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# Microbiological Investigation in Teeth with Persistent/Secondary Endodontic Infection in Different Stages of Root Canal Retreatment

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## ABSTRACT

**Objective:** The present clinical study investigated the microbiota of teeth with persistent secondary endodontic infection in the different phases of root canal retreatment.

**Methods:** Twenty filled single-rooted teeth with apical periodontitis were included. Samples were collected with sterile paper points before chemo-mechanical preparation (CMP) (S1), after CMP (S2) and after 30 days of calcium hydroxide-based intracanal medication (ICM) (S3). Cultivable bacteria were assessed by colony forming units count (CFU/mL). DNA was extracted and assessed by using nested PCR. Paired t-test and repeated measures ANOVA were applied for intragroup analysis in the stages of endodontic therapy at a significance level of 5%.

**Results:** Cultivable bacteria were detected in all initial samples. CMP reduced bacteria by 99.4% and ICM by 99.5%. The most prevalent species found in the initial samples were *E. faecalis* (20/20), *P. gingivalis* (20/20), *F. nucleatum* (17/20) and *A. actinomycetemcomitans* (10/20), whereas *D. pneumosintes*, *F. alocis*, *P. nigrescens* and *T. socranskii* were not detected. After CMP, *A. israelii*, *A. naeslundii*, *G. morbillorum*, *T. forsythia* and *T. denticola* were not detected ($P<0.05$) either. *E. faecalis* and *P. gingivalis* had a low reduction ($P>0.05$) and *F. nucleatum* had its DNA significantly reduced after CMP ($P<0.05$). ICM had no additional effect on microbial reduction.

**Conclusion:** The microbiota of teeth with persistent/secondary endodontic infection consists of a polymicrobial community with Gram-positive and Gram-negative species, bacillus and cocci, facultative and strict anaerobes. *E. faecalis* and *P. gingivalis* were frequently detected in all stages of root canal retreatment, evidencing their great resistance to endodontic procedures. The endodontic procedures were effective in reducing the levels of bacteria from teeth presenting with persistent/secondary endodontic infection.

**Keywords:** Bacteria, endodontic treatment, intracanal medication, nested PCR, persistent endodontic infection

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## HIGHLIGHTS

- The microbiota of teeth with persistent/secondary endodontic infection is a polymicrobial community with Gram-positive and Gram-negative species, bacillus and cocci, facultative and strict anaerobes
- *Enterococcus faecalis* (Gram-positive) and *Porhyromonas gingivalis* (Gram-negative) were the most detected species in teeth with persistent endodontic infection.
- Nested PCR was effective identifying difficult-to-grow bacteria, including *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.

## INTRODUCTION

The main objective of the root canal treatment is to prevent, reduce or eliminate apical periodontitis by means of shaping, cleaning and filling the root canal system (RCS). Bacteria remaining in the RCS can contribute to poor outcomes in the endodontic therapy (1-4). Thus, reduction/elimination of intracanal bacteria has been considered a key step in the root canal therapy (2, 3, 5, 6).

Chemo-mechanical preparation (CMP) and intracanal medication (ICM) are stages of root canal treatment aimed at promoting great microbial reduction. The use of sodium hypochlorite (NaOCl) at different concentrations (1% to 5.25%) during CMP is well-established in the literature (7). More recently, studies have proposed the use of more concentrated solutions (i.e. 6% NaOCl) for a

more effective microbial elimination (2, 3). Calcium hydroxide [$Ca(OH)_2$] is the most used ICM (3, 8), but due to its limited antimicrobial activity against some resistant species, its association with 2% chlorhexidine (CHX) has been suggested (3, 8, 9).

Previous literature has investigated the microbial composition of root canal with persistent/secondary infections by using traditional culture and molecular methods (e.g. Polymerase Chain Reaction (PCR) assay) (1, 5, 10, 11). Culture methods for microbial identification cannot reliably assess the bacterial profile in these cases because of technical limitations (i.e. culture medium, culture techniques and gaseous requirements) (12). Several bacterial species are difficult to culture or maintain *in vitro* (12). Therefore, the prevalence of some oral pathogens may be underestimated (12).

