PCR identification of endodontic pathogens and DNA quantification in samples from teeth with post-treatment apical periodontitis

• **Marcos Sergio Endo** Discipline of Endodontics, Department of Restorative Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil • **Fernanda Graziela Corrêa Signoretti** Discipline of Endodontics, Department of Restorative Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil • **Ariane Cássia Salustiano Marinho** Discipline of Endodontics, Department of Restorative Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil • **Frederico Canato Martinho** Discipline of Endodontics, Department of Restorative Dentistry, São José dos Campos Dental School, State University of São Paulo, SP, Brazil • **Brenda Paula Figueiredo de Almeida Gomes** Discipline of Endodontics, Department of Restorative Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil

ABSTRACT | *Aim:* The aim of this clinical study was to quantify the concentration of DNA and to detect selected bacterial species from samples of infected root-filled teeth with post-treatment apical periodontitis after removal of gutta-percha (S1), after chemo-mechanical preparation at the first appointment (S2), 5 days after the canal was filled with sterile physiological solution (S3), after reinstrumentation at the second appointment (S4), and 14 days after an intracanal dressing was placed at the third appointment (S5). *Methods:* Fifteen root-filled teeth were selected. Removal of gutta-percha was performed using the crown-down technique. Chemo-mechanical preparation was performed with hand files associated with 2% chlorhexidine gel. An intracanal dressing based on $Ca(OH)_2$ was used. DNA was extracted from the samples and 14 endodontic 16S rDNA species-specific primers were tested. The concentration of DNA was quantified using a NanoDropTM 2000 spectrophotometer. *Results:* Bacteria were present in all cases at all sampling times, as revealed by a universal primer. DNA was isolated from all samples, with an average concentration of 4.24 ± 2.9 ng/µL (S1), 3.39 ± 1.54 ng/µL (S2), 4.0 ± 1.94 ng/µL (S3), 2.66 ± 0.98 ng/µL (S4) and 3.97 ± 2.32 ng/µL (S5). *Parvimonas micra* and *Enterococcus faecalis* (S1), *P. micra* (S2), *Porphyromonas endodontalis* and *E. faecalis* (S3), *E. faecalis* and *Prevotella nigrescens* (S4/S5) were the species most frequently deteced. DNA concentration reductions were detected between S3 and S4 (p = 0.0256), whereas an increase was found between S4 and S5. *Conclusion:* A wide variety of bacterial species was detected in root-filled teeth with post-treatment apical periodontitis. Moreover, the use of an intracanal dressing was unable to further reduce the concentration of bacterial DNA.

DESCRIPTORS | Endodontics; Treatment Failure; Periapical Periodontitis; Bacteria; Polymerase Chain Reaction; DNA; Chlorhexidine.

RESUMO | **Identificação de patógenos endodônticos por PCR e quantificação de DNA em amostras extraídas de dentes com periodontite apical pós-tratamento** • *Objetivo:* O objetivo deste estudo clínico foi quantificar a concentração de DNA e detectar algumas espécies bacterianas de amostras de dentes tratados endodonticamente com periodontite apical após a remoção da guta-percha (S1), após o preparo químico-mecânico na primeira sessão (S2), 5 dias após o preenchimento do canal com solução fisiológica estéril (S3), após reinstrumentação na segunda sessão (S4), e 14 dias após a inserção da medicação intracanal na terceira sessão (S5). *Métodos:* Quinze dentes tratados endodonticamente foram selecionados. A remoção da guta-percha foi realizada por meio da técnica coroa-ápice. Utilizaram-se limas manuais associadas à clorexidina gel a 2% durante o preparo químico-mecânico. A medicação intracanal selecionada foi à base de hidróxido de cálcio. DNA foi isolado das amostras e foram investigadas 14 espécies bacterianas (primer espécie-específico16S rDNA). A concentração de DNA foi quantificada utilizando o espectrofotômetro NanoDrop™ 2000. *Resultados:* Em todos os casos foram detectadas bactérias, como revelado por meio do primer universal. DNA foi isolado de todas as amostras, com uma concentração média de 4,24 ± 2,9 ng/µL (S1), 3,39 ± 1,54 ng/µL (S2), 4,0 ± 1,94 ng/µL (S3), 2,66 ± 0,98 ng/µL (S4) e 3,97 ± 2,32 ng/µL (S5). *Parvimonas micra* e *Enterococcus faecalis* (S1), *P. micra* (S2), *Porphyromonas endodontalis* e *E. faecalis* (S3), *E. faecalis* e *Prevotella nigrescens* (S4/S5) foram as espécies mais frequentemente detectadas. A concentração de DNA diminuiu entre S3 e S4 (p = 0,0256), ao passo que um aumento foi observado entre S4 e S5. *Conclusão:* Uma ampla variedade de espécies bacterianas foi detectada em canais radiculares de dentes tratados endodonticamente com periodontite apical. Além disso, o uso da medicação intracanal não potencializou a redução da concentração de DNA bacteriano.

DESCRITORES | Endodontia; Falha de Tratamento; Periodontite Periapical; Bactérias; Reação em Cadeia da Polimerase; DNA; Clorexidina.

