Microbial Analysis in Primary and Persistent Endodontic Infections by Using Pyrosequencing

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Abstract

Introduction: The aim of this study was to investigate the bacterial community profile of intracanal microbiota in primary and persistent endodontic infections associated with asymptomatic chronic apical periodontitis by using GS-FLX Titanium pyrosequencing. The null hypothesis was that there is no difference in diversity of overall bacterial community profiles between primary and persistent infections. **Methods:** Pyrosequencing analysis from 10 untreated and 8 root-filled samples was conducted. **Results:** Analysis from 18 samples yielded total of 124,767 16S rRNA gene sequences (with a mean of 6932 reads per sample) that were taxonomically assigned into 803 operational taxonomic units (3% distinction), 148 genera, and 10 phyla including unclassified. Bacteroidetes was the most abundant phylum in both primary and persistent infections. There were no significant differences in bacterial diversity between the 2 infection groups ($P > .05$). The bacterial community profile that was based on dendrogram showed that bacterial population in both infections was not significantly different in their structure and composition ($P > .05$). **Conclusions:** The present pyrosequencing study demonstrates that persistent infections have as diverse bacterial community as primary infections. (*J Endod* 2013;39:1136–1140)

Key Words

Diversity, operational taxonomic unit, persistent infection, primary infection, pyrosequencing, richness

Apical periodontitis is a common bacterial biofilm-induced disease caused by infection of the root canal system (1). Primary endodontic infection is caused by microorganisms that initially invade and colonize the necrotic pulp, which is characterized by a polymicrobial infection dominated by anaerobic bacteria (2, 3). In contrast, persistent infection is caused by microorganisms that were members of a primary or secondary infection and that, in some way, resisted intracanal antimicrobial procedures and endured periods of nutrient deprivation in treated canals (4). The microbial diversity between primary and secondary endodontic infection was previously surveyed by culture-dependent methods, and later it was reported that culture-independent molecular approaches were able to identify putative endodontic pathogens that had not been found previously (5). Thus, the bacterial communities in primary endodontic infections have been reported to be more diverse than those in persistent infections (4, 5). Previous molecular studies have shown that viable but non-culturable microorganisms are still numerous in infected root canals (6, 7). However, most molecular methods are limited to identification of only the most predominant community members (7). A seminal microbial ecology study demonstrated that low-abundant bacteria might serve as keystone species within complex mixed consortia (8).

Keystone species may occupy critical niches within a complex microbial community and thus are potentially important in maintaining the stability and virulence of a microbial community (9). Therefore, identification of all species in endodontic infection would not only be relevant from an ecological perspective but could provide understanding in the formation/development of these microbial communities in the root canal.

Massive parallel pyrosequencing technique is a rapid, high-throughput technology that allows for extensive sequencing of microbial populations, avoiding the biases inherent in cloning procedures (10). So far, 5 pyrosequencing studies have investigated the endodontic microbiota in primary infected root canals (11–15), and these have disclosed a greater bacterial diversity than non–pyrosequencing-based microbial studies (2, 7). However, compared with primary infections, there is a lack of in-depth pyrosequencing-based microbial studies in persistent infection. The microbial

diversity and community profiles related to persistent infections may be of utmost importance to gain a better understanding of disease pathogenesis and to further develop effective intracanal disinfection modalities. Therefore, the purpose of this study was to compare the bacterial community profiles of endodontic microbiota in primary and persistent infections with asymptomatic chronic apical periodontitis. The hypothesis was that there is no difference in diversity of overall bacterial community profiles between primary and secondary endodontic infections.

