Microbiome Changes during Regenerative Endodontic Treatment Using Different Methods of Disinfection

ABSTRACT

Introduction: The purpose of this study was to characterize qualitatively and quantitatively the changes in the endodontic microbiome, in teeth with necrotic pulp, open apexes, and apical periodontitis, with 3 antimicrobial protocols, undertaken in a multicenter clinical trial.

Methods: Microbiological samples were collected from 116 regenerative endodontic teeth, and 97 qualified for inclusion. The teeth were randomly divided into 3 treatment groups: apexification (APEX), regeneration (REGEN), and revascularization (REVASC), all in 2 appointments. The group variables in the first appointment irrigants, and second appointment irrigants and medicaments were as follows: APEX: 5.25%–6% NaOCl, 5.25%–6% NaOCl + 17% EDTA and calcium hydroxide; REGEN: 1.25% NaOCl, 17% EDTA, and 0.1 mg/mL triple antibiotic paste (TAP); and REVASC 5.25% NaOCl, saline, and 1 g/mL TAP, respectively. Sampling was done upon access (S0), after irrigation in the first appointment (S1), and after using medication and irrigation in the second appointment (S2).

Results: Quantitative polymerase chain reaction analysis of the 16S ribosomal RNA gene showed significant reduction in bacterial load from S0 to S2 in all groups; however, the APEX and REVASC groups had significantly less residual DNA than the REGEN group (P = .0045). The relative abundance of Bacteroidetes, Fusobacteria, Spirochaetes, and Synergistetes were reduced with the treatment rendered. However, relative abundance of Firmicutes and Actinobacteria was not changed, and that of Proteobacteria increased. LEfSe analysis showed that reduction in bacterial taxa was more in REVASC than APEX, which in turn was more than in REGEN.

Conclusion: Enhanced antimicrobial protocols lead to better reduction in quantitative and qualitative parameters of the endodontic microbiota. (J Endod 2022;48:1273–1284.)

KEY WORDS

Regenerative endodontic therapy; endodontics; microbiome; Bacteriome; next generation sequencing; triple antibiotic paste

Regenerative endodontic treatment (RET) has been defined as biologically based procedures designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as cells of the pulp-dentin complex. To achieve these objectives, effective disinfection of the necrotic pulp space is of paramount importance. Strategies to enhance the effectiveness of antimicrobial therapy necessitate the understanding of the full extent of root canal infection and the efficacy of disinfection using contemporary methods during treatment.

Endodontic microbiology has gone through many phases of evolution since it was first described in the late 19th century. Contemporary molecular analysis using next generation sequencing (NGS) has provided increased sensitivity and great depth of coverage compared with traditional culture-based or targeted molecular methods. For a polymicrobial infection with wide diversity of microflora, housed in a closed environment, microbial analysis using NGS may reveal sufficient data on the quantity and constituents of the bacterial components to inform newer disinfection approaches that can effectively target a broad spectrum of microbial taxa.

The disinfection of root canals in teeth with necrotic pulp and open apexes undergoing RET is complicated by several factors. Bacterial penetration in dentinal tubules of young patients is enhanced by their wider diameter compared with that of older patients. The canal space is frequently larger, thus

SIGNIFICANCE

A comprehensive microbiological analysis was performed in a large sample of regenerative endodontic therapy in teeth with necrotic pulp, open apexes, and apical periodontitis, in which different protocols were used. The results showed that enhanced disinfection protocols lead to better reduction in several measures of the endodontic microflora.
providing a larger surface area for microbial biofilms and interfering with adequate cleaning and shaping. In addition, the mechanical instrumentation of the root canal walls, which disrupts the microbial biofilm and removes a layer of infected dentin, is not recommended because of concerns about further weakening of the tooth. Furthermore, residual microbial biofilm interferes with the growing vital tissues, and root development. Finally, the antimicrobial agents, such as sodium hypochlorite, chlorhexidine, or antibiotics used during treatment, may be toxic at higher concentrations to the stem cells of the apical papilla and to the proteins from the dentin matrix, which are thought to mediate the revascularization process. Considering these restrictions, the decision of which protocols for disinfection to be used in a randomized controlled trial is rather challenging. On the one hand, one needs to select the best approaches for disinfection currently approved for this purpose. On the other hand, a more biocompatible approach would potentially encourage true regeneration of the dental pulp and other dental tissues should be considered.

Few previous studies have examined the microbiological status of teeth with necrotic pulp and open apexes, and the changes that occur after the use of antimicrobial agents. Histologically, the root canals and immediate periapical area in these teeth were shown to harbor bacterial biofilms, similar to those found in teeth with mature apexes. A previous study described the presence of bacteria, identified by culture and targeted polymerase chain reaction (PCR), preoperatively and after 5 stages of RET. A more recent study from the same group showed the relationship of the treatment variables in the original study to the outcomes of treatment; however, this study had a small sample size and used only paper points (which mainly sample planktonic bacteria) for sampling. It also used very large concentrations of triple antibiotic paste (TAP), or calcium hydroxide combined with 2% chlorhexidine as medicaments, and examined only a few taxa of microorganisms. A more extensive analysis in this area, with lower concentrations of antimicrobials, and without chlorhexidine, may present an adequate characterization of preoperative infections in teeth with necrotic pulp and open apexes and provide comprehensive data on potential sensitive and resistant taxa of bacteria. This would enhance our understanding of the microbiology of endodontic infections in these cases and provide insight into the optimal methods of antimicrobial therapy.

