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Bacterial examination of endodontic infections by clonal analysis in concert with denaturing high-performance liquid chromatography

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Background/aims: The aim of this study was to examine the diversity of bacterial species in the infected root canals of teeth associated with endodontic abscesses by cloning and sequencing techniques in concert with denaturing high-performance liquid chromatography.
Methods: Samples collected from five infected root canals were subjected to polymerase chain reaction (PCR) with universal 16S ribosomal DNA primers. Products of these PCRs were cloned and sequenced. Denaturing high-performance liquid chromatography (DHPLC) was used as a screening method to reduce the number of clones necessary for DNA sequencing.
Results: All samples were positive for the presence of bacteria and a range of 7–13 different bacteria were found per root canal sample. In total, 48 different oral clones were detected among the five root canal samples. *Olsenella profusa* was the only species present in all samples. *Porphyromonas gingivalis*, *Dialister pneumosintes*, *Dialister invisus*, *Lachnospiraceae* oral clone, *Staphylococcus aureus*, *Pseudoramibacter alactolyticus*, *Peptostreptococcus micros* and *Enterococcus faecalis* were found in two of the five samples. The majority of the taxa were present in only one sample, for example *Tannerella forsythia*, *Shuttleworthia satelles* and *Filifactor alocis*. Some facultative anaerobes that are frequently isolated from endodontic infections such as *E. faecalis*, *Streptococcus anginosus* and *Lactobacillus* spp. were also found in this study.
Conclusion: Clonal analysis of the microflora associated with endodontic infections revealed a wide diversity of oral species.

Key words: anaerobic bacteria; clonal analysis; DHPLC; endodontic infections

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More than 500 bacterial species are recognized as members of the oral microbiota. However, only a limited number of species are able to colonize the root canal system and induce pulpal and periradicular diseases. Species usually isolated from primary root canal infections belong to the genera *Fusobacterium*, *Prevotella*, *Porphyromonas*, *Eubacterium*, *Peptostreptococcus*, *Propionibacterium*, *Actinomyces* and *Streptococcus* (13, 16). However, recent studies using sensitive molecular diagnostic methods have detected microorganisms that are difficult or even impossible to culture in root canal infections, such as *Tannerella forsythia* and oral treponemes (3, 12, 28).

Molecular methods for microbial analysis of endodontic infections include the

DNA probe method based on the ability of single-stranded DNA to bind complementary sequences, and polymerase chain reaction (PCR), generally targeting the 16S ribosomal RNA (rRNA) gene. However, these techniques only show a qualitative description of the endodontic microbial composition because they search for specific microorganisms. PCR amplification of conserved regions of the 16S ribosomal DNA (rDNA) followed by cloning and sequencing of PCR products has been widely used for unravelling the microbiota associated with different human healthy and diseased sites, including endodontic infections (9, 10, 19, 24–26). However, cloning and sequencing are laborious, time-consuming and expensive techniques.

Denaturing high-performance liquid chromatography (DHPLC) is a new technology designed for the detection of unknown DNA sequence variation, including single-nucleotide substitutions, deletions and insertions. It uses a combination of partial heat denaturation and reverse-phase chromatography to detect sequence polymorphisms within DNA fragments. DHPLC has been used in the discovery of genetic variations in diploid genomes and also for the identification of microbial pathogens from mixed samples of genitourinary infections (6). Depending on the sequence variation within PCR products of a particular gene, differential retention times are exhibited by the products when separated by DHPLC.

The aim of this study was to examine the bacterial diversity of root canal infections by cloning and sequencing the 16S rRNA PCR products obtained from DNA extracted from endodontic samples, in conjunction with DHPLC. The DHPLC was used to identify clones from the same sample that exhibited identical retention times, thus reducing the number of clones that needed to be sequenced.

Material and methods

Patient selection

This study was based on five patients who attended the State University of Campinas – UNICAMP – for treatment of infections of dental root canals. (Ethical approval was obtained before the study.) The selection of patients was accomplished by anamnesis, and by clinical and radiographic examination. The following features were noted for each patient: age, gender, tooth and pulpal status, nature of pain, previous pain, tenderness to percussion, pain on palpation, mobility, presence of a sinus and its origin, presence of swelling of the periodontal tissues, depth of periodontal pocket, previous antibiotic therapy and internal condition of the root canal (e.g. presence of clear, haemorrhagic or purulent exudates). Patients who had not received antibiotic therapy in the last 6 months and who had teeth with primary endodontic infections, periapical lesions and absence of periodontal diseases were selected for this study. All the cases selected showed clinical features of endodontic abscesses.

