Characterization of the microbiota of root canal-
treated teeth with post-treatment disease

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Running headline: Microbiota of root canal-treated teeth

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ABSTRACT
This study evaluated the microbiota of root canals undergoing retreatment. The most prevalent taxa detected by checkerboard included Propionibacterium species, Fusobacterium nucleatum, streptococci, and Pseudoramibacter alactolyticus. Quantitative real-time PCR detected Enterococcus faecalis and streptococci in 38% and 41% of the cases, comprising 9.76% and 65.78% of the total bacterial counts, respectively. Findings question the status of E. faecalis as the main pathogen and suggest that other species can be candidate pathogens associated with persistent/secondary infections.

Key words: 16S rRNA gene; endodontic retreatment; post-treatment apical periodontitis; DNA-DNA hybridization; real-time polymerase chain reaction.

SHORT-FORM PAPER
Post-treatment apical periodontitis is as an inflammatory disease caused by a persistent/secondary infection of the dental root canal (8, 11, 13, 15, 26, 30). Enterococcus faecalis has been the most frequently detected species in root canal-treated teeth as revealed by both culture and molecular studies (5, 8, 11-12, 16, 23-24, 26, 30-31), but recent studies have questioned its etiologic role in post-treatment disease (7, 15, 20, 31). Moreover, taxa related to several other genera, including Streptococcus, Dialister, Fusobacterium, Filifactor, Parvimonas, Prevotella, Propionibacterium, and Pyramidobacter, have also been detected in treated teeth (5, 8, 11-12, 15, 20, 22, 26-27, 29-30).
The purpose of this molecular study was two-fold. First, the samples from root canal-treated teeth with post-treatment apical periodontitis undergoing retreatment were surveyed for the presence of 28 bacterial taxa using the reverse capture-checkerboard DNA-DNA hybridization approach. Then, the total bacterial counts and the presence, levels and proportions of *E. faecalis* and streptococci were determined by using a quantitative real-time polymerase chain reaction (qPCR) assay.

Forty-two teeth were selected from patients (30 females and 12 males; aged 16-70 years; mean age 41 years) who had been referred for root canal retreatment to the Department of Endodontics, Estácio de Sá University. All the root canal-treated teeth were asymptomatic, showed radiographic evidence of apical periodontitis and had endodontic therapy completed more than 2 years earlier. All teeth were coronally restored and no direct exposure of the root canal filling material to the oral cavity was evident. Terminus of the root canal fillings ranged from 0 to 4 mm short of the radiographic apex. No case was overfilled. Selected teeth showed absence of periodontal pockets >4mm. Approval for the study protocol was obtained from the Ethics Committee of the Estácio de Sá University.

Procedures for disinfecting the operative field and taking samples from treated root canals were as described previously (22, 26). Eight participants (out of 50 patients that started the experiment) were excluded because control samples from the operative field were positive for bacterial DNA as determined by broad-range PCR. DNA was extracted from clinical samples using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA), following the protocol
recommended by the manufacturer. To maximize DNA extraction, a step of pre-incubation with lysozyme for 30 min was added. DNA from a panel of several oral bacterial species was also prepared to serve as controls (27).

The reverse-capture checkerboard assay was conducted to determine the presence and levels of 28 bacterial taxa as described previously (9, 14, 19, 25). Probes were based on 16S rRNA gene sequences of the target bacteria and were described and validated elsewhere (1, 9, 12, 14). A semi-quantitative analysis of the checkerboard findings was performed as reported previously (14, 17).

To quantify total bacterial load and the prevalence and levels of *E. faecalis* and streptococci, 16S rRNA gene-targeted qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 Real-time PCR instrument (Applied Biosystems) in a total reaction volume of 20 μl. Primers and the respective annealing temperatures are shown in Table 1. Primers in a concentration of 0.5 μM each and DNA extract volume of 2 μl were added to the PCR master mix in MicroAmp Optical 96-well reaction plates. Plates were sealed, centrifuged and then subjected to amplification. Cycling conditions for the qPCR included: 95°C/10 min; 40 repeats of the following steps: 95°C/1 min, annealing for 1 min (specific temperatures are shown in Table 1), and 72°C/1 min. Double-stranded DNA product was measured at 78°C. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence of the reporter dye. All measurements were done in triplicate for samples and standards. In all experiments, triplicates of appropriate negative controls containing no template.
DNA were subjected to the same procedures. Following amplification, melting curve analysis was performed to determine the specificity of the amplified products. Melting curve was obtained from 60°C to 95°C, with continuous fluorescence measurements taken at every 1% increase in temperature. Data acquisition and analysis were performed using the ABI 7500 software v2.0.4 (Applied Biosystems).

