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Molecular Identification of Microorganisms from Endodontic Infections

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A relatively wide range of bacteria have been isolated from root canals using standard culture techniques. However, only 50% of the bacteria in the oral cavity are cultivable (S. S. Socransky et al., Arch. Oral Biol. 8:278–280, 1963); hence, bacterial diversity in endodontic infections is underestimated. This study used a PCR-based 16S rRNA gene assay, followed by cloning and sequencing of 16S rRNA amplicons from a small subset of samples to assess the diversity of bacteria present in infected root canals. A total of 41 clinical samples from 15 de novo and 26 refractory cases of endodontic infections were assessed. Of these samples, 44% were positive by culture and 68% were positive by PCR. Eight samples were selected for further analysis. Of these, the two de novo cases yielded sequences related to those of the genera *Enterococcus, Lactobacillus, Propionibacterium*, and *Streptococcus* and two clones were related to previously uncultivated bacteria, while the sinus-associated, de novo case yielded sequences related to those of the genera *Lactobacillus, Pantoea, Prevotella*, and *Selenomonas*. The five refractory cases produced clones which were related to the genera *Capnocytophaga, Cytophaga, Dialister, Eubacterium, Fusobacterium, Gemella, Mogibacterium, Peptostreptococcus, Prevotella, Propionibacterium, Selenomonas, Solobacterium, Streptococcus*, and *Veillonella* and two clones representing previously uncultivated bacteria. The phylogenetic positions of several clones associated with the *Clostridiaceae* and *Sporomusa* subgroups of the *Firmicutes* grouping are also shown. This study demonstrates that molecular techniques can detect the presence of bacteria in endodontic infections when culture techniques yield a negative result and can be used to identify a wider range of endodontic-infection-related bacteria including the presence of previously unidentified or unculturable bacteria.

It is now well established that the etiology of periradicular periodontitis is microbiological (18). Microorganisms most commonly infect the root canal system by ingress from the oral cavity through dental caries or defective restorations. The dentine-pulp complex of the tooth may react in a number of ways to the presence of microorganisms, but irreversible inflammatory changes may ultimately occur with the development of an inflammatory front in the periradicular tissues causing a chronic periradicular periodontitis. Periradicular periodontitis is treated by root canal therapy, whereby the root canal system is cleaned, chemomechanically shaped, and then obturated, which allows healing to take place. The objective is to reduce the microflora to a minimum and prevent recontamination, which usually occurs coronally.

The development of effective strategies for root canal therapy is dependent upon understanding the composition of the pathogenic flora of the root canal system. Identification of the root canal isolates from previous studies has traditionally been performed using standard microbiological and biochemical techniques. These methods have shown that the polymicrobial infections are mainly caused by obligate and facultative anaerobes (19, 40). However, correlation of the microbiological findings from these studies is affected by certain limitations of the culture techniques, leading to the underestimation of bacterial diversity within the root canal system.

It is estimated that less than 20% of bacteria in the environment are cultivable (45), with that percentage increasing to 50% for clinical cultivation techniques for bacteria from the oral cavity (37), leading to the suggestion that a large number of bacteria are still uncultivable using conventional techniques. Since it is recognized that uncultivable species may be present in root canals and contribute to the disease process (5), it is imperative to identify these species so that their contribution to the disease process can be assessed.

Some bacteria from clinical isolates are fastidious in their growth requirements (44) and may give variable results with commercially available biochemical tests and are therefore not always detected or may be misidentified when they are detected (8).

The 16S rRNA gene has provided a new tool for estimating bacterial phylogeny, which has led to rapid changes in bacterial taxonomy (29). Many of these changes in taxonomy have occurred within the anaerobic bacterial genera (16). In papers which predate these taxonomic updates, many clinical isolates are recorded as belonging to bacterial species which have been subsequently split into further taxa or reassigned to new ones, hence underestimating biodiversity within endodontic infections and making correlation of results from different studies very difficult.

Molecular techniques have been used to detect bacteria in endodontic infections using oligonucleotide probes (17) and checkerboard DNA-DNA hybridization analysis (35). How-

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ever, the use of specific DNA probes limits the boundaries of the detection technique, as it assumes that these probes target the species of importance. The species selected are based on culture studies and do not account for any uncultivated bacteria or uncultivable biotypes of known species. There are inherent problems with checkerboard analysis, which stem from the lack of specificity of the whole genomic probes used. Neither technique can be used to determine the true diversity of potential pathogens from infected root canals.

Techniques utilizing the 16S rRNA gene sequence data have been developed for use in the field of microbial ecology to evaluate the members of diverse microbial communities including uncultivable microorganisms (13, 15, 45). These techniques have been adapted to study uncultivable microorganisms involved in disease (32); to study the bacterial diversity in dentoalveolar abscesses (9), subgingival plaque (21), and saliva (34); and to investigate the eubacterial and spirochaete species involved in periodontitis (4, 38). The aim of this study was to use these techniques to examine the diversity of bacterial species in infected root canals of teeth with associated periradicular periodontitis.