CORRESPONDING AUTHOR | • **Marcos Sergio Endo** Department of Restorative Dentistry, Piracicaba Dental School, State University of Campinas • **Av. Limeira, 901** Piracicaba, SP, Brazil • **13414-903** E-mail: marcossendo@gmail.com

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INTRODUCTION

Failure of endodontic treatment is acknowledged to be the continuing presence of bacteria within the root canal system, even in well-treated teeth.[1] Endodontic treatment failure that is attributable to remaining microorganisms will only occur if these microorganisms possess pathogenicity, reach sufficient numbers, and gain access to the periradicular tissues to induce or maintain periradicular disease.[1]

In most cases where endodontic treatment fails, failure is the result of treatment procedures not having met a satisfactory standard for control and elimination of infection.[2] Modern endodontic treatment procedures aim to eliminate microorganisms during root canal preparation and disinfection. Follow-up studies examining the outcome of endodontic therapy revealed a very high success rate when a negative bacterial culture was a prerequisite before root filling,[3] although there is clear evidence that a negative culture does not correlate with a bacteria-free root canal system.[4]

Most clinical trials evaluating the antibacterial effectiveness of intracanal procedures and the bacterial species persisting after treatment have been based on traditional culture-dependent methods.[5-7] Recent molecular biology studies have suggested that the microbiota in root-canal-treated teeth with apical periodontitis is more complex than previously shown by culture-dependent methods.[4,8] Bacteria have been detected in almost all treated canal associated with persistent disease and a higher mean number of taxa per case has been observed.[8,9]

Essentially, endodontic infections are treated by chemo-mechanical preparation supplemented or not by an interappointment intracanal medication. Although a substantial reduction in intracanal microbial communities is usually reached after chemo-mechanical procedures with antimicrobial irrigants, it has been shown that predictable disinfection in most cases can only be achieved after an interappointment intracanal medication.[6-7] Calcium hydroxide is arguably the most used substance between treatment sessions, but studies have shown inconsistent results as to its efficacy in significantly enhancing disinfection.[6-7,10] Then intracanal dressing was used to test if the concentration of DNA and bacterial species that might have survived after chemo-mechanical preparation would decrease.

The aim of this clinical study was to quantify the total concentration of DNA and to detect bacterial species from samples of infected root-filled teeth with post-treatment apical periodontitis after removal of the gutta-percha (S1), after chemo-mechanical preparation with 2% chlorhexidine (CHX) gel in the first appointment (S2), 5 days after the canal was filled with sterile physiological solution (S3), after reinstrumentation in the second appointment (S4), and 14 days after an intracanal dressing was placed in the third appointment (S5).

MATERIAL AND METHODS

Patient selection

Fifteen patients were selected from those who attended the Piracicaba Dental School, SP, Brazil, with a need for nonsurgical endodontic retreatment. The Human Volunteers Research and Ethics Committee of the Piracicaba Dental School approved a protocol describing the specimen collection for this investigation, and all patients signed an informed consent to participate. A detailed medical and dental history was obtained from each patient. Patients who had received antibiotic treatment during the last 3 months or had a general disease were excluded from the study. The age of the patients ranged from 19 to 65 years. All of the selected teeth were single-rooted. The teeth had been previously root-filled and showed radiographic evidence of apical periodontitis. Failure of the root canal treatment was determined on the basis of clinical and radiographic examinations. All teeth had been endodontically treated and filled

more than 2 years previously, and the patients were asymptomatic. All teeth had enough crown structure for adequate isolation with a rubber dam, and had no periodontal pockets deeper than 4 mm.

Microbial sampling

The teeth were isolated with a rubber dam. The crown and the surrounding rubber dam were disinfected with 30% H_2O_2 (v/v) for 30 s followed by 2.5% NaOCl for an additional 30 s. Subsequently, 5% sodium thiosulphate was used to inactivate the disinfectant agents.[11-12] A swab sample was taken from the surface and streaked on blood agar plates to test for disinfection. An access cavity was prepared with sterile high-speed diamond burs under irrigation with sterile physiological solution. Before entering the pulp chamber, the access cavity was disinfected following the same protocol as above, and sterility was checked again by taking a swab sample of the cavity surface and streaking it onto blood agar plates. Aseptic techniques were used throughout root canal treatment and sample acquisition. The samples (pre- and post-clinical procedures) were collected with three sterile paper points, which were consecutively placed into each canal to the total length calculated from the pre-operative radiograph, kept in place for 60 s and then pooled in a sterile tube containing 1 mL of VMGA III transport medium.[11] The samples were immediately frozen at -20°C.

Clinical procedures

The same endodontic specialist performed all retreatments, intracanal dressing and sampling procedures. The tooth was anesthetized and, after accessing the pulp chamber, the root filling materials were removed using the crown-down technique. No solvent was used at any time to avoid a negative effect on microbial viability. Buccolingual and mesiodistal radiographs of each tooth were taken to confirm gutta-percha removal.

First appointment

The root filling was removed using Gates-Glidden drills (Dentsply Maillefer, Ballaigues, Switzerland) of sizes 5 (1.3 mm), 4 (1.1 mm), 3 (0.9 mm) and 2 (0.7 mm) up to 6 mm shorter than the working length. Irrigation with sterile physiological solution was performed in order to remove any remaining materials and to moisten the canal prior to sample collection. A K-file of size #15 (Dentsply Maillefer, Ballaigues, Switzerland) was inserted to the full length of the root canal calculated from the pre-operative radiograph. This file was inserted to full length until canal patency was confirmed. Working length (at the apical foramen) was confirmed by an apical locator (Novapex, Forum Technologies, Rishon le-Zion, Israel). After removal of the gutta-percha with hand files, the first sample was taken with three paper points, which were then pooled in VMGA III. The apical preparation was performed using K-files ranging from size #40 to #45 followed by step-back instrumentation, which ended after the use of three files larger than the last file used for the apical preparation.