Therefore, the purpose of this study was to characterize qualitatively and quantitatively the endodontic microbiome using the 16S ribosomal RNA (rRNA) gene analysis, in teeth with necrotic pulp and open apexes and determine the efficacy of 3 antimicrobial protocols in patients undergoing RET.

**MATERIALS AND METHODS**

Institutional review board (IRB) approvals were obtained from the University of Texas, Health Sciences Center at San Antonio (UTHSCSA) (IRB-HSC2013046714), Loma Linda University (LLU) (IRB-5130231), the University of Maryland Baltimore (UMB) (IRB-HCR-HP-00056544–1), University of North Carolina at Chapel Hill (UNC) (IRB-15–3061), and the University of Alabama at Birmingham (UAB) (IRB-300006518) for conducting and/or analyzing the data for this multicenter clinical trial. The protocol had been registered in ClinicalTrials.gov (ID: NCT01976065). Patients between the ages of 6 and 20 were recruited for this study if they had clinical diagnosis of pulp necrosis and apical periodontitis in 1 or more teeth with immature apexes, according to the inclusion and exclusion criteria listed in Table 1. Following consent and assent, the patients were randomized to 1 of 3 treatment groups using the UTHSCSA-based IDEAS (Informatics Data Exchange and Acquisition System) clinical trial management system. This is a research portal in which data were entered to register each patient when he or she fulfilled the inclusion/exclusion criteria, and the system automatically randomly assigns the patient to one of the study groups. This study had been designed and powered to determine the clinical outcomes of RET. Sample size determination for that analysis will be published separately. In this article, only the microbiological information is described.

**Microbiological Sampling and Clinical Protocols**

The study intervention consisted of 3 groups: REGENDO, REVASC, and APEX treatments followed by placement of permanent composite restorations. Table 2 summarizes the protocols used. For all treatment arms, each appointment lasted from 60 to 90 minutes. The treatments were performed either by one of the investigators assisted by the endodontic residents or by the endodontic residents under the direct supervision of one of the endodontist study investigators; all providers were trained using video demonstrations and calibrated using Real-T endo teeth models (Acadental, Overland Park, KS). Investigators and residents were calibrated at the completion of standardized video training using criteria on aseptic technique, microbiological sampling, placement of Gelfoam (Pfizer, Kent, United Kingdom) and collagen barriers (Collatap; Zimmer Biomet Dental, Palm Beach Gardens, FL), as well as placement of Mineral Trioxide Aggregate Cement (ProRoot MTA; Dentsply Sirona, Charlotte, NC).

The study medications and the dental materials used in this study were maintained centrally at the lead study site at UTHSCSA. The TAP consisted of US Pharmacopeia (USP) ciprofloxacin, minocycline, and metronidazole. These Food and Drug Administration–approved antibiotics were used in an off-label manner in this study, as we had obtained a Food and Drug Administration Investigational New Drug #104599 approval for this study. The TAP was ordered pre-mixed from Champs Compounding Pharmacy (San Antonio, TX) by a certified compounding pharmacist using USP grade drugs to create a 1:1:1 powdered mixture. The capsules were stored in individual moisture-tight, heat-sealed plastic packaging that was sequentially numbered for inventory control and distribution. For the REGENDO protocol, the TAP was mixed in sterile water to achieve a 0.1 mg/mL concentration; this concentration is consistent with the pharmaceutically active antibiotic concentrations in plasma. The REVASC protocol, the TAP was mixed using sterile water to achieve a concentration of 1 g/mL, which has a runny paste consistency. The individual MTA packages were sequentially numbered for inventory control. To permit distribution under blinded basis, envelopes containing either of the antibiotic concentrations or of calcium hydroxide treatments (UltraCal XS; Ultradent Products Inc., South Jordan, UT) were placed inside identical opaque packages identified only by a random number sequence. All medicaments were slowly injected into the canal space using a 3-mL syringe fitted with a capillary tip (Ultradent Products Inc) inserted to working length.

For each case included, tooth isolation was achieved with the dental rubber dam according to strict criteria ensured by the calibrated investigators. The tooth involved and the surrounding rubber dam and retainer were disinfected with a sterile cotton pellet that had been soaked in 5.25% to 6% sodium hypochlorite. This was followed by inactivation of the hypochlorite using a sterile cotton pellet soaked in 5% sodium thiosulfate (Boston Bioproducts Inc., Milford, MA). A surface sterility sample was obtained before access preparation (ST1) and placed in a vial with
Into the Vial, Followed by 3 Sterile Paper Points

Details of Disinfection Procedures in the 3 Groups: All samples were obtained using 1 loose file (usually size 25 K-file) repeatedly touching the canal walls, without filing, and aseptically separated into the vial, followed by 3 sterile paper points. The file handle was held firmly, and then the file shaft was separated by repeated bending with a sterile hemostat. S0 was obtained immediately following access preparation before any irrigation was performed. For S1 and S2, irrigation with 1 mL 5% sodium thiosulfate was done to inactivate any hypochlorite present before sampling. S0, S1, and S2 were also transported in vials with anaerobic transport medium. Calibration of all investigators and providers was performed before any clinical procedures, using a video recording of the steps involved made by the first author.