MATERIALS AND METHODS

Sample details. For the initial culture and PCR screening assay, 41 samples were taken from 24 patients, comprising 17 females and 7 males. Subjects were patients with teeth exhibiting chronic periradicular periodontitis with necrotic pulps (de novo cases) or where root canal treatment was judged to have failed (refractory cases). For the detailed molecular analysis, eight samples were taken from the above set, representing three de novo and five refractory cases. One tooth in the de novo group was associated with a sinus discharging buccally.

Collection of endodontic samples. After local anesthesia had been administered, the tooth to be treated was isolated with a rubber dam. The tooth and surrounding dam and clamp were cleaned with 30% hydrogen peroxide and then swabbed with 5% potassium iodide. The surface was then swabbed with 5% sodium thiosulfate solution to inactivate the iodine solution.

(i) De novo cases. Access to the root canal system was gained using a diamond bur in an air rotor. Specimens were taken as soon as the pulp chamber was reached. A sterile file was used to explore the openings of the root canals. A sterile paper point previously soaked in sterile saline solution was placed in the root canal and left for 30 s. These paper points were transferred to 200 μl of sterile cell suspension solution from the Puregene DNA Purification kit (Flowgen, Ashby de la Zouch, United Kingdom) and taken immediately to the microbiology laboratory.

The root canal system was prepared using GT Rotary files (Dentsply Maillefer, Ballaigues, Switzerland) using a crown-down technique. Each root canal was irrigated with 2 ml of 4% NaOCl solution between each file size, and finally, 4 ml of a chelating agent (15% EDTA) was used to remove the smear layer followed by 4 ml of 4% NaOCl to remove the EDTA solution. The canal system was dried with paper points and then dressed with a nonsetting calcium hydroxide (Rootcal; Ellman Company). Cotton wool was placed in the pulp chamber, and a coronal seal of Coltosol (Coltene, Altstatten, Switzerland) that was at least 2.5 mm thick was placed on top of the pulp chamber.

At the second appointment, after the coronal dressing was isolated with a rubber dam and disinfected as described in "Sample details" (above), the dressing was removed with a sterile round steel bur in a slow-speed handpiece. The cotton wool pledget was removed, and two sterile paper points that had been soaked in sterile saline were placed in the root canal system for 30 s. These paper points were transferred to the sterile cell resuspension solution as described above.

(ii) Refractory cases. All aspects of gaining access to, sampling from, and treating the root canals from the refractory cases were identical to that for the de novo cases, except for the fact that when the root canals were initially found, an ISO 35 orifice shaper (Dentsply Maillefer) was used to remove coronal gutta-percha.

Cultural analysis. Each sample was sent immediately to the microbiology laboratory where it was mixed on a vortex shaker (Gallenkamp, Loughborough, England) for 30 s. Ten-microliter aliquots of the sample were pipetted onto two fastidious anaerobe agar plates (Bioconnections, Leeds, United Kingdom) supplemented with 7.5% (vol/vol) sterile defibrinated horse blood and streaked using a sterile loop. The plates were incubated as follows. One plate was incubated in an atmosphere of 5% carbon dioxide and 95% air, and the other sample was incubated in a Mark III anaerobic incubator (Don Whitley Scientific, Shipley, United Kingdom). Each plate was incubated for a maximum of 10 days and examined daily for evidence of bacterial growth. Each different colony type from positive cultures was subcultured for purity and identification. The results from Gram staining and atmospheric growth requirements of each colony type were used to determine the additional biochemical tests required to identify the cultures. API 32 Strep (Biomerieux, Basingstoke, England) tests were used to identify catalase-negative facultative gram-positive cocci, and API 32A tests were used to identify anaerobic bacteria and, facultative gram-positive bacilli. Other conventional tests for different bacteria were used where appropriate.

DNA extraction. A crude DNA lysate of gram-positive and gram-negative bacterial DNA was prepared for each sample as follows. Two microliters of lytic enzyme solution from the Puregene DNA Purification Kit (Flowgen) was added to each sample, which was then briefly mixed and incubated at 37°C for 30 to 45 min. Samples were pelleted and resuspended in 100 μl of sterile distilled water, boiled for 10 min, and then stored at −20°C until required. Ten microliters of sample was used as a template in each PCR.

PCR primers. The PCR primers which target the 16S rRNA gene had previously been published (22). The primers used were 27F (5′-AGAGTTTGATC [A/C]TGGCTCAG-3′) and 1492R (5′-TACGG[C/T]TACCTTGTTACGACTT-3′).

PCR amplification. PCR amplification was performed in a volume of 50 μl consisting of 5 μl of concentrated lysate or 10 μl of 1:10 and 1:100 dilutions of the lysate in sterile MilliQ-grade water (Millipore, Boston, Mass.). The remainder of the reaction mixture contained 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, 1.5 mM $MgCl_2$), 0.2 mM each of the four deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 1.0 U of *Taq* DNA polymerase (Promega UK Ltd., Southampton, United Kingdom) and 0.2 μM (each) PCR primer. Thirty-five microliters of DyNAwax (Finnzymes Oy, Riihitontuntie, Finland) was used to separate the primers and lysate from the rest of the reaction mixture to reduce the incidence of nonspecific PCR products and also improve the yield of the desired DNA fragments. The PCR was performed in an Omni-Gene thermal cycler (Hybaid, Teddington, United Kingdom). The cycling conditions were as follows: (i) an initial denaturation step at 94°C for 5 min; (ii) 35 cycles, with 1 cycle consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min; and (iii) a final extension step at 72°C for 10 min.

