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Investigation of bacterial communities associated with asymptomatic and symptomatic endodontic infections by denaturing gradient gel electrophoresis fingerprinting approach

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The purpose of the present study was to investigate the bacterial communities associated with asymptomatic and symptomatic endodontic infections and to compare denaturing gradient gel electrophoresis (DGGE) fingerprinting patterns of these two clinical conditions. The root canal microbiota of teeth associated with asymptomatic or symptomatic periradicular lesions was profiled by the PCR-DGGE method and then compared, taking into consideration the banding patterns. Bacteria were present in all examined cases. Comparative analysis of the two clinical conditions revealed bands that were common to both symptomatic and asymptomatic cases, but most DGGE bands appeared to be unique for each clinical condition. No single band occurred in all profiles. The mean number of bands detected in the 16S rDNA community profiles were 12.1 ± 9.4 (range 2–29) for symptomatic samples and 6.7 ± 2.7 (range 2–11) for asymptomatic ones. Clustering methods and principal component analysis of DGGE banding pattern placed the samples according to the presence or absence of symptoms. Four intense bands that were excised from the gel and sequenced showed similarities to species of the *Campylobacter* genus (found in 5/12 asymptomatic and in 3/11 symptomatic cases), *Fusobacterium* genus (4/11 symptomatic cases), *Acinetobacter* genus (5/12 asymptomatic cases), and *Enterobacteriaceae* family (11/12 asymptomatic and 2/11 symptomatic cases). The profiles of the predominant bacterial community appeared to be unique for each individual. These findings confirm that endodontic infections are polymicrobial and showed that there are significant differences in the predominant bacterial composition between asymptomatic and symptomatic cases.

Key words: 16S rRNA gene; endodontic microbiology; molecular biology; symptoms

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Periradicular diseases are symptomatic or asymptomatic inflammatory lesions caused by microbial infection of the root canal system (27). To evaluate the composition of the endodontic microbiota, a large number of studies have been conducted using light and electron microscopy as well as cultivation and molecular identification (27). All techniques have shown that the microbiota associated with primary endodontic infections comprises a complex mixture of bacterial species. Several bacterial species, particularly gram-negative anaerobic bacteria, have been implicated in disease etiology (32, 35). Nonetheless, no single pathogen or group of pathogens has been clearly identified as the causative agent of periradicular diseases (29). Although some species have been suggested to be involved with symptomatic teeth, including abscessed teeth (13, 33, 34), it has been revealed that the same species may be present in asymptomatic cases (2, 11, 31, 32). Therefore, it appears that factors other than the mere presence of a given putative pathogenic species can play a role in the etiology of symptomatic endodontic infections (28). The structure of the microbiota and the resulting interactions among the bacterial members of the consortium may be one of these factors.

Genetic fingerprinting techniques represent a powerful tool for the investigation of the structure of microbial communities in a given ecosystem. A commonly used strategy for genetic fingerprinting of complex microbial communities is the amplification of the 16S rRNA gene and analysis of polymerase chain reaction (PCR) products by a genetic fingerprinting technique, such as denaturing gradient gel electrophoresis (DGGE) (19). DGGE was originally developed for mutation detection (4, 22, 23), but environmental microbiologists adapted the method to generate culture-independent profiles of complex microbial communities (20). DGGE entails electrophoresis of DNA fragments at high temperature (50–60°C) in a polyacrylamide gel containing a gradient of denaturant (a mixture of urea and formamide) (21). As the DNA fragment migrates in the gel, it encounters increasing concentrations of denaturant. At some position in the gel, it will become partially or fully denatured. Partial denaturation causes a significant decrease in the electrophoretic mobility of the DNA molecule. The position in the gel at which the DNA melts is determined by its nucleotide sequence and composition (8). Thus, DNA fragments of the same size but differing in sequence melt at different positions within the gradient of the gel and then separate. In general, AT-rich sequences denature at lower denaturant concentrations than GC-rich sequences (8). By this method, a single-base change in a given sequence can be resolved (4). Consequently, PCR-DGGE has great potential to identify closely related species based on 16S rRNA gene sequence divergence.