Materials and Methods
Participant Recruitment, Sample Collection, and DNA Extraction

All clinical protocols were approved by the Ethics Committee of the Seoul National University Dental Hospital (CRI09014), Seoul, South Korea, and written informed consent was obtained from the patients. Subjects with marginal periodontitis or those who had undergone antibiotic therapy within 2 months before collection and those with cancer, diabetes, and other immunodeficiency disorders were not included. Eighteen teeth from 33 adult patients (age 23–76 years) with radiographic evidence of apical periodontitis were finally included. Ten teeth (upper anterior 2, lower anterior 1, upper premolar 3, lower molar 2, and upper molar 2) with primary endodontic infections exposed to the oral cavity because of caries showed untreated necrotic root canals associated with asymptomatic chronic apical periodontitis. Eight root-filled teeth (upper anterior 1, lower anterior 1, upper premolar 3, lower molar 2, and upper molar 1) with persistent infections were diagnosed as asymptomatic chronic apical periodontitis. The teeth showed intact coronal restorations and no direct exposure of the filling materials to the oral cavity, and all the root canals had been filled more than 2 years earlier. Termini of the root canal fillings ranged from 0–2 mm short of the radiographic apex. Microbial samples from the primary and the persistent infected root canals were collected under strict aseptic conditions by using paper point method, which included rubber dam isolation and a 2-step disinfection protocol of the operative field with 2.5% NaOCl (6, 13). Sterility was checked by taking a swab sample of the access cavity and the tooth surface and streaking onto blood agar plates incubated at 37°C under both aerobic and anaerobic conditions; all samples were negative. DNA was extracted with the use of a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. To maximize DNA extraction from gram-positive bacteria, samples were preincubated in lysozyme for 30 minutes. DNA samples were quantified by using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Montchanin, DE).

Pyrosequencing

The following universal 16S ribosomal RNA primers were used for the polymerase chain reaction (PCR) reactions: 27F (GAGTTT-GATCMTGGCTCAG) and 518R (WTTACCGCGGCTGCTGG) (16). Primers were barcoded with 8 or 10 nucleotides for sorting individual samples. Each PCR reaction was carried out with 2 of the 25-μL reaction mixtures containing 60 ng bacterial DNA, 10 μmol/L of each primer (Macrogen, Seoul, Korea), and 1.25 U AccuPrime *Taq* DNA Polymerase High Fidelity (Invitrogen, Madison, WI), 50 mmol/L MgCl$_2$, and 10$\times$ PCR buffer. AC1000 thermal cycler (Bio-Rad, Hercules, CA) was used for the PCR according to the manufacturer's instructions. To avoid any biased results or possible contamination, negative control was included. After PCR amplification, the amplicons were visualized by gel electrophoresis and purified by 2 purification steps by using a QIAquick Gel Extraction Kit and a QIAquick PCR Purification Kit (Qiagen). To recover a sufficient amount of purified amplicon from the purification steps, two 25-μL reaction mixtures were combined into one mixture before amplicon purification. All amplicons were pooled and sequenced by using a 454/Roche GS-FLX titanium instrument (Roche, Nutley, NJ). Triplicate amplification and pyrosequencing were performed to obtain enough sequencing reads for each sample.

Analysis of Microbial Community and Diversity

Analysis of sequencing data was conducted by using Mothur software and the pipeline adapted from SOP from Schloss et al (17). By using multiplex barcodes, the flowgram files with more than 1 mismatch to the barcode, 2 mismatches to the primer, an ambiguous nucleotide, flows between 450 flows and 720 flows were sorted. Flowgrams were corrected and translated to DNA sequences by using shhh.flows command as implemented version of Pyronoise in Mothur (18). Chimera sequences were removed by applying the UCHIME algorithm with self-references (19). To remove and/or reduce PCR amplification and sequencing errors, sequences were denoised by using shhh.seqs as implemented version of denoise in AmpliconNoise (18, 20). Sequences were collected together from all samples, and a group file was created from which subject each sequence was generated. Unique sequences were aligned by using Silva reference aligned sequences (21), and sequences were trimmed including comparable region. To generate operational taxonomic units (OTUs) at 0.03 distances, distance matrix was created, and sequences were clustered by using the furthest algorithm. Then, sequences were classified by using the Human Oral Microbiome Database (HOMD). Classified sequences were analyzed into phylotype at phylum and genus levels. To equalize the sequencing efforts to compare microbial diversity, each total read was randomly resampled to 4700 reads. To compare bacterial diversity and richness between 2 infection groups, an observed number of OTUs, Shannon index, and rarefaction curve were measured as adapted in Mothur (22, 23). Statistical analysis of the bacterial diversity and richness was performed by *t* test. To compare bacterial communities from the 18 individual samples, 16S rRNA-based dendrogram was created in Mothur to visualize distance among individual samples. Statistical analysis of the bacterial community profiles between 2 infection groups was performed by analysis of molecular variance (24).

