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Análise Microbiológica e Imunológica de Lesões Refratárias ao Tratamento Endodôntico

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Análise Microbiológica e Imunológica de Lesões Refratárias ao Tratamento Endodôntico

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LISTA DE ABREVIATURAS

1- ATCC: *American Type Collection*

2- CD4: *cluster of differenciation 4*

3- CD8: *cluster of differenciation 8*

4- DNA: *Deoxyribonucleic acid*

5- GAPDH: *Glyceraldehyde 3-phosphate dehydrogenase*

6- IL-: *Interleukin*

7- IFN-: *interferon*

8- MCP-1: *monocyte chemotactic protein-1*

9- MDA: *Multiple displacement amplification*

10- mRNA: *Messenger RNA*

11- PCR: *polymerase chain reaction*

12- RNA: *Ribonucleic acid*

13- SCR: Sistema de canais radiculares

14- SEM: *Standard error of the mean*

15- TCD4+: *Tcell CD4*

16- TCD8+: *Tcell CD8*

17- TCLE: Termo de consentimento livre e esclarecido

18- TGF: *Transforming growth factor*

19- Th: *T helper cell*

20- TNF: *Tumor necrosis factors*

21- Treg.: *Regulatory T cells*

22- UFC: unidades formadoras de colônias

Resumo

Introdução: Falhas no tratamento endodôntico são geralmente causadas pela persistência de microorganismos no sistema de canais radiculares que são capazes de induzir uma resposta imunológica perirradicular com a produção de citocinas e quimiocinas.

Objetivos: Avaliar o perfil microbiológico em infecções de dentes refratários ao tratamento endodôntico utilizando-se a associação das técnicas do Multiple Displacement Amplification (MDA) e Hibridização DNA-DNA (checkerboard), e caracterizar, por PCR em tempo real, a expressão gênica de citocinas e quimiocinas no periápice desses dentes.

Metodologia: Dentes de 40 pacientes apresentando lesões refratárias ao tratamento endodôntico (grupo experimental) e de 20 pacientes apresentando vitalidade pulpar (grupo controle) foram avaliados. Recuperaram-se as amostras microbianas utilizando-se limas tipo K # 10, que foram inseridas nos SCR após a remoção do material obturador pré-existente. Posteriormente estas amostras foram amplificadas pelo MDA e analisadas pelo checkerboard. Avaliou-se a presença de 107 espécies microbianas. Utilizando-se o PCR em tempo real avaliou-se a expressão gênica das citocinas IFN-γ, TNF-α, IL-1-β, IL-17A, IL-10 e da quimiocina MCP-1 nas amostras coletadas do periápice dos dentes do grupo experimental e controle. A significância das diferenças foi avaliada utilizando o teste Mann Whitney em relação aos achados microbiológicos e imunológicos ($p<0,05$).

Resultados: A população dominante (>4%) constitui-se das espécies *Corynebacterium diphtheria, Streptococcus constellatus, Porphyromonas gingivalis, Granulicatella adjacens* e *Prevotella denticola.* Por sua vez encontram-se na população sub dominante (>2%a 4%) o *Streptococcus mutans*, *Actinomyces georgiae*, *Helicobacter pylori*, *Dialister pneumosintes* e *Eikenella corrodens.* A maioria das espécies se encontram na população residual (<2%), inclusive o *Enterococcus faecalis.* Diferenças significativas nos níveis de expressão gênica de IFN-γ, TNF-α, IL-17A, e MCP-1 foram observados nas amostras de lesões refratárias ao tratamento endodôntico quando comparados ao grupo controle. Não foram encontradas diferenças significativas nos níveis de expressão gênica de IL-1β entre os dois grupos. A expressão gênica da IL-10 não foi detectada em ambos os grupos experimental e controle.

Conclusões: A microbiota de infecções refratárias ao tratamento endodôntico demonstrou ser mais complexa do que antes observado. Apesar da maioria dos estudos vincularem a presença do *E. faecalis* ao insucesso do tratamento endodôntico, seu papel, nessas infecções, necessita ser revisto. As respostas imuno-periapicais à infecção presente nos dentes refratários ao tratamento endodôntico apresentou um perfil pró-inflamatório.

Abstract

Introduction: Endodontic treatment failure is often caused by the persistence of microorganisms in the root canal system by developing an periapical immune response with the production of cytokines and chemokines.

Aims: To combine MDA and Checkerboard DNA–DNA hybridization to qualitatively and quantitatively evaluate the microbiota of infections refractory to endodontic treatment, and to determine the relative mRNA expression of IFN-γ, TNF-α, IL-1β, IL-17A, IL-10, and MCP-1 in periapical area.

Methodology: Subjects were 40 patients presenting periapical lesions refractory to endodontic treatment (experimental group) and 20 patients presenting pulp vitality (control group). Microbial samples were taken by scraping or filing the root canal walls with a #10 K-type hand file. MDA was performed and Checkerboard DNA–DNA hybridization was employed to assess the levels of for levels of 107 microbial taxa. Immunological samples were taken from experimental and control groups and analysed by the quantitative real time PCR. Mann-Whitney test was used to determine the statistical significance of microbiological and immunological findings ($p < 0.05$).

Results: The dominant taxa were *Corynebacterium diphtheria, Streptococcus constellatus, Porphyromonas gingivalis, Granulicatella adjacens,* and *Prevotella denticola.* Among sub dominant population were *Streptococcus mutans, Actinomyces georgiae, Helicobacter pylori, Dialister pneumosintes,* and *Eikenella corrodens.* Most species were included in the residual

population. *E. faecalis* was detected in low mean proportion. Significant differences in the levels of IFN-γ, TNF-α, IL-17A, and MCP-1 mRNA expression were observed in cases refractory to endodontic treatment compared to the control group. The expression of IL-1β mRNA was not significantly different between the groups. Expression of IL-10 mRNA was not detected in both experimental and control groups.

Conclusions: The microbiota of infections refractory to endodontic treatment is far more complex than previously shown. Although most studies have linked the presence of *E. faecalis* in endodontic failure, its role in these infections need to be revised. The periapical immune responses to these infections presented a pro-inflammatory profile.

