Bacterial diversity in persistent periapical lesions on root-filled teeth

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Background: The purpose of this study was to analyze the bacterial diversity in persistent apical lesions on root-filled teeth by using culture-independent molecular methods.
Design: Twenty surgically removed apical lesions from therapy-resistant teeth were examined for the presence of bacterial DNA using PCR targeting the 16s ribosomal RNA gene, followed by cloning and sequencing.
Results: Bacterial DNA was detected in 17 of the 20 samples (85%). A total of 236 clones were analyzed. Seven different bacterial phyla were represented and a total of 75 different bacterial taxa were identified; 36% of the species have not yet been cultivated. Commonly detected bacterial species included *Fusobacterium* spp., *Prevotella* spp., *Tannerella forsythia*, *Porphyromonas endodontalis*, *Treponema denticola*, *Bacteroidetes* spp., *Peptostreptococcus* spp., and *Streptococcus* spp.
Conclusions: A wide range of bacteria was identified in periapical lesions on therapy-resistant teeth. These bacteria may contribute in the etiology of periapical infection and impede healing of these lesions.

Keywords: *bacterial phyla*; *endodontic infection*; *therapy-resistant teeth*; *16s ribosomal RNA*; *sequencing*

Teeth with apical periodontitis are highly prevalent amongst adults (1). The majority of apical periodontal lesions are located in previously root-filled teeth (1–6). The proportion of root-filled teeth with apical periodontitis was 43% in a study comprising 250 35-year-old inhabitants in Norway in 2003 (7).

Bacteria play the major role in the etiology of periapical lesion formation (8, 9), resulting in bone resorption which is an active process carried out by osteoclasts. Bacteria can gain access to the dental pulp through the crown or root surfaces in association with processes such as caries, periodontal disease, or trauma. A select group of microorganisms colonizing the normally sterile root canal space can cause pulp necrosis and inflammation in the surrounding bone. In well-controlled studies, where the root canal is properly instrumented and obturated, a success rate of 80–90% has been shown (10, 11). However, if bacteria are resistant to host defense mechanisms and/or therapy, they will survive within the root canal system and periapical tissue. In such cases, healing of the periapical lesion is compromised.

Culture-independent analysis using the bacterial ribosomal RNA (rRNA) genes has provided insights into the composition of mixed microbial communities in the root canals (12–19). It has been demonstrated that several uncultivated phylotypes and newly named species are present in the root canals associated with asymptomatic primary or persistent endodontic infections (18), and these can also take part in the root canal flora associated with symptomatic endodontic infections (15).

While several studies have shown that *Propionibacterium* and *Actinomyces* spp. can survive and thrive in persistent periapical lesions (20, 21), it is controversial whether or not a consortium of different species can live in apical lesions. At present there is scarce information about the role of microorganisms in apical lesions on therapy-resistant teeth. One recent culture-independent study (22) identified bacterial DNA, including unidentified and uncultured species, in samples from extraradicular infections. Studies employing molecular techniques have shown that the bacterial diversity in most environments is strongly underestimated in surveys using cultivation-based techniques (23–25), in some cases with less than 1% of the organisms being cultivable (26). As only 50% of the oral bacteria have been cultivated, it is likely that at least some members of the uncultivable flora may contribute to disease. Molecular assays have shown that 700–1,000 species can colonize different oral biofilms, far more than

detected by cultivation (25, 27). The development of 16s rRNA phylogenetic methodology has widened the scope of detectable microorganisms to include uncultivable organisms that may play a significant, as yet undefined role in the pathogenesis of oral disease (25, 27, 28).

Since tissue invasion is considered a major step in the pathogenesis and complete healing of the periapical lesion is the ultimate goal in the treatment of apical periodontitis, the understanding of endodontic disease requires knowledge on the entire bacterial population. The aim of the present study was to further investigate the bacterial diversity in persistent apical lesions on root-filled teeth by using culture-independent molecular methods.

