

Highly Diverse Microbiota in Dental Root Canals in Cases of Apical Periodontitis (Data of Illumina Sequencing)

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Abstract

Introduction: Chronic apical periodontitis (CAP) is a frequent condition that has a considerable effect on a patient's quality of life. We aimed to reveal root canal microbial communities in antibiotic-naive patients by applying Illumina sequencing (Illumina Inc, San Diego, CA). **Methods:** Samples were collected under strict aseptic conditions from 12 teeth (5 with primary CAP, 3 with secondary CAP, and 4 with a periapical abscess [PA]) and characterized by profiling the microbial community on the basis of the V6 hypervariable region of the 16S ribosomal RNA gene by using Illumina Hi-Seq2000 sequencing combinatorial sequence-tagged polymerase chain reaction products. **Results:** Root canal specimens displayed highly polymicrobial communities in all 3 patient groups. One sample contained 5–8 (mean = 6.5) phyla of bacteria. The most numerous were *Firmicutes* and *Bacteroidetes*, but *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, *Spirochaetes*, *Tenericutes*, and *Synergistetes* were also present in most of the patients. One sample contained 30–70 different operational taxonomic units; the mean (\pm standard deviation) was lower in the primary CAP group (36 ± 4) than in the PA (45 ± 4) and secondary CAP (43 ± 13) groups ($P < .05$). The communities were individually different, but anaerobic bacteria predominated as the rule. *Enterococcus faecalis* was found only in patients with secondary CAP. One PA sample displayed a significantly high proportion (47%) of *Proteobacteria*, mainly at the expense of *Janthinobacterium lividum*. **Conclusions:** This study provided an in-depth characterization of the microbiota of periapical tissues, revealing highly polymicrobial communities and minor differences between the study groups. A full understanding of the etiology of periodontal disease will only be possible through further in-depth systems-level analyses of the host-microbiome interaction. (*J Endod* 2014;40:1778–1783)

Key Words

Bacteria, *Janthinobacterium lividum*, next-generation sequencing, oral microbial ecology, periapical abscess, periodontal diseases

Apical periodontitis is an inflammatory disease of periradicular tissues that is caused by microbial infection within the root canal system of the implicated tooth. This is mainly the consequence of dental caries when the root canal system is infected by oral microbiota (1, 2). Apical periodontitis is a frequent condition that has a considerable effect on a patient's quality of life. Endodontic treatment is essentially aimed at the elimination of microorganisms from the root canal system, and endodontic success is directly related to the presence or absence of microorganisms before root canal filling (3). Because the etiology and pathogenesis of apical periodontitis have not been finally elucidated, the current treatment options are not always successful. Therefore, additional research is urgently needed.

Microbial diversity in infected root canals has been widely explored by culture and, afterward, by molecular technology. Molecular methods have revealed a higher complexity of the endodontic microbiota than previously reported by cultivation approaches. In addition to detecting some cultivable species in increased prevalence, these methods have also expanded the list of putative endodontic pathogens by the inclusion of some fastidious bacterial species or even uncultivated bacteria that have never been previously found in endodontic infections. Therefore, there is a current trend to move away from the concept that a single pathogen causes a disease toward a more holistic concept that the community is indeed the unit of pathogenicity (1, 4). Recently, the first studies have been published that describe the results of pyrosequencing of root canal samples (2, 5–9). Furthermore, the latter have expanded the list of putative endodontic pathogens. In this context, the recognition of community profiles involved with some type of disease may represent an important step toward a better understanding of the pathogenesis of the disease in addition to setting the grounds for the establishment of more effective therapeutic protocols. We aimed to reveal root canal microbial communities in cases of chronic apical periodontitis by using Illumina sequencing.

Materials and Methods

Subject Population and Clinical Examination

The subject population was composed of 12 antibiotic-naive patients (ages ranging from 27–66 years) attending the Clinic of Stomatology at the University of Tartu, Estonia. Patients came to the clinic for root canal treatment or extraction between September 2010 and April 2011. Thorough anamnesis (systemic and local diseases, previous treatment, hygiene habits, allergy, and so on), intraoral status, and periapical

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x-rays were taken, which were all necessary for the upcoming treatment. To be included in the study, subjects had to have good systemic health.