All root canals were irrigated with a syringe (27-gauge needle) containing 1 mL of the chemical auxiliary substance (2% CHX gel; Endogel, Itapetininga, SP, Brazil) before the use of each instrument and immediately rinsed afterwards with 4 mL of sterile physiological solution. The CHX gel consisted of a gel base (1% natrosol) and CHX gluconate at pH 7.0. Natrosol gel (hydroxyethyl cellulose) is a nonionic, highly inert and water-soluble agent. After the instrumentation, CHX activity was inactivated with 5 mL of a solution containing 5% Tween 80 and 0.07% (w/v) lecithin during a 1-minute period, which was removed with 5 mL of sterile physiological solution. Retreatment was deemed complete when the last file reached the working length, there was no filling material covering the instrument, and the canal walls were smooth and free of visible debris. Furthermore, a close inspection was conducted

with a dental operating microscope (DF Vasconcellos S/A, São Paulo, Brazil) under high magnification to confirm the complete removal of gutta-percha.

After the root canal preparation had been completed, the canal was irrigated for 3 minutes with 5 mL of 17% EDTA. Then, the root canal was rinsed with 5 mL of sterile physiological solution. Subsequently, the second (first chemo-mechanical) sample (S2) was taken with three paper points, which were pooled in VMGA III. The canal was then thoroughly rinsed with sterile physiological solution using a syringe (27-gauge needle). The access cavity was then temporized with cement to a thickness of at least 2 mm (Coltosol, Coltène/Whaledent Inc., Cuyahoga Falls, OH, USA) and a second layer of Filtek Z250® (3M ESPE, St. Paul, MN, USA) in combination with a single-bond adhesive (3M ESPE, St. Paul, MN, USA).

Second appointment

Rubber dam isolation and access cavity procedures were performed following the same protocol as described above. After access was obtained and coronal disinfection was performed, a third sample (S3) was taken with three paper points, which were pooled in VMGA III. Re-instrumentation was performed with the last file up to working length and following the same irrigation protocol. After this second chemo-mechanical preparation was performed, a fourth sample (S4) was taken with three paper points, pooled in VMGA III. An intracanal dressing with calcium hydroxide [$Ca(OH)_2$] associated with 2% CHX gel was placed over the entire length of the prepared canal using lentulo spiral fillers. The paste was packed at the level of the canal entrance and a radiograph was taken to check for adequate placement (homogeneous filling throughout the entire extent of the prepared canal). The access cavity was then temporized with temporary cement to a thickness of at least 2 mm (Coltosol, Coltène/Whaledent Inc., Cuyahoga Falls, OH, USA) and a second layer of Filtek Z250® (3M ESPE, St. Paul, MN, USA) in combination with a single-bond adhesive (3M ESPE, St. Paul, MN, USA).

Third appointment

The third appointment was scheduled for 14 days later. At this time, the tooth was isolated with a rubber dam, the operative field was disinfected, as previously described for the first visit, and a control bacteriological sample was obtained from the operating field. The composite restoration and temporary cement were removed with a sterile high-speed carbide bur and the canal was irrigated with sterile physiological solution. The canal walls were cleaned with a hand K-file one size greater than that of the master apical file, and irrigated with 5 mL of sterile physiological solution, dried with paper points, and irrigated with 5 mL of a solution containing 5% Tween 80 and 0.07% (w/v) lecithin during a 1-minute period to inactivate the CHX. After removal of the intracanal dressing, a fifth sample (S5) was taken with three paper points, which were pooled in VMGA III.

Finally, all teeth were filled using vertical and lateral compaction of the gutta-percha cones (Konne, Belo Horizonte, MG, Brazil) and with Endomethasone® sealer (Septodont, Saint-Maur-des-Fossés, France). The access cavities were restored with a 2 mm layer of Coltosol® (Coltène Whaledent, Cuyahoga Falls, OH, USA) and Filtek Z250® (3M Dental Products, St Paul, MN, USA).

DNA extraction

Microbial DNA from samples of all stages of endodontic retreatment (S1, S2, S3, S4, S5), from the control sample as well as from ATCC bacteria were extracted and purified by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

DNA quantification

The extracted DNA samples were quantified by

using a NanoDrop spectrophotometer (Thermo Scientific NanoDrop™ 2000/2000c, Wilmington, DE, USA). A blank sample was established using AE buffer AE (Elution buffer; Qiagen, Hilden, Germany). Each sample (1.5 µL) was placed sequentially in the spectrophotometer. The DNA concentration was calculated from the 260 nm absorbance value for each replicate using the DNA-50 settings. The software automatically calculated the DNA concentration in ng/µL.

Bacterial detection (polymerase chain reaction - PCR 16S rDNA)

The reference bacteria strains used in this study were purchased from the American Type Culture Collection (ATCC) and are listed as follows:

- *Aggregatibacter actinomycetemcomitans* (ATCC 43718),
- *Enterococcus faecalis* (ATCC 4034),
- *Filifactor alocis* (ATCC 35896),
- *Fusobacterium nucleatum* (ATCC 25586),
- *Gemella morbillorum* (ATCC 27824),
- *Parvimonas micra* (ATCC 33270),
- *Porphyromonas endodontalis* (ATCC 35406),
- *Porphyromonas gingivalis* (ATCC 33277),
- *Prevotella intermedia* (ATCC 25611),
- *Prevotella nigrescens* (ATCC 33536),
- *Prevotella tannerae* (ATCC 51259),
- *Tannerella forsythia* (ATCC 43037),
- *Treponema denticola* (ATCC 35405) and
- *Treponema socranskii* (ATCC 35536).

