Microbial Analysis of Endodontic Infections in Root-filled Teeth with Apical Periodontitis before and after Irrigation Using Pyrosequencing

Homan Zandi, DDS,[*†] *Anne K. Kristoffersen, MSc, PhD,*[†] *Dag Ørstavik, DDS, PhD,*[*] *Isabela N. Rôças, DDS, MSc, PhD,*[‡] *José F. Siqueira, Jr, DDS, MSc, PhD,*[‡] *and Morten Enersen, DDS, PhD*[†]

Abstract

Introduction: The root canal microbiota in root-filled teeth with post-treatment apical periodontitis before and after chemomechanical instrumentation and irrigation with either 1% sodium hypochlorite (NaOCl) or 2% chlorhexidine digluconate were analyzed by using the pyrosequencing method. **Methods:** Samples from 10 root-filled teeth with apical periodontitis undergoing retreatment were taken before (S1) and after (S2) preparation using irrigation with either NaOCl (n = 5) or 2% chlorhexidine digluconate (n = 5). DNA was extracted, and the 16S rRNA gene (V3-V5) variable regions were amplified and subjected to pyrosequencing (GS junior 454) to determine the bacterial composition. **Results:** Pyrosequencing yielded 43,797 sequence reads in S1 and 9196 in S2 samples. Overall, 125 bacterial species belonging to 68 genera (S1, 59; S2, 38) and 9 phyla were found. The most abundant and prevalent phyla in S1 and S2 samples were Firmicutes, Fusobacteria, Bacteroidetes, and Actinobacteria. The most represented, abundant, and prevalent genera in S1 and S2 samples were *Streptococcus* and *Fusobacterium.* The most prevalent species in S1 and S2 samples were *Fusobacterium nucleatum ss. vincentii, Streptococcus oralis/mitis, Streptococcus intermedius*, and *Streptococcus gordonii.* The mean number of species per root canal was 20 (range, 4–37) in S1 and 9 (range, 4–15) in S2, respectively. **Conclusions:** A high interindividual diversity was observed in both S1 and S2 samples, with no difference between the two irrigation groups. *F. nucleatum ss. vincentii* and some *Streptococcus* species were the most prevalent species in pre-preparation and post-preparation samples during retreatment of root-filled teeth with infection. *(J Endod 2017;■:1–7)*

Key Words

Chlorhexidine, endodontic retreatment, post-treatment apical periodontitis, pyrosequencing, 16S ribosomal RNA gene, sodium hypochlorite

Significance

This study analyzes the composition of the microbiota before and after irrigation with 2% chlorhexidine (CHX) and 1% sodium hypochlorite (NaOCl) in treatment of infected root-filled teeth. We show a high interindividual diversity and a high prevalence of streptococci and fusobacteria in the microbiota before and after irrigation, with no differences between the irrigants.

Post-treatment apical periodontitis is primarily caused by bacteria persisting in or reinfecting the root canal system after initial endodontic therapy (1). Bacteria associated with endodontic infections have been identified by culture and molecular methods (2). During the last decade, several studies have used advanced high-throughput sequencing approaches, including the pyrosequencing technique, to evaluate the bacterial composition of endodontic infections (3–11). Some of these studies have evaluated the endodontic microbiota in persistent infections (9–11) and reported that they may be at least as diverse as primary infections (9, 11). Pyrosequencing provides a large number of reads per run, resulting in large sampling depth and detection not only of the dominant bacterial community members but also of low-abundant species (3). This is important because low-abundant species, which are difficult to detect by less sensitive techniques, may be as relevant for the ecology and pathogenicity of the bacterial community as high-abundant species (4).

Endodontic treatment entails the reduction or elimination of microbial infection by disinfecting irrigation solutions. Sodium hypochlorite (NaOCl) and chlorhexidine digluconate (CHX) in various concentrations have been widely used for this purpose (12–14). Several *in vitro* and *in vivo* culture-dependent studies have compared the antibacterial effects of these irrigation solutions on the endodontic microbiota and reported conflicting results (15–18). Culture-independent methods have also been used to compare the antimicrobial clinical performance of NaOCl and CHX during treatment (14, 19) or retreatment (13) and reported no significant difference between them. The *in vivo* studies evaluated the levels and prevalence of total bacteria or some bacterial groups, but they did not include broad-range analysis of the bacterial community

*Department of Endodontics, Institute of Clinical Dentistry, University of Oslo, Oslo, Norway; †Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway; and ‡Department of Endodontics, Estácio de Sá University, Rio de Janeiro, Brazil.

Address requests for reprints to Dr Homan Zandi, Department of Endodontics, Institute of Clinical Dentistry, University of Oslo, PO Box 1109 Blindern, N-0317 Oslo, Norway. E-mail address: homanz@odont.uio.no

0099-2399/$ - see front matter

https://doi.org/10.1016/j.joen.2017.11.019

composition. To our knowledge, no study has so far evaluated the clinical antibacterial effects of endodontic retreatment with NaOCl or CHX irrigation by using sensitive, high-throughput sequencing methods for bacterial identification.

The purpose of this study was to analyze the bacterial community in root-filled teeth with post-treatment apical periodontitis before and after chemomechanical preparation using either 1% NaOCl or 2% CHX as irrigants by means of the pyrosequencing method.

