Comparison of endodontic bacterial community structures in root-canal-treated teeth with or without apical periodontitis

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Bacterial occurrence in treated root canals, even in patients without post-treatment apical periodontitis, raises the possibility that factors other than mere bacterial presence can be determinants for a favourable outcome of endodontic treatment. Because these factors may be related to the bacterial communities colonizing the root canal, including virulence, density and interactions, the objective of this study was to compare the community structures found in root-canal-treated teeth with (12 samples) and without (11 samples) apical periodontitis lesions by means of a PCR-denaturing gradient gel electrophoresis fingerprinting approach. Results confirmed a polymicrobial composition even in treated patients without post-treatment disease. A large microbial community diversity was observed for treated teeth both with or without disease, but no specific pattern was detected for diseased teeth. Nevertheless, the number of bands from samples with apical periodontitis lesions was statistically significantly higher ($P=0.04$) than that from samples collected from root-canal-treated teeth without post-treatment apical periodontitis. Furthermore, predominant bands in samples from patients with apical disease were also observed.

INTRODUCTION

Endodontic procedures, such as root canal instrumentation and irrigation, intracanal medication and obturation, are intended to eradicate infection from the root canal system and prevent further reinfection (Sakamoto et al., 2007; Siqueira et al., 2007). Nevertheless, such procedures are not efficient for the complete elimination of endodontic infections in a large majority of cases. What may be achieved with such procedures is a reduction of the bacterial populations inside the root canal to a level below that necessary for maintenance of the disease process. This is confirmed by the fact that some apical periodontitis lesions may heal even when bacteria are found in the root canal at the time of filling (Fabricius et al., 2006; Sundqvist et al., 1998). Even considering that bacterial persistence can be an important risk factor for post-treatment disease (Sjögren et al., 1997; Sundqvist et al., 1998), there is no single specific species described as the culprit for endodontic failures, even with several reports of Enterococcus faecalis colonizing treated canals (Kaufman et al., 2005; Rôças et al., 2004b; Zoletti et al., 2006).

Although bacteria present in treated canals are arguably involved in post-treatment disease (Molander et al., 1998; Siqueira & Rôças, 2004; Sundqvist et al., 1998), there are reports showing that even treated teeth with no discernible disease may harbour bacteria (Kaufman et al., 2005; Zoletti et al., 2006). Therefore, rather than mere bacterial presence, other related factors may play a role as determinants of disease causation. These factors may include density and/or virulence of the bacterial community as a whole, bacterial localization in the root canal, and interactions between community members persisting in the root canal. In this context, a great challenge is to define whether specific bacterial community profiles are linked to the success/failure of endodontic treatment (Siqueira & Rôças, 2008).

Recently, the denaturing gradient gel electrophoresis (DGGE) technique has been used to evaluate the oral microbial community (Li et al., 2005, 2006) and specifically the endodontic microbiota in diverse clinical conditions (Alves et al., 2009; Rôças et al., 2004a; Siqueira et al., 2004).
This technique allows the visualization of the structure of bacterial communities in multiple clinical specimens at a time, including culture-difficult or as-yet-uncultivated taxa in the fingerprints (Siqueira et al., 2004). The present study was undertaken to compare the bacterial communities present in treated root canals of teeth with or without apical periodontitis by using a 16S rRNA gene-based broad-range PCR-DGGE approach.

**METHODS**

**Case description and sample taking.** Samples were collected from 23 root-canal-treated teeth from adult patients (ages ranging from 19 to 75 years) who had been referred to the Endodontic Clinic at two universities (Federal University of Rio de Janeiro and Estácio de Sa University) for root canal retreatment. Eleven teeth had no radiographic evidence of apical periodontitis and were referred for endodontic retreatment because of long exposure of the root canal filling material to the oral cavity as a consequence of loss of the coronal restoration or when an extensive coronal restoration had to be placed and the technical quality of the endodontic treatment was considered inadequate. The other 12 teeth presented post-treatment apical periodontitis as revealed by conventional periapical radiographs. All the root-canal-treated teeth had endodontic therapy completed more than 1 year previously, and termini of the root canal filling ranging from 0 to 5 mm short of the radiographic root apex. Selected teeth showed an absence of periodontal pockets deeper than 4 mm.

All samples were collected by one of the authors (G.O.Z.) for a previous study (Zoletti et al., 2006). Samples were stored frozen and were available for reanalysis. Sample taking and DNA extraction procedures were as described previously (Zoletti et al., 2006).

**PCR amplification.** A 16S rRNA gene fragment corresponding to nucleotide positions 968–1401 (Escherichia coli numbering) was amplified from DNA extracts of clinical samples using the following universal bacterial primers: 968f (5'-AAC GCG AAG AAC CTT AC-3') containing a 40 bp GC clamp (5'-CGC CCG CCG GGC GGC GGG GGG GCA CGG GGG G-3') added to its 5'-end, which makes it suitable for DGGE, and 1401r (5'-CGG TGT GTA CAA GAC CC-3') (Nübel et al., 1996).