Methods

Subjects and sample collection

Twenty patients (age 27–93, mean age 56) with persistent apical periodontitis were surgically treated with apicectomies by the same oral surgeon. Each patient was referred to the oral surgeon for the treatment of one tooth (Fig. 1). None of the teeth had previously responded to conventional endodontic therapy. Fistulous tracts or endo-perio-like lesions related to the teeth to be treated were not present. No patients had pain at the time of surgery and none had taken antibiotics the previous two months before surgery. Radiographically, it was evident that the patients referred for treatment had root-filled teeth with periapical radiolucencies of diameters varying
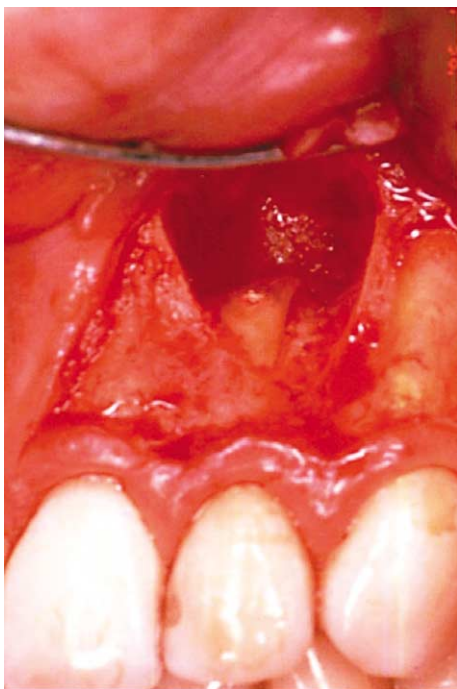

Fig. 1. Apical surgery with a submarginal incision to avoid contamination of the surgical area.

between 4 and 12 mm. Clinical inspection showed that all teeth had satisfactory coronal restorations.

All patients had an oral rinse with 0.2% chlorhexidine gluconate solution for 30 sec immediately before surgery. The present investigation used a full-thickness mucoperiosteal flap, reflected without involving the sulcular area of the periodontium. A submarginal incision was applied in order to avoid contamination of the periapical lesion with microorganisms present in the marginal area. Care was taken to avoid contamination from saliva. The whole lesion was enucleated before apicectomy. Samples were transferred to 200 μl of lysis buffer from the extraction kit QiaAmp® DNA Mini Kit (Qiagen, GmbH, Germany) and subsequently kept at −70°C prior to DNA extraction. The study was approved by the Norwegian Ethical Board. Informed consent was received from all patients.

Preparation of DNA and PCR amplification of 16s ribosomal RNA (rRNA) genes

DNA was extracted using the QiaAmp® DNA Mini Kit (Qiagen) according to the manufacturer's recommendation. PCR amplification of 16s rRNA gene was performed using previously published universal eubacterial primers (PA and PD) (MWG-Biotech, GmbH, Ebersberg, Germany) (29). Amplicons were purified using the QIAquick Gel Extraction Kit (Qiagen).

Cloning procedures and 16s ribosomal RNA (rRNA) sequencing

The amplicons were ligated into the plasmid vector pCR4-TOPO and transformed into *Eschericia coli* one Shot Top10 chemically competent cells (Invitrogen, Oslo, Norway). The transformed cells were plated onto Luria Bertoni agar plates, supplemented with kanamycin and incubated overnight at 37°C. From each sample, 12–36 clones were randomly selected to investigate bacterial species. A total of 236 clones were investigated. Correct sizes of the inserts were determined by PCR with M13 forward (−20) and M13 reverse primer (Invitrogen) followed by electrophoresis on 1% agarose gel. DNA amplicons (7 μl) were purified in a mixture containing 1 μl exonuclease and 1 μl shrimp alkaline phosphatase, and incubated for 15 min at 37°C and 15 min at 80°C, as recommended by the manufacturer (Amersham Biosciences, Cleveland, OH). The purified product was sequenced on both strands using the primers PB and PC (MWG-Biotech, GmbH) (29) and the Big-Dye Terminator mix (Applied Biosystems, Foster City, CA), according to the manufacturer's instruction. The sequencing reactions were run on an ABI 3730 DNA sequencer (Applied Biosystems).

The partial 16s rDNA sequences of approximately 500 bp were used to determine bacterial identity. For identification of closest relatives, the consensus sequences were

compared with 16s rDNA sequences in GenBank data-bases using the Blast 2.1 program from the GenBank Online Service. Clone sequences with 98–100% identity were considered to be of the same bacterial species. The sequences were aligned with the CLUSTAL W program. A neighbor-joining phylogenetic tree was constructed using the package MEGA, version 4.1 (30). Two hundred bootstrap trees were generated, and bootstrap confidence levels were determined with the MEGA program.