A thermal test was performed using cold and hot. A cold test was performed with Endo-Frost (−50°C) (Roeko, Langenau, Germany) and a cotton pellet (size 00) (Roeko); the frozen cotton pellet was held on the isolated and dried tooth on the restoration-free surface for about 2–5 seconds or until pain was felt. The hot test was performed using silicone polisher (HiLuster; Kerr Corp, Orange, CA) with a 1:1 contra-angle handpiece (W&H, Bürmoos, Austria) without air and water cooling by touching the restoration-free tooth surface with about 4000 rpm for about 5 seconds or until the patient felt pain.

The electric pulp test or the vitality test was performed with the Elements Diagnostic Unit (Sybron Endo, Orange, CA) according to the manufacturer’s instructions or until pain was felt. The probe was touched to the restoration-free part of the isolated tooth until pain was felt.

A percussion test was performed with a mirror handle using gentle and uniform tapping on the occlusal and horizontal side of each tooth, and sound teeth were registered as zero feeling; the same kind of tapping was performed on the accused tooth, and the feeling of the patient was compared and described.

The palpation test was performed using uniform and solid pressure with the right index finger on the tip area of the root on both sides of the alveolar bone; the tooth was palpated by applying pressure on the tooth both vertically and horizontally. This was done bilaterally on both sides of the jaw to consider anatomic differences.

Periapical radiographs were taken by an experienced radiologist using the Planmeca Prostile Intra X-Ray unit (Planmeca OY, Helsinki, Finland) with the RVG 6100 sensor (Carestream Dental LLC, Atlanta, GA) at a parallel angle with RINN yellow (posterior) or blue (anterior) sensor holder (Dentsply Rinn, Elgin, IL). PA radiographs were analyzed using the Trophy DICOM program (Kodak).

Exclusion criteria were as follows: the presence of periodontal pockets greater than 5 mm, horizontal and vertical root fracture, deep carious lesions that made the tooth unrestorable, roots with previously apicectomized root tips with or without retrograde fillings, or any severe systemic condition like diabetes or immune suppression. In addition, subjects who received antibiotic or anti-inflammatory therapy in the previous 6 months were excluded. None of the sampled teeth presented posts, crowns, or bridges.

Five of the investigated teeth were diagnosed with primary chronic apical periodontitis (pCAP) and 3 with secondary apical periodontitis (sCAP). Four teeth with a periapical abscess (evolved from apical periodontitis) were included as the controls. Clinical data are presented in Table 1.

Sample Collection

Samples were collected from each of the 12 teeth under strict aseptic conditions as described previously (10). Initially, the tooth was cleaned with pumice and isolated with a rubber dam. The tooth and the rubber dam were cleaned with a solution of 3% hydrogen peroxide and then disinfected with 2.5% sodium hypochlorite (NaOCl) solution. The coronal access was made with the use of sterile round burs without water spray. The pulp chamber and the operator field were disinfected again using a swab soaked in 2.5% NaOCl. This solution was inactivated with sterile 5% sodium thiosulfate. Samples were collected from the root canal by means of a #08-25 H-type file (Dentsply Maillefer, Ballaigues, Switzerland) with a firm filing motion introduced as apically as possible but ~1 mm short of the apical foramen. This length was determined by means

