Analysis of Metabolically Active Bacteria After Adjunctive Steps for Disinfection of Teeth With Primary Endodontic Infections: RNA- and DNA-based Molecular Study

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STUDY PROTOCOL

BACKGROUND

Bacteria and their products in the root canals of necrotic teeth are the main cause of apical periodontitis. Therefore, the success of endodontic treatment in teeth with apical periodontitis (*i.e.* apical repair) depends on the reduction of viable microorganisms of root canals by endodontic disinfection procedures (1). Since the endodontic microbial community comprises many as-yet-uncultivated/difficult-to-culture bacteria, molecular methods that target viable cells are the choice methods for endodontic microbiology studies (2). As active bacteria present higher abundance of ribosomal rRNA than DNA (rRNA gene), the rRNA/DNA ratio is considered a useful strategy for monitoring bacterial load during endodontic treatment (3-6).

The endodontic treatment is usually performed at 2 visits. Root canal preparation at the 1^{rst} visit represents the main strategy for bacterial reduction by the mechanical action of the endodontic instrument along with irrigation (7). On the other hand, the use of an inter-appointment medication to maximize root canal disinfection after root canal preparation is one of the most controversial issues in Endodontics (8). DNA-based studies to evaluate the antimicrobial effectiveness of calcium hydroxide [$Ca(OH)_2$] as an intracanal medicament, have shown divergent results (9-13). Comparing DNA and rRNA may cast some new light on this issue, as these methods allow bacterial levels and activity to be compared.

OBJECTIVES

This study aimed to use the rRNA/DNA ratio to assess the total viable bacterial load in root canals during a treatment protocol, which uses a new irrigation tool during root canal preparation at the 1^{rst} visit, and a subsequent two-visit antibacterial approach with the use of an intracanal medicament for 14 days. Moreover, the success (apical repair) of the treatment protocol was assessed

after 1 year. In addition to the total bacteria, the metabolic activity of the following species/taxa was investigated: *Bacteroidaceae* sp. HOT-272 (synonym, *Bacteroidetes* oral clone X083), *Cutibacterium acnes* (formerly known as *Propionibacterium acnes*), *Selenomonas* spp. and *Enterococcus faecalis*. Moreover, the composition of the active microbiome was investigated by Next Generation Sequencing (NGS) analysis.

STUDY DESIGN

This interventional study compared the antimicrobial effects of endodontic procedures (clinicaltrials.gov ID: NCT03537664). The interventions tested at the first and second visits are described in **Figure 1**.

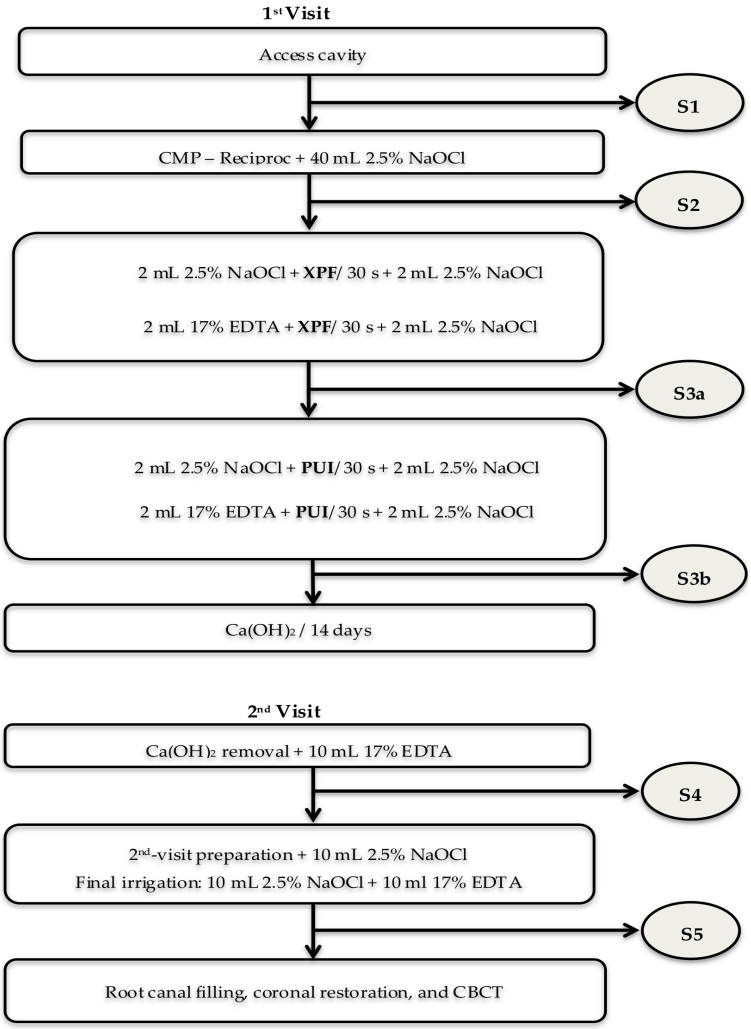

Figure 1. Flow chart of the interventions.