Results

In the present study, no PCR product was obtained from three samples (nos. 5, 11, and 20) using the primers PA and PD while 17 of 20 (85%) lesions were positive for bacterial DNA. Bacterial species from seven different phyla were represented (Table 1). *Bacteroidetes*, *Fusobacteria,* and *Firmicutes* dominated, followed by *Spirochaetes*, *Actinobacteria*, *Proteobacteria,* and *Synergistes.*

All specimens harbored 1–11 different bacterial species with a mean of seven species per lesion. The bacterial profiles of patients differed in diversity and bacterial dominance (Table 2). One patient (no. 15) presented with monoculture (*Fusobacterium* spp.), two patients had three species (no. 3: *Fusobacterium* spp., *Selenomonas*-like spp., uncultured bacterial species; no. 4: *Fusobacterium* spp., *Treponema* spp., *Propionibacterium* spp.), otherwise 4–11 different species were found within the patient samples. *Fusobacterium* spp. were the most prevalent (38% of taxa), detected in all but two lesions (88%). *Fusobacterium nucleatum* was the only bacterium that could be identified to species level within this genus. Several other strains of *Fusobacterium* were also found, including yet uncultivable strains. *Bacteroidetes* was the second most prevalent phylum with *Prevotella* as the most commonly detected genus (present in 35% of the lesions). Other well-known endodontic bacterial species such as *Porphyromonas endodontalis*, *Bacteroidetes* spp., and *Tannerella forsythia*

Table 1. Bacterial phyla in patients with refractory apical periodontitis

Phylogenetic group	No. of taxa	No. of clones (%)
Fusobacteria	13	90 (38.1)
Bacteroidetes	19	53 (22.5)
Firmicutes	19	32 (13.6)
Spirochaetes	4	10 (4.2)
Proteobacteria	2	5 (2.1)
Actinobacteria	3	4 (1.7)
Synergistes	1	1 (0.4)
Other[a]	18	41 (17.4)
Total	75	236 (100)

[a]Eighteen of the taxa were not assigned to any phylogenetic group.

Table 2. Distribution of bacterial taxa detected in the apical lesions

Subject no.	Bacteria[a] (no. of clones if >1)
1	*Fusobacterium* sp. (2)
	Prevotella sp. (2)
	Porphyromonas sp. (3)
	Bacteroidetes sp. (3)
	Uncultured *Bacteroidetes*
	Uncultured bacterium
2	*Fusobacterium* sp. (3)
	Prevotella sp. (3)
	Treponema sp.
	Campylobacter rectus (2)
	Synergistes-like sp.
	Uncultured bacterium
	Unidentified oral bacterium
3	*Fusobacterium* sp. (6)
	Selenomonas-like sp.
	Uncultured bacteria (3)
4	*Fusobacterium* sp. (6)
	Treponema sp. (4)
	Propionibacterium sp. (2)
6	*Fusobacterium* sp. (4)
	Peptostreptococcus sp. (4)
	Streptococcus sp.
	Uncultured bacteria (2)
	Unidentified oral bacterium
7	*Fusobacterium* sp. (11)
	Porphyromonas-like sp. (2)
	Tannerella sp. (4)
	Peptostreptococcus sp.
	Campylobacter sp.
	Eubacterium sp.
	Uncultured bacterium
8	*Prevotella* sp. (5)
	Porphyromonas-like sp.
	Treponema sp. (5)
	Peptococcus sp. (2)
	Eubacterium sp.
	Unidentified oral bacteria (2)
9	Uncultured bacteria (4)
	Unidentified oral bacteria (6)
10	*Fusobacterium* sp (2)
	Uncultured *Propionibacterineae*
	Streptococcus sp.
	Uncultured *Streptococcus* sp. (3)
	Corynebacterium sp.
	Uncultured bacteria (3)
	Uncultured *Bradyrhizobium* sp.
12	*Fusobacterium* sp. (2)
	Prevotella sp.
	Porphyromonas sp. (2)
	Porphyromonas-like sp. (3)

Table 2 (*Continued*)