The development of novel molecular methods has allowed the detection of unculturable bacteria or those difficult to grow (13). Nested PCR is a molecular technique used for identification of new endodontic pathogens (5, 14), including difficult-to-grow strains and fastidious bacteria, especially in cases of unsuccessful root canal treatment. The sensitivity of nested-PCR is higher compared to culture and single round PCR (5, 15-17). As in the culture method, nested PCR is also an important tool for monitoring the effects of endodontic procedures on some microbial species (17).

It is of major relevance to know microbial species present in failed endodontically treated teeth in the different stages of root canal re-treatment so that endodontic procedures can be improved, which consequently increases the predictability of the endodontic treatment. Thus, the objective of this study was to investigate the microbiota of teeth with persistent/secondary endodontic infection by using the nested PCR method in the different stages of the endodontic re-treatment. The null hypothesis tested was there is no difference in the microbiota before and after endodontic re-treatment.

## MATERIALS AND METHODS

### Patients information and selection of cases

A total of 20 patients seeking non-surgical root canal re-treatment were included in this investigation. The Research Ethics Committee approved the study according to protocol number 018/2014, in which the sample collection was also described. All patients signed an informed consent form prior to their inclusion in the study. All teeth were single-rooted and had been endodontically treated. Additionally, radiographic examination showed evidence of apical periodontitis.

The indication for endodontic re-intervention was based on clinical and radiographic examinations, as previously suggested (1-3). Signs and/or symptoms including tenderness to percussion, pain on palpation, presence of sinus tract, presence of periapical radiolucency and voids in/ around the root canal filling were recorded.

Exclusion criteria were as follows: patients who received antibiotic therapy in the past three months, who had severe systemic disease (ASA-III, American Society of Anaesthesiologists), who had teeth that could not be properly isolated with rubber dam, absence of coronal restoration and periodontal pockets ≥3 mm.

### Clinical protocols and sample collection

The protocols for operative field and sampling procedures have been described in previous studies (2, 3, 5). All clinical procedures were performed by a single experienced operator. After local anesthesia (2% lidocaine with epinephrine 1:100000; Nova DFL, Rio de Janeiro, RJ, Brazil), a two-stage access cavity preparation was performed under abundant irrigation with syringe containing sterile saline solution and a diamond bur at high-speed for removal of contaminants. Next, prior to accessing the pulp chamber, the tooth was isolated with rubber dam and disinfection protocol was performed (2, 3, 5). The crown and surrounding structures were disinfected with 30% hydrogen peroxide (volume/volume) for 30 seconds followed by 2.5% sodium hypochlorite for the same period before being inactivated with 5% sodium thiosulfate. The disinfection of the tooth surface was monitored by taking a swab sample from both external and internal surfaces of the crown and its surrounding areas before streaking it on blood agar plates, which then were incubated aerobically and anaerobically. Following, DNA extraction from the swab and PCR run by using universal bacterial primers were performed. If any positive culture or presence of bands on the agarose gel was detected, then the patient was excluded from the study (18).

After taking a pre-operative radiograph, the root filling materials were removed by using Reciproc R25 files (VDW, Munich, Germany) according to the manufacturer's instructions in a crown-down technique with no chemical solvent. Next, the working length (WL) was established by using an apex locator (Novapex; Forum Technologies, Rishon le-Zion, Israel) at zero point.