1. INTRODUÇÃO E RELEVÂNCIA

Das mais de 700 espécies microbianas que habitam a cavidade oral humana, 35% permanecem não cultivadas (Paster *et al.*, 2001, Paster *et al.*, 2002). Essa microbiota vem sendo associada a doenças como a endocardite bacteriana (Berbari *et al.*, 1997), osteomielites em crianças (Dodman *et al.*, 2000), doenças respiratórias (Scannapieco, 1999) e doenças cardíacas (Beck *et al.*, 1996). Sabe-se que a capacidade dos microrganismos em se implantar ou não em certos sítios dependerá, além de outros fatores, de seu número, virulência e da resistência do hospedeiro (Socransky & Haffajee 2005). Quanto ao estudo das infecções de origem endodôntica, muitas lacunas ainda precisam ser preenchidas na busca de diagnóstico e tratamentos precisos.

O insucesso no tratamento endodôntico é, na maioria das vezes, consequência da persistência de microrganismos nos sistemas de canais radiculares (SCR) (Gomes *et al.*, 2004). Alguns fatores como cavidade de acesso deficiente, canais não localizados, instrumentação insuficiente, irrigação deficiente, e infiltração de restaurações temporárias podem ser enumerados como perpetuadores desse processo (Nair, 2006). A microbiota presente em dentes com lesões refratárias ao tratamento endodôntico ainda necessita ser mais bem avaliada para se estabelecer se a mesma é similar ou não à de dentes portadores de infecção primária.

Até recentemente, imputava-se a uma única espécie ou a um pequeno número de espécies bacterianas a responsabilidade pelo insucesso do tratamento endodôntico em dentes com lesões persistentes. Predominavam microorganismos Gram-positivos, com igual distribuição entre anaeróbios

facultativos e estritos (Molander *et al.*, 1998, Sunqvist *et al.*, 1998). Esse padrão de contaminação diferia do de infecções primárias, em que a microbiota constituía-se tipicamente de um consórcio polimicrobiano com proporções iguais entre espécies Gram-positivas e Gram-negativas, prevalecendo microorganismos anaeróbios obrigatórios (Moller, 1966, Molander *et al.*, 1994). Os estudos demonstravam maior prevalência de *Enterococus* e *Estreptococus* em dentes portadores de lesões refratárias ao tratamento endodôntico (Molander *et al.*, 1998). Outros microrganismos encontrados em grandes proporções nesse tipo de infecção eram dos gêneros *Lactobacillus*, *Actinomyces* e *Peptostreptococcus*, associados ao *Pseudoramibacter alactolyticus, Propionibacterium propionicum, Dialister pneumosintes*, *Filifactor alocis*, e *Candida albicans* (Molander *et al.*, 1998, Sundqvist *et al.*, 1998, Moller, 1966, Peciuliene *et al.*, 2000, Hancock *et al.*, 2001, Peciuliene *et al.*, 2001, Siquiera *et al.*, 2004).

Recentemente, com o advento das técnicas de biologia molecular, houve melhora significativa na sensibilidade, especificidade e custo benefício das análises microbiológicas associadas à cavidade bucal (Socransky *et al.*, 1998, Siqueira *et al.*, 2000b, Rocas *et al.*, 2001, Fouad *et al.*, 2002, Siqueira *et al.*, 2002, Kawada *et al.*, 2004, Socransky *et al.*, 2004, Siqueira *et al.*, 2005, Seol *et al.*, 2006, Brito *et al.*, 2007, Teles *et al.*, 2007b, Haffajee *et al.*, 2009, Tavares *et al.*, 2011). Muitas espécies microbianas, que se acreditava não estarem presentes nessas infecções, são hoje detectadas e confirmadas como integrantes dessa microbiota (Paster *et al.*, 2001). Essas técnicas vêm sendo utilizadas na detecção de microrganismos nos SCR infectados portadores de

infecções primárias (Gatti *et al.*, 2000, Siqueira *et al.*, 2000a, Siqueira *et al.*, 2000b, Rocas *et al.*, 2001, Fouad *et al.*, 2002, Siqueira *et al.*, 2002, Siqueira & Rocas, 2004a, Siqueira & Rocas, 2004b, Siqueira & Rocas, 2006, Brito *et al.*, 2007, Tavares *et al.*, 2011). Utilizando-se essas técnicas nos dentes com lesões refratárias ao tratamento endodôntico, demonstrou-se que a microbiota aí presente é mais complexa do que anteriormente observado (Rolph *et al.* 2001, Siqueira & Roças 2004). A recuperação de um consórcio bacteriano nesses dentes demonstra que bactérias incultiváveis e outras espécies, além do *E. faecalis*, também estão aí presentes (Sakamoto *et al.*, 2008).

Com o objetivo de aperfeiçoar a técnica do "checkerboard", utilizou-se o "Multiple Displacement Amplification" (MDA), para amplificar o DNA bacteriano, antes da análise pela Hibridização DNA-DNA (Teles *et al.*, 2007a). Pesquisas que associaram o "MDA" ao "Chekerboard" foram conduzidas com sucesso e permitiram demonstrar que a média de espécies por canal radicular é muito superior à da tradicionalmente encontrada (Brito *et al.*, 2007, Tavares *et al.*, 2011).

Uma vez instalados nos SCR, os microrganismos induzem uma resposta de defesa nos arredores do ápice radicular (Stashenko, 1990). A resposta inflamatória, que aí se processa, recruta células imunocompetentes para conter e impedir a disseminação dessa infecção para outros sítios, culminando com a formação de uma lesão crônica e a concomitante reabsorção dos tecidos de suporte periodontal adjacentes (Fukada *et al.*, 2009).

Nas últimas décadas, houve fortes evidências de que muitos dos efeitos patogênicos microbianos sobre os tecidos periapicais operam-se de forma

indireta, via estimulação de mediadores solúveis derivados do hospedeiro, como as citocinas e quimiocinas (Stashenko *et al.,* 1998). Daí o grande interesse em se conhecer esses mediadores e seus efeitos sobre as células imunocompetentes (Silva *et al.*, 2005).

Têm-se detectado uma grande variedade de células nas lesões perirradiculares humanas, dentre elas: os linfócitos $TCD4^+$ e $TCD8^+$, macrófagos, células plasmáticas, mastócitos, eosinófilos. As células T entretanto são as mais numerosas nessas lesões (Colic *et al.*, 2009b).