DGGE allows the simultaneous analysis of multiple samples, making it possible to compare the diversity of different communities. An additional feature of this technique is the possibility of identifying community members by sequencing of excised bands (21). Although DGGE has been widely used for analysis of bacterial diversity in various natural habitats, including marine, lake or soil (17, 18, 21, 37), only in a few studies has it been used to investigate polymicrobial communities in clinical specimens; for example, those associated with corneal ulcer (26), gastrointestinal tract or feces (3, 15, 38, 42), oral biofilms (25) and subgingival plaque from healthy or periodontitis patients (7, 41). As far as we are aware, DGGE has never been used to fingerprint the microbiota associated with primary endodontic infections. The purpose of the present study was therefore to investigate the bacterial communities associated with asymptomatic and symptomatic endodontic infections and to compare the DGGE fingerprinting patterns of these two clinical conditions.

Material and methods

Subjects and sampling procedures

This study was carried out in accordance with the guidelines of, and after approval by, the Ethical Committee at Estácio de Sá University, Rio de Janeiro, Brazil. Samples were taken from patients who had been referred for root canal treatment or emergency treatment to the Department of Endodontics, Estácio de Sa University. Only teeth from adult patients (ages range 19–65 years) with carious lesions, necrotic pulps, and radiographic evidence of periradicular diseases were included in this study. Overall, 23 samples were obtained with the following clinical diagnoses: asymptomatic teeth with chronic periradicular lesions (n=12) and symptomatic teeth, clinically diagnosed as acute periradicular abscesses and showing localized or diffuse swellings along with fever, lymphadenopathy, or malaise (n=11). No apparent communication from the abscess to the oral cavity or the skin surface was observed. Selected teeth showed no significant gingival recession and were free of periodontal pockets >4 mm deep.

Samples from teeth with asymptomatic periradicular lesions were taken from the root canals. After the tooth crown was cleansed with pumice, a rubber dam was placed and the tooth and the surrounding field were cleansed with 3% hydrogen peroxide and disinfected with a 2.5% sodium hypochlorite (NaOCl) solution. Complete access preparations were made using sterile burs without water spray. The operative field, including the pulp chamber, was again swabbed with 2.5% NaOCl, which was then inactivated with sterile 5% sodium thiosulfate. If the root canal was dry, a small amount of sterile saline solution was introduced into the canal. Samples were initially collected by means of a #15 K-type file with the handle cut off. The file was introduced to a level approximately 1 mm short of the tooth apex, based on diagnostic radiographs, and a gentle filing motion was used. Afterwards, two sequential paper points were placed to the same level and used to soak up the fluid in the canal. Each paper point was retained in position for 1 min. The cut file and the two paper points were transferred to cryotubes containing 1 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6) and immediately frozen at − 20°C.

Samples from teeth with symptomatic periradicular lesions were taken by aspiration of the purulent exudate from the swollen mucosa over each abscess. The overlying mucosa was disinfected with 2% chlorhexidine, and a sterile disposable syringe was used to aspirate pus, which was immediately injected into cryotubes containing TE buffer. Pus samples were then immediately frozen.

DNA extraction

Samples in TE buffer were thawed to 37°C for 10 min and vortexed for 30 s. Microbial suspension was washed three times with 100 µl of sterile milliQ water by centrifugation for 2 min at 2500 × *g*. Pellets were then resuspended in 100 µl of milliQ water, boiled for 10 min and chilled on ice. After centrifugation to remove cell debris for 10 s at 9000 × *g* at 4°C, the supernatant was collected and used as the template for PCR amplification. Reference DNA from *Porphyromonas endodontalis* (ATCC 35406), *Propionibacterium propionicum* (ATCC 14157), and *Treponema denticola* (B1 strain, Forsyth Institute, Boston, MA) was also extracted to be used as a standard DGGE mixture.

PCR amplification

A 16S rDNA fragment corresponding to nucleotide positions from 968 to 1401 (*Escherichia coli* numbering) was amplified using the following universal bacterial primers: 968f (5′-AAC GCG AAG AAC CTT AC-3′), containing a 40-bp GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) added to its 5′-end, which makes it suitable for DGGE, and 1401r (5′- CGG TGT GTA CAA GAC CC -3′). Primers were as described by Nübel et al. (24).

The PCR mixture was made up of 5 μl of the supernatant from clinical samples, 25 pmol of universal primers, 5 μl of 10X PCR buffer (Biotools, Madrid, Spain), 3.8 mM $MgCl_2$, 2.5 U of *Tth* DNA polymerase (Biotools), 0.2 mM concentration of each deoxynucleoside triphosphate (Biotools) and sterile filtered milliQ water to a final volume of 50 μl. Negative controls consisting of sterile milliQ water instead of sample were included with each batch of samples analyzed.