Subject no.	Bacteria[a] (no. of clones if > 1)
	Bacteroidetes sp. (3)
	Tannerella sp.
	Unidentified oral bacteria (3)
13	*Fusobacterium* sp. (8)
	Porphyromonas sp. (2)
	Peptostreptococcus sp.
	Uncultured *Veillonella* sp. (2)
14	*Fusobacterium* sp. (2)
	Prevotella sp.(6)
	Peptostreptococcus sp.
	Streptococcus intermedius (2)
	Streptococcus sp.
	Uncultured bacterium
15	*Fusobacterium* sp. (28)
16	*Fusobacterium* sp. (8)
	Peptostreptococcus sp.
	Uncultured *Bradyrhizobium* sp.
	Uncultured bacterium
17	*Fusobacterium* sp. (3)
	Prevotella sp.
	Selenomonas sp.
	Solobacterium moorei strain N407
	Uncultured bacteria (6)
	Unidentified oral bacterium
18	*Fusobacterium* sp. (2)
	Prevotella sp.
	Porphyromonas sp. (5)
	Bacteroidales genomosp.
	Streptococcus sp.
	Uncultured bacterium clone bE15-57
	Unidentified oral bacteria (2)
19	*Fusobacterium* sp. (2)
	Tannerella sp. (2)
	Peptostreptococcus sp.
	Streptococcus sp. (2)
	Uncultured *Streptococcus* sp.
	Solobacterium sp.
	Uncultured bacteria (2)
	Unidentified oral bacterium

[a]Species name given only when 100% homology was seen.

were also identified. *Peptostreptococcus* sp. oral clone FG014 and *Streptococcus* spp. were the most prevalent within *Firmicutes* (41 and 29% of lesions, respectively). Additionally, *Selenomonas* spp., *Eubacterium* sp., and *Solobacterium moorei* were identified. Amongst *Spirochaetes*, *Treponema denticola* and strains that could not be identified at species level were found. *Campylobacter rectus* and the less commonly encountered bacterial species *Bradyrhizobium* spp. and *Synergistes*-like sp. oral clone BH007 were detected in two lesions. No association

of bacterial phyla or taxa could be observed using the chi-square test (Fisher's exact test, data not shown).

A high bacterial diversity, with 75 different bacterial taxa, were identified amongst the 236 clones investigated (Fig. 2). All 16s rDNA sequences, except for three, showed 98–100% similarity to sequences deposited to GenBank. Three isolates (uncultured bacterium clone 2:3 [FJ472593], uncultured bacterium clone 7:8 [FJ472594], and uncultured bacterium clone 14:12 [FJ472595]) were not closely related to any sequence previously in the GenBank database and, thus, might represent new phylotypes. Notably, 36% of the taxa represented yet uncultivable/unidentified species and these were present in 88% of the lesions comprising 23% of the total clones investigated.

Discussion

The present study assessed the occurrence of cultivable and also uncultivated phylotypes in persistent apical lesions on root-filled teeth using culture-independent molecular methods. Most studies of the bacterial etiology of periapical lesions have used either culture-based or targeted DNA approaches (31–33), and it is therefore likely that pathogens remain undiscovered. Sampling of microorganisms in the periapical area during surgery is difficult because the samples are easily contaminated with microorganisms from the marginal area and the indigenous oral microbiota unless care is taken to avoid such contamination (31–33). During surgery, a submarginal incision was performed in order to avoid contamination in the surgical field. It was, therefore, assumed that the bacterial species recovered in this study were present in the refractory lesions before surgery. Microbiological profiling has previously indicated that following a marginal incision, bacteria from the periodontal pocket reach the underlying tissues by surgeon-released bacteremia or direct translocation (32).