Sampling procedure

The method followed for the microbiological procedures has been described previously (13, 16). After a two-stage cavity access preparation, which was made without the use of water spray but under manual irrigation with sterile saline solution and employing sterile burs, the teeth involved were individually isolated from the oral cavity with a previously disinfected rubber dam. Disinfection of the rubber dam and teeth was carried out using 30% hydrogen peroxide first, then 2.5% sodium hypochlorite. The solution was inactivated with 5% sodium thiosulphate to avoid interference with the bacteriological sampling. Aseptic techniques were used throughout endodontic therapy and sample acquisition. After initial entry to the pulp space the patency of the root canal was established with minimal instrumentation, where possible, and without the use of any chemically active irrigant.

For microbial sampling, a sterile paper point was introduced into the full length of the canal (as determined with a preoperative radiograph), and kept in place for 60 s. In cases where a dry canal was identified, a further sterile paper point, moistened in sterile saline solution, was used to ensure viable sample acquisition. In cases of wet canals (or those that had been previously irrigated with saline) as many paper points as possible were used to absorb all the fluid inside the canal. The paper point sample from the root canal was immediately transferred to a tube containing 1 ml transport medium-Viability Medium Göteborg Agar (VMGA III) (4) for transport to a laboratory in a different country (UK), where the molecular investigation was undertaken.

DNA extraction from the clinical samples (paper points)

An RTP Spin Bacteria DNA Mini Kit (Invitek GmbH, Berlin, Germany) was used for DNA extraction from the paper points following the manufacturer's instructions. The paper points were transferred to an extraction tube L (tube Lysis) followed by the addition of 400 µl of a resuspension buffer R. The sample was incubated in a Thermomixer (Eppendorf, Netheier, Hamburg, Germany) at 65°C for 10 min followed by incubation at 95°C for another 5 min. To this was added 400 µl of a Binding buffer (B6) and the sample was loaded into a spin filter placed in a 2-ml tube, incubated for 1 min at room temperature and centrifuged at 15,680 ***g*** for 1 min. The suspension was washed twice with 500 and 700 µl washing buffers I and II, respectively, for 1 min at 15,680 ***g***. The spin filter was then placed into a new 1.5-ml tube and 200 µl elution Buffer D was added. After incubation for 1 min and centrifugation at 10,035 ***g*** the samples were frozen at −20°C.

Amplification of the 16S rRNA

The V6 to V8 region of the 16S rRNA gene was amplified by PCR using the 16S forward primer 0933 F, 5′-GCA-CAAGCGGTGGAGCATGTGG-3′ and reverse primer 1407R 5′-GAC-GGG CGG-TGT-GTA-CAA-G-3′ (6).

PCR were performed in a total volume of 50 µl, containing 3 µl DNA from clinical samples (or 1 µl DNA from reference strains), 2 µl of each primer (40 pmol) and 25 µl master mix (Bioline, London, UK) containing BioTaq DNA Polymerase, 32 mM $(NH4)_2SO_4$, 125 mM Tris–HCl (pH 8.8 at 25°C), 0.02% Tween-20, 2 mM dNTPs and a stabilizer.

PCR amplification was carried out in a DNA thermocycler (Primus 96-MWG-Biotech, Ebersberg, Germany). Cycling parameters for the PCR included an initial denaturation step at 94°C for 7 min, followed by 25 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 40 s and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. The amplification products were analysed by gel electrophoresis with a horizontal 1.5% agarose gel for 1 h at 150 V at room temperature in 1× TBE (Tris Borate EDTA) running buffer. The gel was stained for 15 min with 0.5 µg/ml ethidium bromide and recorded under ultraviolet light with the Gel Doc 200 System (BioRad Laboratories, Herts, UK). Reactions were deemed positive if bands of the appropriate sizes were present using the 100-base-pair DNA ladder (Promega, Madison, WI).