PCR assay

The PCR reaction was performed in a thermocycler (My-Cycler; Bio-Rad, Hercules, CA, USA) with a total volume of 25 µL containing 2.5 µL of 10× Taq buffer (1×; MBI Fermentas, Mundolsheim, France), 0.5 µL of dNTP mix (25 µmol/L of each deoxyribonucleoside triphosphate – dATP, dCTP, dGTP, and dTTP; MBI Fermentas, Hanover, MD, USA), 1.25 µL of 25 mmol/L $MgCl_2$, 0.25 µL of forward and reversal universal primers (0.2 µmol/L; Invitrogen, Eugene, OR, USA), 1.5 µL of sample DNA (1 µg/50 µL), 1.5 µL of Taq DNA polymerase (1 U; MBI Fermentas), and 17.25 µL of nuclease-free water. The primer sequences and PCR cycling parameters were previously optimized[12] and are listed in Table 1.

Statistical analysis

The concentration of DNA from each treatment step was calculated based on quantitative data obtained from samples S1, S2, S3, S4 and S5. Quantitative data were statistically analyzed for differences by using the Mann-Whitney *U* test comparing pairs of groups. The significance level was always set at 5% ($p < 0.05$).

RESULTS

DNA quantification

DNA was isolated from all samples, with an average concentration of 4.24 ± 2.9 ng/µL (S1), 3.39 ± 1.54 ng/µL (S2), 4.0 ± 1.94 ng/µL (S3), 2.66 ± 0.98 ng/µL (S4) and 3.97 ± 2.32 ng/µL (S5; Table 2). The highest DNA amount was found in the initial samples (S1). No significant statistical difference was detected between the DNA concentration of samples S1 and S2 (Mann-Whitney test, $p = 0.3937$); S2 and S3 (Mann-Whitney test, $p = 0.1548$); and S1 and S5 (Mann-Whitney test, $p = 0.4017$). However, a statistically significant reduction in DNA concentration from S3 to S4 (Mann-Whitney test, $p = 0.0256$) and an increase from S4 to S5 (Mann-Whitney test, $p = 0.0445$) were detected.

Overview of microbial composition by 16S rDNA PCR

Bacteria were present in all samples (S1, S2, S3, S4, S5), confirming the infectious etiology of post-treatment disease after PCR amplification using broad-range 16S rDNA gene primers.

The bacteria recovered by species-specific

Table 1 | PCR primer pairs and cycling parameters used for detection of bacterial species in samples from root-filled teeth with post-treatment apical periodontitis.

Target bacteria	Primer pairs (5'–3')	Amplicon size	Cycles
Universal (16S rDNA)	Forward: TCC TAC GGG AGG CAG CAG T Reverse: GGA CTA CCA GGG TAT CTA ATC CTG TT	466 bp	Initial denaturation at 95°C for 10 min and 40 cycles of 95°C for 10 s, 60°C for 10 s, and a final extension step at 72°C for 25 s
Actinobacillus actinomycetemcomitans	Forward:AAA CCC ATC TCT GAG TTC TTC TTC Reverse: ATG CCA ACT TGA CGT TAA AT	557 bp	Initial denaturation at 94°C for 30 s and 36 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min
Prevotella intemedia	Forward: TTT GTT GGG GAG TAA AGC GGG Reverse: TCA ACA TCT CTG TAT CCT GCG T	575 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min
Prevotella nigrescens	Forward: ATG AAA CAA AGG TTT TCC GGT AAG Reverse: CCC ACG TCT CTG TGG GCT GCG A	804 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min
Tannerella forsythia	Forward: GCG TAT GTA ACC TGC CCG CA Reverse: TGC TTC AGT GTC AGT TAT ACC T	641 bp	Initial denaturation at 95°C for 1 min and 36 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 2 min
Prevotella tannerae	Forward: CTT AGC TTG CTA AGT ATG CCG Reverse: CAG CTG ACT TAT ACT CCC G	550 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min
Treponema denticola	Forward: TAA TAC CGA ATG TGC TCA TTT ACA T Reverse: TCAAAGAAGCAT TCC CTC TTC TTC TTA	316 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min
Treponema socranskii	Forward: GAT CAC TGTATA CGGAAGGTAGACA Reverse: TAC ACT TAT TCC TCG GAC AG	288 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min
Enterococcus faecalis	F: CCG AGT GCT TGC ACT CAA TTG G R: CTC TTA TGC CAT GCG GCA TAA AC	138 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 95°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 7 min
Filifactor alocis	F: CAG GTG GTT TAA CAA GTT AGT GG R: CTA AGT TGT CCT TAG CTG TCT CG	594 bp	Initial denaturation at 95°C for 2 min and 26 cycles of 95°C for 30 s, 58°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 2 min
Fusobacterium nucleatum	F: AGT AGC ACA AGG GAG ATG TAT G R: CAA GAA CTA CAA TAG AAC CTG A	1000 bp	Initial denaturation at 95°C for 5 min and 30 cycles of 94°C for 30 s, 40°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min
Gemella morbillorum	F: GAC TAC CAG GGT ATC TAA TCC R: TAT GAG GTT GGC TGA CTC TCG	781 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 52°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min
Parvimonas micra	F: AGA GTT TGA TCC TGG CTC AG R: ATA TCA TGC GAT TCT GTG GTC TC	207 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min
Porphyromonas endodontalis	F: GCT GCA GCT CAA CTG TAG TC R: CCG CTT CAT GTC ACC ATG TC	672 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min
Porphyromonas gingivalis	F: AGG CAG CTT GCC ATA CTG CG R: ACT GTT AGC AAC TAC CGA TGT	404 bp	Initial denaturation at 95°C for 2 min and 36 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 2 min