Os linfócitos $TCD4^+$ e $CD8^+$, após seu contato com antígenos ou serem estimulados por outras células inflamatórias, podem produzir uma grande variedade de citocinas (Marton & Kiss, 2000). As células $TCD4^+$ atualmente se subdividem em vários subgrupos que incluem as células: Th1, Th2, Th17 e T regulatórias (T_{reg}) (McGeachy & Cua, 2008). A resposta Th1 caracteriza-se pela produção de IFN-γ, IL-12, IL-2, e TNF, envolvendo-se na progressão e destruição óssea perirradicular (Stashenko *et al.*, 1998, Colic *et al.*, 2009b). A resposta Th2 induz a síntese e atividade das citocinas IL-4, IL-5, IL-6, IL-9, e IL-13, relacionando-se com a cicatrização e regeneração dos tecidos perirradiculares (Akamine *et al.*, 1994, Stashenko *et al.*, 1998, Kawashima & Stashenko, 1999, Sasaki *et al.*, 2000, Teixeira-Salum *et al.*, 2010). O subgrupo Th17 produz a IL-17, citocina pró-inflamatória com atuação em várias células da resposta inata, e é considerado ponte entre esta e a resposta adaptativa. (Yu & Gaffen, 2008). As células T_{reg}, produtoras de TGF-β e IL-10, possuem um efeito inibitório sobre a reabsorção óssea durante a formação e diferenciação dos osteoclastos, além de atuarem na regulação da resposta

imune contra a infecção (Colic *et al.*, 2009a).

As quimiocinas participam do processo inflamatório ao promoverem a ativação de selectinas que, por sua vez, estão envolvidas na adesão de células às paredes endoteliais. A expressão localizada de quimiocinas nos tecidos gera gradientes quimiotáticos que são responsáveis pela migração guiada e manutenção de células inflamatórias nesses locais (Mantovani et al., 1998, Silva *et al.*, 2005). A Proteína Quimiotática para Monócitos (MCP-1) tem sido descrita em granulomas periapicais e associada à modulação de lesões periapicais humanas (Kabashima et al., 2001).

Este estudo se ateve à análise microbiológica de dentes com lesões refratárias ao tratamento endodôntico, e avaliou a expressão imunológica de citocinas, em resposta à infecção.

2. OBJETIVOS

2.1. Objetivos gerais

- Caracterizar a microbiota das infecções endodônticas refratárias ao tratamento endodôntico;

- Identificar a expressão gênica de citocinas e quimiocinas nos tecidos perirradiculares de indivíduos portadores de infecções refratárias ao tratamento endodôntico.

2.2. Objetivos Específicos

- Avaliar o perfil microbiológico de amostras recuperadas de dentes com lesões refratárias ao tratamento endodôntico, utilizando-se as técnicas: Multiple Displacement Amplification (MDA) e hibridização DNA-DNA (checkerboard);

- Caracterizar, por PCR em tempo real, a expressão gênica das citocinas IFN-γ, TNF-α, IL-1-β, IL-17A, IL-10, e da quimiocina MCP-1 no periápice de dentes com lesões refratárias ao tratamento endodôntico.

3. TRABALHOS CIENTÍFICOS

Trabalho 1- Microbial ecosystem analysis in Root Canal Infections Refractory to Endodontic Treatment

Trabalho 2- Cytokine Analysis in Lesions Refractory to Endodontic Treatment

Microbial Ecosystem Analysis in Root Canal Infections Refractory to Endodontic Treatment

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Abstract

Aims To combine MDA and Checkerboard DNA–DNA hybridization to qualitatively and quantitatively evaluate the microbiota of infections refractory to endodontic treatment

Methodology Subjects were 40 patients presenting periapical lesions refractory to endodontic treatment. Samples were taken by scraping or filing the root canal walls with a #10 K-type hand file, MDA-amplified and analysed by Checkerboard DNA–DNA hybridization for levels of 107 bacterial taxa. The taxa were divided in three distinct microbial populations, depending on their mean proportion (%DNA probe counts ± SEM) as follow: dominant (>4%), sub dominant (>2 to 4%) and residual (< 2%) populations. Significance of differences was sought using the Mann Whitney test.

Results The dominant taxa were *C. diphtheria* (7.35 ± 1.22), *S. constellatus* (6.85 ± 1.15), *P. gingivalis* (6.19 ± 2.20), *G. adjacens* (5.94 ± 0.99), and *P. denticola* (5.79 ± 0.97). Among sub dominant population were *S. mutans* (4.12 ± 0.61), *A. georgiae* (4.02 ± 0.60), *H. pylori* (3.11 ± 0.30), *D. peneumosintes* (2.25 ± 0.54), and *E. corrodens* (2.16 ± 0.36). In the residual population, *E. coli* (0.05 ± 0.01), and *L. acidophilus* (0.02 ± 0.01) showed the lowest mean proportions. *E. faecalis* was detected in low mean proportion (0.55 ± 0.27).

Conclusion: The microbiota of infections refractory to endodontic treatment is far more complex than previously shown and, despite features of *E. faecalis* that allow it to survive and persist in treated root canals, this species is only a small part of this microbiota.

Keywords: bacteria, checkerboard DNA–DNA hybridization, endodontic treatment failure, endodontic infection, multiple displacement amplification, root-canal-treated teeth.

Introduction

Failure in endodontic therapy is often caused by the persistence of microorganisms in root canal system or recontamination after inadequate coronal sealing. When the treatment fails a bone resorption may develop as a consequence of the periapical immune/inflammatory responses (Sundqvist 1994; Stashenko *et al.* 1998; Nair, 2004, Henriques *et al.* 2011). Whenever possible, the endodontic retreatment might be performed in an attempt to achieve the teeth healthy.

Over the years, most of the studies have shown the microbiota recovered from teeth refractory to endodontic treatment is predominantly consisted of Gram-positive bacteria, specially *Enterococcus faecalis* (Molander *et al.* 1998, Pinheiro *et al.* 2003, Sundqvist *et al.* 1998, Siqueira & Roças 2004). Nevertheless the high detection of this species may have been influenced by significant limitations of microbial technique procedures, such as low sensitivity, inability to detect fastidious and as-yet-uncultivated phylotypes, which underestimate the bacterial diversity in diverse oral ecosystems (Paster *et al.* 2006).