METHODS

Patient selection

The study was conducted in accordance with the Declaration of Helsinki and the research protocol was approved by the Institutional Ethical Committee (#2.201.768). All the selected patients gave signed informed consent before the treatment. Inclusion criteria were patients with single- rooted teeth and asymptomatic apical periodontitis. Exclusion criteria were: patients who had received antibiotic therapy during the previous 3 months or had general disease, teeth that could not be properly isolated with a rubber dam, non-restored teeth, periodontal pockets depths greater than 4 mm, and radiographic evidence of previous root canal filling, open apex, crown/ root fracture, root resorption, or narrow canals.

Interventions and microbiologic samples

After the initial root canal samples **(S1, baseline)**, chemo-mechanical preparation (CMP) was performed using Reciproc NiTi instruments (VDW GmbH, Munich, Germany) according to the manufacturer's instructions. A total of 40 mL of 2.5% NaOCl was used by the end of root canal preparation. Then, the root canal was irrigated with 5 mL of 5% sodium thiosulfate and filled with sterile saline before the post-instrumentation sample was taken (**S2**).

The root canal was filled with 2 mL of 2.5% NaOCl and then activated for 30 s with the XPF instrument followed by irrigation/aspiration with 2 mL of 2.5% NaOCl. Next, 2 mL of 17% EDTA was inserted into the root canal and activated with XPF for 30 s, again followed by irrigation/aspiration with 2 mL of 2.5% NaOCl. The canal was dried using paper points and flushed with 5 mL of 5% sodium thiosulfate for 1 min. The root canal was filled with sterile saline and a root canal sample was taken (**S3a**).

The Passive Ultrasonic Irrigation (PUI) protocol following XPF activation was similar to that described above. Both 2.5% NaOCl and 17% EDTA were activated

using a smooth wire with 0.2 mm diameter and .01 taper (Irrisonic - Helse, Ribeirão Preto, SP, Brazil), driven by an piezoelectric ultrasonic device (Piezo Light D5 Led, Olsen, SC, Brazil) in accordance with the manufacturer's recommendations. Finally, 2.5% NaOCl was inactivated using 5% sodium thiosulfate and a new sample was taken at the end of the first visit (**S3b**).

UltraCal XS Calcium Hydroxide Paste (Ultradent Products Inc., South Jordan, UT, EUA) was used as an intracanal medication for 14 days. The access cavities were filled with 2 mm of temporary restorative material (Dentalvile, Joinville, SC, Brazil) and glass ionomer cement (Riva light cure, SDI limited, Bayswater, Victoria, Australia).

At the second visit, the intracanal medicament was removed using 10 mL of 17% EDTA and agitation with K-files, and a fourth sample was taken (**S4**). Next, the root canal was irrigated with 10 mL of 2.5% NaOCl and the second visit instrumentation was performed using the same Reciproc file selected for the first visit. A final irrigation was performed with 10 mL of 2.5% NaOCl followed by 10 mL of 17% EDTA. A root canal sample was obtained at the end of the treatment, as described above (**S5**).

Root canal filling was performed, and the access cavity restored. An intraoral radiograph and cone beam computed tomography (CBCT) scans were taken to allow future analysis of the treatment outcome (after 1 year).

Nucleic Acids Extraction and cDNA synthesis

DNA and RNA were extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Technologies, Madison, WI), as described previously. The complementary DNA (cDNA) was synthesized using the SuperScript® III First-Strand Synthesis System (Invitrogen) for reverse transcription (RT), in accordance with the manufacturer's instructions. The DNA and cDNA were stored at −20 °C until use.

Total bacteria analysis: DNA levels and metabolic activity

Root canal samples from 20 patients were used to assess differences in the

total bacteria between the first and second visit endpoints (S3b and S5).