PCR amplification was performed in a DNA thermocycler (Primus 25/96, MWG-Biotech, Ebersberg, Germany). The temperature profile included an initial denaturation step at 94°C for 2 min, followed by 35 cycles of a denaturation step at 94°C for 1 min, a primer annealing step at 55°C for 1 min, an extension step at 72°C for 2 min and a final step of 72°C for 10 min. Prior to DGGE analysis, the presence of PCR products was confirmed by electrophoresis in a 1.5% agarose gel conducted at 4 V/cm in Tris-borate-EDTA buffer. The gel was stained for 15 min with 0.5 μg/ml ethidium bromide and viewed under 300-nm ultraviolet light. A 100-bp DNA ladder digest (Biotools) served as the molecular size standard.

DGGE assay

DGGE of PCR products generated with 968f–GC/1401r primer set was performed using the Dcode Universal Mutation Detection System (Bio-Rad Dcode, Richmond, VA) at 75 V and 60°C for 16 h in 0.5X TAE buffer [20 mM Tris-acetate (pH 7.4), 10 mM sodium acetate, 0.5 mM disodium EDTA]. The PCR products (30 μl) were loaded onto 6% (w/v) polyacrylamide gels containing a linear gradient ranging from 45% to 70% denaturant [100% denaturant corresponded to 7 M urea and 40% (v/v) formamide] and increasing in the direction of electrophoresis. A 10-ml stacking gel without denaturant was added on top. A mixture of PCR products obtained from strains of *P. endodontalis, P. propionicum*, and *T. denticola* was run with each gel to facilitate comparison between gels. After electrophoresis, gels were stained with SYBR green I nucleic acid gel stain (Molecular Probes, Leiden, the Netherlands) for 40 min and then scanned using a Storm PhosphorImager (Amersham Biosciences, Uppsala, Sweden).

DGGE analysis

Individual lanes of the DGGE gel images were straightened and aligned using Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA), as described by Yang et al. (40). The DGGE banding patterns were converted to a binary matrix using presence–absence data. Dendrograms for comparison of DGGE banding patterns were constructed with the unweighted pair group method using arithmetic averages (UPGMA) following calculation of the Pearson coefficient. Cluster analysis was conducted to determine whether the samples revealed a nonrandom pattern and whether they clustered according to the presence or absence of symptoms. Principal component analysis (PCA) was used to investigate the variation in the DGGE banding patterns. PCA allows ordering of samples and bands along axes (principal components) on the basis of the banding patterns alone. Samples were plotted along the first (*x*-axis) and second (*y*-axis) principal components. PCA was also performed by adding the third principal component (*z*-axis). Student's *t*-test was used for statistical analysis of comparison between the number of bands in symptomatic and asymptomatic teeth.

Sequence analysis of products

Four intense bands were excised from the gels, purified, and re-amplified by PCR with 968f–GC/1401r primers set under the same conditions described above. Bands were cut out from the DGGE gel with a fresh sterile scalpel blade, placed in 50 μl of milliQ water, and left at 4°C for 24 h. A total of 5 μl of the resulting solution was added to a PCR mixture under the same PCR conditions and with the primers mentioned above. Products were then checked on an agarose gel and purified using a PCR purification system (Wizard PCR Preps, Promega, Madison, WI). PCR products were sequenced directly with the 1401r primer on the ABI 377 automated DNA sequencer using dye terminator chemistry (Amersham Biosciences). Sequences and chromatograms were checked by using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (12). Sequences were then analyzed using the BLAST program in the GenBank (1) and checked for chimeric molecules by using the CHECK CHIMERA tool of the Ribosomal Database Project (http://rdp.cme.msu.edu/html). Nucleotide sequences of close evolutionary relatives of our sequences were retrieved from the National Center for Biotechnology Information World Wide Web ENTREZ browser, which maintains and distributes the GenBank sequence database. Each sequence was aligned to the 10^{th} to 15^{th} closest matched sequences with ClustalX software, which is a windows interface for the ClustalW multiple sequence alignment tool (36). Neighbor-joining phylogenetic trees were constructed from the alignments using the Molecular Evolutionary Genetics Analysis package (MEGA version 2.1) (14). A distance matrix was constructed using a Tamura-Nei model without Gamma correction. The robustness of the phylogeny was tested by bootstrap analysis with 500 iterations.

Nucleotide sequence accession numbers

The sequences of the bands excised from the DGGE gels have been deposited in the GenBank database under accession numbers AY538595 to AY538598.