Traditionally, the study of infectious diseases has focused on one or a small number of pathogens in a given infectious disease. Examination of complex mixtures of microorganisms has been hampered by the tradition of focusing on a small number of species thought to be pathogenic or by the absence of useful, rapid identification techniques to evaluate (Socransky & Haffajje, 2005). However, new concepts of biofilms infections become established, indicating microbial communities greater than the simple sum of its

parts (Siqueira & Roças 2009). In this regards, molecular assays have shown that the microbiota in teeth refractory to treatment is much more complex than previously known (Rolph *et al.* 2001, Siqueira & Roças 2004). A mixed consortium was recovered from those teeth, revealing that as-yet-uncultivated bacteria and taxa other than *E. faecalis*, might participate in these infections (Sakamoto *et al.* 2008).

The quantity of bacteria in the samples is an important factor in the checkerboard DNA-DNA hybridization technique, since its level of detection is about 10^4 bacterial cells of a given species (Socransky *et al.* 1994). Recently, to overcome these limitations, that would underestimate the presence of some taxa in the root canal microbial ecosystem which contain very few bacterial cells (Zavistoski *et al.* 1980), the researchers have used multiple-displacement amplification (MDA) before hybridizing the samples. MDA has provided a simple and reliable method to amplify the sample DNA with minimal bias (Hawkins *et al.* 2002, Nelson *et al.* 2002, Yan *et al.* 2004, Brito *et al.* 2007, Teles *et al.* 2007, Tavares *et al.* 2011). The association of these techniques has contributed to recognize that endodontic microbiota is far more complex than previously thought (Brito *et al.* 2007, Tavares *et al.* 2011). The aim of the present study was to combine MDA and Checkerboard DNA–DNA hybridization to qualitatively and quantitatively evaluate the microbiota of infections refractory to endodontic treatment, determining dominant, sub dominant and residual microbial population in this ecosystem.

Materials and Methods

Human subjects

Subjects were 40 patients presenting periapical lesions refractory to endodontic treatment. Subjects were drawn from patients that were referred to the School of Dentistry at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil). Patients were excluded from this study if they had taken antibiotics in the three months prior to the initiation of endodontic therapy. All participants signed the Free Agreement Formulary. This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais (ETIC 0011.0.215.203-10).

Sample collection

All selected teeth had clinical crowns that permitted effective rubber dam isolation. There was no history of trauma associated with the selected teeth or periodontal involvement. Samples from multi-rooted teeth were taken from the largest root canal always associated with the periapical lesion.

The selection and preparation of the teeth was performed as previously described (Brito *et al.* 2007, Tavares *et al.* 2011). In brief, the pre-existing root canal filling was removed using retreatment ProTaper NiTi files (Dentsply, Ballalgues, Switzerland) without the use of any solvent solution. Samples were taken by scraping or filing the root canal walls with a #10 K-type hand file (Maillefer, Ballaigues, Switzerland). The file was introduced into the canal to a level approximately 1 mm short of the tooth apex. After removal from the canal, the final 4 mm of the file was removed using a sterile pair of surgical scissors and placed in a microcentrifuge tube containing 20 µl of alkaline lysis buffer

(400 mM KOH, 100 mM dithiothreitol, 10 mM EDTA). After 10 min of incubation on ice, 20 µl of neutralization solution (400 mM HCl, 600 mM Tris-HCl, pH 0.6) was added. Samples were kept at 4°C until analysis.

Multiple displacement amplification (MDA) of root canal samples

Multiple displacement amplification was performed as previously described. (Brito *et al.* 2007, Teles *et al.* 2007, Tavares *et al.* 2011). The Illustra™ GenomiPhi™ V2 DNA Amplification Kit (GE Healthcare, USA) was used for whole genomic amplification as described by the manufacturer. In brief, 1 µL of each of the DNA templates (i.e. endodontic samples) was added to 9 µL of sample buffer (50 mM Tris-HCl pH 8.2, 0.5 mM EDTA containing random hexamer primers) in 200 µL microcentrifuge tubes (Stratagene, La Jolla, CA, USA). Templates in sample buffer were heat denatured at 95°C for 3 minutes in a Perkin-Elmer Thermocycler and cooled to 4°C. One µL of phi 29 DNA polymerase mix including additional random hexamers was mixed on ice with 9 µL of reaction buffer containing dNTPs. The mixture was then added to the denatured sample to make a final volume of 20 µL and incubated at 30°C for 2 hours. Ten ng of Lambda DNA (contained in 1 µL) was used as a control. The amplification reaction was terminated by incubation of the samples at 65°C for 10 min. The amplified material was either immediately used, stored short-term at 4°C or at –20°C for longer storage.

The DNA content of the samples was measured prior to and after amplification using the Picogreen™ dsDNA quantification assay (Invitrogen, Carlsbad, CA, USA). Picogreen™ is a fluorescent nucleic acid stain that allows the quantification of as little as 25 pg/mL of double stranded DNA in samples.

The microbiological content of the amplified samples was analyzed using checkerboard DNA-DNA hybridization.

Bacterial strains and growth conditions, DNA isolation, preparation of DNA probes and Checkerboard DNA-DNA hybridization

The 107 reference strains used for the preparation of DNA probes are listed in Table 1. The grown conditions of the selected bacterial strains have been described earlier (Socransky *et al.* 2004, Brito *et al.* 2007, Teles *et al.* 2007, Tavares *et al.* 2011).

Preparation of probes and standards for quantification

Checkerboard DNA-DNA Hybridization was performed as previously described (Socransky *et al.* 1994, Socransky *et al.* 2004). To prepare probes and standards, each species listed in Table 1 was grown on agar plates (except the two spirochetes, which were grown in broth) for 3–7 days. The cells were harvested and placed in 1.5 mL microcentrifuge tubes containing 1 mL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6). Cells were washed twice by centrifugation in TE buffer at 1300×g for 10 min. The cells were resuspended and lysed with either 10% SDS and Proteinase K (20 mg/mL) for Gram-negative strains or in 150µL of an enzyme mixture containing 15 mg/mL lysozyme (Sigma) and 5 mg/mL achromopeptidase (Sigma) in TE buffer (pH 8.0) for gram-positive strains. The pelleted cells were resuspended by 15 s of sonication and incubated at 37°C for 1 h. DNA was isolated and purified using the method of Smith et al. (1989). The concentration of the purified DNA was

determined by spectrophotometric measurement of the absorbance at 260 nm. The purity of the preparations was assessed by the ratio of the absorbances at 260 and 280 nm. Whole genomic DNA probes were prepared from each of the 107 test strains by labeling 1 – 3μg of DNA with digoxigenin (Boehringer Mannheim, Indianapolis, IN, USA) using a random primer technique (Feinberg & Vogelstein, 1983).