Cloning of 16S rDNA fragments from clinical samples

The 16S rDNA amplification products from the five samples were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) into the vector pCR 4-TOPO and transformed into *Escherichia coli* One-Shot TOP10 cells (Invitrogen) according to the manufacturer's instructions. In summary, 4 µl fresh PCR product plus 1 µl salt solution (provided with the kit) were added to 1 µl TOPO vector and incubated at room temperature for 1 h. After the TOPO cloning reaction, transformation of the pCR4-TOPO construct into the competent *E. coli* cells was performed by adding 2 µl TOPO Cloning Reaction into a vial of One Shot electrocompetent *E. coli* cells. Immediately, 250 µl SOC (Super Optimal Catabolite) medium was added and the solution was incubated for at least 1 h at 37°C. From 10 to 200 µl of the transformation was spread on a prewarmed selective plate and incubated overnight at 37°C.

For analysis of the transformants, 96 individual colonies from each sample were picked with a plastic tip and resuspended in 20 µl sterile distilled water in a 96-well microtitre plate. Ten microlitres of this suspension was used as template for a 50-µl PCR with the M13 forward primer and M13 reverse primer. The cycling conditions were: initial denaturation at 94°C for 2 min, 25 cycles of denaturation for 1 min at 94°C, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 1 min. Five microlitres of the PCR product was analysed by agarose gel electrophoresis.

Denaturing high-performance liquid chromatography

DHPLC analysis of the PCR products obtained from the 96 clones of individual samples was performed on the WAVE 3500 system as previously described by Domann et al. (6) with minor modifications. We employed the DNASep HT cartridge, which uses alkylated non-porous polystyrene-divinylbenzene copolymer microspheres for high-performance nucleic acid separations. The gradient was formed by buffer A, consisting of 0.1 M triethylammonium acetate (TEAA), pH 7.0, and buffer B, consisting of 0.1 M TEAA and 25% acetonitrile, pH 7.0. Buffer C, consisting of 25% water and 75% acetonitrile, was used for washing the column. The elution of DNA fragments in the system is influenced by essential factors such as column temperature, pump flow rate, gradient rate of buffer B, and time of elution. A range of temperatures was investigated under a fixed gradient that enables maximum resolution of the amplicon for known endodontic pathogens (FIG. 1). PCR products amplified from known bacterial species and environmental samples using the 16S primers were mixed together and analysed by DHPLC to optimize the following conditions: column temperatures ranging from 50 to 70°C, gradient rate of buffer B from 40% to 55%, elution durations of 5–40 min, and pump flow rates of 0.2–0.9 ml/min. All buffer solutions were obtained from Transgenomic (Omaha, NE). The analysis was accomplished with NAVIGATOR software version 1.5.4.

Sequencing procedures

PCR products from the clones that showed different retention times on DHPLC were selected for sequencing. The M13 forward primer and M13 reverse primer were used for sequencing both strands of the PCR products. The sequencing was performed on the Beckman Coulter CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) as recommended by the vendor. The resulting sequences were