TABLE 1. Clinical Information of the Study Group

Sample no	Age/sex	Tooth	Group diagnosis	Clinical diagnosis	Palpation	Percussion	Pulp testing	Restoration	Root filling	Radiographic changes	Pain intensity	
											without manipulation	Other
VV5	43M	36	sCAP	PAC	No pain	No pain	No reaction	None present	Part	PR	AS	Caries reaching pulp
VV7	23F	16	PA	AAC	No pain	+	No reaction	Composite	Part	PR	AS	Sinus tract
VV10	27M	11	pCAP	PAC	++	++	No reaction	None present	None present	PR	+++	Opened root canals visible orally
VV11	29F	36	sCAP	PAC	No pain	+	No reaction	Composite	Part	PR	AS	PR mesial root
VV13	27M	36	PA	SA	+++	+++	No reaction	None present	None present	PR	++	Swelling + pus
VV15	66F	34	pCAP	PAC	No pain	+	No reaction	None present	None present	PDL	AS	Caries reaching pulp
VV16	54M	21	PA	SA	+++	+++	No reaction	None present	None present	PR	+++	Swelling + pus
VV17	54M	11	PA	SA	+++	+++	No reaction	None present	None present	PR	+++	Swelling + pus
VV20	31F	36	sCAP	PAC	No pain	+	No reaction	Composite	Part	PR	AS	PR mesial root
VV23	33M	33	pCAP	PAC	No pain	+++	No reaction	None present	None present	PDL	+	Caries reaching pulp
VV24	41F	43	pCAP	PAC	+++	+++	No reaction	None present	None present	PR	++	
VV27	54M	22	pCAP	PAC	++	++	No reaction	Composite	None present	PR	+	

+, discomfort feeling or slight pain; ++, medium pain; +++, very painful to palpation/percussion; AAC, chronic apical abscess; AS, asymptomatic; F, female; M, male; No reaction, no reaction to all types of pulp testing (including thermal and electric pulp test); PA, periapical abscess; PAC, chronic apical periodontitis; Part, partially filled root canal system with poor quality; pCAP, primary chronic apical periodontitis (no root filling inside root canal system); PR mesial root, periapical radiolucency only around mesial root tip (other root tips not involved radiologically); SA, subperiosteal and submucosal abscess; sCAP, secondary chronic apical periodontitis (root filling inside root canal system); sinus tract, visible sinus tract on mucosa associated to the root tip.

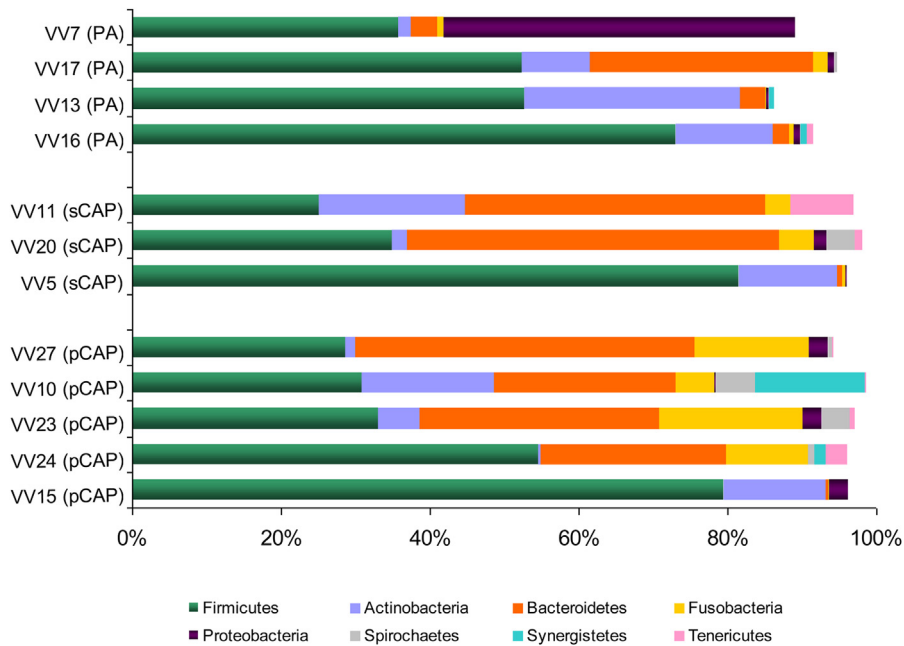


Figure 1. Microbial communities in root canals in case of chronic apical periodontitis and periapical abscess. Distributions (relative abundances) of phyla in samples arranged by diagnosis of the tooth. pCAP, primary chronic apical periodontitis; sCAP, secondary chronic apical periodontitis; PA, periapical abscess.

of a periapical radiograph and a plastic ruler (Dentsply Maillefer) and apex locator (Root ZX, Morita, Japan). Subsequently, 1–4 sterile paper points were introduced in the root canals at about the same level of the file, and each was left in place for 20 seconds to soak up the fluid. Both the file and paper points were then transferred to Eppendorf tubes containing 1 mL Brucella broth (Oxoid, Basingstoke, UK) as the transport medium. The samples were transported to the laboratory and processed within 2 hours.