TABLE 1. Pyrosequencing Result Summary and Normalized Comparison of OTU Richness/Diversity Estimates in Primary and Persistent Endodontic Infections

	Sample number	Filtered reads*	Average length (base pair)	Subsampled reads	Richness/diversity[†] at 0.03 distance	
					Observed OTUs	Shannon index
Primary	10	67,870	326 ± 5	4,700	97.9 ± 55.8	0.5 ± 0.2
Persistent	8	56,897	332 ± 9	4,700	122.0 ± 47.2	0.6 ± 0.1

*Excluding chimeric and low quality (<300 base pair) sequences.

[†]Richness was observed from number of OTUs in each subject (average ± standard deviations), and diversity was measured from Shannon index estimates (average ± standard deviations). The *t* test showed there were no significant differences between 2 infection groups (*P* > .05).

Results

Bacterial Richness and Diversity

Pyrosequencing of the 18 samples with asymptomatic chronic apical periodontitis yielded 232,801 raw sequence reads. After removing low-quality sequence reads and the chimera, the final data contained 124,767 sequence reads (>300 base pairs) with an average length of 329 base pairs, corresponding to 6,932 reads/sample (range, 4,700–14,658). Each refined pyrosequencing read was first taxonomically assigned by aligning it to the sequences in the HOMD. In total, 803 OTUs at 0.03 distances were obtained, and from the OTUs, 10 bacterial phyla including unclassified and 148 genera were assigned by the HOMD. In addition, the mean number of species-level OTUs per sample was 97.9 ± 55.7 in primary infections and 122.0 ± 47.2 OTUs in persistent infections, respectively. The comparison of the sequence-size–normalized diversity estimates is shown in Table 1. According to the sequence-size–normalized OTU richness, there was no significant difference in diversity between 2 infection groups ($P > .05$). The rarefaction curves indicated bacterial richness of individual samples against sampling intensity (Fig. 1). The Shannon diversity index at 3% distinction showed no significant difference on diversity between 2 groups ($P > .05$, Table 1).

Bacterial Community Structure and Population Composition

Ten phyla were represented in the 18 root canal samples (Fig. 2). The most abundant phylum was Bacteroidetes (29.6%), followed by Firmicutes (23.2%), Actinobacteria (10.5%), Fusobacteria (13.1%), Proteobacteria (8.8%), Synergistetes (6.3%), unclassified (6.2%), and Spirochaetes (1.6%). The remaining 2 phyla (Chloroflexi and TM7) were of relatively low abundance (0.8% in total). Bacteroidetes was the most abundant phylum in both primary and persistent infections.

