). The application of culture-independent molecular biology methods to the analysis of the oral microbiota has led to increased precision and greater reliability in the detection and identification of oral bacteria (20). Such analyses have disclosed several taxa related to *D. pneumosintes* that are associated with different oral infections. One of these, designated *Dialister* E1, was found in samples obtained from cases of asymptomatic primary endodontic infections (4) and further proposed as a new species; *D. invisus* (15). Phylogenetic characterization of 10 asaccharolytic anaerobic Gram-negative coccobacilli isolated from endodontic infections and periodontal pockets revealed that seven of these isolates were identified as *D. invisus* (21).

As with other oral infections, *Dialister* species have been consistently detected and identified in endodontic infections only after the advent of molecular biology techniques. Both *D. pneumosintes* and *D. invisus* have been frequently present in the microbiota associated with asymptomatic and symptomatic primary endodontic infections (4–6, 22–24). Moreover, culture-independent molecular biology studies have revealed the occurrence of several as-yet-uncultivated phylotypes belonging to the *Dialister* genus in the oral cavity, some of them, including *Dialister* oral clones GBA27 and BS095, being found in significant association with periodontal diseases and halitosis, respectively (3, 25). Studies of the diversity of the endodontic microbiota using 16S rRNA gene clone library and terminal-restriction fragment length polymorphism analyses have detected *Dialister* species other than the named species *D. pneumosintes* and *D. invisus*, including phylotypes 55A-29, 9N-7, and BS016/MCE7134 (4, 26).

Because of the emergence of *Dialister* species as candidate endodontic pathogens, this study intended to investigate the presence and identity of *Dialister* species in

primary root canal infections associated with apical periodontitis lesions using a devised group-specific heminested PCR assay followed by sequencing of PCR products.

Materials and Methods

Subjects and Sample Collection

Root canal samples were taken from 21 patients who had been referred for root canal treatment to the department of Endodontics at the Estácio de Sá University, Rio de Janeiro, RJ, Brazil. Only single-root teeth from adult patients older than 22 yr, all of them having carious lesions, necrotic pulps and radiographic evidence of periradicular bone destruction were included in this study. All cases were asymptomatic and showed an absence of periodontal pockets deeper than 4 mm. Samples were taken from the root canals. Sampling procedures and DNA extraction protocol were as described earlier (6). Approval for the study protocol was obtained from the Ethics Committee of the Estácio de Sá University.

Primer Design

A reverse primer specific for the genus *Dialister* was designed from regions of identity within the 16S rRNA gene sequences from the following oral *Dialister* species/phylotypes (GenBank accession numbers in parentheses): *D. invisus* (AY162469), *D. pneumosintes* (X82500), *Dialister* sp. oral strain GBA27 (AF287788), *Dialister* MCE7134 E2 (AF481210), *Dialister* sp. oral clone BS016 (AF287786), *Dialister* sp. oral clone BS095 (AF287787), and *Dialister* sp. oral clone FY011 (AY134907). Sequences were retrieved from GenBank and aligned with CLUSTAL X (27) together with sequences from representatives of each bacterial phylum found in endodontic infections, as follows: *Enterococcus faecalis* (Y18293), *Filifactor alocis* (AJ006962), *Escherichia coli* (J01695), *Fusobacterium nucleatum* subsp. *polymorphum* (AJ810282), *Campylobacter gracilis* (L04320), *Porphyromonas endodontalis* (L16491), *Propionibacterium propionicum* (AJ315953), *Synergistes* sp. oral clone BA121 (AY005444), *Tannerella forsythia* (L16495), *Treponema denticola* (AF139204), and *Veillonella atypica* (X84007). Primers were designed taking into consideration the nucleotide mismatches with other bacteria, the complementarity with the target at the 3′ end, a G+C content of >50%, and a size of ca. 18 to 20 nucleotides. The specificity of the primer sequences was determined by BLAST (28) by comparing each primer sequence with all available sequences in the GenBank database. Based on BLAST search, a reverse primer sequence Dial649r (5′-CTC TCC GAT ACT CCA GCT-3′, corresponding to base position 666-649 of the *E. coli* 16S rRNA gene sequence, accession no. J01695) was selected and used in this study (Fig. 1). This reverse primer was used in individual PCR reactions along with forward universal bacterial primer 27f (5′-AGA GTT TGA TYM TGG CTC AG-3′, *E. coli* base position 8-27, accession no. J01695), producing an amplicon of ca. 650 bp. The specificity of the primer was confirmed by sequencing of the PCR products obtained from positive clinical samples.

PCR Identification

Whole-genomic DNA extracts from clinical samples were used as templates in a 16S rRNA gene-based heminested PCR method devised to detect *Dialister* species in endodontic samples. In the first PCR reaction, a practically full-length 16S rRNA gene fragment was amplified using universal 16S rRNA gene primers 27f and 1492r (29). Aliquots of 5 μl of the DNA extracts from clinical samples were used as target in the first PCR reaction. PCR reaction mixture and temperature profile for the first amplification were as presented previously (5).