The PCR mixture comprised 5 μl of the supernatant from clinical samples, 25 pmol universal primers, 5 μl 10 × PCR buffer (Biotools), 3.8 mM MgCl₂, 2.5 U Taq DNA polymerase (Biotools), 0.2 mM concentration of each deoxynucleoside triphosphate (Biotools) and sterile ultrapure water to a final volume of 50 μl. Negative controls consisting of sterile ultrapure water instead of sample were included with each batch of samples analysed. PCR amplification was performed in a DNA thermocycler (Mastercycler Personal; Eppendorf). The temperature profile included an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of a denaturation step at 94 °C for 1 min, a primer annealing step at 55 °C for 1 min, an extension step at 72 °C for 2 min and a final step of 72 °C for 10 min. Before the DGGE analysis, the presence of PCR products was checked by electrophoresis in a 1.5% agarose gel conducted at 4 V cm⁻¹ in Tris–borate–EDTA buffer. The gel was stained for 15 min with 0.5 μg ethidium bromide ml⁻¹ and viewed under 300 nm wavelength UV light. A 100 bp DNA ladder digest (Biotools) served as the molecular size standard.

**DGGE assay.** DGGE of PCR products generated with the 968f-GC/1401r primer set was performed using the Dcode Universal Mutation Detection System (Bio-Rad) at 75 V and 60 °C for 16 h in 0.5 × TAE buffer (20 mM Tris/acetate 1⁻, pH 7.4; 10 mM sodium acetate 1⁻; 0.5 mM disodium EDTA 1⁻). The PCR products from clinical samples (30 μl) were loaded on 6% (w/v) polyacrylamide gels containing a linear gradient ranging from 45% to 70% denaturant (100% denaturing solution contains 7 mol urea l⁻¹ and 40%, w/v, formamide) and increasing in the direction of electrophoresis. In each gel, samples were loaded in alternate slots according to the presence or absence of apical periodontitis. For the sake of alignment and comparisons between gels, a sample from a pool of reference DNA from Enterococcus faecalis (ATCC 51299), Staphylococcus epidermidis (ATCC 35984) and Pseudomonas aeruginosa (ATCC 27853) showing multiple DGGE bands in earlier experiments was loaded in the first and last slot of each gel.

**DGGE analysis.** Individual lanes of the DGGE gel images were straightened and aligned with GelCompar II software, version 5.10 (Applied Maths). Dendrograms for diverse comparisons of DGGE banding patterns were constructed with the unweighted pair group method using arithmetic averages following calculation of the Pearson coefficient. A similarity level of 60% was arbitrarily considered for cluster preview. The number of bands in patients with and without post-treatment disease was also compared by using Student’s t-test (SPSS for Microsoft Windows, version 9.0). The prevalence of the most dominant bands was also recorded.

**RESULTS**

All clinical samples yielded an ampiclon of the expected size after 16S rRNA gene-based broad-range PCR, indicating the presence of bacterial DNA. Fig. 1 depicts the community profiles of the clinical samples clustered in a dendrogram according to the similarities among them. There was a large inter-individual variability and it was not possible to determine any specific pattern associated with the clinical condition.

The mean number of bands, indicative of the number of bacterial species, was higher in samples collected from patients with apical periodontitis (mean 10.9 ± 2.4, ranging from 7 to 16) than in those from patients with no disease (mean 8.9 ± 1.9, ranging from 5 to 11). This difference was statistically significant (P = 0.04).

All samples presented several bands (strong and weak), indicating a polymicrobial community. No band was found to occur in all samples. However, six different bands were visualized in more than 50% of the samples taken from root canals of teeth with disease. One of these bands was visualized in 15 out of 23 (65%) samples analysed. This band was observed more frequently in clinical samples collected from patients with apical periodontitis (9/12, 75%) than in samples without disease (6/11, 54.5%). As for the other five most prevalent bands in samples from patients with disease, the respective prevalences in patients with and without apical periodontitis were 67% and 18%; 67% and 36%; 58% and 18%; 50% and 36%; and 50% and 27%. Moreover, in 11/12 (92%) root canals of teeth with post-treatment disease, at least three of these five bands were found together in the same bacterial community.

**DISCUSSION**

The DGGE technique has been widely used to profile bacterial communities from different environments and has...
been recently used for the study of endodontic infections (Muyzer et al., 1993; Siqueira et al., 2005). Some studies observed differences in the endodontic bacterial community profiles associated with apical periodontitis when considering patients from distinct geographical regions (Machado de Oliveira et al., 2007) or according to the presence/absence of symptoms (Siqueira et al., 2004). The rationale for bacterial species to be distinguished by the DGGE approach lies in the fact that different bacterial species present different nucleotide sequences within the variable regions of the 16S rRNA gene, making PCR amplicons migrate differently in the DGGE gel. Theoretically, each band in the polyacrylamide DGGE gel represents a certain species, although it must be recognized that there are several factors that may overestimate or underestimate the community diversity as revealed by DGGE (Siqueira & Rôças, 2005).