DNA Extraction

Genomic DNA was extracted from the samples using the DNA QIAamp DNA Mini Kit (Qiagen, GmbH, Germany) according to the manufacturer’s instructions and stored at -80°C .

Illumina Sequencing

The samples were characterized by profiling the microbial community on the basis of the 16S ribosomal RNA gene by using the Illumina HiSeq2000 sequencing combinatorial sequence-tagged polymerase chain reaction products. Forward (5’-CAACGCGARG AACCTTACC-3’) and reverse (5’-ACAACACGAG CTGACGAC-3’) primers were used to amplify the bacterial-specific V6 hypervariable region of the 16S ribosomal RNA gene (11). Details of the sequencing method and data analysis are provided in Supplemental Table S1 (Supplemental Table S1 is available online at www.jendodon.com).

Statistical Analysis

For statistical analyses, SigmaStat software (Systat Software, Chicago, IL) was used. The differences between the 3 groups were analyzed using the Kruskal-Wallis 1-way analysis of variance by ranks and the Fisher exact test. A principal coordinate analysis performed in PAST software (Hammer, Harper & Ryan, Oslo, Norway) was used to visualize the similarities between samples. Statistical significance was assumed at $P < .05$ for all parameters.

Ethical Consideration

Participation in this study was voluntary. All subjects were informed about the study’s nature, and after signing an ethics committee–approved informed consent form, they were entered into the study. This work was approved by Ethics Review Committee on Human Research of the University of Tartu (protocol no: 195/T-11). All of the collected data were coded and isolated; personal data and measurements data were kept separately.

Results

All root canals specimens displayed highly polymicrobial communities in all 3 patient groups. One sample contained 5–8 (mean = 6.5) phyla of bacteria. The most numerous were *Firmicutes* and *Bacteroidetes*, but *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, *Spirochaetes*, *Tenericutes*, and *Synergistetes* were also present in most of the patients (Fig. 1).

The detected bacteria in the patients are provided in Table 2. Most of them were identified at the species level; however, some bacteria were identified to genus or higher taxonomic level. One sample contained 30–70 different operational taxonomic units (OTUs); the mean (\pm standard deviation) was lower in the pCAP group (36 ± 4) than in the PA (45 ± 4) and sCAP (43 ± 13) groups ($P < .05$). The communities were individually different, but anaerobic bacteria predominated as the rule. Figure 2 shows the clustering of microbial community data in different patient groups, revealing the most remarkable differences between the communities with pCAP diagnosis, whereas the sCAP and PA communities are more uniform. *Enterococcus faecalis* was found only in patients with sCAP. One periapical abscess sample displayed a significantly high proportion (47%) of *Proteobacteria*, mainly (40%) at the expense of *Janthinobacterium lividum*.

Discussion

In this study, we obtained microbiota samples from root canals having different diagnoses and sequenced the 16S ribosomal RNA

TABLE 2. Prevalence of Different Bacteria in Root Canals in Case of pCAP, sCAP and Periapical Abscess

