# Bacterial diversity in primary and secondary/persistent endodontic infections by Checkerboard DNA-DNA Hybridization technique

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## ABSTRACT

**Introduction:** One of the main advantages of the molecular diagnostic methods is detecting microorganisms using the bacterial DNA, leading to a more accurate microbial characterization. **Objective:** This paper aims to study the bacterial diversity present in primary and secondary/persistent endodontic infections, comparing the profile of the existing microbial communities before and after endodontic therapy. **Methods:** Microbiological samples were collected using sterile/non-pyrogenic paper points in teeth with primary endodontic infections (n = 10) and teeth with persistent/secondary endodontic infections (n = 10), before ($T_1$) and after endodontic therapy ($T_2$). The presence and levels of 40 bacterial species in endodontic infections were investigated by checkerboard DNA-DNA hybridization. **Results:** In primary endodontic infections, higher levels of *P. micra, F. nucleatum sp. nucleatum, S. constellatus, P. gingivalis, G. morbillorum, P. endodontalis, T. denticola, P. acnes, S. gordonii, S. mitis, V. parvula* and *C. rectus* were found In $T_1$. For $T_2$, the most frequent bacteria were *P. micra*, *S. oralis* and *P. acnes*. The most frequent species found in $T_1$, considering secondary endodontic infections group were: *P. acnes, P. micra, S. constellatus, G. morbillorum, C. rectus, A. naeslundii, S. mitis* and *S. oralis*. In $T_2$, the most frequent species were *E. faecalis* and *P. acnes*. **Conclusion:** This study confirmed the distinctness of microbial communities in primary and secondary endodontic infections. Furthermore, clinical endodontic procedures were significantly effective in reducing the prevalence, the detection levels and bacterial diversity.

**Keywords:** Endodontics. Bacteria. Dental pulp cavity.

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## Introduction

Microorganisms play an important role in the establishment and maintenance of chronic periapical lesion.[1] Most of the bacteria capable of infecting the root canals comes from the oral cavity. However, root canal is a selective environment that allows only a few microorganisms to colonize this system.[2]

Traditionally, the identification of microorganisms in endodontic samples is based on culture methods. However, the prevalence of some oral pathogens may be underestimated by this technique, which contains several stages and may fail to isolate and grow some of the most fastidious bacteria, such as spirochetes.[3,4]

The emergence of molecular diagnostic methods, such as techniques based on DNA-DNA hybridization and PCR (polymerase chain reaction), has led to several advances. One of the main advantages of such methods is to detect uncultivable microorganisms, leading to a more accurate characterization.[5] The checkerboard technique performs the detection of species associated with endodontic infections based on the bacterial DNA structure, allowing the analysis of a large number of DNA samples.[6]

The characteristics of the ecosystem where many microorganisms grow are not yet fully known, impeding the proper cultivation in laboratory.[6] It is necessary the use of advanced and effective molecular methods in the search of knowledge of the microbial community to make the clinical diagnosis and the investigation procedures more effective. However, to date, there is a lack of scientific evidence evaluating the microbial profile involved in primary endodontic infections (PEIs) and persistent/secondary infections (PSEIs) before and after endodontic therapy using molecular methods such as Checkerboard DNA-DNA Hybridization. Thus, the present study investigated the bacterial diversity present in PEIs and PSEIs, comparing the profile of microbial communities existing before and after endodontic therapy.

## Material and Methods

This research was approved by the Research Ethics Committee (CAAE): 17159513.0.0000.0077.

### Sample selection

Twenty patients were selected and divided into two groups based on the initial clinical condition: a) Group 1 (n = 10): uni-radicular teeth with primary endodontic infection (PEI) with periapical lesion; b) Group 2 (n = 10): uni-radicular teeth with persistent/secondary infection (PSEI) with periapical lesion. Group 2 included teeth with a previous filling and chronic periapical lesion, with a previous endodontic treatment time equal to or greater than 3 years, or the presence of signs and/or symptoms.