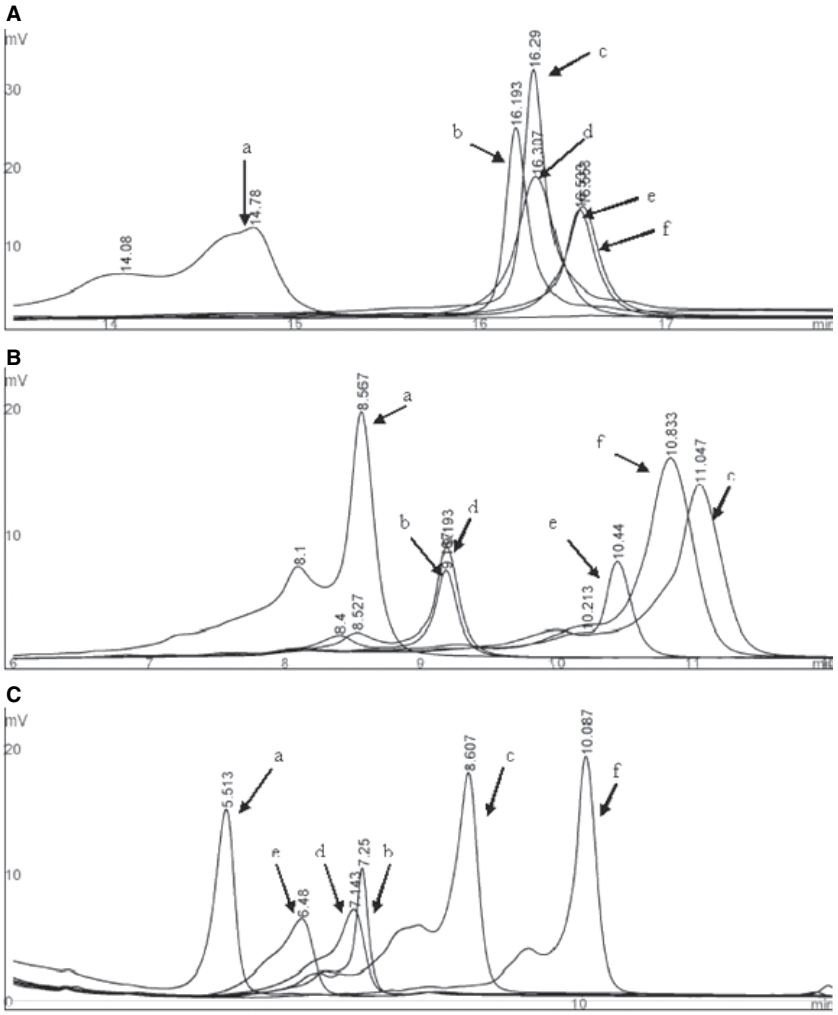

Fig. 1. Optimization of DHPLC parameters to improve the resolution of peak profiles. The profiles and retention times were obtained by analysis of the V6–V8 region of the 16S rRNA gene of six cloned fragments from one root canal sample. The analytical gradient (see Material and methods) was kept constant but the temperature and flow rate were varied. (A) Peak profiles obtained at 62°C with flow rate 0.2 ml/min; (B) Peak profiles at 62°C with flow rate 0.9 ml/min; (C) Peak profiles at 64°C with flow rate 0.9 ml/min. Each peak represents a different species, and numbers above the peak represent the retention times (in min). Peaks are labelled with the corresponding sequencing results: (a) *Flexitipes*-like sp. oral clone, (b) *Eubacterium infirmum* W1471, (c) *Synergistes* sp. P4G_18 P1, (d) *Staphylococcus aureus* sp. *aureus*, (e) *Pseudoramibacter alactolyticus*, (f) *Olsenella profusa.*

Table 1. Identification of bacterial species and phylotypes by sequence analysis of V6–V8 region of the 16S rRNA gene