Molecular Analysis of Samples
Samples at 4 treatment institutions (UTHSCSA, LLU, UMB, and UNC) were immediately frozen at −80°C. Two batches of samples were processed for this analysis that had approximately the same number of samples; the first had samples from UTHSCSA, LLU, and UMB and the second had samples from UTHSCSA, LLU, and UNC.

For DNA extraction, the vials containing the samples were vortexed for 2 minutes to disperse the microbial suspension. The suspension was transferred into sterile vials, and then centrifuged at 7500 rpm (equivalent to 6918 g; all centrifuge procedures carried out with Eppendorf [Westbury, NY] model 5415D) for 10 minutes. The supernatant was removed and not used. The residue of paper point fragments in liquid dental transport medium was transferred to a 2-mL tube containing 200 mg of ≤106 μm glass beads (Sigma, St. Louis, MO) and 0.3 mL of Qiagen ATL buffer (Qiagen, Valencia, CA), supplemented with 20 mg/mL lysozyme (Thermo Fisher Scientific, Grand Island, NY). The suspension was incubated at 37°C for 1 hour with occasional agitation. Subsequently, the suspension was supplemented with 600 IU of Qiagen proteinase K and incubated at 60°C for 1 hour. Finally, 0.3 mL of Qiagen AL buffer was added, and a final incubation at 70°C for 10 minutes was carried out. Bead beating was then used for 3 minutes in a Qiagen Tissuelyser II at 30 Hz. After a brief centrifugation, supernatants were aspirated and transferred to a new tube containing 0.3 mL of ethanol. DNA was purified using a standard on-column purification method with Qiagen buffers AW1 and AW2 as washing agents and eluted in 10 mM Tris (pH 8.0).

Initially, all ST1 and ST2 samples were analyzed using universal 16S rRNA gene broad range PCR assay for any bacterial DNA as previously described17,18. The PCR reaction mix had 50 μL that contained 2.5 μM each of the forward and reverse 10 μM primers, 25 μM HotStar Master Mix, 2.5 U HotStar DNA Polymerase, 1X PCR Buffer, 200 μM of each deoxynucleoside triphosphate, 1.5 mM MgCl2, 10 μL PCR water. Extracted DNA

Table 1 - Inclusion and Exclusion Criteria

Inclusion criteria
- Age 6–20.
- Participant has a permanent tooth with a necrotic pulp (as defined by lack of responsiveness to 2 consecutive electrical pulp tests).
- Tooth in question is restorable (as defined by Class A or Class B using Samet and Jotkowitz classification19) without the need of a stainless steel crown.
- Incomplete (ie, immature) root development defined by apical foramen ≥1.0 mm (all foramina ≥1.0 mm for multi-rooted teeth).
- At least 5 mm of root development (cementoenamel junction to radiographic apex).
- Wiling and able to provide informed assent/consent.
- Legal guardian willing and able to provide informed consent.

Exclusion criteria
- No access to telephone for study contacts.
- Unable to comprehend study materials in English or Spanish.
- Subject not available for follow up at 12 or 24 months.
- Previous allergic response to ciprofloxacin, metronidazole, or minocycline.
- History of systemic diseases with altered immune function including diabetes, immunodeficiency, leukemia, and Addison’s or Cushing’s disease.
- History of taking immunosuppressants or chemotherapeutic agents including glucocorticoids.
- Clinical judgment (with documentation of the reason).
- Tooth in question has a history of avulsion.
- Tooth in question has class III mobility or dens invaginatus.
- Clinical or radiographic evidence of root fracture or alveolar fracture.
- (Infection-related) root resorption.
- History of systemic diseases with altered immune function including diabetes, immunodeficiency, leukemia, and Addison’s or Cushing’s disease.
- Subject not available for follow up at 12 or 24 months.
- Legal guardian willing and able to provide informed consent.
- At least 5 mm of root development (cementoenamel junction to radiographic apex).
- 1.0 mm for multi-rooted teeth).

Table 2 - Details of Disinfection Procedures in the 3 Groups: All samples Were Obtained Using 1 Loose File (Usually Size 25 K-file) Touching All Canal Walls and Aseptically Separated Into the Vial, Followed by 3 Sterile Paper Points

<table>
<thead>
<tr>
<th>Visit</th>
<th>APEX</th>
<th>REGEN</th>
<th>REVASC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>20 mL of 5% NaOCl then 20 mL saline, followed by 1 mL 5% sodium thiosulfate</td>
<td>20 mL of 1.25% NaOCl then 20 mL saline, followed by 1 mL 5% sodium thiosulfate TAP (0.1 mg/mL) is slowly injected into the canal space</td>
<td>20 mL of 5% NaOCl then 20 mL saline, followed by 1 mL 5% sodium thiosulfate TAP (1 mg/mL) is slowly injected into the canal space</td>
</tr>
<tr>
<td>Final</td>
<td>20 mL 17% EDTA and 20 mL 5% NaOCl followed by 1 mL 5% sodium thiosulfate</td>
<td>20 mL saline followed by 20 mL 17% (EDTA) and then 5 mL saline</td>
<td>20 mL saline followed 5 mL saline</td>
</tr>
</tbody>
</table>

APEX, apexification; qPCR, quantitative polymerase chain reaction; REGEN, regeneration; REVASC, revascularization; TAP, triple antibiotic paste.
(10 μL) was then added to each experimental tube. The first tube served as a positive control with 10 μL of extracted DNA from a known bacterial isolate (Streptococcus intermedius ATCC 31412). The final tube served as a negative control with no DNA added but with an extra 10 μL of PCR water. The reactions were run in Applied Biosystems GeneAmp PCR System 9700 at conditions of 94°C for 15 minutes for 25 cycles, followed by 94°C for 15 seconds; 56°C for 15 seconds; 72°C for 45 seconds; 72°C for 5 minutes, held at 4°C and then PCR products were stored at −20°C until analysis by gel electrophoresis. The entire sample set (S0, S1, and S2) for the patient was excluded from further analysis if either ST1 or ST2 for a patient had a positive result.