Phylum	Genus	Species	Primary CAP (n = 5)	Secondary CAP (n = 3)	Periapical abscess (n = 4)	
Actinobacteria	<i>Atopobium</i>		3	3	4	
	<i>Atopobium</i>	<i>A. vaginae</i>	1	1	1	
	<i>Bifidobacterium</i>		2	2	4	
	<i>Corynebacterium</i>	<i>C. matruchotii</i>	3	1	3	
	<i>Corynebacterium</i>	<i>C. amycolatum</i>	1	0	1	
	<i>Gardnerella</i>	<i>G. vaginalis</i>	1	1	2	
	<i>Kocuria</i>	<i>K. palustris</i>	1	0	1	
	<i>Olsenella</i>		2	3	4	
	<i>Renibacterium</i>		3	1	1	
	<i>Scardovia</i>	<i>S. inopinata</i>	1	0	3	
		Family Coriobacteriaceae	1	0	2	
	Bacteroidetes	<i>Flavobacterium</i>	<i>F. succinicans</i>	1	1	1
		<i>Porphyromonas</i>	<i>P. endodontalis</i>	2	1	3
		<i>Porphyromonas</i>	<i>P. gingivalis</i>	1	1	2
<i>Prevotella</i>		<i>P. intermedia</i>	2	1	2	
<i>Prevotella</i>		<i>P. oris</i>	5	3	2	
<i>Prevotella</i>		<i>P. baroniae</i>	2	1	1	
<i>Prevotella</i>			4	3	4	
<i>Prevotella</i>		<i>P. nigrescens</i>	2	3	1	
<i>Prevotella</i>		<i>P. multiformis</i>	2	3	3	
<i>Prevotella</i>		<i>P. tannerae</i>	2	2	4	
<i>Tannerella</i>		<i>T. forsythia</i>	1	2	1	
Firmicutes		<i>Anaeroglobus</i>		4	3	3
		<i>Dialister</i>	<i>D. invisus</i>	5	3	4
		<i>Dialister</i>	<i>D. pneumosintes</i>	5	3	4
	<i>Enterococcus</i>	<i>E. faecalis</i>	0	1	0	
	<i>Filifactor</i>		3	3	3	
	<i>Lactobacillus</i>		2	2	3	
	<i>Lactobacillus</i>	<i>L. crispatus</i>	2	3	3	
	<i>Lactobacillus</i>	<i>L. iners</i>	4	2	3	
	<i>Lactobacillus</i>	<i>L. zeae</i>	3	1	2	
	<i>Lactococcus</i>	<i>L. lactis</i>	2	0	1	
	<i>Mogibacterium</i>		4	3	3	
	<i>Moryella</i>		2	1	1	
	<i>Moryella</i>	<i>M. indoligenes</i>	1	2	1	
	<i>Oribacterium</i>		4	3	4	
	<i>Peptostreptococcus</i>		2	2	1	
	<i>Pseudoramibacter</i>	<i>P. alactolyticus</i>	5	3	4	
	<i>Selenomonas</i>	<i>S. noxia</i>	3	2	3	
	<i>Shuttleworthia</i>	<i>S. satelles</i>	4	3	2	
	<i>Staphylococcus</i>	<i>S. epidermidis</i>	1	1	2	
	<i>Streptococcus</i>	<i>S. infantis</i>	5	3	4	
	<i>Streptococcus</i>		2	2	4	
	<i>Veillonella</i>	<i>V. parvula</i>	4	1	4	
		Family Veillonellaceae	5	3	4	
	<i>Fusobacteria</i>	<i>Fusobacterium</i>		5	3	4
	Proteobacteria	<i>Campylobacter</i>	<i>C. rectus</i>	2	2	3
		<i>Desulfobulbus</i>		2	0	3
		<i>Janthinobacterium</i>	<i>J. lividum</i>	3	1	1
			Family Enterobacteriaceae	1	2	1
	<i>Spirochaetes</i>	<i>Treponema</i>	<i>T. socranskii</i>	5	2	3
	Synergistetes	<i>Pyramidobacter</i>	<i>P. piscolens</i>	2	0	2
		TG5 group		2	2	1
	Tenericutes	<i>Bulleidia</i>	<i>B. extracta</i>	0	2	2
<i>Solobacterium</i>		<i>S. moorei</i>	4	2	3	

gene fragments using a next-generation sequencing platform. To the best of our knowledge, this is the first study investigating root canal microbiota using Illumina sequencing.