The initial sampling (S1) was conducted by introducing three consecutive sterile paper points into the full length of the root canals and retaining in position for 60 seconds. Subsequently, the paper points were pooled in a sterile tube containing 1 mL Viability Medium Göteborg Agar (VMGA III) transport medium for microbial sampling. The samples were transported within 15 minutes to an anaerobic workstation (Don Whitley Scientific, Bradford, UK) for bacterial culture analysis. Following, two sterile paper points (Dentsply Maillefer) were introduced into the same length and period. The papers points were placed into a sterile tube containing 1 mL of Tris-EDTA buffer solution at pH 8.0 (Merck KGaA Darmstadt, Germany) and stored at low temperature (–80°C) for further analysis. In case of a dry canal, 0.85% sterile saline was used to moisten the root canals to allow better sample collection. In case of a wet canal (or those that have been previously irrigated with saline) numerous paper points were used to absorb all the fluid inside the canal. whenever the paper point was unable to reach the full length of the root canal.

The instrumentation of the root canals was performed by using Reciproc R40 files (VDW, Munich, Germany), according to the manufacturer's instructions, in a reciprocating motion generated by electric motor (VDW, Munich, Germany). The endodontic file was used in an in-and-out pecking motion

(approximately 3 mm amplitude) with apical pressure until the instrument reached the working length (i.e. zero displayed on the apex locator). To confirm whether the canals were free of filling material, a dental operating microscope (DF Vasconcelos SA, São Paulo, SP, Brazil) was used.

CMP was performed as described elsewhere (3). In short, the root canals were filled with 1 mL of 6% NaOCl (Drogal, Piracicaba, SP, Brazil) before using each endodontic file and immediately irrigated with 5 mL of 6% NaOCl by using the EndoVac System. After completion of the CMP, 5 mL of 5% sodium thiosulfate (Drogal, Piracicaba, SP, Brazil) was used to inactivate NaOCl for 60 seconds, which was also removed with 5 mL of saline solution. The root canals were irrigated 17% EDTA (3mL) by using ultrasonic device (Satelec/Acteon, Mount Laurel, NJ, USA) with Irrisonic E1 tip (Helse Ultrasonic, Santa Rosa de Viterbo, SP, Brazil), inactivated with 5 mL of 0.5% citric acid, followed by irrigation with sterile saline solution (5 ml). Next, a second sampling (S2) was performed as above-mentioned.

After that, an intracanal medication (ICM) was prepared by mixing $Ca(OH)_2$ with 2% CHX gel at a ratio of 1:1. The resulting paste was inserted into the root canals by using a sterile Lentulo filler (Dentsply Maillefer, Ballaigues, Switzerland) until they were completely filled with the medication. A sterile cotton pellet was used to condense the paste at the level of the canal orifice. The access cavities were restored with a 2-mm layer of temporary cement (Cimpat, Septodont, Saint-Maur-des-Fossés, France) and light-cured resin composite (Filtek Z350 XT, 3M Dental Products, St Paul, MN, USA) (18). A radiograph was taken to confirm the filling of ICM into the full length of the root canal.

After 30 days, the root canals were aseptically accessed and the ICM was removed with 5 mL of saline solution by using a master apical file (# 40) and two other files. Additional irrigation with 17% ethylenediaminetetraacetic acid (EDTA) was ultrasonically activated (as previously described) and the removal of ICM, was checked at high magnification. Next, the root canals were rinsed with 5 mL of sterile saline solution. $Ca(OH)_2$ was neutralized with 5 mL of 0.5% citric acid for 1 minute before being removed with 5 mL of saline solution. A third sampling (S3) was performed as mentioned earlier.

Then, the root canals were rinsed with 3mL of 17% EDTA by using an ultrasonic device, followed by irrigation with 5mL of sterile saline solution. After the root canals were dried and no symptoms reported, root canal filling was performed with single Reciproc gutta-percha cone and Endométhasone sealer (Septodont, Saint-Maur-des-Fossés, France). The access cavities were sealed as previously mentioned.