Quantitative PCR Methods

For the samples that had negative ST1 and ST2, quantitative PCR (qPCR) was carried out with the Fast-Real Time 7500 (Applied Biosystems, Waltham, MA). The final reaction volume was 20 μL and contained each primer at a final concentration of 200 nM, Power SYBR Green (Applied Biosystems) 1X, and 2 μL of template DNA. Samples and standards were run in triplicate. Because of the large number of samples tested, the entire analysis was carried out on 8 plates. We used 1 sample of known copy number per microliter (5000 copies/μL) on each plate (shown as “KNOWN” in each plate).

The following primers were used:

Universal 16S rRNA was used from Maeda et al.10. Expected amplicon size: 150 base pairs. Forward Primer (1048–1067): 5'- GTG STG CAY GGY YGT CGT CA-3'); Reverse Primer (1198–1175): 5'- ACG TCR TCC MCN CCT TC-3'.

The thermal cycling conditions were as follows: 1 cycle at 50°C for 20 seconds, 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, 1 minute at 60°C. Melting curve analysis was carried out using the continuous method from the 7500 Software (Applied Biosystems) conducted at 60°C, with increments of 1°C for 15 seconds. Data analysis was carried out using 7500 Software (Applied Biosystems). The auto threshold and baseline options were used for the calculations of Ct values per well. The linear equation for the standard curve (ie, for preparations containing known quantities of DNA) was then used to interpolate the numbers of copies present in unknown samples.

NGS Protocols

NGS protocols were run on the 2 pools of samples, at least in part, in 2 different sequencing facilities. This was performed to minimize pipeline-related bias and inconsistencies in relative abundance20. The first batch of samples was processed using the homings analysis at the Forsythe Institute in Boston, MA, and at the Molecular Microbiology Core facility at UNC. The second batch was run only at the Molecular Microbiology Core facility at UNC. The final bioinformatic processing of all data was collated and combined at the UNC core facility. The NGS protocols in the 2 sites were run on the Illumina MiSeq platform (Illumina Inc., San Diego, CA), were similar in most other respects, and followed the QIIME 1 pipeline as follows:

16S rRNA amplicon sequencing: 12.5 ng of total DNA was amplified using universal primers targeting the V4 region of the bacterial 16S rRNA gene1. Primer sequences contained overhang adapters appended to the 5' end of each primer for compatibility with Illumina sequencing platform. The primers used were F515: 5'- GTG CCA GCM GCC GCG GTA A-3' /R806: 5'-GGA CTA CHV GGG TWT CTA AT-3'. Master mixes contained 12.5 ng of total DNA, 0.5 μM of each primer and 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA). The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a cycling of denaturing of 95°C for 30 seconds, annealing at 55°C for 30 seconds, and a 30-second extension at 72°C (25 cycles), a 5-minute extension at 72°C, and a final hold at 4°C. Each 16S amplicon was purified using the Ampure XP reagent (Beckman Coulter, Indianapolis, IN). In the next step, each sample was amplified using a limited cycle PCR program, adding Illumina sequencing adapters and dual-index barcodes (Index 1[7] and index 2[5]) (Illumina) to the amplicon target. The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a denaturing cycle of 95°C for 30 seconds, annealing at 55°C for 30 seconds, and a 30-second extension at 72°C (8 cycles), a 5-minute extension at 72°C, and a final hold at 4°C. The final libraries were again purified using the Ampure XP reagent (Beckman Coulter), quantified and normalized before pooling. The DNA library pool was then denatured with NaOH, diluted with hybridization buffer, and heat denatured before loading on the MiSeq reagent cartridge (Illumina) and on the MiSeq instrument (Illumina). Automated cluster generation and paired-end sequencing with dual reads were performed according to the manufacturer’s instructions20,22.

Output sequences were converted to fastq format and demultiplexed using Illumina Bcl2Fastq 2.18.0.12 (Illumina Inc). The resulting paired-end reads were joined using the QIIME 1.9.023 invocation of fastq-join24.