Results

DNA extracted from clinical samples was amplified by using primers directed towards the V6–V8 regions of 16S rDNA. PCR amplicons were detected in all samples, indicating that bacteria were always present and that the PCR reactions were conducted without significant amounts of inhibitors in clinical samples.

The DGGE profiles of the amplified 16S rDNA of endodontic samples are shown in Figs 1 and 2. Distinct banding patterns were observed from different clinical samples. Comparative analysis of the two data sets revealed bands that were common to both symptomatic and asymptomatic cases, but most DGGE bands appeared to be unique for each clinical condition. Most profiles contained intense DNA bands, as well as many faint DNA bands. Some profiles consisted almost exclusively of faint bands. No single band occurred in all profiles.

The mean number of bands in asymptomatic cases was smaller than in symptomatic cases; in the 16S rDNA community profiles there were 6.7 ± 2.7 (range 2–11)

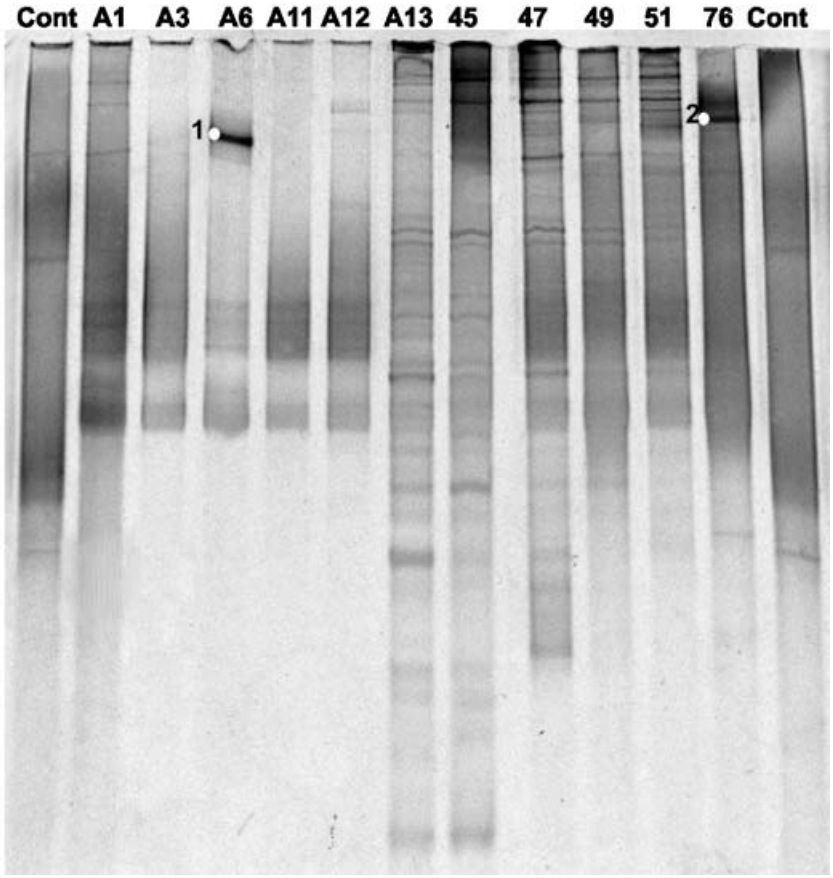

Fig. 1. DGGE profiles of amplified 16S rDNA from pus samples taken from symptomatic periradicular lesions (acute abscesses of endodontic origin). Numbered bands were excised and sequenced.

and 12.1 ± 9.4 (range 2–29), respectively. However, the difference was not significant ($P = 0.069$).

Cluster analysis of DGGE band polymorphism clearly separated asymptomatic from symptomatic cases. Samples from asymptomatic teeth tended to cluster with their respective types, except for sample ASY5N, which was unusual in that it was separate from the asymptomatic clusters. These findings were confirmed by PCA with two or three factors extracted (Figs 3 and 4).

Four representative DGGE bands from clinical samples were excised from the gel, reamplified and the resulting PCR products directly sequenced. Sequences were then processed to give their approximate phylogenetic affiliation. After checking that there was no indication of chimerism, sequences were analyzed for similarities to sequences deposited in GenBank. Band SYM01 was closest to both *Campylobacter rectus and Campylobacter showae* (98% homology). Band SYM02 showed 94% similarity to *Fusobacterium nucleatum*. Band ASY03 showed low-scoring homology (81%) to all of the following sequences: an uncultured bacterium (human infant D1A1) detected in the intestine of newborn babies, *Photorhabdus luminescens*, and *E. coli*. Band ASY04 had a match value of 89% to *Acinetobacter calcoaceticus*. Sequences were aligned with the sequences of close relatives and then used to construct phylogenetic trees (Fig. 5).