In the present study, three periapical samples were negative for bacterial DNA, thus suggesting that in few cases the bacteria causing the lesions may be present exclusively in the root canal system. Alternatively, sometimes DNA extractions and subsequent PCR simply do not work. Eighty-eight percent of the lesions harbored uncultivable/unidentified bacterial species, constituting 34% of the taxa identified. These findings clearly indicate that previously unrecognized bacterial species are present in the apical lesions and may participate in persistent apical periodontitis. This is in concordance with the findings of Noguchi et al. (22) who detected unidentified and uncultivable bacterial species in 11 of 14 samples from extraradicular biofilm. The pathogenicity of the uncultivable microbiota in endodontics is not known. However, it is reasonable that at least some members of this part of the microbiota are pathogenic and may contribute in the disease process.
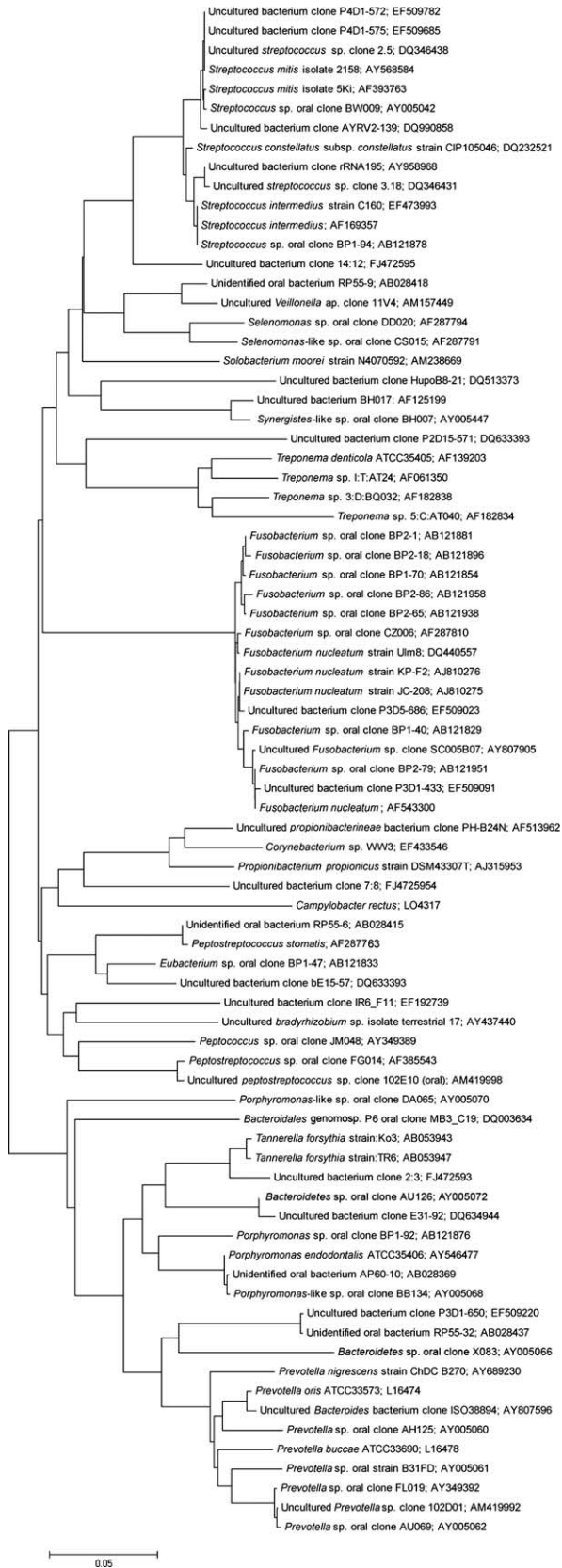
Microbiota in persistent apical lesions on root-filled teeth