Stringent anticontamination procedures were employed when performing PCR as previously described (33). Positive and negative controls were included in every set of PCRs performed. The positive control was a standard reaction mixture containing 10 ng of bacterial DNA instead of sample, whereas the negative control contained sterile water instead of sample. Reaction products were either analyzed immediately or stored at −20°C until required.

Cloning of mixed 16S rRNA gene products from endodontic samples. The mixed 16S rRNA gene products were ligated into the pCR2.1-TOPO vector (Invitrogen BV, Groningen, The Netherlands) and transformed into *Escherichia coli* TOP10 cells (Invitrogen) according to the manufacturer's instructions.

Insert amplification and restriction fragment length polymorphism analysis of 16S rRNA gene clones. Fifty to 100 white colonies from each library were transferred from the transformation plates using sterile toothpicks to Luria-Bertani liquid medium with ampicillin at 100 μg/ml and incubated overnight at 37°C in an orbital shaker (Gallenkamp). One milliliter of the culture from each clone was then pelleted and resuspended in 100 μl of sterile distilled water. The suspensions were then boiled for 10 min, followed by pelleting of the cell debris. Five microliters of the resultant lysate from each clone was used as the template for PCR amplification using primers 27F and 1492R. The amplified insert from each clone was then digested with restriction endonucleases *Cfo*I, *Rsa*I, and *Hin*fI (Promega UK Ltd.) according to the manufacturer's instructions. Clones with identical profiles from all three enzymes were grouped together, and one representative from each group was selected for sequencing.

Sequencing. Plasmid minipreps were prepared from recombinants using the Promega Wizard Plus purification system (Promega UK Ltd.) according to the manufacturer's instructions. Sequencing was performed using the Thermo Sequenase sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Amersham, United Kingdom). The sequencing reactions were set up with 5 μl of plasmid DNA, 1 μl of sequencing primer (M13 Universal [−21] [5′-TGTAAA ACGACGGCCAGT-3′] or M13 Reverse [−29] [5′-GAGCGGATAACAATTT CACACAGG-3′], both labeled with IRD800 dye), 0.7 μl of dimethyl sulfoxide, and 14.3 μl of sterile molecular biology-grade MilliQ-grade water (Millipore). For each clone, 4.5 μl of the sequencing reaction was added to 1.5 μl (each) of

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A, C, G, and T reagent (primer termination mixes for each dideoxynucleotide). Reactions were overlaid with 1 drop of Chill-out 14 wax (Genetic Research Instrumentation, Braintree, Essex, England). Reactions were performed using a Primus96 DNA thermal cycler (MWG AG Biotech, Milton Keynes, United Kingdom) using the following cycling program: (i) initial denaturation at 95°C for 30 s; (ii) 20 cycles, with 1 cycle consisting of 10 s at 95°C, 30 s at 57°C, and 30 s at 70°C; and (iii) 15 cycles, with 1 cycle consisting of 10 s at 95°C and 30 s at 70°C. After thermal cycling, 6 μl of formamide loading dye was added to each reaction mixture. A portion (1.5 μl) of each denatured sequencing reaction mixture was run on a LI-COR Gene ReadIR 4200S DNA sequencing system (MWG AG Biotech) according to the manufacturer's instructions.

Sequence analysis. Sequences obtained from the LI-COR image analysis program were converted to FASTA format and analyzed for chimeric forms using the Chimera-CHECK 2.7 program from the Ribosomal Database Project II (23). After elimination of chimeric sequences, the partial 16S sequences were then compared with 16S rRNA gene sequences from the public sequence databases GenBank, EMBL, and DDBJ databases using the advanced gapped BLAST program, version 2.1 (1, 2). The program was run through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

Clone sequences with 98 to 100% identity with a GenBank sequence were considered to be of the same species as the highest score-matching sequence on the public sequence databases. Sequences with less than 98% identity with public database sequences were compared with close relatives from the BLAST results using the PHYLIP suite of programs and the closest related sequence; certain clones gave sequence identities as low as 90% but were still given in Table 3. Further phylogenetic analysis of particular clusters of sequences was also performed as follows. Sequences were aligned using the CLUSTAL W program (43) (CLUSTAL W Service at the European Bioinformatics Institute [http://www2.ebi.ac.uk/clustalw]; Rodrigo Lopez, Services Programme). A phylogenetically closely related sequence was selected as a suitable outgroup for each data set using the Ribosomal Database Project II phylogenetic tree browser (Center for Microbial Ecology, Michigan State University [http://www.cme.msu.edu/RDP/cgis/phylo.cgi]). After manual editing, a distance matrix was generated for each multiple alignment with the DNADIST program from the PHYLIP suite of programs using the Jukes Cantor algorithm (Phylogeny Inference Package, version 3.5c; [12]). The PHYLIP program NEIGHBOR was used subsequently to generate a tree file. Resultant trees were used to indicate the phylogenetic relatedness of the clone sequences. Reliability of the data was tested for each multiple alignment by bootstrapping with the PHYLIP program SEQBOOT (12) using 100 replicates. Bootstrap tree data sets were analyzed as described above with DNADIST and NEIGHBOR, and a consensus tree was selected using the PHYLIP program CONSENSE. Trees were visualized using the TreeView program (31) (version 1.6.1, Division of Environmental and Evolutionary Biology, Institute of Biomedical Life Sciences, Glasgow University, [http://taxonomy.zoology.gla.ac.uk/rod/treeview.html]).