PCR: polymerase chain reaction

Table 2 | Bacterial frequency for 14 selected target species-specific primers and DNA concentration (ng/μL) in samples from root-filled teeth with apical periodontitis. Initial sample (S1), after first chemo-mechanical preparation (S2), 5 days after the canal was filled with sterile physiological solution (S3), after second chemo-mechanical preparation (S4), and 14 days after placement of intracanal dressing (S5).

	S1	S2	S3	S4	S5
Number of positive samples	31	15	31	20	32
DNA concentration (mean ± SD)	4.24 ± 2.9	3.39 ± 1.54	4.0 ± 1.94 a	2.66 ± 0.98 b,A	3.97 ± 2.32 B

Different lower letters (a,b) indicate a significant difference (Mann-Whitney test, $p < 0.05$). Different capital letters (A,B) indicate a significant difference (Mann-Whitney test, $p < 0.05$).

Table 3 | Frequency of positive samples for selected species in initial sample (S1), after first chemo-mechanical preparation (S2), 5 days after the canal was filled with sterile physiological solution (S3), after second chemo-mechanical preparation (S4), and 14 days after placement of intracanal dressing (S5).

Target species	Time of sample collection				
	S1	S2	S3	S4	S5
Fusobacterium nucleatum	1	0	4	3	4
Prevotella intermedia	2	0	0	2	2
Prevotella nigrescens	4	0	5	6	6
Prevotella tannerae	1	2	1	0	0
Enterococcus faecalis	6	2	5	6	7
Gemella morbillorum	3	0	4	1	6
Treponema denticola	1	0	0	0	0
Treponema socranskii	1	0	1	0	0
Porphyromonas endodontalis	0	2	8	1	3
Porphyromonas gingivalis	0	0	1	0	0
Filifactor alocis	0	0	1	0	0
Parvimonas micra	10	7	0	1	1
Tannerella forsythia	2	1	1	0	1
Actinobacillus actinomycetemcomitans	0	1	0	0	2
Total	31	15	31	20	32

primer 16S rDNA after removal of the gutta-percha (S1), after the first chemo-mechanical preparation (S2), 5 days after the canal was filled with sterile physiological solution (S3), after re-instrumentation (S4), and 14 days after the intracanal dressing was placed (S5) are shown in Table 3. At S1, the most prevalent taxon was *Parvimonas micra* (10 cases), followed by *Enterococcus faecalis* (6 cases) and *Prevotella nigrescens* (4 cases). After the first chemo-mechanical preparation (S2), the most prevalent taxon was *Parvimonas micra* (7 cases), followed by *Enterococcus faecalis* (2 cases). The most prevalent taxon at S3 was *Porphyromonas endodontalis* (8 cases) followed by *Enterococcus faecalis* (5 cases) and *Prevotella nigrescens* (5 cases). After re-instrumentation (S4), the most prevalent taxons were *Prevotela nigrescens* (6 cases) and *Enterococcus faecalis* (6 cases). At S5, the most prevalent taxon was *Enterococcus faecalis* (7 cases). The higher the concentration of DNA, the higher the number of bacterial species detected by 16S rDNA PCR in each step (Table 2).

DISCUSSION

The DNA concentration in samples from root-filled canals with periapical lesions was monitored at five time-points after endodontic procedures. Many studies have shown that microorganisms are the major causative agents of endodontic therapy failure; in contrast, to date no study has monitored the individual concentration of total DNA after gutta-percha removal, chemo-mechanical preparation and intracanal medication. Residual DNA may contribute to disease progression and/or maintenance. Furthermore, properties that enable bacteria to persist after therapy include resistance to disinfection by chemo-mechanical and intracanal medication procedures, and the ability to enter a viable but nonculturable (VBNC) state in response to stress.[13]

In the present study, DNA was measured using the NanoDrop system. It is a full-spectrum spec-

trophotometer for measuring the absorbance of DNA, RNA, protein, and dye, and it functions by combining fiber optic technology and natural surface tension properties for capture. The system employs shorter wavelengths, which result in a broad range of nucleic acid concentration measurements, essentially eliminating the need to perform dilutions. Moreover, molecular genetic studies hold the promise of identifying genetic factors that influence human disease susceptibility and outcome.[14] The accuracy and precision of DNA quantification are critical factors for efficient use of DNA samples in high-throughput genotype and sequence analyses.