### Table 3 - All Groups: qPCR Mean, Median, and Reduction in Successive Stages of Treatment (Friedman Test: *Statistically Significant)

<table>
<thead>
<tr>
<th>Time point</th>
<th>Mean (SD)</th>
<th>Median</th>
<th>Reduction in qPCR</th>
<th>Dunn’s adjusted P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>16,986,886 (102,776,420)</td>
<td>37,629</td>
<td>S0 → S1</td>
<td>.0088*</td>
</tr>
<tr>
<td>S1</td>
<td>64,296 (113,870)</td>
<td>14,711</td>
<td>S1 → S2</td>
<td>.0175*</td>
</tr>
<tr>
<td>S2</td>
<td>40,680 (62,378)</td>
<td>12,447</td>
<td>S0 → S2</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

### Table 4 - qPCR Mean, Median, and Reduction in Successive Stages of Treatment (Friedman Test: *Statistically Significant)

<table>
<thead>
<tr>
<th>Group</th>
<th>Time point</th>
<th>Mean (SD)</th>
<th>Median</th>
<th>Reduction in qPCR</th>
<th>Dunn’s Adjusted P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>APEX</td>
<td>S0</td>
<td>27,992,383 (137,385,854)</td>
<td>32,817</td>
<td>S0 → S1</td>
<td>.025*</td>
</tr>
<tr>
<td>APEX</td>
<td>S1</td>
<td>71,123 (131,290)</td>
<td>12,638</td>
<td>S1 → S2</td>
<td>.1535</td>
</tr>
<tr>
<td>APEX</td>
<td>S2</td>
<td>35,453 (50,442)</td>
<td>14,666</td>
<td>S0 → S2</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>REGEN</td>
<td>S0</td>
<td>36,210,746 (139,518,109)</td>
<td>147,312</td>
<td>S0 → S1</td>
<td>.0185*</td>
</tr>
<tr>
<td>REGEN</td>
<td>S1</td>
<td>53,273 (58,628)</td>
<td>37,375</td>
<td>S1 → S2</td>
<td>&gt;.9999</td>
</tr>
<tr>
<td>REGEN</td>
<td>S2</td>
<td>88,073 (104,936)</td>
<td>52,939</td>
<td>S0 → S2</td>
<td>.0185*</td>
</tr>
<tr>
<td>REVASC</td>
<td>S0</td>
<td>163,867 (320,848)</td>
<td>31,912</td>
<td>S0 → S1</td>
<td>&gt;.9999</td>
</tr>
<tr>
<td>REVASC</td>
<td>S1</td>
<td>62,057 (113,605)</td>
<td>13,069</td>
<td>S1 → S2</td>
<td>.0658</td>
</tr>
<tr>
<td>REVASC</td>
<td>S2</td>
<td>28,937 (43,317)</td>
<td>7,570</td>
<td>S0 → S2</td>
<td>.0265*</td>
</tr>
</tbody>
</table>

APEX, aperifunction; qPCR, quantitative polymerase chain reaction; REGEN, regeneration; REVASC, revascularization.
with the default parameters. Index and linker primer sequences were trimmed, and the reads were subsequently filtered for quality, removing any read in which the percentage of quality scores below the quality threshold of 24 fell below 70%. Quality control of both raw and processed sequencing reads was verified by FastQC25. Sequences were clustered into operational taxonomic units (OTU) based on the de novo OTU picking algorithm using the QIIME implementation of UCLUST26 at a similarity threshold of 97%. OTUs identified as chimeric by vsearch27 of the ChimeraSlayer “gold” reference database28 and those composed of a single read (singletons) were eliminated. The remaining OTUs were assigned taxonomic identifiers with respect to the Greengenes database29 and independently with respect to the Human Oral Microbiome Database (HOMD). The representative sequences were aligned using template alignment through PyNAST30, and a phylogenetic tree was built with FastTree 2.1.331. Alpha diversity was analyzed using the Chao-1 algorithm. Observed species number metrics were estimated using QIIME at a rarefaction depth of 1000 sequences per subsample. Beta diversity estimates were calculated within QIIME using weighted and unweighted UniFrac distances32,33 between samples at a subsampling depth of 1000. Results were summarized and visualized through principal coordinate analysis as implemented in QIIME. Differential abundance was assessed with respect to linear discriminant effect size using LEfSe (LDA [Linear discriminant analysis] Effect Size)34.

Analysis

The mean qPCR values (mean of triplicate values) representing DNA copies at each of the 3 time points, S0, S1 and, S2, were compared using the nonparametric test (Kruskal-Wallis for reduction ratio or Friedman tests for repeated measures), because the combined data did not follow a Gaussian distribution. One-way analysis of variance was used to compare the residual DNA in S2 among the 3 groups. Correlations of qPCR data in S0 with S1 and S2, using Pearson r, was performed to determine if more preoperative bacterial load was associated with residual bacterial loads. Data were obtained from the QIIME-1 pipeline analysis on relative abundance of major taxa, alpha and beta diversity. In addition, data on the statistical significance, biological consistency, and effect relevance of major changes in different groups at different time points were also analyzed using LDA.

RESULTS

The trial from all 4 sites included 126 cases, of which microbiological samples were available for 116 cases. A total of 19 cases were eliminated from further microbiological analysis because of positive ST1 and/or ST2 samples. The remaining 97 cases were from the following groups: REGEN (n = 17), REVASC (n = 42), and APEX (n = 38), and provided a total of 291 specimens for S0, S1, and S2.

![FIGURE 1 – Mean (SD) of residual DNA in S2 in the 3 groups. REGEN had significantly more residual DNA than the 2 other groups (analysis of variance; P = .0045).](image)

![FIGURE 2 – Bacterial phyla present at S0, S1, and S2, with main phyla annotated.](image)
For the qPCR data, 2 REGEN cases were further eliminated because there were no data available for their S0 or S1 read outs, leaving 95 cases that were analyzed for S0, S1, and S2. All cases analyzed had bacterial DNA at all 3 stages of analysis. There was a wide variability in measurement of qPCR, especially at S0 (range 91–8 × 10^8 copies) that did not follow a Gaussian distribution.