**Culture method**

The method for colony-forming units count (CFU) has been previously published (3). Briefly, the tubes containing root canal samples were transported to an anaerobic workstation (Don Whitley Scientific, Bradford, UK) and shaken thoroughly for 60 seconds (Vortex; Marconi. Piracicaba. São Paulo. Brazil). Then, serial 10-fold dilutions were made to 10-4 in tubes containing Fastidious Anaerobe Broth (FAB, Lab M, Bury, UK). Aliquots of 50 µL of the undiluted sample and each dilution were placed on pre-prepared plates containing fastidious anaerobe agar (Lab M, Bury, England) supplemented with 5% defibrinated sheep blood, hemin (1 mg/L) and menadione (1 mg/L). Next, the plates were incubated in an anaerobic chamber at 37°C for up to 14 days to allow the detection of slow-growing microorganisms. After the incubation period, the total CFUs were determined by using a stereoscopic magnifying glass (Lambda Let 2, Hong Kong).

**Microbial identification – nested PCR**

DNA extraction was performed from samples obtained directly with Tris-EDTA buffer solution and by using the QIAamp DNA kit (Qiagen, Valencia, USA) according to the manufacturer's instructions.

The primers investigated were *Aggregatibacter actinomycetemcomitans, Actinomyces israelii, Actinomyces naeslundii, Dialister pneumosintes, Enterococcus faecalis, Filifactor alocis, Fusobacterium nucleatum, Gemella morbillorum, Porphyromonas endodontalis, Porphyromonas gingivalis, Parvimonas micra, Prevotella nigrescens, Prevotella tannerae, Tannerella forsythia, Treponema denticola, Treponema socranskii*.

After extraction, the DNA concentration of the samples collected from the root canals was measured by using a spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, DE, USA) operating at 260 nm wavelength. The first amplification used universal primers for regions covering the genes 16S and 23S rRNA.

PCR reactions were run in a total amount of 50 µL for each sample containing 5 µL of buffer for PCR reaction (10x Reaction Buffer, Invitrogen, São Paulo, SP, Brazil), 2 µl of a mixture of phosphate deoxyribonucleotides (2 mM) (dNTPs, Invitrogen, São Paulo, SP, Brazil), 4 µl of magnesium chloride solution (25 mM) (MgCl2, Invitrogen, São Paulo, Brazil), 1 µL of a 20 mM forward and reverse primer solutions (20mM) (Invitrogen, São Paulo, SP, Brazil), 34.8 µL of ultrapure water free of DNAase and RNAase, 0.12 µL of Taq polymerase enzyme (Invitrogen, Taq Platinum, São Paulo, SP, Brazil) and 2 µL of DNA extracted from the sample collected from the root canal at a CO-concentration of 40 ng/mL. The reaction consisted of initial denaturation (95°C for 2 min), 22 cycles of denaturation (94°C for 1 min), annealing (42°C for 2min) and extension (72°C for 3 min), followed by a final extension (72°C for 10 minutes).

The products of the PCR reaction were analysed by using 1% agarose gel electrophoresis (Invitrogen, São Paulo, SP, Brazil) stained with ethidium bromide, EDTA (pH 8.0) and TBE buffer (5 µg/mL) (Invitrogen, São Paulo, SP, Brazil). A molecular weight standard of 1Kb (DNA ladder, Invitrogen, São Paulo, SP, Brazil) was added to each gel. After each run (60 volts for 40 min), the bands were observed with ultraviolet light transilluminator. Positive or negative identification was based on the presence of clear bands of approximately 1500 bp.

After checking the success of the universal reaction (first reaction), an aliquot of 1 µL of its product was used to perform the second PCR reaction, but now using a specie-specific primer