Analysis of the endodontic microbiota is still focused on the detection and identification of bacteria using different methods, including culture[15] and PCR[4,8] techniques. PCR 16S rDNA assays represent the most sensitive method applied to the study of endodontic bacteria.[16] This technique can readily identify slow growing or uncultivable strains.[17] Among many potential amplification sites, 16S rDNA genes appear to be the most useful target of PCR 16S rDNA genes, are present in every bacterium and are highly conserved within a species.[18] It is noteworthy that all examined samples at the different timepoints contained bacterial DNA. These advantages of molecular methods also help to explain why bacteria were detected in all treated cases with post-treatment disease in this and other studies,[8-9] whereas culture studies have found bacteria in 44% to 85% of the cases.[8,15,19,20] For the present study, we used the PCR (16S rDNA) assay, which has the potential to offer more detailed insights into complex bacterial communities.[21] Furthermore, it seems interesting to evaluate the effects of endodontic procedures against these microorganisms, in case they are still present after root canal filling removal (S1), after the first chemo-mechanical preparation in the first appointment (S2), 5 days after the canal was filled with sterile physiological solution (S3), after re-instrumentation in the second appointment (S4), and 14 days after the intracanal dressing was placed in the third appointment (S5).

Our findings were confirmed by amplification with 16S rDNA universal primers, which generated the predicted amplicon for all samples (S1, S2, S3, S4, S5) from root-filled teeth with apical periodontitis. This assertion lends strong support to the claim that persistent intraradicular infection is the major factor associated with endodontic therapy failure.[3,8] On the other hand, it should be considered that not only the presence of the bacteria is an important factor for development or maintenance of disease, but also the population size.[22] Diagnostic methods using DNA-based tools allow the identification of viable but noncultivable cells that are metabolically active, but not dividing.[16] It is worth pointing out that the ability to detect DNA from dead cells poses a major problem when one is investigating the immediate effectiveness of antibacterial treatment because DNA from cells that have recently died can still be detected.[21] The possibility exists that DNA from dead cells may have been destroyed by the effects of the substances used during treatment.[23] Hydroxyl ions from calcium hydroxide also have oxidative damaging effects on DNA,[23] and may have contributed to the degradation of free DNA from dead cells. Failure to detect microorganisms does not necessarily mean that they are absent. Given the difficulties in taking samples from root-filled teeth, it is possible that many microorganisms can escape detection, particularly when they number below the detection rate of the identification method.[8] The PCR method used in this study only detects targeted microbial species. However, it is possible that species other than those studied could have been present in the examined teeth.

This molecular study also evaluated the reduction in bacterial DNA concentration promoted by intracanal disinfection procedures, and identified the 14 taxa persisting after each step. At S1, *Par-*

vimonas micra was the most frequent anaerobic species detected by PCR[24,25] followed by *Enterococcus faecalis*. The ecological niche of *Parvimonas micra* could be related to its wide range of peptidase activities, making amino acids and peptides available from serum glycoproteins.[26]

The present results also revealed the occurrence of some gram-negative bacteria after clinical procedures. Post-treatment apical periodontitis is almost always associated with intraradicular polymicrobial infection.[8,25] *Porphyromonas endodontalis* and *Parvimonas micra* are also considered as typical bacteria involved in endodontic infection.[25] *Tannerella forsythia*, *Porphyromonas gingivalis*, *Treponema denticola*,[27] *Fusobacterium nucleatum* and *P. gingivalis*[28] have also been described as common endodontic bacterial pathogens. These gram-negative bacteria, which are common members of primary intraradicular infections, are usually eliminated following treatment, though studies have reported that some anaerobic rods, such as *F. nucleatum* and *Prevotella* species, are among the most common species found in post-instrumentation samples.[3,4,10] At S1, many studies have detected gram-negative bacteria in root-filled teeth using PCR;[8,24,25] however, monitoring this species after clinical procedures is a necessity. The finding that several gram-negative species were found in samples (S2, S3, S4, and S5) might also indicate that bacterial persistence can be related to factors other than the intrinsic resistance to treatment procedures and substances by a specific taxon.

After endodontic procedures (S3, S4, and S5), *Enterococcus faecalis* was a species frequently detected. This species can colonize filled root canals and may be involved in endodontic failures.[8] It can survive in an environment in which there are scant available nutrients and in which commensality with other bacteria is minimal.[22] These bacteria, in particular *E. faecalis*, may also survive in the smear layer and other debris inside the root canal, and may be extremely difficult to remove by irrigation and instrumentation.[29] *E. faecalis* was found in a range of 0%–77% and is the predominant species in most of the studies of secondary infection.[8,15,24,30] In only three studies was *E. faecalis* not detected in persistent apical periodontitis.[4] Employing real-time PCR, *E. faecalis* was found to be three times more prevalent in refractory than in primary endodontic infections.[31] *E. faecalis* has already been previously found to endure endodontic treatment procedures in cases with necrotic pulp.[32] This finding is in line with studies showing that gram-positive bacteria might be more resistant to treatment procedures.[10] *E. faecalis* has been shown to have the ability to invade dentinal tubules[33] and adhere to collagen in the presence of human serum,[34] which can allow it to resist chemo-mechanical preparation. Clearly, more effective methodologies for disinfection must be established to eradicate this pathogen in the course of endodontic treatment.

The present study confirms that *E. faecalis* is resistant to endodontic procedures. At S2 and S4, this species could be detected after the chemo-mechanical preparation using 2% CHX gel, and, at S5, it could still be detected after using the combination of $Ca(OH)_2$ with 2% CHX gel for 14 days as intracanal dressing. Moreover, this species is resistant to calcium hydroxide,[35] a commonly used intracanal medicament. Combinations of $Ca(OH)_2$ with CHX[22] are used to enhance antimicrobial properties. All of these factors help to explain why *E. faecalis* is so prevalent in patients in whom endodontic treatment has failed.