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                                  ....|....| ....|....| ....|....|
                                  -------AGC TGGAGTATCG GAGAG-----
Dialister pneumosintes            ACTGGGAAGC TGGAGTATCG GAGAGGAAAG
Dialister invisus                 ACTGGGAAGC TGGAGTATCG GAGAGGAAAG
Dialister BS095                   ACTGGGAAGC TGGAGTATCG GAGAGGAAAG
Dialister GBA27                   ACTGGGAAGC TGGAGTATCG GAGAGGAAAG
Dialister MCE7_134 E2             ACTGGGAAGC TGGAGTATCG GAGAGGAAAG
Dialister BS016                   ACTGAGAAGC TGGAGTATCG GAGAGGAAAG
Dialister FY011                   ACTGGGGAGC TGGAGTATCG GAGAGGAAAG
                                     *** ********** *****
Veillonella atypica               ACTGCTNATC TAGAGTATCG GAGAGGAAAG
Enterococcus faecalis             ACTGGGAGAC TTGAGTGCAG AAGAGGAGAG
Filifactor alocis                 ACTGTTTAAC TTGAGTGCAG GAGAGGAAAG
Escherichia coli                  ACTGGCAAGC TTGAGTCTCG TAGAGGGGGG
Campylobacter gracilis            ACTGATAATC TAGAGTAAGG GAGAGGCAGA
Fusobacterium nucleatum           ACTGTGTAAC TAGAGTACTG GAGAGGTAAG
Porphyromonas endodontalis        ACTNATATGC TAGAGGAGAC CGAAGGTATG
Tannerela forsythia               ACTGGTAGTC TTGAGTATAG RTGAAGTAGG
Synergistes BA121                 ACTGGAGAGC TTGAGAAGCA GAGAGGCAAA
Propionibacterium propionicum     ACGGGCTGAC TTGAGGGAGG TAGGGGAGAA
Treponema denticola               ACTGCTTGAC TTGAATCACG GAGGGGAAAC
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Figure 1. Analogous PCR priming site on 16S rRNA gene sequences of oral *Dialister* species and phylotypes as well as of representatives of each bacterial phylum found in endodontic infections. Asterisks indicate matches among sequences.

Afterwards, 1 μl of the universal reaction was used as template for the second reaction, which was performed in a 50 μl of reaction mixture containing 1 μM concentration of each primer (27f and Dial649r), 5 μl of 10X PCR buffer (Biotools, Madrid, Spain), 2 mM $MgCl_2$, 1.25 U of *Tth* DNA polymerase (Biotools) and 0.2 mM of each deoxyribonucleoside triphosphate (Biotools). Positive and negative controls were included with each batch of samples analyzed. DNA extracted from *D. pneumosintes* ATCC 33048 was used as positive control. Negative control consisted of sterile ultrapure water instead of sample.

Preparations were amplified in a DNA thermocycler (Mastercycler Personal, Eppendorff, Hamburg, Germany). PCR cycling conditions for the second round of amplification specific for *Dialister* species included an initial denaturation step at 95°C for 2 minutes, and a touchdown PCR procedure as follows: denaturing temperature of each cycle at 95°C for 30 s, annealing temperature initially set at 63°C and then lowered 0.5°C every other cycle until it reached 60°C. Twenty-one additional cycles were carried out at 60°C. Primer annealing was performed using this scheme for 30 s, and primer extension was carried out at 72°C for 1 minute. The final extension step was at 72°C for 5 minutes. Amplification products were separated by electrophoresis in a 1.5% agarose gel, which was stained with 0.5 μg/ml ethidium bromide and viewed under ultraviolet transillumination.

Sequencing

To confirm that positive samples did contain *Dialister* species and to speciate them, PCR products from positive clinical samples were subjected to sequencing. Amplicons were purified using a PCR purification system (Wizard PCR Preps, Promega, Madison, WI) and then sequenced directly with primer Dial649r on the ABI 377 automated DNA sequencer using dye terminator chemistry (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Sequence data and electropherograms were inspected and edited by using BioEdit software (30). Sequences were corrected when obvious sequencing software errors were observed, such as when false spacing occurred or when undetermined nucleotides in the sequence could be determined according to the electropherogram. Sequences were then compared with those available in GenBank to identify the closest relatives by using the BLAST algorithm (28). Database sequences with the highest similarities and scorebits to our sequences were chosen as their identification. The criterion to define a novel phylotype was set at sequences that differ from the closest GenBank entry by more than 2%. Nucleotide sequences were then checked for chimeric molecules by using the CHECK CHIMERA tool of the Ribosomal Database Project (31). Sequences of the