(F) combined with L189R primer. Reactions were processed in a total amount of 25 µL for each sample with 2.5 µL of buffer for PCR reaction (Invitrogen, São Paulo, SP, Brazil), 2.5 µl of a mixture of phosphate deoxyribonucleotides (2mM) (Invitrogen, São Paulo, SP, Brazil), 1.5 µl sodium magnesium solution (25 mM) (Invitrogen, São Paulo, SP, Brazil), 0.62 µL of a 100 µM of forward and reverse primer solutions each (20 mM) (Invitrogen, São Paulo, SP, Brazil), 16.14 µL of ultrapure DNAase and RNAase water, 0.12 µL of Taq polymerase enzyme (Invitrogen, Taq Platinum, São Paulo, Brazil) and 1 µl aliquot of the first reaction. The reaction consisted of initial denaturation (95°C for 2 min), 22 cycles of denaturation (94°C for 1 min), annealing (42°C for 2min) and extension (72°C for 3 min), followed by a final extension (72°C for 10 min), track fading (94°C for 1 min), annealing (52°C for 2 min) and extension (72°C for 3 min). The reading was performed following the same pattern as the previous one.

Binding region specificity, synthesis direction and sequence of the primers used in the bacterial species detection before and after CMP and after intracanal medication with nested PCR are shown in Table 1.

### Statistical analysis

The resulting data were statistically analysed by using SAS for Windows (SAS Inc, Cary, NC, USA). The normality of the data was verified by the Shapiro-Wilk test, and those presenting normal distribution were analyzed with 1-way analysis of variance and the post hoc Tukey-Kramer method for intergroup analysis. Paired t-test and repeated measures analysis of variance were also applied for intragroup analysis in the stages of endodontic therapy. All tests were performed at a significance level of 5%.

## RESULTS

### Clinical features

The patients' age ranged from 30 to 60 years old. Sixty percent of the patients were women. None of the patients reported spontaneous pain at the moment of intervention. Tenderness to percussion was present in 12 out of 20 volunteers, and 4 of them reported pain on palpation. Apical periodontitis was present in all cases (size: 3.5±1.8 mm). From the 20 teeth included in this investigation, 6 were maxillary central incisors, 3 maxillary lateral incisors, 1 maxillary canine, 2 maxillary second premolars, 1 mandibular central incisor, 1 mandibular first premolar and 6 mandibular second premolars. All of them were single-rooted teeth.

### Culture method

The presence of cultivable bacteria (101.2±79.2) CFU/mL was detected in all initial RC samples. After CMP, it was observed a significant reduction in the microbial levels by 99.4% (0.6±1.4) CFU/mL. After the use of ICM, the microbial reduction was 99.5% compared to S1 (0.5±0.8) CFU/mL. No significant reduction was observed from samples collected after CMP and after ICM.

### Nested PCR

Microbial DNA was recovered from all infected root canals (20/20). The results of the identification by using nested-PCR before and after CMP and after ICM are shown in Figure 1.

Amongst the sixteen primers tested, the most prevalent species in the initial sampling were *Enterococcus faecalis* (20/20), *Porphyromonas gingivalis* (20/20), followed by *Fusobacterium nucleatum* (17/20) and *Aggregatibacter actinomycetemcomitans* (10/20). Four species were not detected, irrespective

**TABLE 1.** Binding region, specificity, synthesis direction and sequence of the primers used in the bacterial species detection using molecular method Nested PCR