RESULTS

Comparison between standard culture techniques and 16S rRNA PCR for detection of bacteria in root canals of periradicular periodontitis patients. A total of 41 samples, comprising 15 de novo cases and 26 refractory cases, were analyzed using culture techniques and an initial screening with the 16S rRNA-specific PCR. The lower rate of detection of bacteria using both techniques in the refractory second-appointment samples is probably due to the effective cleaning of the root canals.

There were a greater number of positive results identified for the de novo second-appointment and refractory first- and second-appointment cases by the PCR assay than by culture techniques (Table 1), although a larger sample size would be required to determine whether this was a significant difference.

The species isolated using standard culture methods (Table 2) were similar to some species that have been isolated by other researchers (6). They mainly comprised low-G+C- and high-G+C-content gram-positive bacteria from the order *Firmicutes*. In general, the bacteria found in the de novo cases differed from those in the refractory cases. However, many of the species cultured were found only once in the samples studied. There were three exceptions, with *Propionibacterium acnes*, *Streptococcus intermedius*, and a *Veillonella* species being found in both sample types. More samples would have to be analyzed before any conclusions could be drawn as to whether the differences in cultivable microflora from de novo and refractory endodontic infections were significant.

Bacteria identified using 16S rRNA PCR cloning and sequencing techniques. From the 41 samples, 73% of de novo samples were positive by PCR compared with 65% for the refractory samples. A subset of eight samples were selected for further analysis using the 16S rRNA PCR cloning and sequencing method. The samples chosen comprised two de novo cases (009 and 020), one de novo case (016) in which the tooth was associated with a sinus, and five refractory cases (007, 008, 017, 032, and 037). After restriction fragment length polymorphism analysis with three restriction enzymes, 100 clones from the eight libraries were sequenced. Sequence lengths from the clones ranged from 400 to 700 bp. Two pure cultures from one refractory case (032) were sequenced and are included in Ta-

TABLE 1. Positive results from culture and PCR analyses of samples from 15 de novo and 26 refractory cases of periradicular periodontitis

Case and appt[a]	No. of samples		
	Total	Positive by culture	Positive by PCR
De novo appt 1	8	6	6
De novo appt 2	7	3	5
Refractory appt 1	11	5	10
Refractory appt 2	15	4	7

[a] appt, appointment.

TABLE 2. Bacterial species cultivated from 9 de novo and 9 refractory cases of teeth with associated periradicular periodontitis

Bacterial species	No. of samples with the species	
	De novo	Refractory
Actinomyces naeslundii	2	0
Actinomyces viscosus	1	0
Corynebacterium sp.	1	0
Enterococcus faecalis	1	0
Fusobacterium sp.	1	0
Lactobacillus fermentum	1	0
Staphylococcus lentus	1	0
Streptococcus constellatus	1	0
Streptococcus sanguis type I	1	0
Streptococcus sanguis type II	2	0
Viridans group streptococci	1	0
Actinomyces israelii	0	1
Eubacterium sp.	0	1
Lactobacillus acidophilus	0	1
Peptostreptococcus micros	0	1
Peptostreptococcus prevotii	0	1
Porphyromonas endodontalis	0	1
Streptococcus intermedius	1	1
Streptococcus mitis	0	1
Streptococcus oralis	0	1
Veillonella sp.	1	1
Propionibacterium acnes	2	2
Propionibacterium granulosum	0	2

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TABLE 3. Sequencing results from three de novo cases (two cases without a sinus, one case with a sinus) and five refractory cases