The present study disclosed polymicrobial communities in treated root canals, irrespective of the presence or absence of apical periodontitis. Occurrence of mixed communities associated with treated teeth with post-treatment disease has been previously reported by other studies using molecular biology technology (Rôças et al., 2004b; Sakamoto et al., 2008). However, an apparently intriguing finding was the detection of mixed communities also in teeth with no disease.

Considering that bacteria are the primary aetiopathogenic agent of post-treatment apical periodontitis (Molander et al., 1998; Sakamoto et al., 2008; Siqueira & Rôças, 2004; Sundqvist et al., 1998), a question therefore arises as to why some patients harbouring intracanal bacteria do not develop disease. One possible explanation may be related to the intracanal bacterial populational density, i.e. bacteria may be present in quantities below that necessary to provoke significant damage to the periradicular tissues. Further studies using sensitive quantitative molecular biology techniques, such as real-time PCR, are required to compare bacterial counts in treated teeth with or without post-treatment disease and then help elucidate this issue (Espy et al., 2006; Siqueira & Rôças, 2005).

Another explanation may reside in the spatial distribution of the community within the root canal system. For instance, bacteria present in the coronal portions of the canal may have been sampled by the method used in this study, which is widely used for in vivo sampling of endodontic infections. In other words, the paper point sampling technique does not distinguish the regions of the canal where detected bacteria...
were colonizing. Therefore, bacteria present only in the coronal region of the canal are detected but are not clinically relevant as they may have no access to the periapical tissues to cause disease. Ricucci et al. (2009) evaluated histologically 51 root-canal-treated teeth with no evidence of apical periodontitis and observed bacteria in the coronal portion of the root canal in almost all cases. They concluded that despite the presence of bacteria coronally, apical tissue was seldom affected. Bacteria located only at the coronal portion of the canal and not apically are very likely to be a result of saliva leakage through the definitive or temporary coronal restoration, which was a common condition for many of the teeth in the no disease group.

It also seems reasonable to speculate that bacteria under nutrient-scarce conditions may have entered a dormant state or a state of low metabolic activity, waiting to thrive again when the source of nutrients is reestablished (Lleó et al., 1998). This may never happen in well-treated canals, but these bacteria may still be detectable for some time.

Another possibility is that the bacterial community associated with teeth with no disease may present lower virulence than that present in teeth with disease. This may be because of the original composition of the community before treatment or due to perturbations in the community induced by treatment (Siqueira & Rôças, 2008). Finally, one cannot rule out immunological aspects involved in host resistance to infection as a factor influencing the presence or absence of disease in treated canals that are positive for the presence of bacteria (Kuramitsu et al., 2007).

A limitation of the present study relates to the limited diagnostic capacity of periapical radiography for detection of bone changes associated with apical periodontitis (Huumonen & Ørstavik, 2002; Ricucci & Bergenholtz, 2003). Conventional periapical radiography lacks sufficient sensitivity to serve as a reliable means for analysis of endodontic treatment outcome, since lesions restricted to the cancellous bone may pass unnoticed on conventional radiographs, especially in the posterior mandibular area (Bender, 1982). Therefore, the absence of a radiolucency associated with the periapical region of a root-canal-treated tooth does not necessarily prove that the periapical tissues are actually free of disease (Wu et al., 2009). In our study, cases were selected by using periapical radiographs, and the lack of a community structure pattern associated with disease may be related to such radiographic diagnostic limitations.

A great inter-individual variability in endodontic bacterial communities was observed, which is in agreement with previous studies using community profiling techniques (Alves et al., 2009; Rôças et al., 2004a; Siqueira et al., 2004). Although no disease-related community pattern was observed, the present findings revealed that the number of bands (bacterial taxa) in samples from teeth with disease was significantly larger than in those from teeth with no disease. This results in more complex communities, with resulting interactions that may influence the pathogenicity of the bacterial consortium and the initiation or maintenance of apical periodontitis.

Five bands (bacterial taxa) were found with a higher prevalence in teeth with post-treatment disease. At least one of these bacteria was found in all diseased samples analysed, and over 90% of cases harboured at least three of them in the same community. No efforts were made to identify these species by cutting out the bands from the gel and sequencing their DNA, since this was not the scope of this study, which was primarily focused on community analysis. Our further purpose is to look for the identity of species more frequently found in diseased than in non-diseased teeth. Based on our previous reports on the same samples, E. faecalis is very unlikely to be one of these species (Zoletti et al., 2006).

In conclusion, no significant pattern of bacterial community profile was associated with root-canal-treated teeth with or without apical periodontitis. This does not mean that the composition of the microbiota is similar between these conditions, but quite the opposite. They are so dissimilar that apparently very different compositions can cause disease and raises the possibility of functional redundancy in the community behaviour. Identification of community members may refine information to the point of detecting species more associated with disease than with health, since our findings revealed bands more prevalent in samples with apical periodontitis. Diseased teeth harboured a more diverse community and the number of bands was significantly higher in these samples.

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REFERENCES