Our study revealed highly polymicrobial communities in the root canal samples of all diagnoses groups that tended to be dominated by anaerobes. The most prevalent phyla were *Firmicutes* and *Bacteroidetes*; however, *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, *Spirochaetes*, *Tenericutes*, and *Synergistetes* were also present in most of the samples. This coincides with previous data (1, 2, 9, 12). Some previous studies have revealed that the bacterial communities in primary endodontic infections are more diverse than those in persistent infections (13, 14). Our study did not confirm this

association; however, the sample number was quite low. At the same time, the older molecular methods are limited to the identification of only the most predominant community members (15).

Endodontic infections are similar to several other human endogenous infections in that no single pathogen but rather a set of species, usually organized in multispecies biofilm communities, is involved. Although there is little specificity as to the involvement of single-named species in the etiology of apical periodontitis, specificity becomes more evident when bacterial community profiles are taken into account. The concept of the community as pathogen is based on the principle that teamwork is what eventually counts. The behavior of the community and the outcome of the host/bacterial community

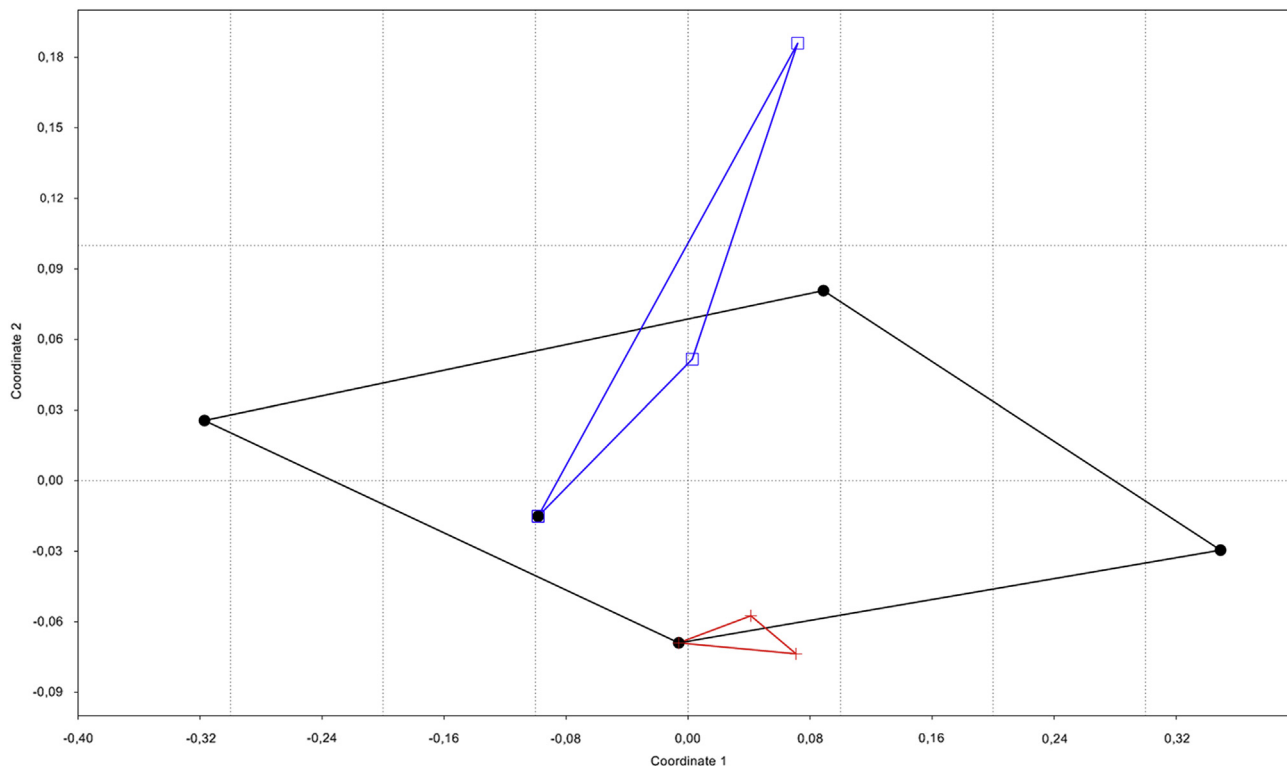


Figure 2. Two-dimensional plot of the principal coordinate analysis (PCoA) showing the clustering of microbial community data in different patient groups. The first 2 principal coordinate axes account for 45.6% and 11.2% of overall data variance, respectively. Black dot, pCAP; red cross, sCAP; dark blue rectangle, periapical abscess.