Clone	% Identity[a]	Genus or species match[b]	Sample[c]		
			De novo	De novo, with sinus	Refractory
020 (09)	99	*Enterococcus faecalis* clinical isolate [AF039902]	+	−	−
020 (79)	99–98	Human oral bacterium C23 [AF202012]; *Streptococcus bovis* ATCC 43143 [AF104114]	+	−	−
009 (09)	91	*Propionibacterium* sp. oral strain FMA5 [AF287756]	+	−	−
020 (61)	99	*Streptococcus anginosus* strain 367 [AF145239]	+	−	−
009 (22)	95–96	*Streptococcus sanguis*-like bacterium [AF003928]; unidentified oral bacterium AP60-3 [AB028364]	+	−	−
009 (39)	90	Uncultured bacterium isolated from adult human fecal matter strain adhufec68 [AF132278]	+	−	−
009 (41)	99	Uncultured human oral bacterium A37 [AF201986]; *Selenomonas sputigena* [AF287793]	+	−	−
020 (63)	99	*Streptococcus mitis* ATCC 49456 [AF003929]; *Streptococcus* sp. oral clone CH016 [AY005044]; human oral bacterium AC25 [AF202015]	+	−	+
016 (38)	100	*Lactobacillus paracasei* JCM 8130 [D79212]; *Lactobacillus casei* ATCC 334 [D86517]	+	+	−
016 (21)	98	*Lactobacillus fermentum* strain KC5b [AF23316]	−	+	−
016 (49)	99	*Lactobacillus* sp. strain KC45a [AF243163]; *Lactobacillus gasseri* strain KC36a [AF243151]	−	+	−
016 (08)	99	*Pantoea* sp. [AF031958]	−	+	−
016 (32)	99	*Pantoea* sp. strain spl [AF199029]	−	+	−
016 (04)	98	*Prevotella* sp. oral clone AH005 [AY005053]	−	+	−
032 (73)	98	*Prevotella oris* ATCC 33573 [L16474]; *Prevotella* sp. oral clone F045 [AY005056]; unidentified *Eubacterium* clone 3.3 from human dentoalveolar abscess [U43698]	−	+	+
007 (19)	98	*Selenomonas infelix* ATCC 43532 [AF287802]	−	+	+
007 (15)	99	*Selenomonas* sp. oral clone CS015 [AF287791]	−	+	+
032 (PC5)[d]	99	*Capnocytophaga gingivalis* ATCC 33624 [X67608]	−	−	+
037 (39)	97	*Cytophaga* sp. strain P1 [AF260716]	−	−	+
037 (73)	98	*Dialister* sp. oral clone BS095 [AF287787]	−	−	+
032 (04)	99	*Eubacterium* sp. oral strain A35MT [AF287761]; *Eubacterium infirmum* W 1471 [U13039]	−	−	+
032 (PC6)	99	*Eubacterium yurii* ATCC 43713 [L34629]	−	−	+
007 (35)	99	*Fusobacterium nucleatum* ATCC 49256 [AJ006964]; *Fusobacterium naviforme* NCTC 11464 [AJ006965]	−	−	+
037 (47)	99	*Gemella haemolysans* ATCC 10379 [L14326]	−	−	+
032 (05)	97	*Mogibacterium* sp. [AB037875, AB021702, AB037874]	−	−	+
032 (03)	99	*Peptostreptococcus* sp. oral clone CK035 [AF287763]	−	−	+
008 (02)	99	*Prevotella nigrescens* NCTC 9336 [X73963]	−	−	+
007 (17)	98–96	*Prevotella* sp. oral strain B31FD [AY005061]; *Prevotella loeschii* ATCC 15930 [PVORR16SQ]	−	−	+
037 (34)	98	*Propionibacterium acnes* [AF073602]	−	−	+
007 (03)	98	*Solobacterium moorei* isolate RCA59-77 [AB031058]	−	−	+
017 (01)	99	*Streptococcus gordonii* ATCC 10558 [AF003931]	−	−	+
037 (29)	99	*Streptococcus intermedius* strain VAMC3276 [AF169357]	−	−	+
017 (51)	99	*Streptococcus salivarius* ATCC 13419 [M58839]	−	−	+
037 (71)	99	Uncultured *Micrococcus* strain MC6 [MSP404531]; *Rothia dentocariosa* ATCC 17931 [ROISSRNA]	−	−	+
032 (16)	95–93	Unidentified oral bacterium AP60-54 [AB028406]; *Eubacterium brachy* ATCC 33089 [Z36272]	−	−	+
007 (36)	98	*Veillonella dispar* DSM 20735 [X84006]; *Veillonella atypica* DSM 20739 [X84007]	−	−	+
007 (01)	98	*Veillonella* sp. oral clone AA050 [AF287782]	−	−	+

[a] Results were based on BLAST similarity scores for cloned sequences approximately 450 to 600 bp long.
[b] Match for partially sequenced clones from three de novo and five refractory cases. Accession numbers are shown in brackets.
[c] +, present in sample; −, not found in sample.
[d] PC, sequence from pure culture.

ble 3. Analysis of the clones using the Chimera-CHECK program indicated that one clone was chimeric, and this clone was discarded from the analysis. The bacteria found in the de novo and refractory cases which were identified from the BLAST searches are shown in Table 3. Where the percent identity scores were very close between the clone and the top two or three public database sequences, all alternative sequences have been stated in the table. Where a sequence appeared in more than one sample, only one clone name is given as an example.

The clone sequences identified came from the *Bacillus-Clostridium* low-G+C-content gram-positive group plus its gram-negative *Sporomusa* subbranch, the *Actinobacterium* high-G+C-content gram-positive group, the *Fusobacterium* group, the *Cytophaga-Flexibacter-Bacteroides* group, and the gamma

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subdivision of the *Proteobacteria* (Table 3). The results echoed those from the culture analysis in that the de novo and refractory cases displayed relatively low similarity in the species found with only five species being found in both case types. However, this difference again may well be due to the small sample size examined, and it would not be possible to conclude that the microflora from the two different types of endodontic infection differed significantly until a much larger sample size had been examined using appropriate statistical methods. *Streptococcus mitis* was found in both de novo and refractory cases, while the de novo case with the tooth associated with a sinus shared a *Lactobacillus* species with one de novo case as well as one *Prevotella* species and two *Selenomonas* species with the refractory cases. Many of the clone sequences had high percent identities with sequences from bacterial species, which have been already reported from infected root canals, such as *Streptococcus anginosus, Streptococcus mitis*, *Propionibacterium acnes*, *Fusobacterium nucleatum*, and *Fusobacterium naviforme* (6). However, other clone sequences had high percent identities with sequences which were identified only to the genus level. Some of these belonged to genera, which had previously been isolated from root canal infections. For example, clone 007(32), from a refractory first-appointment case, had 99% identity with an oral clone from *Selenomonas*, a genus which has been recovered from teeth with necrotic pulps (3). However, other clone sequences were similar to unidentified bacteria, such as clone 009(39), from a first-appointment de novo case, which matched with a bacterial isolate from human fecal matter. The percent identity for this clone was very low, as was the case for three other clones [009(09), 009(22), and 032(16)], and suggests that these may represent new species or even new genera.