Bacterial levels were inferred for each sample based on obtained standard curves. Standard curves for *E. faecalis* and streptococci were constructed using DNA extracted from known concentrations of *E. faecalis* ATCC 29212 and *Streptococcus mutans* ATCC 25175 grown in pure culture. *E. faecalis* was also used to quantify total bacteria using the pair of universal primers. DNA was isolated from fresh pure cultures of these strains using the QIAamp DNA Mini Kit and quantified using a BioPhotometer (Eppendorf, Hamburg, Germany). Knowing the genome size of both *E. faecalis* (3.2 Mb) and *S. mutans* (2 Mb) and the average molecular weight of one base pair (660 Da), the measured DNA value could then be converted into target genomic copy levels per microliter using the formula $m = n[1\text{ mole}/6\times10^{23}(\text{bp})][660\text{ (g)/mole}] = n[1.096\times10^{-21}\text{ (g/bp)}]$, where $m$ is the genomic mass of a single cell and $n$ the genome size. Genome copy levels were considered as numerically equivalent to bacterial cell levels. The standards were then 10-fold diluted from $10^7$ to $10^2$ cells in Tris-EDTA buffer and used for standard curve construction. The levels of total bacteria could not be precisely calculated because of the differences in numbers of rrn operons. Therefore, a pure culture of *E. faecalis*, which contains 4 copies of the gene, was used with 4 being considered as the average copy.
number in the range of most known oral bacteria (http://www.cbs.dtu.dk/services/GenomeAtlas-3.0). Relative amounts were calculated as the percentage of \textit{E. faecalis} and streptococci out of the total bacterial load.

All clinical samples were positive for the presence of bacteria in both assays, confirming the primary infectious etiology of post-treatment apical periodontitis. The checkerboard results revealed that 24 of the 28 taxon-specific probes tested were reactive with one or more samples. The number of selected bacterial taxa detected per sample ranged from 1 to 12. Only 5 cases harbored more than 5 target taxa. Taxa detected more frequently included \textit{Propionibacterium acnes} (22/42 cases-52%), \textit{Fusobacterium nucleatum ss nucleatum} (10/42-24%), \textit{Streptococcus} species (7/42-17%), \textit{Propionibacterium acidifaciens} (6/42-14%), \textit{Pseudoramibacter alactolyticus} (6/42-14%), \textit{Enterococcus faecalis} (5/42-12%), and \textit{Tannerella forsythia} (5/42-12%) (Figure 1). Semi-quantitative analysis showed that no taxon was found at >10^6 and only 8 taxa were present at levels >10^5. Of these, only three occurred in more than one sample: \textit{P. acnes} (7 cases), \textit{Bacteroidetes} clone X083 (2 cases) and \textit{Pseudomonas aeruginosa} (2 cases) (Figure 1).

Samples from 29 root canal-treated teeth were available for qPCR analysis. Correlation coefficient ($r^2$), amplification efficiency ($E$) and $y$-intercept values of the standard curves for each qPCR assay were as follows, respectively: 0.993, 101% and 41.4 for total bacteria; 0.995, 117% and 40.7 for \textit{E. faecalis}; and 0.992, 109% and 43 for \textit{Streptococcus} species. Accuracy of the amplification was confirmed by melting curve analysis.
Analysis using universal primers revealed a mean bacterial load of $3.2 \times 10^5$ (range, $1.27 \times 10^3$ - $7.25 \times 10^6$). These values fit into the range of $10^3$-$10^7$ bacterial cell equivalents revealed by previous culture and molecular studies for treated teeth (2, 10, 24).