### Sample collection

The selected teeth were polished with pumice, isolated with a rubber dam. The crown and rubber dam sheet junction was sealed with cyanoacrylate adhesive to prevent the infiltration of saliva. Antisepsis of the operative field was performed.[7]

### Group 1

In order to collect samples of the root canal before the chemical-mechanical preparation ($T_1$), three paper cones were introduced into the root canal at their working length, remaining in this position for 60s; and then transferred to an Eppendorf tube containing VMGA III. Then, root canals were prepared according to the crown-down technique, using oscillatory instruments (Endo-Eze, Ultradent Endodontics, USA). At the end of the biomechanical preparation, the canals were irrigated with 10 mL physiological saline and then a new sample of the root canal was collected ($T_2$) in a similar way to the previously described collection.

### Group 2

Root canal desobturation was executed with Gates-Glidden drills and endodontic files up to the working length; an x-ray was taken to evaluate the removal of the filling material. Then, the root canal was flooded with physiological saline solution and the first collection ($T_1$) was performed in the same manner as in group 1. After desobturation, the root canal was prepared with Gates-Glidden drills and K-type manual files. At the end of the biomechanical preparation, the irrigation was performed with

physiological saline solution and a new sample of the root canal ($T_2$) was collected in a similar way to the previous collection.

## DNA extraction

DNA extraction was performed with the QiAamp DNA kit (QIAGEN, Chatsworth, CA, USA). This extracted DNA was purified using the ReliaPrep (TM) gDNA Tissue Miniprep System kit.

## Checkerboard DNA-DNA hybridization technique

A positively charged nylon membrane (Amersham Biosciences, Chicago, IL, USA) was assembled in Minislot 30® (Immunetics, Cambridge, MA, USA). Each sample suspension containing free DNA was deposited in the slots of Minislot 30® and the DNA remained deposited on the nylon membrane. The membrane was removed from the apparatus and the DNA, previously deposited therein, was fixed by heating in an oven at 120°C for 20 min.

After DNA attachment to the membrane, this was prehybridized at 42°C for one hour in a solution of 50% formamide, 1% casein, 5 X SSC (Citrate Saline), 25 nM sodium phosphate (pH 6.5) and 0.5 mg/mL yeast RNA. The membrane was then placed in the Miniblotter 45® acrylic plate, rotated 90° from its original position, with the DNA-containing lanes fixed perpendicular to Miniblotter 45® channels.

DNA probes specific for the 40 species that were used in this study are shown in Table 1. These were made using random primer digoxigenin labeling kit (Boehringer Mannheim, Indianapolis, IN, USA).

After hybridization with the probes, the membranes were removed from Miniblotter 45® and washed at 65°C for 40 min. The membranes were immersed for 1 h under stirring in a blocking solution containing 0.1 M maleic acid, 3 M NaCl, 0.2 M NaOH, 0.3% Tween 20, 0.5% casein, pH 8.0, and for 30 min in the same solution containing anti-digoxigenin antibody (Roche Diagnostics GmbH, Mannheim, Germany) conjugated to alkaline phosphatase. The membranes were incubated in CDP-Star Detection Reagent (Amershan Biosciences UK Limited, Buckinghamshire, UK) at 37°C, for 60 min. Finally, the membranes were placed in cassette under an x-ray film, and the films were revealed shortly thereafter.