Table 5 shows the most abundant genera of bacteria at the 3 time points excluding unassigned taxa. The bacterial DNA (measured as mean quantity of qPCR read out) was significantly reduced on sequential sampling from S0 to S1 to S2 (Friedman test; P < .0001). Further analysis showed incremental significant reduction of bacterial load from S0 to S1, from S1 to S2, and from S0 to S2 (Table 3). A similar analysis for each of the 3 groups revealed significant reductions of DNA from S0 to S1 and S0 to S2 in the APEX and REGEN groups but only from S0 to S2 in the REVASC group (Table 4). A comparison of the final residual DNA in the 3 groups (S2) showed that the APEX and REVASC groups had significantly less residual DNA than the REGEN group (Fig. 1). The REGEN group also had a substantial number of early clinical failures of treatment, which led the Data Safety and Monitoring Board for this study to discontinue recruitment in this group at approximately the midpoint of recruitment for the study. This explains the reduced sample size for this group.

There were no significant correlations between qPCR levels in S0, and respective values in S1 or S2 (Pearson r; P > .05). However, there were 3 S0 specimens (2 APEX and 1 REGEN), all from UMB, where the values of the qPCR were 2 to 3 orders of magnitude higher than all other specimens. When these 3 outliers were eliminated, there was a significant positive correlation between the qPCR values in S0 and S2 (Pearson r; P = .0081).

The NGS analysis yielded 21,324,407 total reads from 291 bacterial samples. After chimera and singleton read elimination, the mean reads per sample was 71,914. This resulted in the analysis of 38,953 OTUs, of which 12.72% could not be identified in the HOMD database. This analysis resulted in the identification of 80 known bacterial genera and 262 known species.

Bacterial analysis at the level of phyla showed considerable reduction in relative abundance of Bacteroidetes, Fusobacteria in S1 and S2 compared with S0 (Fig. 2). Spirochaetes and Synergistetes were eliminated by S2. However, the relative abundance of Firmicutes and Actinobacteria did not appear to be changed, and that of Proteobacteria seemed to have increased considerably (Fig. 2).

The most common genera identified at the 3 time points are shown in Table 5 (species provided in Supplemental Table 1 is available online at www.jendodon.com). The comparison among the 3 time points shows reduction of common endodontic pathogens like the gram-negative anaerobes: Fusobacterium spp., Prevotella spp., and Tannerella spp., and increase in the resistant gram-positive and gram-negative facultative or aerobic bacteria, like Acinetobacter spp., Pseudomonas spp., Enterococcus spp., Sphingomonas spp., and Klebsiella spp.

Analysis of the alpha diversity for the 3 treatment groups combined showed no significant differences among the 3 time points (Chao-1; P > .05) (Fig. 3A). However, alpha diversity was significantly increased in S2 compared with S0 in the APEX and REGEN groups (Chao-1; P = .015) (Fig. 3B and C). There were no significant differences among the 3 time points alpha diversity in the REVASC group (Fig. 3D). The beta diversity was determined using weighted UniFrac PCoA plots (Fig. 4). In general, this analysis showed better separation among the 3 time points in the APEX and the REVASC groups than the REGEN group (Fig. 4).

The effects of the treatment procedures in the 3 groups on changes in the abundance of root canal microbiota were determined using the LDA analysis (Fig. 5 and Supplemental Fig. 1 is available online at www.jendodon.com). Considering the LDA differences between S0 and S2 as the overall outcome of the disinfection protocols investigated, the results showed that in the REGEN group only 15 taxa in S0 had their abundance reduced at the 4 (log 10) or higher level. The APEX group had the abundance of 28 S0 taxa reduced, and the REVASC group had the abundance of 35 S0 taxa reduced. In the APEX and REVASC groups, the abundance of 14 and 5 taxa were increased in S2, respectively. Of note, these taxa that increased in the APEX group included Betaproteobacteria, Burkholderiales, Comamonadaeae; [Delftia] acidovorans, which are all members of the phylum Proteobacteria, and this was not the case in the REVASC group.

**DISCUSSION**

As far as we are aware, this study had a larger and more diverse sampling than previously reported in microbiological studies that analyzed endodontic micro flora using NGS of the 16S rRNA gene in teeth with necrotic pulp and periapical lesions^{22,23}. This study also addressed an area that is generally poorly studied, which is the effect of different antimicrobial approaches on the microbiota of teeth with necrotic pulp, open apex, and apical periodontitis using trained investigators following a standardized protocol.

This study identified 80 known bacterial genera and 262 known species in the 291 samples analyzed. There was considerable diversity among different cases; however, there was considerable overlap with taxa that were abundant in traditional preoperative treatment procedures.  