When S3 and S4 were compared, there was a statistically significant reduction in DNA concentration after the second chemo-mechanical preparation. This corroborates the findings of several other studies[5,32] in which bacterial levels and the number of taxa were substantially reduced after chemo-mechanical preparation. During the treatment of infected root canals, two steps assume spe-

cial relevance with regard to bacterial elimination:

- the chemo-mechanical preparation and
- the interappointment dressing.

Studies have revealed that the chemo-mechanical preparation is not sufficient to predictably render root canals bacteria-free, with about 40%–50% of the prepared canals still containing cultivable bacteria.[5,6] To overcome the limitations of chemo-mechanical procedures in disinfecting the entire root canal system, the use of an inter-appointment medication has been advocated.[6,7] However, in the present study intracanal dressing was not able to eliminate or reduce the target species investigated and did not reduce DNA concentration when compared with the initial sample. The present results are in clear agreement with those of Blome *et al.*[25] They observed that a $Ca(OH)_2$ intracanal dressing placed for 14 days failed to achieve a further reduction of total bacterial counts when compared to the values observed immediately after chemo-mechanical preparation.[25] Moreover, this is also in agreement with the findings of Sakamoto *et al.*,[17] who found no significant difference between post-instrumentation samples and the samples collected after placement of the $Ca(OH)_2$ dressing, although these findings were associated with necrotic pulp tissue. The need for intracanal dressings is recognized especially in those cases where endodontic therapy cannot be successfully completed because of the presence of pain, constant exudation and lack of time.

CONCLUSION

A wide variety of bacterial species was detected in root-filled teeth with post-treatment apical periodontitis. Moreover, the use of an intracanal dressing was unable to further reduce the concentration of bacterial DNA.

REFERENCES

1. Siqueira JF Jr. Aetiology of root canal treatment failure: why well-treated teeth can fail. Int Endod J. 2001 Jan;34(1):1-10. doi: 10.1046/j.1365-2591.2001.00396.x.
2. Nair PNR, Sjögren U, Figdor D, Sundqvist G. Persistent periapical radiolucencies of root-filled human teeth, failed endodontic treatments, and periapical scars. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1999 May;87(5):617-27. doi: 10.1016/S1079-2104(99)70145-9.
3. Sjögren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J. 1997 Sept;30(5):297-306. doi: 10.1046/j.1365-2591.1997.00092.x.
4. Rolph HJ, Lennon A, Riggio MP, Saunders WP, MacKenzie D, Coldero L, et al. Molecular identification of microorganisms from endodontic infections. J Clin Microbiol. 2001 Sept;39(9):3282-9. doi: 10.1128/JCM.39.9.3282-3289.2001.
5. Bystrom A, Sundqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. Int Endod J. 1985 Jan;18(1):35-40. doi: 10.1111/j.1365-2591.1985.tb00416.x.
6. Shuping GB, Orstavik D, Sigurdsson A, Trope M. Reduction of intracanal bacteria using nickel-titanium rotary instrumentation and various medications. J Endod. 2000 Dec;26(12):751-5. doi: 10.1097/00004770-200012000-00022.
7. Sjögren U, Figdor D, Spångberg L, Sundqvist G. The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. Int Endod J. 1991 May;24(3):119-25. doi: 10.1111/j.1365-2591.1991.tb00117.x.
8. Siqueira JF Jr, Rôças IN. Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004 Jan;97(1):85-94. doi: 10.1016/S1079-2104(03)00353-6.
9. Rôças IN, Siqueira JF Jr, Aboim MC, Rosado AS. Denaturing gradient gel electrophoresis analysis of bacterial communities associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004 Dec;98(6):741-9. doi: 10.1016/j.tripleo.2004.09.006.