Probable genus or species match	Accession number[1]	Identity (%)[2]	Sample[3]	Average retention time (min)[4]	Number of clones[5]
Eubacterium infirmum W 1471[6]	U13039	98	1	7.18	19
Eubacterium sp. oral strain	AF287761	97			
Synergistes sp. P4G_18 P1[6]	AY207056	99	1	8.57	9
Flexistipes-like sp. oral clone BA121	AY005444	99			
Pseudoramibacter alactolyticus[6]	AB036761/ AB036760	98/99	1/2	6.33/7.85	18/12
Firmicutes sp. oral clone CK057	AF287778	98			
Staphylococcaceae bacterium SM40[6]	DQ195851	98	1	6.98	6
Olsenella profusa[6]	AF292374	99	1/2/3/4/5	10.18/12.56/	17/12/11/10/15
Olsenella sp. N13-17	AY880046	99		11.23/11.42/10.56	
Staphylococcus aureus[6]	BX571857	99	1/4	7.12/7.358	12/6
Staphylococcus aureus subsp. *aureus* COL	CP000046	98			
Uncultured bacterium clone MSFC_2M4H	DQ447795	98			
Staphylococcaceae bacterium SM40	DQ195851	99			
Staphylococcus simiae strain CCM 7229	DQ127902	99			
Staphylococcus haemolyticus JCSC1435 DNA	AP006716	99			
Synergistes sp. P4G_18 P1	AY207056	97	1	5.51	8
Flexistipes-like sp. oral clone BA121	AY005444	97			
Synergistes genomosp. C1 16S	AY278615	96			
Uncultured *Staphylococcus* sp. clone pGA 2	AY601761	97	1/4	5.27/5.25	7/3
Staphylococcus hominis subsp. *novobiosepticus*	AB233326	98			
Uncultured bacterium clone MSFC_2M4H	DQ447795	98			
Enterococcus faecalis[6]	DQ411814/ AY850358	97/99	4/5	9.65/9.766	11/6
Enterococcus sp. T1-2006	DQ462332	97			
Uncultured bacterium clone aab28e07	DQ819332	97			
Lactobacillus gasseri[6]	AY339167	99	4	12.45	11
Lactobacillus sp.	AY094066	99			
Lactobacillus sp. RA2062[6]	AY445129	99	4	10.02	6
Uncultured bacterium clone SM5	DQ318872	99			
Lactobacillus delbrueckii subsp. *bulgaricus*	CR954253	98			
Lactobacillus sp. *rennanqily* f4[6]	AY332396	99	4	10.56	5
Lactobacillus fermentum strain 44197	DQ779203	99			
Lactobacillus sp. CR-6AS clone crev-7	AF349935	99			
Porphyromonas gingivalis[6]	AF287987/ AF285870	99/98	2/4	8.76/9.01	16/10
Porphyromonas gulae	AF285869	99			
Shuttleworthia satelles[6]	AF399956	99	4	9.24	9
Eubacterium sp. oral clone EH006	AF385570	98			
Streptococcus anginosus[6]	AB006120	97	4	11.89	5
Streptococcus infantis[6]	AY485603	99	4	13.37	9
Streptococcus sp. oral strain H3-M2	AF385523	99			
Uncultured *Streptococcus* sp. Clone	DQ346441	99			
Streptococcus pneumoniae strain HK_P55	AY525790	99			
Streptococcus salivarius[6]	AF459433	98	4	12.04	4
Uncultured bacterium clone	DQ905624	98			
Veillonella caviae[6]	AY355140	99	4	6.54	7
Uncultured *Veillonella* sp. Clone	DQ016721	99			
Veillonella sp. oral clone VeillG1	AY995766	99			
Veillonella dispar	AY995770	98			
Bacteroides-like sp. oral clone X083[6]	AY005066	99	3	9.36	15
Bacteroidales oral clone MCE7_20 E1 16S	AF481206	99			
Uncultured bacterium clone CFT112E8	DQ456032	98			
Dialister invisus	AY162469	99	2/5	9.32/9.55	15/12
Dialister sp. E2_20 E1 oral isolate	AF481209	98			
Uncultured bacterium clone 29c-g1	DQ905952	98			
Lachnospiraceae oral clone MCE9_173 E4[6]	AF481221/	99/	2/5	14.09/14.21	17/9
Lachnospiraceae oral clone P4PC_12 P1	AF538857	99			
Firmicutes sp. Oral clone AO068	AF287771	98			
Uncultured rape rhizosphere bacterium wr0200	AJ295561	98	3	5.09	13
Uncultured bacterium clone SBS2w56	AY683256	98			
Fusobacterium nucleatum[6]	AF543300	99	5	6.55	15
Dialister pneumosintes[4]	X82500	99	3/5	8.44/8.61	15/5
Uncultured bacterium clone 014B-E12	DQ905593	99			
Dialister sp. E2_20 E1 oral isolate[6]	AF481209	99	2/3/5	9.79/10.03/10.23	12/5/3
Dialister sp. oral clone BS095	AF287787	97			
Uncultured bacterium clone 29c-g1	DQ905952	99			
Eubacteriaceae oral clone P2PB_46 P3[6]	AF538856	99	3	6.51	7
Peptostreptococcus micros[6]	AY323523	99	3/5	7.86/8.05	13/6
Peptostreptococcus sp. oral clone FG014	AF385543	99			

Table 1. Continued

Probable genus or species match	Accession number[1]	Identity (%)[2]	Sample[3]	Average retention time (min)[4]	Number of clones[5]
Uncultured bacterium clone cadhumucU2bG04	DQ339923	98			
Filifactor alocis[6]	X55406	98	5	6.01	12
Prevotella sp. oral clone F045[6]	AY005056	97	3	12.17	10
Bacteroidales oral clone MCE7_164 E2b[6]	AF481206	98	5	11.34	3
Bacteroidales genomosp. P8 oral clone MB4_G15	DQ003626	98			
Tannerella forsythia[6]	AB053947	98	5	8.87	10
Bacteroides forsythus gene for 16S rRNA	AB035460	98			
Olsenella sp.[6]	AY880047	98	2	12.85	12
Uncultured bacterium clone aab38e07	DQ818830	100	3	8.11	7