Bands corresponding to the same DGGE gel position of specimen SYM01 were visualized in five asymptomatic and in three symptomatic cases. Specimen SYM02 was found only in symptomatic cases (four cases). Specimen ASY04 was found in five asymptomatic cases and in no symptomatic one. An amplicon with identical DGGE band position to specimen ASY03 was present in all but one sample taken from asymptomatic teeth. The same band was found in two symptomatic teeth.

In broad-range PCR assays there is always the risk of contamination of the water and reagents, particularly by *Pseudomonas aeruginosa* (environmental contamination) or *E. coli* (used during industrial production of the DNA polymerase) (9). In the present study, negative controls yielded no bands, which demonstrated that contaminants were not present. This was further confirmed by the fact that no band occurred in all samples.

Discussion

In the present study, the DGGE method was applied to examine the structure of bacterial communities in samples taken from both asymptomatic and symptomatic endodontic infections. Banding patterns of symptomatic and asymptomatic teeth revealed a relative heterogeneity. Fingerprints of root canal samples revealed an average of about seven intense and faint DGGE bands in asymptomatic periradicular lesions and 12 DGGE bands in symptomatic lesions. Admittedly, the number of DGGE bands in a pattern is related to the number of bacterial species in the consortium (20). Although heteroduplex formation and possible divergence within multicopy rDNA families might increase the number of DGGE bands, many other factors should reduce it, including sampling biases, differential spatial distribution of bacteria due to homogenization procedures, differential DNA extraction, PCR biases, and comigration on DGGE gels (9, 10, 39). Therefore, we view the samples studied as complex communities containing a mean of seven bacterial species in asymptomatic endodontic infections and 12 in symptomatic infections.

Samples were not identical, regardless of whether they were from asymptomatic or symptomatic teeth. Each individual harbored a specific endodontic bacterial community, with a few dominant species (represented by more intense bands). However, some bands were shared by many of the DGGE profiles. These bands were intense in some cases and faint in others, indicating that there was no dominant species in the community. One assumption in the interpretation of DGGE fingerprinting is that the band intensity is directly related to the density of corresponding bacterial phylotypes within the sample (6, 18). It is commonly accepted that only the main populations (representing more than 0.1–1% of the target microorganisms in terms of relative proportion) are displayed in the profiles (18, 20). As a consequence, all populations present within a given habitat do not necessarily appear on DGGE banding patterns (21). Even so, the total number