10. Peters LB, van Winkelhoff AJ, Buijs JF, Wesselink PR. Effects of instrumentation, irrigation and dressing with calcium hydroxide on infection in pulpless teeth with periapical bone lesions. Int Endod J. 2002 Jan;35(1):13-21. doi: 10.1046/j.0143-2885.2001.00447.x.
11. Möller AJ. Microbiological examination of root canals and periapical tissues of human teeth. Methodological studies. Odontol Tidskr. 1966 Dec;74(5):Suppl: 1-380.
12. Martinho FC, Chiesa WMM, Leite FRM, Cirelli JA, Gomes BP. Antigenic activity of bacterial endodontic contents from primary root canal infection with periapical lesions against macrophage in the release of interleukin-1beta and tumor necrosis factor alpha. J Endod. 2010 Sept;36(9):1467-74. doi: 10.1016/j.joen.2010.06.012.
13. Figdor D, Davies JK, Sundqvist G. Starvation survival, growth and recovery of Enterococcus faecalis in human serum. Oral Microbiol Immunol. 2003 Aug;18(4):234-9. doi: 10.1034/j.1399-302X.2003.00072.x.
14. Haque KA, Pfeiffer RM, Beerman MB, Struewing JP, Chanock SJ, Bergen AW. Performance of high-throughput DNA quantification methods. BMC Biotechnol. 2003 Oct;3:20. doi: 10.1186/1472-6750-3-20.
15. Pinheiro ET, Gomes BP, Ferraz CC, Sousa EL, Teixeira FB, Souza-Filho FJ. Microorganisms from canals of root-filled teeth with periapical lesions. Int Endod J. 2003 Jan;36(1):1-11. doi: 10.1046/j.1365-2591.2003.00603.x.
16. Siqueira JF Jr, Rocas IN. PCR methodology as a valuable tool for identification of endodontic pathogens. J Dent. 2003 Jul;31(5):333-9. doi: 10.1016/S0300-5712(03)00051-4.
17. Sakamoto M, Siqueira JF Jr, Rôças IN, et al. Bacterial reduction and persistence after endodontic treatment procedures. Oral Microbiol Immunol. 2007 Feb;22(1):19-23. doi: 10.1111/j.1399-302X.2007.00315.x.
18. Schmidt T, Relman DA. Phylogenetic identification of uncultured pathogens using ribosomal RNA sequences. Methods Enzymol. 1994;235:205-22. doi: 10.1016/0076-6879(94)35142-2.
19. Molander A, Reit C, Dahlén G, kvist T. Microbiological status of root-filled teeth with apical periodontitis. Int Endod J. 1998 Jan;31(1):1-7. doi: 10.1046/j.1365-2591.1998.t01-1-00111.x.
20. Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998 Jan;85(1):86-93. doi: 10.1016/S1079-2104(98)90404-8.
21. Siqueira JF Jr, Rôças IN. Exploiting molecular methods to explore endodontic infections: Part 1 - current molecular technologies for microbiological diagnosis. J Endod. 2005 Jun;31(6):411-23. doi: 10.1097/01.don.0000157989.44949.26.
22. Vianna ME, Horz HP, Conrads G, Feres M, Gomes BP. Comparative analysis of endodontic pathogens using checkerboard hybridization in relation to culture. Oral Microbiol Immunol. 2008 Aug;23(4):282-90. doi: 10.1111/j.1399-302X.2007.00425.x.
23. Siqueira JF Jr, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J. 1999 Sept;32(5):361-9. doi: 10.1046/j.1365-2591.1999.00275.x.
24. Gomes BP, Pinheiro ET, Jacinto RC, Zaia AA, Ferraz CC, Souza-Filho FJ. Microbial analysis of canals of root-filled teeth with periapical lesions using polymerase chain reaction. J Endod. 2008 May;34(5):537-40. doi: 10.1016/j.joen.2008.01.016.
25. Blome B, Braun A, Sobarzo V, et al. Molecular identification and quantification of bacteria from endodontic infections using real-time polymerase chain reaction. Oral Microbiol Immunol. 2008 Oct;23(5):384-90. doi: 10.1111/j.1399-302X.2008.00440.x.
26. Ter Steeg PF, van der Hoeven JS. Development of periodontal microflora on human serum. Microbial Ecol Health Dis. 1989;2(1):1-10. doi: 10.3109/08910608909140195.
27. Rôças IN, Siqueira JF Jr, Santos KR, Coelho AM. "Red complex" (Bacteroides forsythus, Porphyromonas gingivalis, and Treponema denticola) in endodontic infections: a molecular approach. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001 Apr;91(4):468-71. doi: 10.1067/moe.2001.114379.
28. Podbielski A, Spahr A, Haller B. Additive antimicrobial activity of calcium hydroxide and chlorhexidine on common endodontic bacterial pathogens. J Endod. 2003 May;29(5):340-5. doi: 10.1097/00004770-200305000-00006.
29. Foschi F, Nucci C, Montebugnoli L, Marchionni S, Breschi L, Malagnino VA, et al. SEM evaluation of canal wall dentine following use of Mtwo and ProTaper NiTi rotary instruments. Int Endod J. 2004 Dec;37(12):832-9. doi: 10.1111/j.1365-2591.2004.00887.x
30. Peciuliene V, Balciuniene I, Eriksen HM, Haapasalo M. Isolation of Enterococcus faecalis in previously root-filled canals

in a Lithuanian population. J Endod. 2000 Oct;26(10):593-5. doi: 10.1097/00004770-200010000-00004.

31. Williams JM, Trope M, Caplan DJ, Shugars DC. Detection and quantitation of E. faecalis by real-time PCR (qPCR), reverse transcription-PCR (RT-PCR), and cultivation during endodontic treatment. J Endod. 2006 Aug;32(8):715-21. doi: 10.1016/j.joen.2006.02.031.
32. Rôças IN, Siqueira JF Jr. In vivo antimicrobial effects of endodontic treatment procedures as assessed by molecular microbiologic techniques. J Endod. 2011 Mar;37(3):304-10. doi: 10.1016/j.joen.2010.11.003.
33. Siqueira JF Jr, de Uzeda M, Fonseca ME. A scanning electron microscopic evaluation of in vitro dentinal tubules penetration by selected anaerobic bacteria. J Endod. 1996 Jun;22(6):308-10. doi: 10.1016/S0099-2399(96)80265-2.
34. Love RM. Enterococcus faecalis – a mechanism for its role in endodontic failure. Int Endod J. 2001 Jul;34(5):399-405. doi: 10.1046/j.1365-2591.2001.00437.x.
35. Siqueira JF Jr, de Uzeda M. Disinfection by calcium hydroxide pastes of dentinal tubules infected with two obligate and one facultative anaerobic bacteria. J Endod. 1996 Dec;22(12):674-6. doi: 10.1016/S0099-2399(96)80062-8.