[1]Accession numbers obtained by Blast interrogation of 16S rRNA sequences against the NCBI database.
[2]The percentage similarity cut-off for species identification was set at 98%.
[3]Frequency of each phylotype from the five samples of infected root canals referred from 1 to 5, accounting for a total of 48 phylotypes.
[4]Average retention times of each species with a variation of 0.4 min.
[5]Number of clones with similar retention times.
[6]Species more likely to be an endodontic pathogen.

edited and aligned using the BIOEDIT (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and CLUSTALW MULTIPLE ALIGNMENT programs (15). The alignment was imported into the BIONUMERICS software package (Applied Maths, St-Martens-Latem, Belgium) and cluster analysis was performed using the unweighted paired group method with arithmetic averages (UPGMA; NEIGHBOR program of PHYLIP). The BLASTn algorithm was used to compare the obtained nucleic acid sequences against a nucleotide sequence database (Table 1) at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/) (1).

Results and discussion

The primer pair 0933F and 1407R was used to amplify the variable regions V6 to V8 of the 16S rRNA to generate a 474-base-pair amplicon from the five samples collected from infected root canals. A number of studies (6, 11) have established that these primers targeting the V6–V8 region of the 16S rRNA gene are most suitable for denaturing gradient gel electrophoresis and DHPLC analysis of complex microbial populations. The current study builds upon these findings and takes the method further to investigate this region of the 16S rRNA gene. However, this region of the 16S rRNA gene presents a lower resolution for species identification and some taxa might not be reliably identified and distinguished from closely related species. Despite this, sequencing of the clones amplified in this study provided valuable information for identification of the majority of the isolates. The range of probable identities of some isolates is given in Table 1.

The predicted 474-base-pair amplicon was detected in all the five root canal samples. This indicated the presence of bacterial DNA in each sample. The study comprised samples from teeth with primary endodontic infections, no periodontal disease, presence of periapical radiolucency, and features of endodontic abscesses. Therefore, a great variety of phylotypes were found among the cases. The number of different bacteria found in each root canal sample varied from 7 to 13. A total of 48 phylotypes were detected, 33 phylotypes belonged to different species. *Olsenella profusa* was the only species present in all the samples. A further seven species, i.e. *Porphyromonas gingivalis*, *Dialister pneumosintes*, *Dialister invisus*, *Staphylococcus aureus*, *Pseudoramibacter alactolyticus*, *Peptostreptococcus micros* and *Enterococcus faecalis* were found in two of the five samples. However, the majority of the taxa were present in only one sample; some of these were species that are very difficult to culture such as *T. forsythia*, *Shuttleworthia satelles* and *Filifactor alocis*. Some facultative anaerobes that are frequently isolated from endodontic infections such as *E. faecalis*, *Streptococcus anginosus* and *Lactobacillus* spp. were also found in this study.

The results of this study confirmed those of previous reports in demonstrating that the microflora of endodontic infections is polymicrobial and dominated by anaerobic bacteria. The greatest advantage of cloning and sequencing of PCR products from polymicrobial infections is the possibility of predicting the general microflora including non-cultivable bacteria, without searching only for target species. Nevertheless, a huge number of clones need to be sequenced for that purpose, which is logistically difficult to perform in a large number of specimens. Fouad et al. (10) were able to clone and sequence only two specimens of the 24 that were used for detecting target species. Another study used restriction fragment length polymorphism analysis to screen 50–100 clones into which DNA amplified from eight specimens from infected root canals had been inserted (24). Clones with similar restriction fragment length polymorphism profiles were grouped, and only one representative from each group was sequenced.

In the present study, 96 clones from each sample were analysed using a DHPLC Wave system at 64°C. At that temperature different species showed different retention times (Fig. 1) and clones of the same species presented the same retention time. Therefore, it was possible to select clones with different retention times to be sequenced, so that a wider range of species could be detected even if the number of samples sequenced was reduced. A dendrogram (Fig. 2) was generated from 16S rRNA sequences obtained from the V6–V8 region of the bacterial species detected in the root canal samples and 16S rRNA sequences from the same region, of type strains of bacteria commonly detected from endodontic infections. The latter were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov). A percentage similarity cut-off of 98% was set for species identification. The dendrogram shows that closely related species clustered together into groups (Fig. 2).