**Table 1.** List of the strains used for the development of bacterial DNA probes.

| SPECIES | STRAINS (ATCC) |
|---|---|
| *Actinomyces gerencseriae* | 23860 |
| *Actinomyces israelii* | 12102 |
| *Actinomyces naeslundii* | 49340 |
| *Actinomyces viscosus* | 43146 |
| *Aggregatibacter actinomycetemcomitans* | 43718 |
| *Campylobacter gracilis* | 33236 |
| *Campylobacter rectus* | 33238 |
| *Campylobacter showae* | 51146 |
| *Capnocytophaga gingivalis* | 33624 |
| *Capnocytophaga ochracea* | 33596 |
| *Capnocytophaga sputigena* | 33612 |
| *Eikenella corrodens* | 23834 |
| *Enterococcus faecalis* | 6569 |
| *Eubacterium nodatum* | 33099 |
| *Eubacterium saburreum* | 33271 |
| *Fusobacterium nucleatum sp. nucleatum* | 25586 |
| *Fusobacterium nucleatum sp. polymorphum* | 10953 |
| *Fusobacterium nucleatum sp. vincentii* | 49256 |
| *Gemella morbillorum* | 27824 |
| *Leptotrichia buccalis* | 14201 |
| *Neisseria mucosa* | 19696 |
| *Parvimonas micra* | 33270 |
| *Porphyromonas endodontalis* | 35406 |
| *Porphyromonas gingivalis* | 33277 |
| *Prevotella intermedia* | 25611 |
| *Prevotella melaninogenica* | 25845 |
| *Prevotella nigrescens* | 33563 |
| *Propionibacterium acnes* | 11827 |
| *Selemonas noxia* | 43541 |
| *Streptococcus anginosus* | 28423 |
| *Streptococcus constellatus* | 27823 |
| *Streptococcus gordonii* | 10558 |
| *Streptococcus intermedius* | 27335 |
| *Streptococcus mitis* | 49456 |
| *Streptococcus oralis* | 35037 |
| *Streptococcus sanguinis* | 10556 |
| *Tanerella forsythia* | 43037 |
| *Treponema denticola* | B1 |
| *Treponema socranskii* | D40dr2 |
| *Veillonella parvula* | 10790 |

Each signal produced by a given probe in the root canals sample was compared to the signal produced by the same probe in the two controls containing $10^5$ and $10^6$.

## Results

### Group 1

In the samples collected before the chemical-mechanical preparation ($T_1$), bacteria were detected in 100% (10/10) of root canals with PEIs. The number of different species per sample ranged from 6 to 11, with a mean value of 8 species. The microbial load of the bacterial species detected varied between $<10^5$ and $10^6$ CFU. The most frequently found species were: *Parvimonas micra* (50%), *Fusobacterium nucleatum sp. nucleatum* (40%), *Streptococcus constellatus* (40%), *Porphyromonas gingivalis* (40%), *Gemella morbillorum* (30%), *Porphyromonas endodontalis* (30%), *Treponema denticola* (30%), *Propionibacterium acnes* (30%), *Streptococcus gordonii* (30%), *Streptococcus mitis* (30%), *Veillonella parvula* (30%) and *Campylobacter rectus* (30%). Particularly, *Enterococcus faecalis* was detected in only 1/10 (10%) root canals studied with primary endodontic infections.

After the chemical-mechanical preparation ($T_2$), bacteria were detected in 8/10 (80%) of root canals with primary endodontic infection. The number of different species per sample ranged from 0 to 8, with a mean value of 5 species. The microbial load of the bacterial species detected ranged from $<10^5$ and $10^5$ CFU. The most frequent species were *Parvimonas micra* (40%), *Streptococcus oralis* (40%) and *Propionibacterium acnes* (30%).

### Group 2

In $T_1$, bacteria were detected in 100% (10/10) of the root canals with PSEIs. The number of different species per sample ranged from 4 to 10, with a mean value of 6 species. The microbial load varied between $<10^5$ and $10^6$ CFU. The most frequent species were *Propionibacterium acnes* (50%), *Parvimonas micra* (40%), *Streptococcus constellatus* (40%), *Gemella morbillorum* (40%), *Campylobacter rectus* (40%), *Actinomyces naeslundii* (40%), *Streptococcus mitis* (40%) and *Streptococcus oralis* (40%). Particularly, *Enterococcus faecalis* was detected in only 2/10 (20%) root canals studied with PSEIs.

In $T_2$, bacteria were detected in 4/10 (40%) of the root canals with PSEIs. The number of different species per sample ranged from 0 to 7, with a mean value of 1.6 species per channel. The microbial load of the bacterial species detected ranged from $<10^5$ to $10^6$ CFU. The most frequent species were: *Enterococcus faecalis* (20%) and *Propionibacterium acnes* (20%).