Sample preparation and microbial analysis

Following amplification and quantification, the amplified endodontic samples were boiled for 10 min. Approximately 1500 ng of DNA (5 μL) of the amplified sample were placed in a microcentrifuge tube containing 1 mL of TE buffer prior to boiling. The samples were placed into the extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA), concentrated onto a nylon membrane (Boehringer Mannheim) by vacuum and fixed onto the membrane by cross-linking using ultraviolet light (Stratalinker 1800, La Jolla, CA, USA) followed by baking at 120 °C for 20 min. The Minislot device permitted the deposition of 28 different samples in individual lanes on a single membrane, as well as two control lanes containing the standards for quantification: 1 and 10 ng of DNA of each bacterial species tested, equivalent to 10^5 and 10^6 cells, respectively.

Checkerboard DNA-DNA hybridization was performed as previously described by Socransky *et al.* (1994, 2004). The membrane with fixed DNA was placed in a Miniblotter 45 apparatus (Immunetics) with the lanes of DNA at 90° to the channels of the device. A 30 × 45 "checkerboard" pattern was produced. Each channel was used as an individual hybridization chamber for separate

DNA probes. Bound probes were detected by anti- digoxigenin antibody conjugated with alkaline phosphatase and a chemifluorescent substrate. Signal intensities of the endodontic samples and the standards (containing 10^5 and 10^6 cells of each species) on the same membrane were measured using a Storm FluorImager (Molecular Dynamics, Sunnyvale, CA, USA). Signals were converted to absolute counts by comparison with standards on the membrane (Socransky *et al.* 2004). Failure to detect a signal was recorded as zero.

Three membranes were run for each sample: one containing the "standard" 40 DNA probes routinely used to examine periodontal samples as well as a probe to detect *Streptococcus mutans.* A second membrane employed 42 probes to species thought to be implicated in endodontic infections. A third membrane was used to assess levels of medically important microbial taxa. Sensitivity and specificity tests were performed for all probes before performing the checkerboard DNA-DNA hybridization analysis, using a protocol similar to that described by Socransky *et al.* (2004).

Data analysis

Since the sample DNA was amplified, absolute numbers could not be determined. Thus, proportions of the total DNA probe count for each species comprised were computed for each sample and then averaged across subjects in each group separately.

Significance of differences between the proportions of test species in samples from selected subjects was sought using the Mann Whitney test.

Results

Quantification of DNA before and after MDA of endodontic samples

DNA from the root canal samples was amplified using MDA. The amount of DNA present in the samples before amplification averaged 4.3ng (± 1.48) ng and 6.6 (± 1.85) µg after amplification, an approximately 1,000-fold amplification.

Microbial species in root canal samples refractory to endodontic treatment

To better analyze the microbial consortium that colonize the refractory teeth to endodontic treatment, we divided the taxa in three distinct microbial populations, depending on their mean proportion (%DNA probe counts ± SEM) as follow: dominant (>4%), sub dominant (>2 to 4%) and residual (< 2%) populations. These mean proportions of the target 107 test species are presented in Fig. 1. The dominant taxa were *C. diphtheria* (7.35 ± 1.22), *S. constellatus* (6.85 ± 1.15), *P. gingivalis* (6.19 ± 2.20), *G. adjacens* (5.94 ± 0.99), and *P. denticola* (5.79 ± 0.97). Among sub dominant population were *S. mutans* (4.12 ± 0.61), *A. georgiae* (4.02 ± 0.60), *H. pylori* (3.11 ± 0.30), *D. peneumosintes* (2.25 ± 0.54), and *E. corrodens* (2.16 ± 0.36). In the residual population, *E. coli* (0.05 ± 0.01), and *L. acidophilus* (0.02 ± 0.01) showed the lowest mean proportions. *E. faecalis* was detected in low mean proportion (0.55 ± 0.27). However, *E. faecalis* was detected as dominant population (10.1%) only in one sample site from 40 teeth analyzed (Table 2). *C. rectus*, *G. haemolysans*, and *C. dificile* were not detected in any sample (Fig 1).

Discussion

Multiple microbial species are necessary to produce apical periodontitis, even in teeth refractory to treatment (Rolph *et al.* 2001, Roças *et al.* 2004; Sakamoto *et al.* 2008). In a developing ecosystem, pioneer organisms colonize first; alter the habitat, making it suitable for colonization by other species (Socransky & Haffajje, 2005). However, the population levels and the distinct microbial species in such climax community are regulated by multifactor process such as changes in the physical or chemical properties of the region or changes in the host (Savage, 1977). In this regards, all microorganisms that act as a pathogen have to be in high levels to play a role in biofilm community as well as in host immune modulation.

Studies that attempted to analyze root canal microbial ecosystem are restricted by the few bacterial cells contained in this site (Zavistoski *et al.* 1980). As a consequence, the lack of detection and the difficult to count some taxa would underestimate their possible role in the endodontic microbial ecosystem. The high sensibility of MDA associated with Checkerboard DNA-DNA Hybridization overcomes this limitation, as demonstrated elsewhere (Teles *et al.* 2007, Brito *et al.* 2007, Tavares *et al.* 2011). As MDA amplified the total DNA of the samples in a 1000 fold, it is likely that even a number of bacterial cells below the level of detection of the Checkerboard DNA–DNA hybridization technique were detected by this approach. Herein, we assessed 40 patients presenting periapical lesions refractory to endodontic treatment using probes to 107 different microbial taxa. Depending on the mean proportion (%DNA probe

counts ± SEM) of each taxa we divided population in dominant (>4%), sub dominant (>2 to 4%) and residual (< 2%) organisms (Fig. 1).