Olsenella spp. has been commonly found in association with infections in the oral cavity (5, 19, 22). *O. profusa* (previously designated *Eubacterium* group D52) is an obligate anaerobe, non-spore-forming, non-motile, gram-positive, short

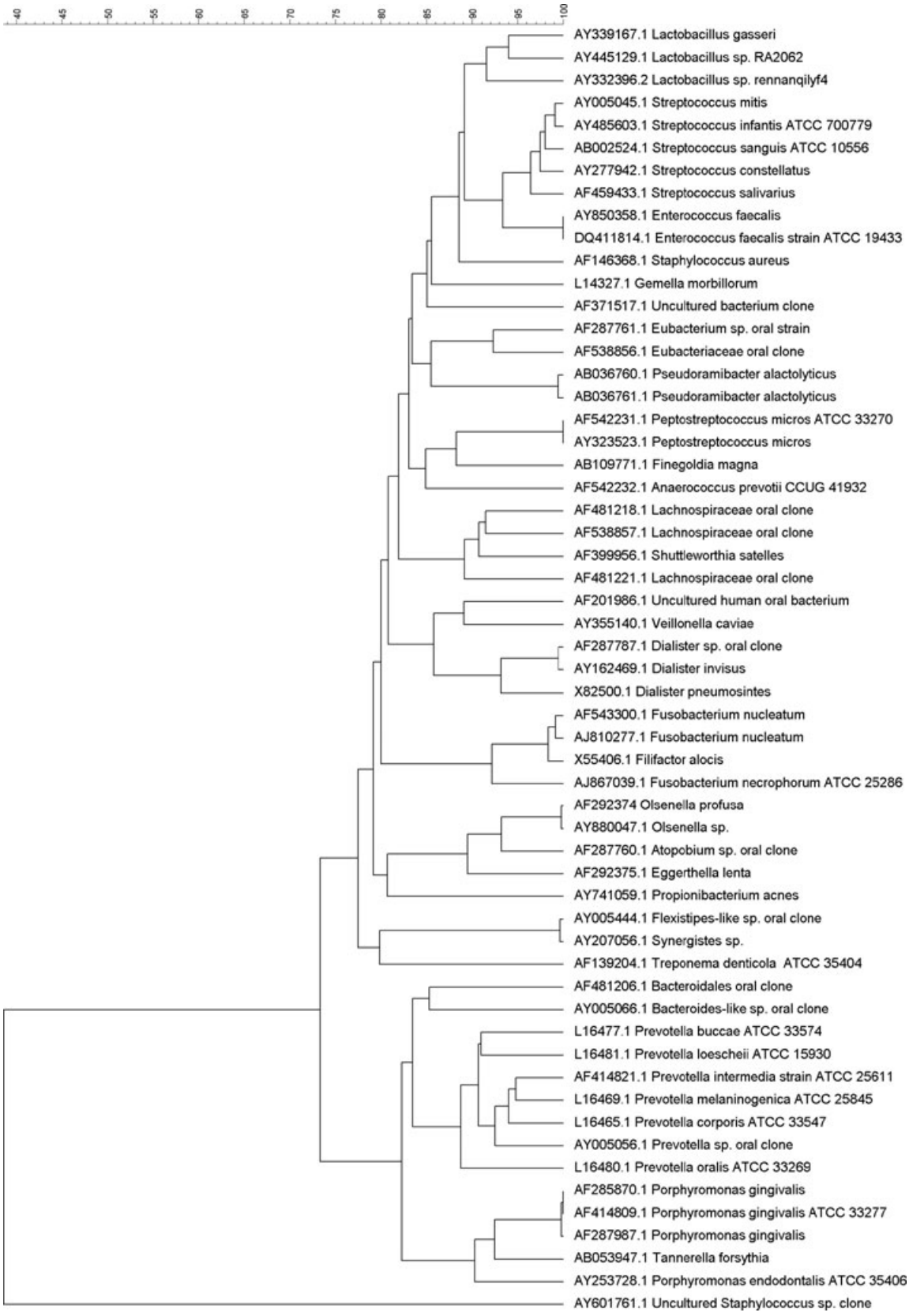

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence comparisons over 500 nucleotide bases of the V6–V8 region. The dendrogram was constructed using the UPGMA method (NEIGHBOR program of PHYLIP) for cluster analysis, in the BIONUMERICS software package. Accession numbers for 16S rRNA sequences are given for each strain, following comparison of sequences against the NCBI database using the BLASTn algorithm. Scale bar represents percentage sequence similarity.