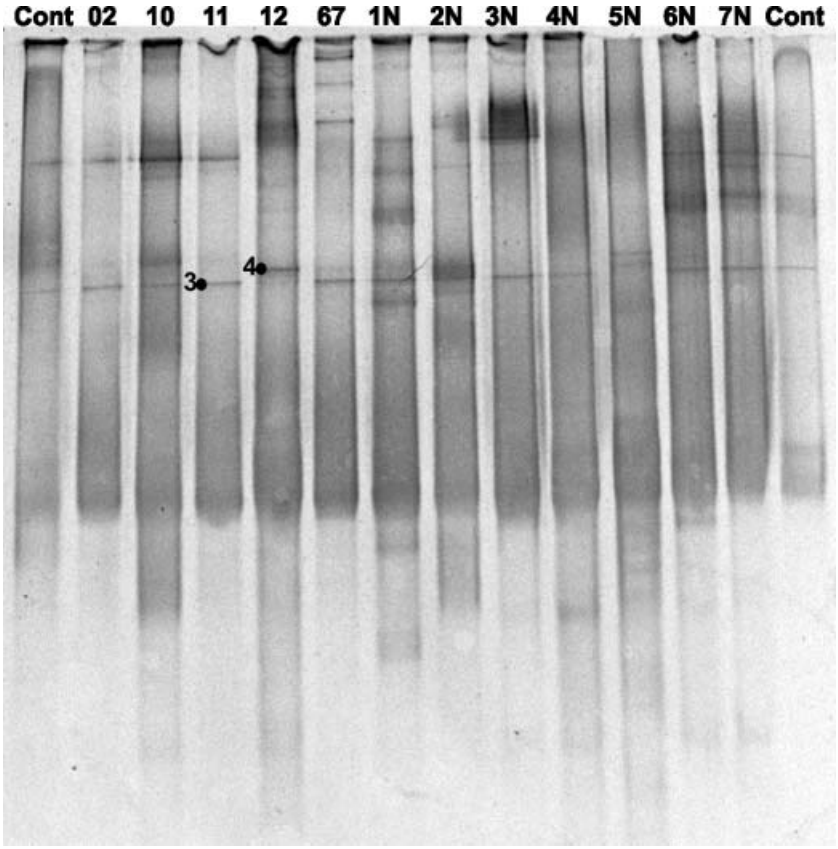

Fig. 2. DGGE profiles of amplified 16S rDNA from samples taken from infected root canals associated with asymptomatic periradicular lesions. Numbered bands were excised and sequenced.

of bands in each sample pattern is related to the number of dominant phylotypes and can be used for comparison purposes (37).

Clustering techniques can be applied to the DGGE profiling to identify samples that generate similar patterns. One advantage of this presentation is that the coherence of the fingerprinting patterns can be assessed rapidly (6). The resulting dendrogram revealed that endodontic samples were clustered according to the presence or absence of symptoms, indicating that the bacterial communities associated with these two clinical situations are clearly different (Fig. 3). The results were also analyzed by PCA, which generates new variables − principal components (linear components of the original variables) − that explain the highest dispersion of the samples. This method has often been used for the interpretation of DGGE community fingerprinting analysis (17, 37, 40). PCA applied to presence/absence of bands within DGGE patterns confirmed that the composition of the endodontic microbiota differed between symptomatic and asymptomatic cases (Fig. 4).

Although a standard mix of PCR products obtained from strains of the putative endodontic pathogens *P. endodontalis, T. denticola, and P. propionicum* was run with each DGGE gel, it was not possible to use it as a marker since most fragments in DGGE migrated to positions where it was difficult to ascertain if they were at the same distance as those of the culture collection strains. Bands present in clinical samples that were supposed to be in the same position as those of the standard mix were rather faint, and even after reamplification the quality of the products was apparently not good for sequencing. In addition, some bands of the species

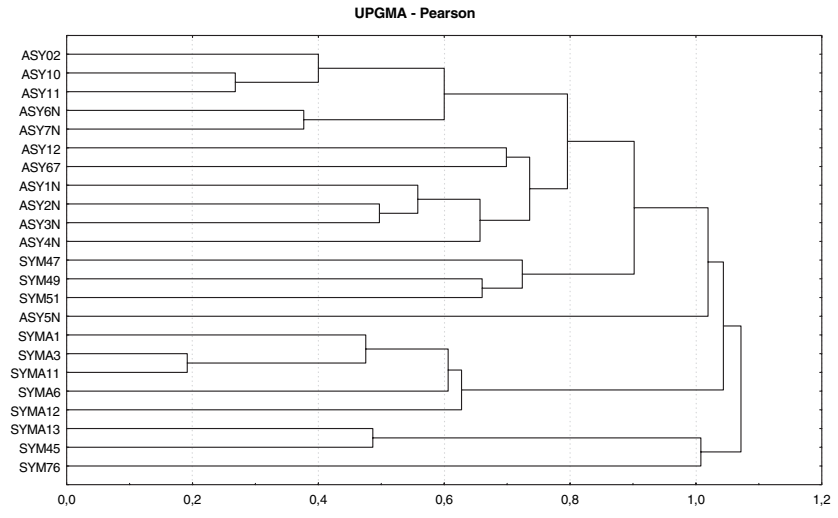

Fig. 3. Dendrogram obtained by UPGMA clustering of DGGE patterns of samples taken from primary endodontic infections associated with asymptomatic (ASY prefix) and symptomatic (SYM prefix) periradicular lesions.

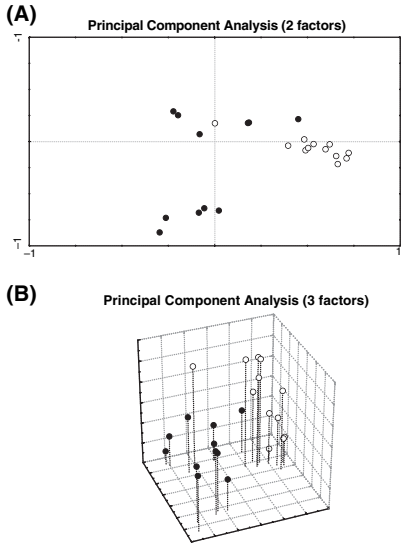

Fig. 4. Principal component analysis score plots of the DGGE banding patterns from samples taken from primary endodontic infections associated with asymptomatic (○) or symptomatic periradicular lesions (●). Samples were plotted (A) along the first (*x*-axis) and second (*y*-axis) principal components, or (B) with the addition of the third principal component (*z*-axis).