DNA and cDNA samples were used as templates for qPCR assays, which targeted conserved regions of the 16S rRNA gene of the *Bacteria* domain. The qPCR reactions (20 µL) contained: 10 µL of Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 2 µL of template, and 100 nM of each primer. Plasmid standard dilutions (from 10^7 to 10 DNA copies), DNA and cDNA samples were run in triplicate. The assay's limit of quantification was 10^2 DNA or cDNA copies; samples below the qPCR limit of quantification were considered negative.

Specific bacterial species analysis: DNA levels and metabolic activity

The analysis of total bacteria in the first 20 patients showed that the root canal preparation was the main step to reduce bacterial levels and activity. Therefore, the analysis of specific bacterial species was performed only in the initial (S1) and post-instrumentation samples (S2). Additional 25 patients were necessary for this analysis (n=45 patients). The metabolic activity of the following species/taxa was investigated: *Bacteroidaceae* sp. HOT-272 (synonym, *Bacteroidetes* oral clone X083), *Cutibacterium acnes* (formerly known as *Propionibacterium acnes*), *Selenomonas* spp. and *Enterococcus faecalis*. The oligonucleotide primers and cycling conditions for qPCR reactions are listed in **Table 1.**

Table 1. Primers sequences and qPCR reactions.

Taxa	Primers Sequences	Cycling conditions for qPCR reactions	Reference
16S rRNA universal	CCA TGA AGT CGG AAT CGC TAG G CT TGA CGG GCG GTG T	95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute	Shelburne et al., 2000 (14)
Bacteroidaceae sp. HOT-272	AGAGTTTGATCCTGGCTCAG ACT TGA GTG GAG GGT AGG	95°C for 10 minutes and 40 cycles of 95°C for 1 minute and 55°C for 1 minute	Rôças et al., 2014 (15)
Cutibacterium acnes	GCGTGAGTGACGGTAATGGGTA TTCCGACGCGATCAACCA	95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute	Eishi et al., 2002 (16)
Selenomonas spp.	TGGCATCATCCCCGGATA GCCCATCGACAGGCGATA	95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 55°C for 1 minute	This study
Enterococcus faecalis	CGCTTCTTTCCTCCCGAGT GCCATGCGGCATAAACTG	95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute	Williams et al., 2006 (17)

Microbiome analysis: Next Generation Sequencing (NGS) analysis

A subset of 20 cDNA samples canals were subjected to PCR with barcoded primers (Bakt_341F CC TAC GGG NGG CWG CAG and Bakt_805R GAC TAC HVG GGT ATC TAA TCC) that amplify the V4–V5 region of the 16S rRNA gene (18). High-throughput sequencing was performed using Illumina MiSeq in a commercial facility (Macrogen, Seoul, Republic of Korea) according to the manufacturer's protocols.

Success rate of the treatment

After 1 year of the endodontic treatment, sinus tract, pain, swelling, tenderness to percussion/ palpation were recorded. The absence of these signs/ symptoms indicated clinical success.

Periapical radiograph images and CBCT scans were analyzed by 2 observers, and the apical lesion area and volume at baseline were compared with those at recall period. The success of the treatment was determined as absence or reduction (≥ 20%) of the radiolucency.

Statistical Analysis Plan

The DNA data was used for bacterial quantification, whereas rRNA data were used to estimate bacterial activity at samples positive for rDNA. Ratios between rRNA and rDNA levels were calculated to search for active bacteria (rRNA/DNA ≥ 1) in root canal samples.

The nonparametric Wilcoxon signed rank test was used to determine differences in the bacterial levels before and after treatment procedures. Cochran's Q test and the Wilcoxon signed rank test were used for qualitative analysis (incidence of qPCR positive samples before and after treatment procedures).

For each qPCR assay, the Wilcoxon signed rank test was used to compare the number of rRNA and DNA copies in root canal samples. McNemar's test was used to compare the rRNA and DNA-based qPCR assays' detection rates. Differences were considered statistically significant if $P < 0.05$.

For the Next Generation Sequencing analysis, sequences were filtered using USEARCH tools (http://www.drive5.com/usearch/). Low quality sequences (score < 30) and reads shorter than 440 bp were excluded. Nonbacterial sequences, chimeras, and singleton reads were also removed. Sequences were

clustered into operational taxonomic units (OTUs) at 97% similarity using the Quantitative Insights Into Microbial Ecology (QIIME) 1.8.0 pipeline (http://qiime.org/1.8.0/). All OTUs occurring <10 times were removed from the dataset. Taxonomic analysis was performed using the BLAST method against the Human Oral Microbiome Database (HOMD) version 15.1 (http://www.homd.org), and the relative abundance was measured using the QIIME pipeline.

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