C.*diphtheriae, S.constellatus, P.gingivalis, G. adjacens*, *P.denticola,* and *E. nodatum* were among dominant taxa in these infections. Interesting, *C. diphtheria* has already been recovered from periodontal disease (Beveridge & Goldner, 1973) and root canal infections (Brito *et al. in press*). C. diphtheriae belong to the family Mycobacteriaceae, and are Gram-positive and aerobic, and it causes the disease diphtheria which primarily affects the upper respiratory tract. The most commons site of infection is the pharynx and tonsils, but the bacteria can also invade the nasal tissues, larynx and skin. However, it is important to point out that toxin production occurs only when the bacillus is itself infected by a specific virus (bacteriophage) carrying the genetic information for the toxin (*tox* gene) (Oram *et al.* 2007). Only toxigenic strains can cause severe disease (Holmes , 2000). Other medical important taxa found in this study were *H. pylori,* present in a subdominant condition. This species is an organism primarily recovered from the stomach and is responsible for certain forms of gastritis or peptic ulcers, and has been found in oral cavity, including endodontic infections (Brito *et al. in press*) and periodontal diseases (de Souza Gonçalves *et al.* 2009, Silva *et al.* 2010, Gao *et al.* 2011). *Granulicatella adjacens*, previously referred to as nutritionally variant streptococci, *Streptococcus adjacens*, causes infective endocarditis. This taxon was described as new type of viridans group streptococci and colonizes the oral cavity and the intestinal and genitourinary tracts as normal flora (Ohara-Nemoto *et al.* 1997). Like other viridans group streptococci, *Granulicatella* species cause sepsis and

bacteremia and are also one of the major pathogens of infective endocarditis (Bouvet, 1995).

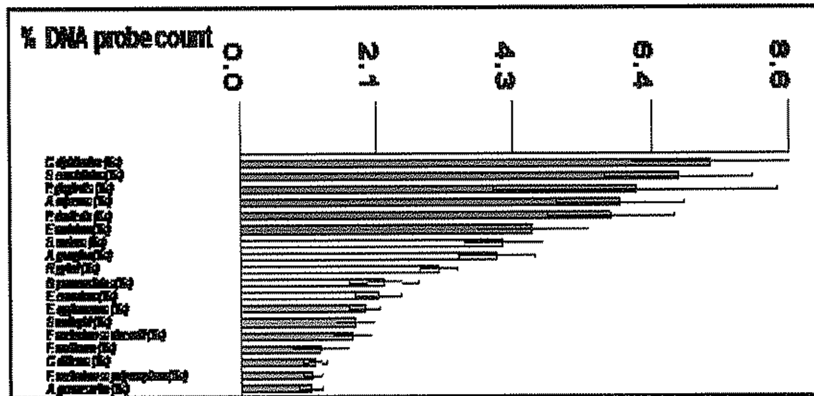
Black-pigmented anaerobic rods such as *P.gingivalis* and *P.denticola* are involved in the etiology and perpetuation of endodontic infections. They were detected in permanent and deciduous teeth (Brito *et al.* 2007, Tavares *et al.* 2011), as well as in teeth refractory to endodontic treatment (Sakamoto *et al.* 2008). *Eubacterium* species, such as *E. nodatum*, although no virulence factors have been described, may form part of a consortium involved in the degradation of host tissue or could be an organism that favor the nutritional conditions in infected sites (Spratt *et al.* 1999). It has been found in root canal infections (Vianna *et al.* 2005, Brito *et al.* 2007, Tavares *et al.* 2011).

S. constellatus, member of the *Streptococcus milleri* group, is normally found in the oral cavities, being associated with abscess formation in respiratory tract (Sibley *et al.* 2008). It has been detected in root canal infections (Vianna *et al.* 2005, Siqueira *et al.* 2001, Brito *et al.* 2007, Tavares *et al.* 2011) and, in this study, it was among those dominant organisms. Additionally, as demonstrated herein, *S.mutans* has already been recovered from teeth refractory to endodontic treatment (Sakamoto *et al.* 2008)

Residual habitants comprised the larger amount of bacterial species in teeth refractory to root canal treatment, similarly what was previously found in studies of indigenous gastrointestinal microflora (Berg, 1996). Despite their presence in low rates, its relevance could not be underestimated. Even low rate members might provide the community with advantageous properties, serving as keystones species within complex communities (Sogin *et al.* 2006, Huse *et*

al. 2008). Associated to this, disturbances in local factors such nutrients sources and competitive process might influence a shift in the microbial composition, allowing them to become dominant (Siqueira & Roças 2009). In accordance, previous findings from studies using PCR-DGGE and PCR demonstrated high interindividual variability in the bacterial community profiles in treatment failure cases (Rôças *et al.* 2004, Sakamoto *et al.* 2008), revealing that dominant populations in one individual can correspond to low abundant populations in another one (Roças *et al.* 2004, Siqueira *et al.* 2005).

During the last decades, it has been attributed to *E. faecalis* for the failure of root canal treatment (Molander *et al.* 1998, Sundqvist *et al.* 1998, Hancock *et al.* 2001, Pinheiro *et al.* 2003, Gomes *et al.* 2008). In this study, *E. faecalis* was detected in low mean proportion (0.55 ± 0.27), being among those residual organisms (Fig 1). Very interesting, in 40 samples analyzed *E. faecalis* was absent in only six subject. It was dominant taxa in one sample site though (Table 2). These findings reinforce the previously reports (Rôças *et al.* 2004, Sakamoto *et al.* 2008), which have questioned about its relevance in endodontic treatment failures. It seems to us that, despite *E. faecalis* features allow it to survive and persist in treated root canals (Portenier *et al.* 2003, Zoletti *et al.* 2006), this species is lone part of a mixed infection that prevail in such endodontic microbiota in teeth refractory to root canal treatment. However, more studies focusing on microbial ecology of refractory apical periodontitis may be invigorated to aid the design of more efficient endodontic retreatments.