| Primer/specificity | Size (pb)/Direction | Sequence |
|---|---|---|
| L422/Universal Forward (23S) | 422/5´→3´ | GGAGTATTTAGCTT |
| 785/Universal Forward (16S) | 785/5´→3´ | GGATTAGATACCCTGGTAGTC |
| L189/Universal Forward (23S) | 1500/5´→3´ | GGTACTTAGATGTTTCAGTTC |
| *A. actinomycetemcomitans* (16S) | 1500/5´→3´ | GAAGAAGAACTCAGAGATGGGTTT |
| *A. israelii* (16S) | 1500/5´→3´ | TGGGCCGGCTGCTCCTGGA |
| *A. naeslundii* (16S) | 1500/5´→3´ | TGGAGACGGGGTTTCCTCCTTTGG |
| *D. pneumosintes* (16S) | 1500/5´→3´ | CCTTGACATTGATCGCAATCCATAGAAATAT |
| *E. faecalis* (16S) | 1500/5´→3´ | GTCGCTAGACCGCGAGGTCATGCA |
| *F. alocis* (16S) | 1500/5´→3´ | ACATACCAATGACAGCCTTTTAA |
| *F. nucleatum* (16S) | 1500/5´→3´ | TTCGGGGAAACCTAAAGACAGGTGG |
| *G. morbillorum* (16S) | 1500/5´→3´ | CGAGAGTCAGCCAACCTCATA |
| P. endodontalis (16S) | 1500/5´→3´ | TTTAGATGATGGCAGATGAGAG |
| *P. gingivalis* (16S) | 1500/5´→3´ | CATCGGTAGTTGCTAACAGTTTTC |
| P. micra (16S) | 1500/5´→3´ | AACGAGAAGCGAGATAGAGATGTTA |
| *P. nigrescens* (16S) | 1500/5´→3´ | CGTTGGCCCTGCCTGCGG |
| P. tannerae (16S) | 1500/5´→3´ | CCAAGAGTGCGGAGTGCAGAGATGCGC |
| *T. forsythia* (16S) | 1500/5´→3´ | TGCGATATAGTGTAAGCTCTACAG |
| *T. denticola* (16S) | 1500/5´→3´ | CAAGAGCAATGACATAGAGATATGG |
| *T. socranskii* (16S) | 1500/5´→3´ | ATGTACACTGGGCGTGTGCG |