E. faecalis was detected by qPCR in 11/29 (38%) cases, with a mean number of cells of $1.28 \times 10^3$. In the samples positive for E. faecalis, this species comprised from 0.3% to 91% of total bacterial counts (median, 1.1%; mean, 9.76%) (Table 2). In 6 cases, it corresponded to >1% of the population, an in only one this species comprised >10% of the community (91%). The overall findings for E. faecalis are very similar to those reported by Sedgley et al. (24), who also used qPCR and reported that E. faecalis comprised from 0.14% to 100% of the total counts (median, 0.98%; mean, 9.84%).

Our findings are in line with recent studies that have questioned the status of E. faecalis as the main pathogen in post-treatment apical periodontitis (7, 15, 22, 31). In the checkerboard assay, of the only 5/42 cases positive for E. faecalis, in none this species was the most dominant taxon. In the qPCR experiment, which is more sensitive than the checkerboard, E. faecalis was detected in a higher frequency, but in terms of proportion, in only one case it was the main component of the community.

Analysis by qPCR revealed streptococci in 12/29 (41%) cases, with a mean number of $2.45 \times 10^5$ cells. These bacteria comprised from 9% to 99% of the total bacterial counts (median, 75.5%; mean 65.78%) (Table 2). In 11 cases, streptococci corresponded to >10% of the overall community and in 8 cases they comprised >50% of the community.
Streptococcus species have been commonly detected in samples taken immediately after endodontic treatment procedures (3-4, 18-19, 21, 28) and in root canal-treated teeth undergoing retreatment (6, 8, 11-12, 26, 30). This suggests that these bacteria may be involved with persistent infections that result in persistent disease. Because most studies show no specific species involved with post-treatment disease, we decided to use primers and probes to detected Streptococcus as a group. Our findings indicate that streptococci may be dominant in the bacterial community associated with many cases of post-treatment disease. Further studies are warranted to elaborate on the specific Streptococcus species participating in the process.

Over the last decade, the knowledge of the bacterial diversity associated with post-treatment apical periodontitis has been substantially refined and redefined by molecular microbiology methods. The present findings contribute to this knowledge by questioning the status of E. faecalis as the main pathogen in post-treatment apical periodontitis, reinforcing the role of streptococci in persistent/secondary infections, and allowing the inclusion of some new species in the set of candidate pathogens associated with post-treatment disease.
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The authors deny any conflicts of interest.
REFERENCES


FIGURE LEGEND

Figure 1. Stacked bar chart of frequency of detection and levels of bacterial species/phylotypes in root canal samples of treated teeth with post-treatment apical periodontitis from 43 individuals. Total length of each bar stack indicates percentage of positive samples; i.e., prevalence of bacterial species/phylotypes. Different colors within each bar indicate the percentage of samples containing different levels of the species.
Table 1
PCR primers used for bacterial quantification in samples from root canal-treated teeth with posttreatment apical periodontitis

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Primer sequences</th>
<th>Annealing temp (°C)</th>
<th>Fragment length (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| **Enterococcus faecalis** | 5’ – GTT TAT GCC GCA TGG CAT AAG AG – 3’  
5’ – CCG TCA GGG GAC GTT CAG – 3’ | 60                  | 310                  | 30        |
| **Streptococcus species** | 5’ – AGA GTT TGA TYM TGG CTC AG – 3’**  
5’ – TTA GCC GTC CCT TTC TGG T – 3’ | 58                  | 502                  | 12        |
| **Universal 16S rRNA gene** | 5’ – GAT TAG ATA CCC TGG TAG TCC AC – 3’  
5’ – TAC CTT GTT ACG ACT T – 3’ | 52                  | 733                  | 1         |

* universal forward primer
Table 2
Quantitative real-time PCR data for total bacteria, Enterococcus faecalis and streptococci in root canal-treated teeth with apical periodontitis

<table>
<thead>
<tr>
<th>Total bacteria</th>
<th>Enterococcus faecalis</th>
<th>Streptococcus species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell numbers</td>
<td>% of total bacteria</td>
<td>% of total bacteria</td>
</tr>
<tr>
<td>Mean</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>3.2 × 10^5</td>
<td>9.76</td>
<td>0.3 - 91</td>
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