% DNA probe count
0.0
2.1
4.3
6.4
8.6

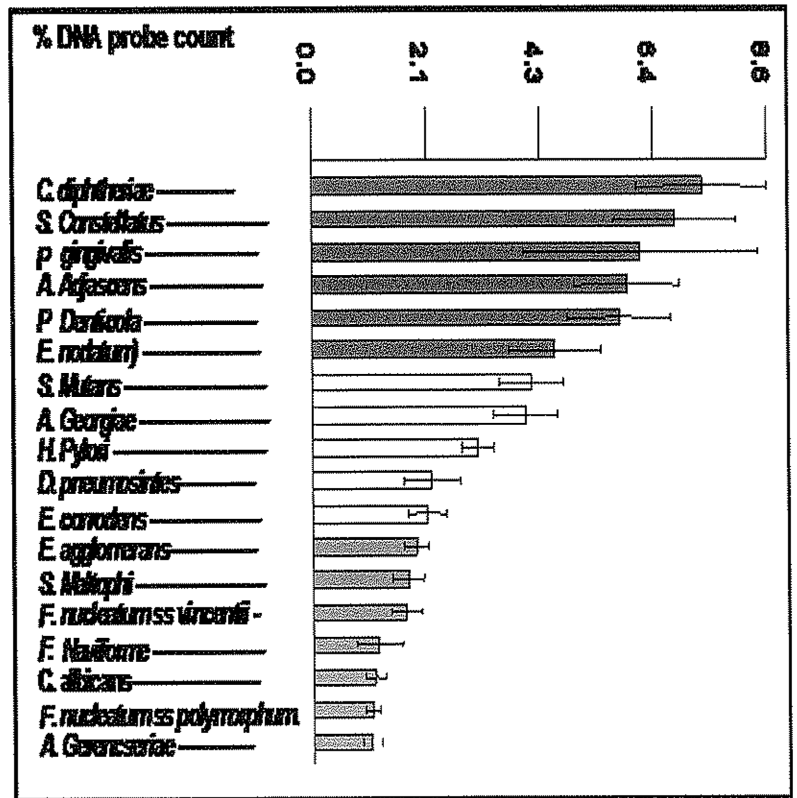

% DNA probe count
0.0
2.1
4.3
6.4
8.6
C. diphtheriae
S. Constellatus
p. gingivalis
A. Adjasens
P. Denticola
E. nodatum)
S. Mutans
A. Georgiae
H. Pylori
D. pneumosintes
E. corrodens
E. agglomerans
S. Maltophi
F. nucleatum ss vincentii
F. Naviforme
C. albicans
F. nucleatum ss polymorphum
A. Gerencseriae

FIGURE 1 . Quantitative analysis of Microbiota of 40 refractory endodontic infections analysed by multiple displacement amplification (MDA) and Checkerboard DNA–DNA hybridization represented by the percentage of mean proportion DNA probe counts (±SEM) of test species. The percentage of the DNA probe count was computed for each species for each sample and averaged across samples. The data are ordered in descending order of mean percentages of DNA probe counts detected in amplified samples.

TABLE 1 . Bacterial species.

Bacterial strains (a)	
Acinetobacter baumannii (19606)	*Legionella pneumophila (33152)*
Actinomyces georgiae (49285)	*Leptotrichia buccalis (14201)*
Actinomyces gerencseriae (23860)	*Mobiluncus mulieris (35243)*
Actinomyces israelii (12102)	*Mogibacterium timidum (33093)*
Actinomyces meyeri (35568)	*Neisseria gonorrhea (21823)*
Actinomyces naeslundii (12104)	*Neisseria meningitidis (13077)*
Actinomyces odontolyticus (17929)	*Neisseria mucosa (19696)*
Actinomyces viscosus (43146)	*Olsenella uli (49627)*
Aggregatibacter actinomycetemcomitans (b)	*Peptostreptococcus anaerobius (27337)*
Atopobium parvulum (33793)	*Parvimonas micra (33270)*
Bacteroides fragilis (25285)	*Porphyromonas endodontalis (35406)*
Bacteroides ureolyticus (33387)	*Porphyromonas gingivalis (33277)*
Campylobacter gracilis (33236)	*Prevotella denticola (35308)*
Campylobacter rectus (33238)	*Prevotella heparinolytica (35895)*
Campylobacter showae (51146)	*Prevotella intermedia (25611)*
Capnocytophaga gingivalis (33624)	*Prevotella loescheii (15930)*
Capnocytophaga ochracea (33596)	*Prevotella melaninogenica (25845)*
Capnocytophaga sputigena (33612)	*Prevotella nigrescens (33563)*
Clostridium difficile (9689)	*Prevotella oris (33573)*
Corynebacterium diphtheriae (13812)	*Prevotella tannerae (51259)*
Corynebacterium matruchotii (14266)	*Propionibacterium acnes (c)*
Dialister pneumosintes (GBA27)	*Propionibacterium propionicum (14157)*
Eikenella corrodens (23834)	*Rothia dentocariosa (17931)*
Enterococcus faecalis (10100)	*Salmonella enterica (27870)*
Enterobacter aerogenes (13048)	*Selenomonas artemidis (43528)*
Enterobacter agglomerans (27155)	*Selenomonas noxia (43541)*
Enterobacter cloacae (10699)	*Selenomonas sputigena (35185)*
Enterobacter gergoviae (33028)	*Serratia liquifasciens (11367)*
Enterobacter sakazakii (12868)	*Slackia exigua (700122)*
Escherichia coli (10798)	*Staphylococcus aureus (14458)*
Eubacterium limosum (8486)	*Staphylococcus epidermidis (14990)*
Eubacterium nodatum (33099)	*Staphylococcus warneri (27836)*
Eubacterium saburreum (33271)	*Stenotrophomonas maltophilia (13637)*
Eubacterium saphenum (49989)	*Streptococcus anginosus (33397)*
Filifactor alocis (35896)	*Streptococcus constellatus (27823)*
Fusobacterium naviforme (25832)	*Streptococcus gordonii (10558)*
Fusobacterium necrophorum (25286)	*Streptococcus intermedius (27335)*
Fusobacterium nucleatum ss. nucleatum (25586)	*Streptococcus mitis (49456)*
Fusobacterium nucleatum ss. polymorphum (10953)	*Streptococcus mutans (25175)*
Fusobacterium nucleatum ss. vincentii (49256)	*Streptococcus oralis (35037)*
Fusobacterium periodonticum (33693)	*Streptococcus parasanguinis (15912)*
Gardnerella vaginalis (49145)	*Streptococcus pneumoniae (49619)*
Gemella hemolysans (10379)	*Streptococcus salivarius (27945)*
Gemella morbillorum (27824)	*Streptococcus sanguinis (10556)*
Granulicatella adiacens (49175)	*Streptococcus sobrinus (33478)*
Haemophilus aphrophilus (33389)	*Streptococcus vestibularis (49124)*
Haemophilus influenzae (33533)	*Tannerella forsythia (43037)*
Haemophilus paraphrophilus (29242)	*Treponema denticola (B1)*
Haemophilus segnis (33393)	*Treponema socranskii (S1)*
Hafnia alvei (13337)	*Veillonella dispar (17748)*
Helicobacter pylori (43504)	*Veillonella parvula (10790)*
Klebsiella oxytoca (12833)	**Fungal strains (a)**
Lactobacillus acidophilus (4356)	*Candida albicans (10231)*
Lactobacillus casei (393)	*Candida tropicalis (750)*