rod that shows good growth in culture media. Previous studies have already detected the presence of this species in endodontic infections in association with *Olsenella uli* (10, 22). Cultural and molecular analyses of samples from infected root canals revealed the occurrence of *O. uli* in three of five cases and *O. profusa* in one of five cases (19). In this study, while *O. profusa* was the most frequently found species; *O. uli* was not detected at all. The high frequency of *O. profusa* suggests an involvement in the pathogenesis of periradicular diseases. However, further studies with larger samples should confirm such involvement and look for specific virulence factors of this species.

Some important endodontic species frequently isolated by culture methods, such as *P. gingivalis*, *Fusobacterium nucleatum* and *P. micros*, were also detected in this study. *P. gingivalis* is a black-pigmented bacterium that is frequently isolated from symptomatic primary endodontic infections (17). *F. nucleatum* has been associated with flare-ups (32) and symptomatic endodontic infections (26) and was shown to increase the pathogenicity of other organisms in mixed culture, especially *P. gingivalis* and *P. intermedia* (2). *P. micros*, a gram-positive strict anaerobic coccus, has been associated with dentoalveolar abscesses (31). Our results disagree with those of Saito et al. (25), Munson et al. (19) and Rolph et al. (24), who did not find *F. nucleatum*, *P. gingivalis* and *P. micros* in primary endodontic infections using clonal analysis.

Dialister spp. have been identified in oral infections with increasing frequencies (18, 20). Saito et al. (25) detected *Dialister invisus*, a recently described oral strict anaerobic gram-negative coccobacillus (8), in five of seven endodontic samples using cloning and sequencing techniques. *Dialister pneumosintes*, a non-motile non-fermentative non-spore-forming gram-negative obligate anaerobe frequently associated with purulent infections has been considered a putative pathogen in endodontic infections (27, 29). It was usually detected in mixed infections and positively associated with other species such as *Treponema denticola*, *Porphyromonas endodontalis*, *F. nucleatum* and *P. micros*. In this study *Dialister* spp. were found in three cases, one case presented both *D. invisus* and *D. pneumosintes*, while two cases presented either *D. invisus* or *D. pneumosintes* associated with a *Dialister* spp. oral clone. However, in both cases where *D. pneumosintes* was found, it was associated with *P. micros*, and in one case *D. pneumosintes* was associated with *T. forsythia.*

The microflora of root canal infections includes a diversity of species that are difficult or impossible to culture using current methods. However, studies using molecular techniques have detected species such as *T. forsythia*, *F. alocis* and *P. alactolyticus* in primary endodontic infections (12, 30), which is in agreement with the findings of the present study. *S. satelles* is an obligate anaerobe, non-spore-forming, non-motile, slightly curved, gram-positive, short bacillus. It was originally isolated from the human subgingival plaque and periodontal pockets of patients with periodontitis and described by Downes et al. (7). To our knowledge, this is the first report of this species from infected root canals. In this study, *S. satelles* was isolated in one case. Further studies investigating its role on the pathogenesis of endodontic infections will be necessary.

E. faecalis has been widely associated with failed endodontic treatments either by culture (21) or molecular methods (23). However, Gomes et al. (14) using PCR of the 16S rDNA detected this species at a higher frequency in primary than in secondary endodontic infections. In the present study, where only primary endodontic infections were assessed by clonal analysis, *E. faecalis* was isolated from two cases.

In conclusion, the clonal analysis of primary endodontic infections revealed a wide diversity of species infecting the root canals. The usual species isolated in culture studies plus species that are difficult to culture but have been proven to be endodontic pathogens, by reports employing molecular approaches, were also isolated in this study. The previous analysis of the oral clones on the DHPLC helped to reduce the number of clones to be sequenced. Future studies should be undertaken using this methodology with an expanded patient population with different clinical features to investigate possible associations between specific species and signs and symptoms of infections as well as positive and negative associations between species colonizing the root canal.

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