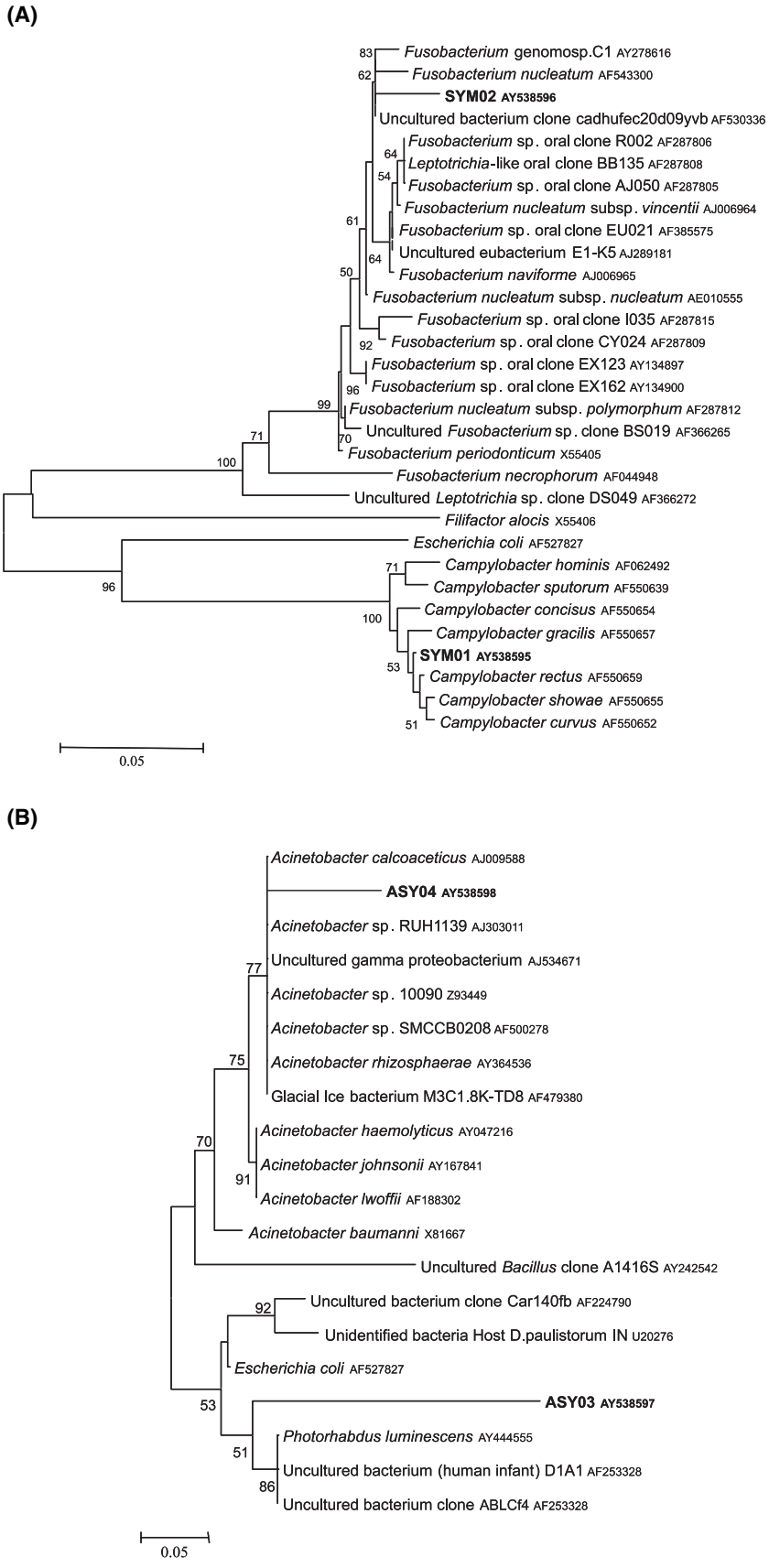

Fig. 5. Neighbor-joining tree showing phylogeny of 16S rRNA gene sequences obtained directly from cases of primary endodontic infection associated with symptomatic (A) or asymptomatic (B) periradicular lesions. Sequences were aligned with ClustalW, and distances were calculated with the Tamura–Nei algorithm. The numbers at the nodes of the tree indicate bootstrap values for each node out of 500 resamplings (values below 50 are not shown). Accession numbers for each 16S rRNA gene sequence are given. The scale bar indicates 5% sequence divergence.