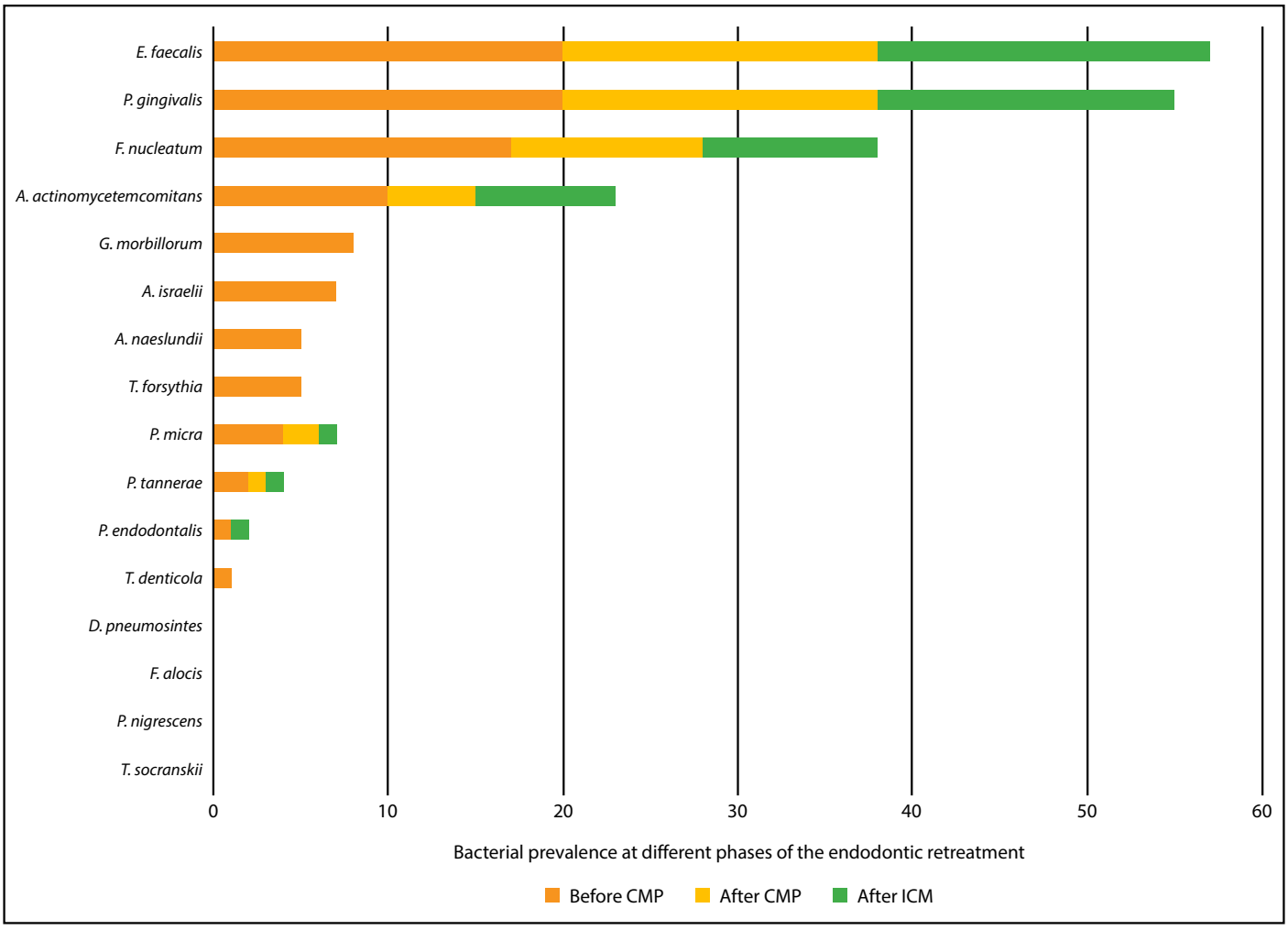


**Figure 1.** Detection of the 16 microbial species investigated in infected root canals at different phases of endodontic retreatment in teeth with persistent/secondary endodontic infection by using nested PCR method

of the endodontic re-treatment stage (*Dialister pneumosintes, Filifactoralocis, Prevotella nigrescens and Treponema socranskii*).

The microbial reduction after CMP varied among the primers tested. *Actinomyces israelii, Actinomyces naeslundii, Gemella morbillorum, Tannerella forsythia* and *Treponema denticola* were not detected after CMP ($P<0.05$). *Enterococcus faecalis* and *Porphyromonas gingivalis* were reduced by 10% ($P>0.05$) and *Fusobacterium nucleatum* by 64.7% after CMP ($P<0.05$). On the other hand, ICM had no additional effect on the reduction of the bacterial species.

## DISCUSSION

Traditionally, culture-dependent studies have associated the failure of the root canal treatment with the presence of Gram-positive bacteria, especially *E. faecalis*. Phenotype-based procedures for identification of bacteria in the root canal system have been used over the years (1, 2). However, technique limitations can underestimate the microorganisms present in a specific micro-ecosystem (5). This study was conducted to investigate the microbiota of teeth with endodontic treatment failure by using nested PCR method in the different stages of endodontic retreatment.

The method for sampling infectious contents of root canals by using sterile paper points is well-established in the literature (2, 3, 12, 19). It is worth mentioning that all floating species present in the main root canal are or were associated with biofilm (20), validating the sampling technique used.

Nested PCR assay identified difficult-to-grow strains, including fastidious bacteria. This technique was shown to be effective in identifying the microbiota in the different stages of endodontic re-treatment of persistent/secondary infections. The sensitivity of nested-PCR is higher (10 cells) compared single round PCR ($10^2$ cells) and culture ($10^4$-$10^5$ cells) methods (5, 15, 16). Our results showed a high prevalence of *E. faecalis* detected in root canals with persistent endodontic infection by using nested PCR (97.5%), being 100% in the initial samples, 90% after CMP and 95% after ICM. A previous study detected *E. faecalis* DNA in endodontic samples, ranging from 0 to 90% (5, 11, 12). Variations in obtaining samples from distinct clinical conditions (i.e. persistent infections or acute apical abscesses) and different methods of analysis explain the differences in the detection of *E. faecalis*.