**TABLE 5 - Most Abundant Genera of Bacteria at the 3 Time Points Excluding Unassigned Taxa**

<table>
<thead>
<tr>
<th>Genera</th>
<th>S0</th>
<th>S1</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusobacterium</td>
<td>Acinetobacter</td>
<td>Sphingomonas</td>
<td>Enterobacteriaceae, Other</td>
</tr>
<tr>
<td>Prevotella</td>
<td>Enterococcus</td>
<td>Pseudomonas</td>
<td>Enterococcus</td>
</tr>
<tr>
<td>Peptostreptococcaceae</td>
<td>Pseudomonas</td>
<td>Sphingomonas</td>
<td>Enterococcus</td>
</tr>
<tr>
<td>Campylobacter</td>
<td>Fusobacteriaceae</td>
<td>Fusobacteriaceae</td>
<td>Pseudomonas</td>
</tr>
<tr>
<td>Sphingomonas</td>
<td>Bacteroidaceae</td>
<td>Stenotrophomonas</td>
<td>Stenotrophomonas</td>
</tr>
<tr>
<td>Tannerella</td>
<td>Rhizobiales; Other</td>
<td>Rhizobiales; Other</td>
<td></td>
</tr>
<tr>
<td>Porphyromonas</td>
<td>Bacillus</td>
<td>Bacillus</td>
<td>Streptococcus</td>
</tr>
<tr>
<td>Treponema</td>
<td>Klebsiella</td>
<td>Bergeyella</td>
<td>Granulicatella</td>
</tr>
<tr>
<td>Bacteroidales</td>
<td>Stenotrophomonas</td>
<td>Lactobacillales; Other</td>
<td></td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>Prevotella</td>
<td>Caulobacteriales; Other</td>
<td></td>
</tr>
<tr>
<td>Selenomonas</td>
<td>Enterobacteriaceae; Other</td>
<td>Caulobacteriales; Other</td>
<td></td>
</tr>
<tr>
<td>Capnocytophaga</td>
<td>Bergeyella</td>
<td>Delftia</td>
<td>Delftia</td>
</tr>
<tr>
<td>Dialister</td>
<td>Treponema</td>
<td>Rhizobiales; Other</td>
<td></td>
</tr>
<tr>
<td>Peptostreptococcus</td>
<td>Enterobacteriaceae; Other</td>
<td>Micrococcaceae; Other</td>
<td></td>
</tr>
<tr>
<td>Acinetobacter</td>
<td>Fretibacterium</td>
<td>Erysipelothrix</td>
<td>Bacillaceae; Other</td>
</tr>
<tr>
<td>Parvimonas</td>
<td>Tannerella</td>
<td>Sphingomonas</td>
<td>Lachnospiraceae</td>
</tr>
<tr>
<td>Fretibacteriaceae</td>
<td>Caulobacteriales; Other</td>
<td>Sphingomonas</td>
<td></td>
</tr>
<tr>
<td>Filifactor</td>
<td>Delftia</td>
<td>Erysipelothrix</td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Alloprevotella</td>
<td>Bacillaceae; Other</td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae; Other</td>
<td>Carnobacteriaceae; Other</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Other: designation at the family level (most abundant species shown in Supplemental Table 1 is available online at www.jendodon.com). Other designations, such as family, order, etc, are not italicized.

This study identified 80 known bacterial genera and 262 known species in the 291 samples analyzed. There was considerable diversity among different cases; however, there was considerable overlap with taxa that were abundant in traditional preoperative treatment procedures.
endodontic samples, such as in *Fusobacterium*, *Prevotella*, Peptostreptococcaceae, *Tannerella*, *Porphyromonas*, *Treponema*, Bacteroidaceae, and *Dialister*. *Actinomyces* spp. did not have a high abundance in this study as in the previous study on teeth with necrotic pulp and immature apaxes, presumably because that study had cultured bacteria that were then sequenced, and had a small sample size.

The findings of this study of teeth with necrotic pulp and immature apaxes are consistent with data from NGS studies on teeth with mature apaxes, regarding the bacterial diversity and many of the taxa described. With respect to the persistence of members of the phylum Firmicutes, and the genera *Streptococcus* and *Enterococcus* following chemo-mechanical preparation, this is consistent with the findings of a recent systematic review on this subject. These gram-positive bacteria appear to be harder to eradicate from the root canal environment. In addition, members of the Proteobacteria phylum, such as *Acinetobacter* and *Pseudomonas* were also previously shown in persistent samples.

In this study, no filling of the canal wall was performed, to minimize further weakening of the root in these cases. However, sampling was performed with light strokes with an endodontic file #25 (K-file in the *Materials and Methods* section) to touch the canal walls, that was then collected, with the intent of sampling adherent biofilm, together with 3 paper points. This may explain why all specimens had some residual bacteria in S1 and S2, in all groups, which was different from other studies in which only paper point sampling was used. Paper point sampling may identify mostly planktonic bacteria, which are easier than biofilm to eliminate with antimicrobial irrigation and intracanal medicaments from the root canal environment; however, consistent with many other previous studies, the bacterial load was significantly reduced in all groups from S0 to S1, from S1 to S2, and from S0 to S2.

qPCR and NGS are very sensitive tools in contemporary microbiology, in that they detect very low amounts of bacteria and can identify as-yet-uncultivable, or viable but not cultivable (and difficult to cultivate) bacteria. In so doing, these tools provide sufficient depth of coverage to identify a high diversity of endodontic microflora, compared with previous technologies. These technologies are so sensitive that they may detect circulating bacteria in blood vessels of healthy

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**FIGURE 3** – Alpha diversity measured by the Chao1 method. (A) Three groups combined: Boxplots showing no significant differences among the 3 time points. Rarefaction curves showing adequate degree of sequencing coverage. (B) APEX and (C) REGEN groups showing significant increase in S2 compared with S0 ($P < .015$). (D) REVASC group showing no significant differences among the 3 time points ($P > .05$).
In the samples analyzed in this study, these molecular tools may also identify dead bacteria, especially in S1 and S2 samples.