composing the standard mixture migrated up to the same position and overlapped in the gel. Difficulties have already been reported in identifying species based on band position in the DGGE gel; sequencing of such bands revealed they were rather different from the standards, even though they were apparently in the same gel position (41). Thus, DGGE profiles generated from the standard mixture was used only to facilitate alignment and comparison between gels.

Comparison of four obtained sequences with sequences listed in the GenBank database revealed that they had an 81–98% homology to sequences of known genera. Because obtained sequences were only parts (about 434 bp long) of the approximately 1500-bp-long 16S rDNA, a clear phylogenetic affiliation could be inferred only to the genus, not to the species level. Generally, a reliable phylogenetic identification based on partial 16S rDNA analysis is often only possible to the genus level (5). Specimen SYM01 showed 98% similarity to both *C. rectus* and *C. showae*. *C. rectus* has been frequently detected in endodontic infections, both associated and not associated with symptoms (30). Bands corresponding to specimen SYM02 occurred only in symptomatic cases. Its sequence was close to members of the genus *Fusobacterium*, and it is conceivably an unidentified species of this genus. Members of the *Fusobacterium* genus, particularly *F. nucleatum*, have been commonly detected in both symptomatic and asymptomatic endodontic infections (16, 35).

A clear identification to the genus level was not possible for the other two sequences obtained from asymptomatic cases. One specimen (ASY04) showed 89% similarity to sequences of the genus *Acinetobacter*, from the *Moraxellaceae* family. Another specimen (ASY03) showed a low similarity to known members of the genera *Photorhabdus* and *Escherichia*, both from the *Enterobacteriaceae* family. The low sequence similarity

value to known bacteria reveals that these two sequences represent new genera related to the respective families, both from the *Proteobacteria* phylum. Because the number of 16S rDNA sequences in public databases is constantly increasing, a better identification of so far unknown bacteria may be possible in the near future.

The events involved in the conversion of an asymptomatic periradicular lesion to a symptomatic lesion are not clear. The possibility exists that, at a given moment in the endodontic infectious process, the microbiota reaches a certain degree of pathogenicity that causes an acute inflammation at the periradicular tissues, with consequent development of pain and sometimes swelling. In the present study, DGGE profiles of bacterial communities in symptomatic cases were significantly different from those of asymptomatic teeth. Although no study has as yet microbiologically examined the transition from an asymptomatic periradicular lesion to a symptomatic condition, differences in the dominant bacterial communities as reported by this study suggest that a shift in the structure of the microbial community is likely to occur before the appearance of symptoms. Such a shift is probably a result of the arrival of new pathogenic species or of variations in the number of members of the consortium. New bacterial species can gain entry into the root canal system via pulpal exposure and become established after competing for space and nutrients with the pioneer species. Fluctuations in the number of cells of certain species may be due to perturbation of the community induced by changes in environmental conditions (e.g. oxygen tension, nutrient availability, bacterial interactions) related to the time of infection, arrival of new species or other factors. Differences in the type and load of dominant species and the resulting bacterial interactions may be responsible for differences in the degree of pathogenicity of the whole bacterial consortium. There does not seem to be a key pathogen involved with symptomatic infections, since no band was found to occur in all profiles. Certain bacterial combinations apparently participate in the development of symptomatic periradicular lesions. Still to be clarified are the reasons for shifts in the composition of the microbiota and the consequent influence on symptomatology, as well as the role exerted by the host in the process.

In conclusion, our study demonstrated that DGGE analysis is useful for assessment of the diversity of the endodontic microbiota and rapid comparison of the community structure among individuals and between different types of endodontic infections. DGGE analysis showed an unexpected difference in the diversity of the amplicons in the profiles from the different individuals. In fact, the profiles of the predominant bacterial community appeared to be unique for each individual, and no specific amplicon was found in all cases examined. These findings confirm that endodontic infections are polymicrobial and several species can be implicated in the pathogenesis of periradicular diseases. Furthermore, there were significant differences in the predominant bacterial composition between asymptomatic and symptomatic cases. This suggests that the structure of the bacterial community may play a role in the development of symptoms.

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