Materials and Methods

Patient Data and Retreatment Procedures

Root canal samples from patients with persistent apical periodontitis and included in a previous randomized clinical study (13) were stored frozen and available for this study. Only samples showing positive polymerase chain reaction (PCR) results for bacteria in both initial and post-preparation samples and still having sufficient DNA for re-analysis were included. Details on patient allocation, randomization, retreatment procedures, and bacteriologic sampling, including initial (S1), post-irrigation (S2), and post-medication (S3) samples, were as described previously (13). Briefly, retreatment and sampling procedures were as follows. The operative field was disinfected twice with hydrogen peroxide and NaOCl, the latter was neutralized with 5% sodium thiosulfate (Sigma-Aldrich, St Louis, MO), and sterility controls samples were taken from the access cavity and operative field. These samples yielded negative results after PCR analysis. Coronal gutta-percha was removed, an endodontic instrument used to remove the filling material in the apical canal was cut, and the fragment with attached root-filling material was placed in a cryotube containing Tris-EDTA buffer (10 mmol/L Tris-HCL, 1 mmol/L EDTA, pH = 7.6) (Sigma-Aldrich). The canal was filled with saline and sampled with sterile paper points, which were also transferred to Tris-EDTA buffer (sample S1). Apical preparation was completed to the working length with hand nickel-titanium files (NitiFlex; Dentsply Maillefer, Ballaigues, Switzerland) ranging from sizes 40 to 60. The irrigants used were 1% NaOCl (n = 5 teeth) or 2% CHX solution (n = 5 teeth). After preparation, the irrigant was neutralized with either 5% sodium thiosulfate (for NaOCl) or a mixture of 0.07% lecithin, 0.5% Tween 80, and 5% sodium thiosulfate solutions (Sigma-Aldrich) (for CHX). Next, the canal walls were filed, and a post-instrumentation sample (S2) was taken by using sterile paper points.

The patients' characteristics are shown in Table 1. One incisor, 4 premolars, and 5 molars were included. Radiographically, the diameter of the periapical radiolucency ranged from 2 to 7 mm. Termini of the previous root canal fillings ranged from 0 to 4 mm short of radiographic apex, with no overfilling. The teeth had intact coronal restorations, with no obvious exposure of the root-filling material to the oral cavity.

TABLE 1. Distribution of Patients' Characteristics

Sample	Tooth no. (ADA)	Sex	Age (y)	Symptoms
NaOCl				
3	13	M	64	No
8	4	F	46	No
18	31	M	91	No
35	25	M	45	No
55	13	M	68	No
CHX				
56	15	F	29	Yes
9	19	M	54	No
11	14	M	51	Yes
45	19	M	49	No
46	13	M	80	Yes

ADA, American Dental Association.

DNA Extraction and Amplification. DNA extraction from clinical samples was performed by using the MasterPure DNA isolation kit from Epicentre (MCD85201; Epicentre Biotechnologies, Illumina, Madison, WI). The 16S ribosomal RNA gene fragments from bacterial DNA were amplified with PCR by using the following universal eubacterial primers: forward primer 334f (5′-CCAGACTCCTACGGGAGGCAGC-3′) and reverse primer 939r (5′-CTTGTGCGGGCCCCCGTCAATTC-3′) targeting the V3-V5 hypervariable region (20, 21). PCR reactions were performed with 32 cycles in 20 μL mixture of OneTaq mastermix (New England Biolabs, Ipswich, MA) in an Applied Biosystem (Foster City, CA) PCR cycler. A second PCR with the index fusion adaptor primer A with 16S *rRNA* 334f sequence and adaptor primer B with 16S *rRNA* 939r sequence was performed with 26 cycles. Amplicons were purified with Agencourt Ampure XP Beads (Agencourt Bioscience Corporation, Beckman Coulter Company, Indianapolis, IN), followed by DNA quantification and quality examination in the 2100 Bioanalyzer and the High Sensitivity DNA Assay kit (Agilent Technologies, Santa Clara, CA). The final amplicon products were used in emulsion PCR via Roche GS Lib-L kit (Roche Diagnostics Gmbh, Mannheim, Germany) with the use of molecules-per-bead ratio of 0.7. All steps were followed according to the manufacturer's instructions.

DNA Sequencing, Data Processing, and Taxonomic Classification. The data analysis workflow based on the Quantitative Insights into Microbial Ecology (QIIME) pipeline was implemented (22). Pyrosequencing data sff file was first transformed into FASTA file and then demultiplexed with lower and upper cutoff values of 300 and 600 bases, respectively. To increase accuracy, homopolymers were removed. Chimera filtering was performed afterwards by using the UCHIME algorithm by either reference-based or de novo method (23). Reads that were classified as chimeric by both methods were removed. Reads were clustered into operational taxonomic units (OTUs) with 16S *rRNA* gene reference database from Silva database (SSU Ref NR 119) and from the Human Oral Microbiome Database (HOMD) (*16S rRNA* RefSeq Version 13.2 FASTA file in QIIME format). Each OTU contains a group of reads that align with at least 97% similarity to the reference *16S rRNA* gene sequence. The command "core_diversity_analyses.py" was used to evaluate the microbial community diversity within a sample (α-diversity) and the diversity between samples (β-diversity). Species diversity in each sample was determined by blasting individual sample sequence directly in the HOMD *16S rRNA* database (cutoff at 98.5%). Each bacterial species were identified by blasting the sequences against HOMD 16S *rRNA* database and aligned with 99%–100% identity to the reads with base length of 380–550 nt.

Results

OTU Analysis of Bacterial Communities

Pyrosequencing of the samples with post-treatment disease resulted in 71,220 raw sequence reads. After removing low-quality sequence reads and chimeras, the final data contained 52,993 sequence reads with an average length of 500–550 nt. Of these, 43,797 sequence reads were from S1 (range, 283–7176), and 9196 sequence reads were from S2 samples (range, 59–5864), respectively.

Table 2 depicts data from diversity and richness estimate calculations. The overall alpha diversity was higher in S1 than S2 samples as indicated by Chao and Shannon indices. The mean numbers of observed OTUs at 3% dissimilarity were 152 and 28 in S1 and S2 samples,