The bacterial load was significantly reduced in the overall study from each step to the next. In the group analysis, all 3 groups had significant reduction from S0 to S2, but only the APEX and REGEN groups had significant reduction from S0 to S1. These effects could be attributed to the combination of the irrigant, and the medicaments used in these groups. Although the REVASC group had the same irrigation regimen in the first appointment as the APEX group and had a higher concentration of NaOCl than the REGEN group, it was the only group with no significant reduction of DNA from S0 to S1. This could be because the REVASC group had much lower bacterial DNA in S0 (Table 4) than the other groups, and the reduction of bacteria with mere irrigation could reach a threshold more easily when fewer bacteria are present in the samples preoperatively. With respect to the role of the medicament alone, the REVASC group was the only group that had a trend toward significance from S1 to S2 ($P = .0658$), whereas the other groups did not. This may be because of the higher concentration of the antibiotics in this group. If this group had received hypochlorite irrigation in the second appointment, the combined effects of the medicament and the second appointment hypochlorite might have produced a significant reduction from S1 to S2. Likewise, using TAP in the APEX group may have also yielded better disinfection. Consistent with this hypothesis, both the REVASC and APEX groups had significantly less residual DNA than the REGEN group (Fig. 1), wider separation of the colonies analyzed for beta diversity (Fig. 4), and more elimination of bacterial taxa in S2 as analyzed by LDA (Fig. 5). It is likely that these findings explain why the REGEN group had more cases with early clinical failures than other groups, which justified the decision to discontinue this group in the investigation. The early failures in the REGEN group would be consistent with the findings of reduced bacterial elimination in this group according to residual qPCR in S2 as well as the findings of the LDA analysis.

Bacterial alpha diversity was significantly increased from S0 to S2 in APEX and REGEN groups (Fig. 3B and C). These 2 groups had significantly more bacterial DNA levels in S0 compared with S2 (Table 4). Higher bacterial load may be associated with less bacterial...
diversity. It has been previously demonstrated that reduced bacterial alpha diversity in NGS samples from persistent endodontic infections was associated with increased endodontic symptoms44. Reduced diversity may favor increased abundance of fewer virulent bacteria. However, the REVASC group also had significant reduction in bacterial load from S0 to S2, but this was not accompanied by a significant increase in diversity. Given that the REVASC group had a higher concentration of TAP, the reduction of bacteria may have covered a wider range of taxa than in the other groups.

An analysis was performed of the correlation of bacterial DNA quantification in S0 with that in S1 and S2. The only significant correlation found was between S0 and S2, after elimination of 3 samples from 1 institution that were considered outliers. A recent study of endodontic retreatment found that the higher bacterial DNA detected by qPCR in preoperative samples correlated with the persistence of residual bacterial DNA before obturation40 and with retreatment outcomes 4 years after treatment45. An analysis of the role of preoperative and residual bacteria in the outcome of RET in this study is currently under way.

An extensive qualitative NGS analysis was done in this study of bacterial taxa present at different stages of treatment in the different groups. The relative abundance of phyla that include common gram-negative anaerobic endodontic pathogens was reduced with the treatment rendered, whereas that of gram-positive facultative Firmicutes and Actinobacteria was unchanged, and mostly aerobic gram-negative Proteobacteria increased. Proteobacteria are not commonly found in endodontic or oral microflora; however, recently, they have been identified in several other NGS studies5. They may reach the oral cavity from environmental sources, drinking tap water or eating raw food, and then reach the root canal systems through the gingival sulcus, lateral canals, or the periodontal circulation43. Some of the genera that had high relative abundance, like Pseudomonas spp., Acinetobacter spp., and Klebsiella spp., may be associated with important nosocomial and multidrug-resistant species46 and have been described in hospital-based as well as dental aerosols17. These taxa appear to be resistant to endodontic irrigants and medicaments as well. Alternatively, the rise in relative abundance of these taxa may be a result of contamination in

![FIGURE 5 – LDA charts for the comparison of major changes in taxa between S0 and S2 in (A) APEX, (B) REGEN, and (C) REVASC groups.](image-url)
the sequencing pipelines. Other commonly resistant endodontic microorganisms, such as Enterococcus spp. and Streptococcus spp., also had an increase in their relative abundance in S1 and S2.

Taken together, these findings showed that higher concentration antimicrobial regimens was more effective in reducing endodontic microflora quantitatively and qualitatively, and that higher concentration TAP may have broader spectrum antimicrobial action than calcium hydroxide medicament. Nonetheless, the ideal concentration of TAP to promote maximum disinfection while minimizing toxicity is not yet determined.

TAP may have broader spectrum antimicrobial effects on regeneration of dental tissues. Furthermore, analyses of the full endodontic microbiome, which includes viruses and fungi, such as with whole genome shotgun sequencing, should be conducted in the future, to determine the role of all these microorganisms together with bacteria in endodontic infections.

**REFERENCES**


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**SUPPLEMENTARY MATERIAL**

Supplementary material associated with this article can be found in the online version at www.jendodon.com (https://doi.org/10.1016/j.joen.2022.07.004).