At the genus level, most genera occurred in relatively low abundance. In total, 129 genera were identified in primary endodontic infections, whereas 133 genera were identified in persistent endodontic infections. The top 50 genera ranked by their abundance (number of sequences) and richness (number of different OTUs) are shown in Supplemental Table S1 (available online at www.jendodon.com). *Prevotella*, *Propionibacterium*, and *Pyramidobacter* were abundant in primary endodontic infections, whereas *Fusobacterium*, *Porphyromonas*, and *Prevotella* were abundant in persistent infections. The largest numbers of OTUs were found in *Propionibacterium*, *Prevotella*, and *Fusobacterium* (>10,000 OTUs). Some genera such as *Enterococcus* and *Camphylo-*
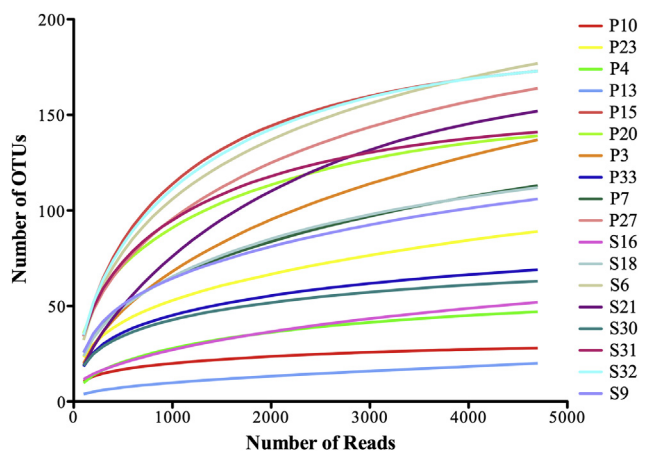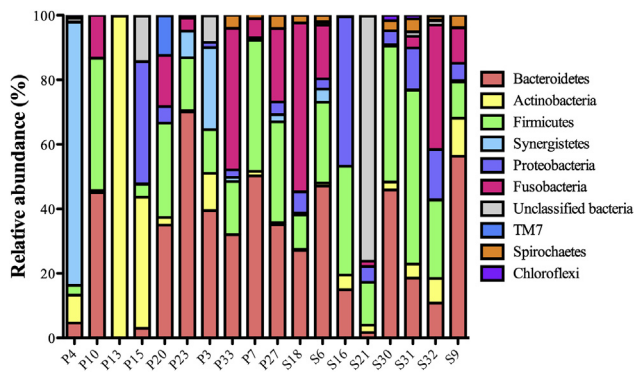

Figure 2. A comparison of community profiles between primary and persistent endodontic infection groups at phylum level. P indicates primary infection group, and S indicates persistent infection group in the x-axis.

bacter were identified as low abundant (<1%). *Bifidobacterium*, *Scardovia*, and *Parascardovia* were not found in the phylum Actinobacteria.

Dendrograms based on theta-YC (Fig. 3*A*) and Jaccard (Fig. 3*B*) showed that both community structure and population composition were not significantly different between primary and persistent infections ($P > .05$).

Discussion

The present study demonstrated that deep-coverage pyrosequencing technology facilitated access to low-abundance bacteria in primary and persistent endodontic infections and that the overall diversity and richness of persistent infections appear to be much higher and more complex than previously reported. This contradicts the results of previous studies where primary infections had more diverse and complex community profiles than persistent infections (4, 5). In addition, the mean numbers of OTUs per sample and OTU richness in primary and persistent infections were higher than those of previous culture and molecular studies (3, 5). These results might be due to the resolution and deep coverage of sequence identification by pyrosequencing (25).

At the phylum level, previous culture or molecular studies reported that Firmicutes was the most dominant phylum in the primary and persistent endodontic infections (2, 3). In previous pyrosequencing studies performed mostly in primary infected canals, the most dominant phyla were Bacteroidetes (11), Firmicutes (13–15), or Proteobacteria (12). In the present study, Bacteroidetes was the most abundant phylum in both primary and persistent infections. These differences and the degree of agreement might be due to the difference of sampling areas (11, 12, 14), sampling methods (11, 14, 24), sequencing platforms used (12), geographical location (26), and sequence abundances (27).

Regarding the accuracy of bacterial sampling methods for microbial analysis, a recent pyrosequencing study mentioned that extraction and cryopulverization might be the most complete microbial sampling method (14). However, this technique cannot be applied in all clinical cases because selected tooth should be extracted for microbial analysis. The paper point method used in the present study may not represent the whole microbial information in complex root canal systems including dentinal tubules, isthmus, and lateral (accessory) canals, especially at apical third areas (28). This indicates that the overall bacterial diversity in both endodontic infections may be considerably higher than currently identified.