A culture-independent molecular technique detected Gram-negative species (i.e. *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *T. forsythia* and *T. denticola*). It is import-

ant to know the role of these bacteria in cases of persistent/secondary infections, since their main virulence factors (endotoxin) may justify the persistence of periradicular inflammation after root canal filling and, in some cases, the presence of symptomatology (11). Additionally, the presence of Gram-negative species may indicate low effectiveness of the previous root canal treatment, since the microbiota is similar to that found in primary infection. Culture-independent methods have been used to compare the antimicrobial effect of CMP on teeth with post-treatment apical periodontitis (21). In our clinical investigation, CMP provided reductions of the microbial content for *A. actinomycetemcomitans* by 50%, *A. israelii* by 100%, *A. naeslundii* by 100%, *F. nucleatum* by 35.3%, *G. morbillorum* by 100%, P. micra by 50%, *T. forsythia* for 100% and *T. denticola* by 100%). These findings are supported by the literature, which indicates a microbial reduction ranging between 80 to 95% after CMP (11). Unfortunately, CMP was not effective in reducing the levels of *E. faecalis* and *P. gingivalis* (10% reduction), which emphasizes the resistance of these bacteria.

In the present investigation, CMP reduced the levels of viable bacteria in 99.4%, while the ICM in 99.5% agreeing with the literature (11, 22, 23). On the other hand, observing the results achieved by Nested PCR, species such as *A. actinomycetemcomitans*, *E. faecalis*, *F. nucleatum* and *P. gingivalis* remained after CMP and ICM, probably due to the high sensitivity of the molecular method used in this investigation.

Red complex bacteria (*P. gingivalis*, *T. denticola*, *T. forsythia*) are frequently detected in deep periodontal pockets (12), with these species being even more virulent when they interact synergistically with each other (24). Our results revealed the presence of red complex species in persistent/secondary endodontic infections, with an increased detection of *P. gingivalis* in all stages of endodontic retreatment, and *T. forsythia* and *T. denticola* in the initial samples. *P. gingivalis* has been shown to be a resistant species to endodontic procedures, as previously reported elsewhere (12, 17).

*P. micra*, a Gram-positive strict anaerobic coccus (25), is an important species investigated here for being associated to apical periodontitis, implantitis and other infections in the brain as well as abdominal, peritoneal and pelvic regions (25, 26). *P micra* can co-aggregate with different species, including *P. gingivalis* and *F. nucleatum* (both Gram-negative strict anaerobes), leading to a synergistic biofilm formation highly associated with apical periodontitis (27). The current study revealed different detection levels of P. micra, *P. gingivalis* and *F. nucleatum* in all stages of endodontic retreatment, but none of the mentioned species was eliminated.

Overall, the endodontic re-treatment modified the microbial composition in teeth with endodontic treatment failure; therefore, the null hypothesis was rejected. The present study has shown that a combination of culture and molecular methods was effective in monitoring the effectiveness of the endodontic retreatment in the root canal system of teeth with endodontic treatment failure. Additionally, it also detected key species comprising specific strains very resistant to endodontic procedures and involved in the development and/or maintenance of apical periodontitis. Therefore, clinical procedures should be cautiously performed during all stages of endodontic treatment in order to increase the predictability of the root canal treatment.

## CONCLUSION

The microbiota of teeth with persistent/secondary endodontic infection is a polymicrobial community with Gram-positive and Gram-negative species, bacillus and cocci, facultative and strict anaerobes. Enterococcus faecalis and Porphyromonas gingivalis are highly detected in all stages of endodontic retreatment, thus evidencing their great resistance to endodontic procedures. The endodontic procedures were effective in reducing the levels of bacteria from teeth presenting with persistent/secondary endodontic infection.

### Disclosures

**Conflict of Interest:** The author declares no conflict of interest.

**Ethics Committee Approval:** All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. The Research Ethics Committee of the Piracicaba Dental School, University of Campinas (UNICAMP, Piracicaba, São Paulo, Brazil) approved the study according to protocol number 018/2014, in which the sample collection was also described.

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