## Discussion

Using the checkerboard DNA-DNA hybridization method, this study initially allowed ($T_1$) the identification of 38 different bacterial species in cases of PEI. Soon after the chemical-mechanical preparation ($T_2$), 29 species were identified. While in the cases collected from PSEIs, the method allowed the identification of 30 different bacterial species in $T_1$, and in $T_2$ this number of species was reduced to 13.

In endodontics, studies using culture and/or molecular methods show that primary endodontic infections are polymicrobial,[2,8,9] dominated by strict anaerobic Gram-negative bacilli.[8-11]

In this study, the most prevalent species in primary endodontic infections were: *Fusobacterium nucleatum sp. nucleatum, Streptococcus constellatus, Porphyromonas gingivalis, Gemella morbillorum, Porphyromonas endodontalis, Treponema denticola, Propionibacterium acnes*, *Streptococcus gordonii, Streptococcus mitis, Veillonella parvula and Campylobacter rectus*. Other studies have also found these species in primary endodontic infections.[8,9,12-15] The number of bacterial species in each sample was, on average, 8, whereas in the findings of Siqueira Jr et al,[5] the value was 4.7.

The microbiological collections after the endodontic treatment in PEIs ($T_2$) demonstrated the presence of microorganisms in 8 samples, with the prevalence of *Parvimonas micra*, *Streptococcus oralis* and *Propionibacterium acnes*, with a significant reduction in the prevalence and the detection levels in the samples taken after endodontic therapy.

Differently from the studies of Siqueira et al[16] and Rôças et al[14], the present study encompassed a comparative analysis of teeth with PEIs and PSEIs, confirming a distinct microbial profile between these two environments. In secondary endodontic infections, traditional methods of microbial culture revealed the prevalence of facultative anaerobic

Gram-positive bacteria.[17-20] After the use of molecular methods, it was observed the presence of gram-negative bacteria, belonging to the genera *Prevotella spp., Porphyromonas spp., Fusobacterium spp., Peptostreptococcus spp.* and *Treponema spp.*[20-22] In the present study, the majority of the most prevalent bacterial species found in PSEIs in $T_1$ are Gram-positive: *Propionibacterium acnes*, *Parvimonas micra*, *Streptococcus constellatus*, *Gemella morbillorum*, *Actinomyces naeslundii*, *Streptococcus mitis* and *Streptococcus oralis*, except for *Campylobacter rectus*, which is Gram-negative.

Bacterial diversity was significantly reduced by clinical endodontic procedures, after instrumentation ($T_2$) of the cases of PSEIs. The most prevalent microorganisms were: *Enterococcus faecalis* and *Propionibacterium acnes*. These microorganisms emerge as a potential risk for persistent diseases, which continues to be determined by longitudinal studies.[17,18,23,24]

The checkerboard technique enables to identify difficult-to-grow microorganisms in clinical samples using traditional methods of microbiological culture and biochemical tests[25,26] and does not require bacterial viability; however, the detection of microorganisms is limited to species whose samples are available.[6,25] Compared with other molecular techniques, such as conventional polymerase chain reaction (PCR), nested-PCR and real time PCR, DNA-DNA hybridization is considered a fast, efficient and relatively low-cost method. Hybridization technology has advantage over other molecular methods because it does not amplify the sample, because if there is a microbial contaminant, such agent would be below the detection limits of the method ($10^3$ to $10^4$ cells). However, non-contaminating microorganisms that are also in small numbers are also not detected.[5,27,28] Also, it is a precise and faster method than the one employed in the PCR technique, since it uses several DNA probes at once and the samples can be stored for long periods.[5,26]

This study demonstrated that the microbiota of teeth with PEIs and teeth with PSEIs are polymicrobial in nature, but they present distinct communities. However, it is prudent to carry out investigations using other molecular methods to confirm the data found in this study.

## Conclusions

The present study confirmed the distinctness of microbial communities in PEIs and PSEIs. In addition, clinical endodontic procedures proved to be effective in significantly reducing the prevalence, detection levels, and bacterial diversity.

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