At the genus level, most genera appeared in relatively low abundance. *Prevotella*, *Propionibacterium*, and *Pyramidobacter* were frequently found in primary infections, which is consistent with previous studies (3, 6, 15). *Fusobacterium* was highly found in persistent infections, which is consistent with the results of previous studies (5,

Figure 1. Rarefaction curves of partial 16S rRNA gene sequences calculated for OTUs at 3% divergence in primary and persistent endodontic infections. P indicates primary infection group, and S indicates persistent infection group.
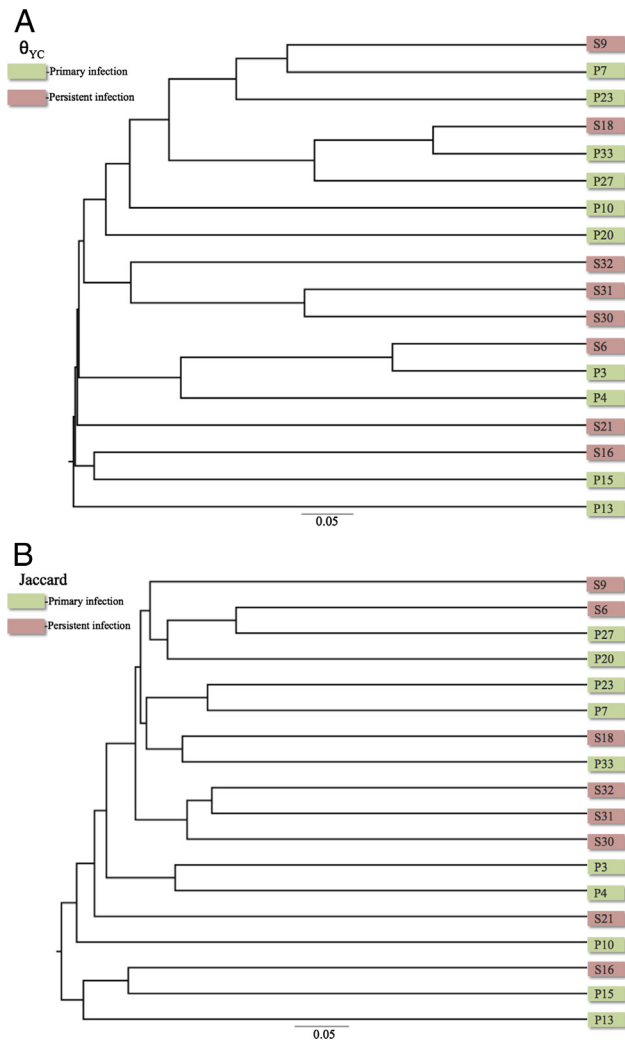
Figure 3. A 16S rRNA-based dendrogram for comparing bacterial communities from the 18 individual samples, which demonstrates that both community structure and population composition were not significantly different between primary and persistent infections. (*A*) Theta-YC sample tree generated by using Mothur (θ_{YC}); (*B*) Jaccard sample tree generated by using Mothur. P indicates primary infection group, and S indicates persistent infection group.

29, 30). Interestingly, the genus *Bifidobacterium* was not found in primary and persistent endodontic infections in the present study. *Bifidobacteria* were previously reported to be present in child endodontic samples, subgingival, and dentin/root caries (31). A possible explanation for this difference of the present results might be due to the bias from the primers used (32). *Enterococcus,* which is the most frequently detected bacterium in root-filled teeth with persistent infections (4, 33), was detected as a low-abundant (0.7%) genus of persistent endodontic infections in our study. The status of *E. faecalis* as the main causative pathogen of endodontic treatment failure has been recently put into question because it has not been detected (34) or is rarely one of the most dominant species in root canal–treated teeth (5). This species was considered as a secondary invader through coronal leakage or opening for drainage (35). Our sampling method might not completely touch the original biofilms in infected canals, and no existence of coronal leakage might be the reason for the low prevalence of *Enterococcus* in the present study.

Careful comparison of diversity estimators revealed that persistent infections did not show significant difference in their diversity compared with primary infections. Also, bacterial community profiles analyses from dendrograms based on distance among microbiome from each individual showed that bacterial population in persistent infection is not significantly less diverse as those in primary infections in terms of both structure and composition. These data will contribute to a further understanding of the microbial etiology and pathogenesis of persistent endodontic infections.

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.2013.05.001).

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