Figure 1 shows the relatedness of the partial 16S rRNA sequences of eight clones from one de novo and two refractory cases of periradicular periodontitis with sequences which gave the highest BLAST similarity scores from the public sequence databases. All bootstrap values are indicated from 100 resampled data sets. The diagram is based on an alignment of 432 bases from 26 public database sequences and eight clone sequences from *E. coli* positions 54 to 486. In order to determine whether the topology of the tree was altered when the clone sequences were removed, a test tree was constructed (data not shown) from the database sequences by the same construction method. The length of the alignment was 532 bases, accounting for the fact the some of the sequences chosen from public databases for Fig. 1 were not complete. Nodes that had previously given low bootstrap values in Fig. 1 did so on this test tree, when the clone sequences were excluded and the overall tree topology was not affected. The only significant difference was the position of the node subtending the species *Mitsuokella multiacidus* and *Selenomonas ruminatum*. In Fig. 1 it grouped with the *Selenomonas sputigena-Anaerovibrio lipolytica* cluster. However, in the test tree it shifted to the *Dialister-Veillonella* cluster. In both Fig. 1 and the test tree, the nodes for the *Selenomonas sputigena-Anaerovibrio lipolytica* cluster and the *Mitsuokella multiacidus-Selenomonas ruminatum* branch had low bootstrap values in both cases.

DISCUSSION

In this study, there were more total samples that gave positive test results when the initial 16S rRNA gene PCR assay was used than when standard culture techniques were used. From the de novo first-appointment cases, the rates of detection of positive samples were the same for both techniques. For the de novo second-appointment cases, the PCR assay gave more positive results than culture techniques, and this was also the case for the first-and second-appointment samples from refractory cases. However, a greater number of samples would have to be analyzed to statistically determine that PCR is a significantly more sensitive technique for the detection of bacteria in root canal samples than culture analysis.

The results also indicated that for both de novo and refractory cases, fewer second-appointment samples were positive for the presence of bacteria than first-appointment samples. The reduced level of bacteria in the root canals by the second appointment was probably due to effective root canal therapy. However, whether the DNA came from viable bacteria cannot be inferred using whole genomic DNA as a template for PCR. Detection of viable bacteria would have to be determined using cDNA as a template for PCR and would therefore require a reverse transcription-PCR method. Such techniques are common to studies on environmental isolates but have also been used recently on a large scale to detect potential uncultivable bacteria in infected synovial tissue from patients with arthritis (20).

There are several stages within this study where it is possible to "lose" bacteria from the original sample or introduce contaminating bacteria from the environment. Some are common to all studies using these techniques. For example, it is possible that certain species in low abundance may not have been detected in the PCR due to competition from higher-titer templates (42). Other complicating factors are the various genome sizes and copy numbers of the *rrn* operons present in different bacteria (11), which may create biased results. The possibility of sequence variation between the *rrn* operon copies in one genome is also a complicating factor, as not all of these variant sequences are known for all species. The choice of DNA extraction procedure is also very important to ensure all species are lysed. The DNA extraction procedure used in this study involved a standard kit developed for the extraction of DNA from gram-positive and gram-negative bacteria, which should have lysed all but the most resistant microorganisms in the samples. All DNA extractions were performed in a laboratory separate from the laboratory where PCR was done, and the extracts were stored in a separate freezer. Stringent procedures were used to reduce the risk of contamination in the PCR to an absolute minimum, with the result that none of the negative-control reactions indicated the presence of bacterial DNA. One reason for the reduction of detected bacterial diversity in the samples in this study was that not every clone was analyzed from each sample because of the logistics involved. The study also found that many isolates designated as belonging to the genus *Enterobacter* were difficult to distinguish using 16S rRNA gene sequencing alone and other techniques would be required to distinguish the enteric bacteria (28). Finally, the limits to the breadth of phylogenetic diversity discovered using a single set of universal PCR primers was shown in this study. The pres-