interaction will depend on the species composing the community and how the myriad of associations that can occur within the community affect and modulate the virulence of involved species. The virulence of a given species is allegedly different when it is in pure culture, in pairs, or part of a large bacterial “society” (community). In mixed communities, a broad spectrum of relationships may arise between the component species. Bacterial species that individually may have low virulence and are unable to cause disease can do so when in association with others as part of a mixed consortium (pathogenic synergism) (1).

We revealed many of the known root canal pathogens including gram-negative anaerobes *Prevotella* sp, *Porphyromonas* sp, *Fusobacterium* sp, *Tannerella* sp, and *Pyramidobacter piscolens* as well as gram-positive *Dialister* sp, oral spirochete *Treponema socranskii*, *Solobacterium moorei*, and many others (16, 17). We also noted an increased species count and the appearance of *E. faecalis* after treatment failure. The latter is gram-positive cocci that normally inhabits the intestinal tract but can also inhabit the oral cavity and cause several opportunistic infections. Being very resistant to environmental factors, this microorganism is hard to eliminate (18). It has been also shown that *E. faecalis* isolates from endodontic infections have a genetic and virulence profile different from pathogenic clusters of hospitalized patients’ isolates, which is most likely because of niche specialization conferred mainly by variable regions in the genome (19). It has been also shown that several difficult-to-culture or nonculturable bacteria may be involved in treatment failure including *S. moorei*, *Renibacterium* sp, *Oribacterium* sp, and others (20); these bacteria were found also in our samples.

At the same time, we also revealed some novel OTUs that have not been associated with apical periodontitis like *Gardnerella vaginalis*, TG5, and *J. lividum*. *G. vaginalis* is typically found from genital samples

and associated with bacterial vaginosis; however, it has been also found in cases of osteomyelitis, retinal vasculitis, acute hip arthritis, and other infections. Its main virulence factors are good adhesiveness, the ability to degrade mucin, the production of several enzymes, and pore-forming toxin (21). Like *G. vaginalis*, *J. lividum* was found from the samples of all patient groups. One abscess sample displayed a significantly high proportion of this gram-negative aerobic rod. *J. lividum* is a major constituent of the human skin microbiota that is able to produce the antibacterial, antiviral, and antifungal compound violacein. Therefore, it has been suggested also as probiotic bacterium against cutaneous infections (22). The high antimicrobial activity may be a reason for its very high concentration in the community. The poorly classified TG5 group belongs to the phylum *Synergistetes*. These taxa are exclusively oral, but none have ever been cultivated in the laboratory despite their widespread prevalence when molecular methods are used for their detection. The study of such bacteria is clearly important in order to improve our understanding of this complex environment, its bacterial inhabitants, their interactions, and their potential role in disease (23, 24).

The present study investigated the microbiological profile of 12 cases of apical periodontitis and periapical abscess using the next-generation sequencing Illumina technique. Our study confirmed the knowledge obtained from pyrosequencing studies that the bacterial diversity in these samples is far greater than previously described by culture methods. This study, like other microbiome studies, can certainly benefit from a larger sample size and a time series study design to get more information on the microbiota dynamics. It is possible that a shift in microbiota can occur in infected sites as the disease progresses, and such a shift can be very informative for prognosis and the design of treatment regimens (5).

In summary, this study provided an in-depth characterization of the microbiota of periapical tissues, revealing highly polymicrobial communities and minor differences between the study groups. A full understanding of the etiology of periodontal disease will only be possible through further in-depth systems-level analyses of the host-microbiome interaction.

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The authors deny any conflicts of interest related to this study.

Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (<http://dx.doi.org/10.1016/j.joen.2014.06.017>).

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