Uncultured bacterium clone P4D1-572; EF509782
Uncultured bacterium clone P4D1-575; EF509685
Uncultured *streptococcus* sp. clone 2.5; DQ346438
Streptococcus mitis isolate 2158; AY568584
Streptococcus mitis isolate 5Ki; AF393763
Streptococcus sp. oral clone BW009; AY005042
Uncultured bacterium clone AYRV2-139; DQ990858
Streptococcus constellatus subsp. *constellatus* strain CIP105046; DQ232521
Uncultured bacterium clone rRNA195; AY958968
Uncultured *streptococcus* sp. clone 3.18; DQ346431
Streptococcus intermedius strain C160; EF473993
Streptococcus intermedius; AF169357
Streptococcus sp. oral clone BP1-94; AB121878
Uncultured bacterium clone 14:12; FJ472595
Unidentified oral bacterium RP55-9; AB028418
Uncultured *Veillonella* ap. clone 11V4; AM157449
Selenomonas sp. oral clone DD020; AF287794
Selenomonas-like sp. oral clone CS015; AF287791
Solobacterium moorei strain N4070592; AM238669
Uncultured bacterium clone HupoB8-21; DQ513373
Uncultured bacterium BH017; AF125199
Synergistes-like sp. oral clone BH007; AY005447
Uncultured bacterium clone P2D15-571; DQ633393
Treponema denticola ATCC35405; AF139203
Treponema sp. I:T:AT24; AF061350
Treponema sp. 3:D:BQ032; AF182838
Treponema sp. 5:C:AT040; AF182834
Fusobacterium sp. oral clone BP2-1; AB121881
Fusobacterium sp. oral clone BP2-18; AB121896
Fusobacterium sp. oral clone BP1-70; AB121854
Fusobacterium sp. oral clone BP2-86; AB121958
Fusobacterium sp. oral clone BP2-65; AB121938
Fusobacterium sp. oral clone CZ006; AF287810
Fusobacterium nucleatum strain Ulm8; DQ440557
Fusobacterium nucleatum strain KP-F2; AJ810276
Fusobacterium nucleatum strain JC-208; AJ810275
Uncultured bacterium clone P3D5-686; EF509023
Fusobacterium sp. oral clone BP1-40; AB121829
Uncultured *Fusobacterium* sp. clone SC005B07; AY807905
Fusobacterium sp. oral clone BP2-79; AB121951
Uncultured bacterium clone P3D1-433; EF509091
Fusobacterium nucleatum; AF543300
Uncultured *propionibacterineae* bacterium clone PH-B24N; AF513962
Corynebacterium sp. WW3; EF433546
Propionibacterium propionicus strain DSM43307T; AJ315953
Uncultured bacterium clone 7:8; FJ4725954
Campylobacter rectus; LO4317
Unidentified oral bacterium RP55-6; AB028415
Peptostreptococcus stomatis; AF287763
Eubacterium sp. oral clone BP1-47; AB121833
Uncultured bacterium clone bE15-57; DQ633393
Uncultured bacterium clone IR6_F11; EF192739
Uncultured *bradyrhizobium* sp. isolate terrestrial 17; AY437440
Peptococcus sp. oral clone JM048; AY349389
Peptostreptococcus sp. oral clone FG014; AF385543
Uncultured *peptostreptococcus* sp. clone 102E10 (oral); AM419998
Porphyromonas-like sp. oral clone DA065; AY005070
Bacteroidales genomosp. P6 oral clone MB3_C19; DQ003634
Tannerella forsythia strain:Ko3; AB053943
Tannerella forsythia strain:TR6; AB053947
Uncultured bacterium clone 2:3; FJ472593
Bacteroidetes sp. oral clone AU126; AY005072
Uncultured bacterium clone E31-92; DQ634944
Porphyromonas sp. oral clone BP1-92; AB121876
Porphyromonas endodontalis ATCC35406; AY546477
Unidentified oral bacterium AP60-10; AB028369
Porphyromonas-like sp. oral clone BB134; AY005068
Uncultured bacterium clone P3D1-650; EF509220
Unidentified oral bacterium RP55-32; AB028437
Bacteroidetes sp. oral clone X083; AY005066
Prevotella nigrescens strain ChDC B270; AY689230
Prevotella oris ATCC33573; L16474
Uncultured *Bacteroides* bacterium clone ISO38894; AY807596
Prevotella sp. oral clone AH125; AY005060
Prevotella buccae ATCC33690; L16478
Prevotella sp. oral strain B31FD; AY005061
Prevotella sp. oral clone FL019; AY349392
Uncultured *Prevotella* sp. clone 102D01; AM419992
Prevotella sp. oral clone AU069; AY005062

0.05

Fig. 2. Phylogenetic tree of bacterial phylotypes detected in persistent periapical lesions. The marker bar represents a 5% difference in nucleotide sequences.