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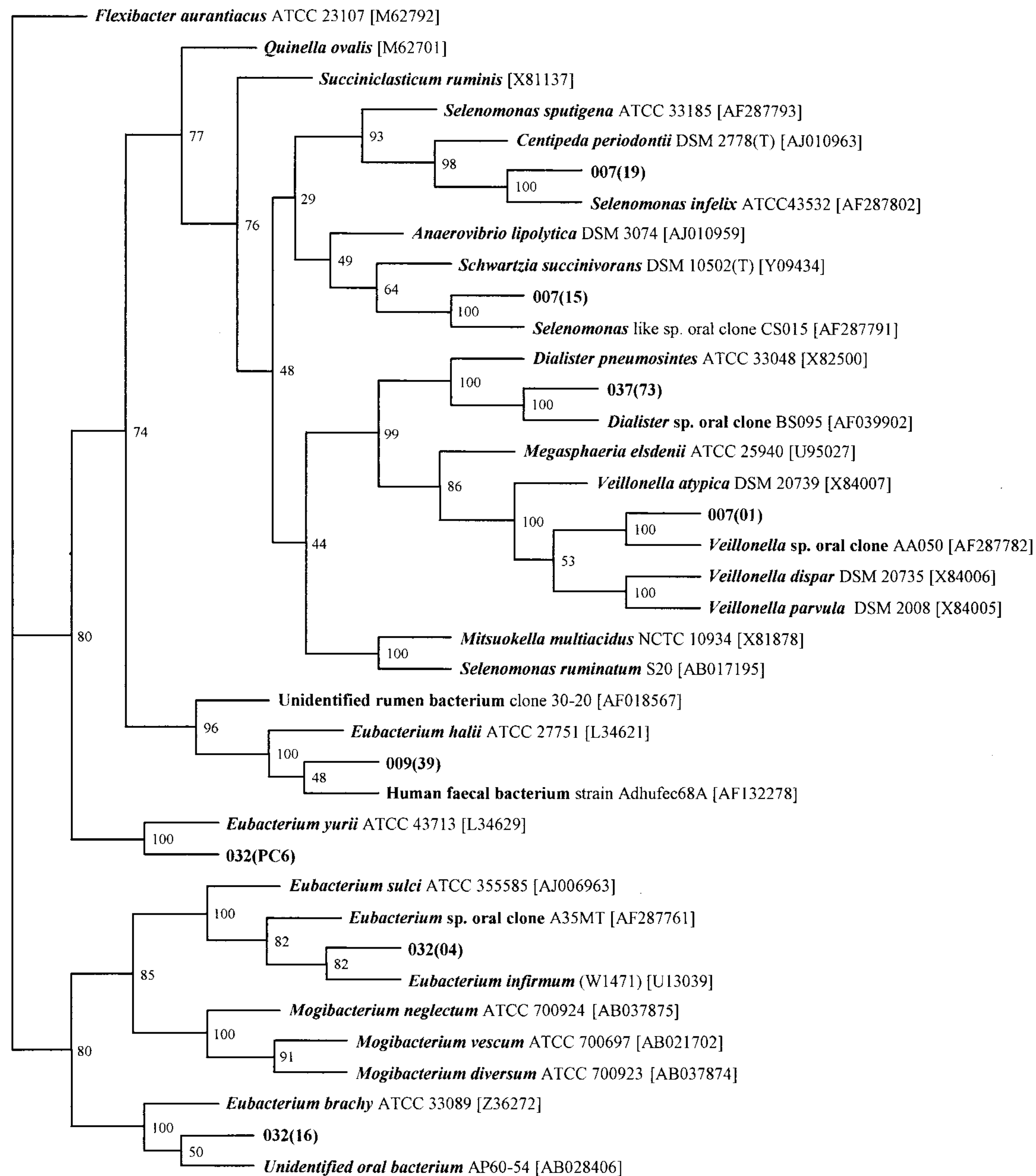

FIG. 1. Phylogenetic analysis showing relationships of cloned partial 16S rRNA gene sequences from one de novo and two refractory cases of teeth with periradicular periodontitis with identical regions of bacterial sequences from species within the Clostrideaceae and Sporomusa subgroups from the Firmicutes grouping. The eight clones are from one de novo case (009) and two refractory cases (007 and 032). One clone is from a pure culture (sample 032).

ence of *Actinomyces* species in culture but not in the cloned PCR products suggests the utility of using a comprehensive set of PCR primers for a more complete study of the microbial community of endodontic infections (7, 24). However, the molecular techniques used in this study were still able to detect sequences related to designated unculturable or uncultivated bacteria, which had not been previously associated with endodontic infections. There have been both positive and negative associations made for certain combinations of cultivable bacteria in root canal infections (39). However, the presence of uncultivable bacteria associated with endodontic infections means that these bacteria should be considered in further evaluations of potential endodontic pathogens.

For the 18 clinical samples with positive culture results from the 41 samples tested, there were differences between the microbial flora of the de novo and refractory cases. Each root canal had its own distinct profile of cultivable bacteria (data not shown), a finding which is prevalent in most studies of endodontic infections, and of the 22 species isolated, only 3 were common to both the de novo and refractory cases. Although it is not possible to make any definite conclusions from the small sample size examined here, previous studies have

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suggested that the microbial flora associated with de novo cases of periradicular periodontitis can differ from that associated with refractory cases (25, 26, 27, 41).

The genera detected through culture techniques on the nine refractory cases were largely similar to those found in refractory cases in previous studies (25, 41) except for the lack of detection of *Enterococcus faecalis*. The 9 de novo cases yielded genera which matched those most frequently isolated from endodontic infections prior to root canal treatment (30) with three exceptions. The exceptions were *Staphylococcus lentus*, a *Corynebacterium* species, and *E. faecalis*, which has been implicated along with other enteric species in persistent endodontic infections (10, 14, 36). The prevalence of *E. faecalis* in cultured samples from persistent endodontic infections ranges from 29% (27) to 46% (25). In this study, *E. faecalis* was not found in any refractory cases using culture or molecular methods, although it was isolated in one de novo case using both techniques. The results from this present study contradict previous findings that *E. faecalis* is found more frequently in refractory cases of apical periodontitis than in de novo cases. As more samples are analyzed, the frequency of *E. faecalis* in refractory cases may increase. If, however, they do not, another explanation is required for the difference. One possibility is that different populations have correspondingly different compositions of microbial flora in refractory root canal infections.