Many of the species identified in the present study were the same that have been reported in extraradicular disease based on cultivation and DNA–DNA hybridization techniques. *Fusobacterium* is commonly isolated from root canal infections, and in the present study all except one lesion where bacterial DNA was detected harbored this organism. *F. nucleatum* was recently found in high proportions and levels in necrotic root canals with multiple displacement amplification, checkerboard DNA–DNA hybridization, and reverse-capture checkerboard hybridization (17, 34, 35). *Fusobacterium* species are known to co-aggregate with many species of oral bacteria and they are common participants in oral disease (36). To our knowledge, the study by Noguchi et al. (22) is the only published of extraradicular infection using PCR-based 16s rRNA gene assay. They identified bacterial DNA in 14 of 20 lesions and 113 bacterial species were isolated. *F. nucleatum* (14 of 14) and *Porphyromonas gingivalis* (12 of 14) were frequently detected. However, some teeth were extracted before examination which can give false positive results from the periodontal or indigenous oral microbiota. This might explain the high levels of the periodontopathogen, *P. gingivalis,* as opposed to our study where it was absent. However, *P. gingivalis* has been detected in small areas of periapical lesions by fluorescence *in situ* hybridization and DNA–DNA hybridization (31, 37).

Organisms belonging to *Porphyromonas* spp., *Treponema* spp., and *T. forsythia* are well-known pathogens in periodontal diseases. In the recent years, these species have also been detected in periapical tissue by DNA–DNA and *in situ* hybridization techniques (31, 33, 37). *T. forsythia* is difficult to culture and has not yet been recovered from root canals or periapical tissue with cultivation techniques. This organism is, however, frequently detected in necrotic canals with molecular methods (34, 38, 39). Although some authors have indicated that treponemes are important pathogens in endodontic disease (40–43), their fastidious growth has led these organisms to be underestimated in endodontic disease. It is only recently that molecular genetic analyses confirm their presence in infected root canals (14, 34, 44, 45) and periapical tissue (22, 31, 33, 37). Based on their frequent detection by molecular techniques, they might be potential endodontic pathogens. *Prevotella* spp. have frequently been found in different types of endodontic infections (46, 47), but have only been detected in the apical tissue with DNA–DNA hybridization and *in situ* hybridization (31, 37). A *Synergistes* phylotype was detected in one lesion in the present study. This phylotype has recently been shown to be a frequent member of the endodontic microbiota (18). Most isolates of *Solobacterium* are found in the oral cavity, particularly in periodontitis (16, 48, 49) and in patients with halitosis (50).

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A remarkably high diversity of species was identified in the periapical lesions. Although traditional views suggest that organisms surviving root canal treatment comprise a select group of the most robust organisms, the application of ecological parameters indicates that bacterial survival after root canal treatment will depend not only on the robustness of the organisms, but on how good an adaptor the organism is to the new limiting factors in their corresponding niches. Surface adherence by bacteria to form biofilms is an example of bacterial adaptation. Increasing amount of information is now available on the existence of polymicrobial biofilm communities on root tips (51) and also in the apical tissue (37). The foundation for this ecological approach to endodontic infections suggests disease is caused by a polymicrobial microbiota that undergoes physiological and genetic changes triggered by changes in the root canal environment. The frequent recovery of *Enterococcus faecalis* in root canals, associated with persistent infections, brought an intense research interest in this bacterium. Actually, *E. faecalis* is seldom identified in persistent apical lesions (22), and was not recovered in any lesion in the present study, indicating that this organism may not be as important when it comes to invading the apical area.

Molecular methods have expanded the list of endodontic pathogens by inclusion of some fastidious bacterial species and even uncultivated bacterial species that have never previously been found in endodontic infections by cultivation procedures. It has been shown that bacteria form biofilms in the root canal and extraradicular area, both on root tips, extruded gutta percha points, and directly in the periapical tissue (37, 51, 52). It is reasonable that extraradicular infections are caused during the acute phase of apical periodontitis or because of overinstrumentation by the dentist during root canal therapy. However, from a biofilm infectious viewpoint, it is possible that extraradicular sites are infected both during the acute or chronic phase of apical periodontitis. Bacterial species in the infected root canals may invade extraradicular sites and form biofilm which in turn can function as a focus of acute infections.

In conclusion, a wide variety of bacterial species, including a high percentage of uncultured/unidentified bacteria were found to colonize periapical lesions. Since endodontic infections develop in a previously sterile place which does not contain a normal microbiota, every bacterial species present in the mixed consortium has the potential to play a role in the infectious disease process. The present study has expanded the list of bacterial species present in the extraradicular area suggesting that the microbial etiology of periradicular disease is far more complex than previously anticipated.

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