The cloning and sequencing results from the eight endodontic samples reflected the culture results in that each root canal had its own distinct microflora (data not shown). However, as stated previously, a greater number of samples would have to be examined in order to determine whether the species found in refractory cases differ significantly from those in the de novo cases. The few species common to both case types (Table 3) were as follows: the *Lactobacillus casei* or *Lactobacillus paracasei* clones from one de novo sample and the sinus-associated, de novo sample; the *Streptococcus mitis*-related clones from one de novo case and one refractory case; two *Selenomonas*-related clones and the *Prevotella oris*-related clones from the sinus-associated, de novo case and several refractory cases. The *Prevotella oris-Prevotella* sp. oral clone-unidentified *Eubacterium* 3.3 group of sequences was particularly prevalent, as it was found in three refractory cases and the sinus-associated, de novo case. The *Eubacterium* sequence was obtained using molecular techniques similar to those used in this study on samples from dentoalveolar abscesses (9), while the oral strain of *Prevotella* came from subgingival plaque. There were also clones isolated from two refractory cases with identities to the *P. loeschii* or oral strain of *Prevotella* from subgingival plaque sequence group. The level of relatedness of these clones was approximately 96 to 98% from partial sequence matches. As the full sequence is determined, this percentage may increase; however, if it does not, this too may represent a new *Prevotella* species. Kroes et al. (21) used similar molecular techniques on human subgingival plaque and detected clones related to *P. oris* and *P. loeschii* with identities of 96.3 and 97.5%, respectively. The identities for the clones were again based on partial sequences. It will not be until a greater number of full-length *Prevotella* sequences are deposited on the public databases that more thorough phylogenetic analyses can be performed to determine whether these sequences represent new phylotypes at the species level.

The main difference between the culture and molecular results was the greater level of species diversity detected per root canal, including potential uncultivable bacteria, detected by the molecular techniques. Table 3 indicated that the majority of the clone sequences represented genera and species similar to those found in previous culture studies on endodontic microflora using culture techniques. Most clones gave high matches over the 400 to 700 bp sequenced. However, certain other clones gave much lower percentage matches, and these are more definite candidates for new phylotypes at the generic level. For example, the closest related sequence to clone 009(39) was a bacterium isolated from human fecal matter (AF132278), with an identity of 90%. On Fig. 1, both sequences were located on the same node in a small cluster containing *Eubacterium halii* and an unidentified ruminal bacterium (AF018567), both of which were supported by high bootstrap values of 100 and 96, respectively. Kroes et al. (21) used maximum-likelihood phylogenetic methods to assess the relatedness of clone sequences amplified from human subgingival plaque. Some of their clones also matched unidentified ruminal bacteria (AF001716 and AF001743), which were in clusters related to *Sporomusa* species. There were two other clones on Fig. 1 of low sequence identity, 032(16) and 009(09), with low identities to *Eubacterium brachy* and an oral *Propionibacterium* species, respectively. Further sequencing may reveal these low-percent-identity clones to be new phylotypes, possibly representing new genera.

Several clones, 007(19), 007(15), and 009(41), shared similarities with those from the genus *Selenomonas*. However, in Fig. 1 all the *Selenomonas* database sequences and the two *Selenomonas*-related clone sequences did not cluster together but were scattered across three clusters, which contained other members of the *Sporomusa* subbranch. The nodes had low bootstrap values and also shifted slightly between Fig. 1 and also the test tree constructed to test the topology of Fig. 1. This may have been due to one or a combination of the following reasons. The low bootstrap values encountered were probably due to the high level of divergence between the clones and the nearest related sequences available for comparison from the public access databases. This was also affected by the fact that only partial sequences were aligned. During the analysis, certain clones were consequently forced into a phylogenetic position that was not entirely appropriate. These phylogenetic gaps will be filled only when more closely related sequences from other sequencing projects are added to the public databases and are therefore available for comparison. The phylogenetic positions of the fully sequenced clones can then be tested using a range of phylogenetic techniques, from distance matrix to maximum likelihood. The discovery of any cultivable bacteria with similar sequences would allow proper description and designation of new genera within the *Sporomusa* subgroup.

In summary, PCR produced a greater number of positive results for the de novo second-appointment and refractory first- and second-appointment cases than the culture techniques. However, the ability of PCR to be more sensitive than culture in detecting bacteria in root canals, the extent to which the microflora of de novo and refractory cases may differ, and the level of diversity per root canal will all require further investigation using molecular techniques with a larger data set of clinical samples. This study has indicated that the microbial

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consortium in any single infected root canal is much more diverse than has been shown using cultural techniques alone and can contain potentially uncultivable bacteria. Some of these bacteria may represent potential new bacterial phylotypes, which may be involved in endodontic infections and ultimately, the disease process of periradicular periodontitis and should therefore be considered in any future studies involved in defining endodontic pathogens.

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