(a) All strains were obtained from the American Type Culture Collection (ATCC number in parentheses) except for Treponema denticola B1 and Treponema socranskii S1, which were obtained from The Forsyth Institute.
(b) ATCC strains 43718 and 29523 *(c) ATCC strains 11827 and 11828*

TABLE 2. *E. faecalis* proportions (% DNA probe counts) per sample

0.3	0.2	0.4	0.4	0.3	0.3	0.1	0.0
0.0	0.2	0.1	0.1	0.1	0.7	0.4	**10.1**
0.8	0.7	0.5	0.7	0.5	0.5	0.2	0.1
0.1	0.3	0.0	0.7	0.3	0.1	0.3	0.1
0.0	0.0	0.0	0.1	0.5	0.3	0.1	0.1

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Cytokine Analysis in Lesions Refractory to Endodontic Treatment

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Abstract

Introduction: Failure in endodontic treatment is often caused by the persistence of microorganisms in the root canal after therapy. When treatment fails, an immune response develops that is characterized by an extensive network of immunologic mechanisms that lead to the production of cytokines and chemokines. **Methods:** The objective of this study was to determine the relative mRNA expression of IFN-γ, TNF-α, IL-1β, IL-17A, IL-10, and MCP-1 in periapical dental lesions refractory to treatment. Clinical samples were taken from teeth presenting periapical lesions refractory to endodontic treatment (experimental group) or from healthy teeth with pulp vitality (control group). Three paper points passing through the root apex (2 mm) were used to collect the samples. Total RNA was extracted from each sample, cDNA was synthesized, and quantitative PCR analysis was performed. The Mann-Whitney test was used to determine the statistical significance of our findings ($p < 0.05$).

Results: Significant differences in the levels of IFN-γ, TNF-α, IL-17A, and MCP-1 mRNA expression were observed in cases refractory to endodontic treatment as compared to the control group. The expression of IL-1β mRNA was not significantly different between the groups. Expression of IL-10 mRNA was insignificant in both the experimental and control groups. **Conclusions:** Significantly increased expression of TNF-α, IFN-γ, IL-17A and MCP-1 mRNA was observed in the periapical immune response in cases of endodontic failure. These results suggest that a pro-inflammatory cytokine profile predominates in these types of dental lesions. **Key Words:** Cytokine, endodontic retreatment, apical periodontitis, chemokine

Introduction

A growing demand for endodontic retreatment has been observed, as there is an interest in preserving teeth whenever possible. Failure in endodontic treatment is often caused by the persistence of microorganisms in the root canal system after therapy or recontamination of the root canal due to inadequate sealing (1, 2). The retreatment procedure requires removal of the original root canal filling, further instrumentation, disinfection, and refilling. When the treatment fails and microorganisms persist in the root canal, an immune response is initiated to impede the dissemination of bacteria beyond the periapical tissues (1-3).

The host immune response is complex and involves the recruitment of inflammatory cells and production of cytokines and chemokines. Ultimately, this process can result in alveolar bone destruction (1). According to the current concept of CD4+ T helper (Th) cell development, at least four different Th subsets exist: Th1, Th2, Th17, and Treg (4). A type 1 immune response, characterized by the production of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), is involved in the progression, bone destruction and remodeling of periapical lesions (5). In contrast, immunosuppressive mechanisms mediated by Treg- or Th2-derived cytokines are responsible for healing and restricting the inflammatory immune mechanisms (6, 7). IL-10, initially described as a Th2 cytokine, exhibits strong anti-inflammatory properties. It is now recognized that Th1 and Th17 cells also produce IL-10, in addition to Th2 and Treg cells (8).

Th17 cells, in humans, are characterized by their expression of IL-17 and IL-22. They reside mainly at barrier surfaces, where they function to protect the host from microorganisms (9-10). IL-17A may play a role in exacerbating inflammation in periapical lesions by stimulating the production of other pro-inflammatory cytokines (11) and might be an important factor in both the initiation and development of periapical lesions (12). Chemokines participate in the inflammatory process by promoting the activation of selectins that are involved in the adhesion of cells to blood vessels. In addition, the localized expression of chemokines in tissues generates chemotactic gradients that are responsible for the guided migration to and maintenance of cells at these sites (13). Monocyte chemotactic protein (MCP-1) is expressed in higher levels in periapical cysts when compared to healthy tissues (14, 15).

Although the immune response is known to participate in the formation of periapical lesions, the cytokine response profile in endodontic failures has not been clearly defined. The aim of this study was to assay and to compare the mRNA expression levels of the cytokines IFN-γ, TNF-α, IL-1β, IL-17A, and IL-10 as well as the chemokine MCP-1 in periapical lesions refractory to endodontic treatment to that in healthy periapical tissues.

Materials and Methods

Human subjects

Subjects were 20 patients presenting periapical lesions refractory to endodontic treatment and 20 patients with healthy teeth. Control subjects were drawn from patients with prosthetic indications for endodontic treatment that were referred to the School of Dentistry at the Universidade Federal de Minas Gerais (Belo Horizonte, MG, Brazil). Patients were excluded from this study if they had taken antibiotics in the three months prior to the initiation of endodontic therapy. All participants signed the Free Agreement Formulary. This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais (ETIC 0011.0.215.203-10).

Sample collection

Clinical samples were taken from teeth presenting periapical lesions refractory to endodontic treatment (experimental group) and teeth with vital pulp treated by prosthetic indication (control group). The criteria used to determine the pulp diagnosis were based on clinical and radiographic analyses and pulp sensitivity tests. Teeth were isolated using a rubber dam followed by a complete asepsis. Cleaning and shaping of the root canals were completed using retreatment ProTaper NiTi files (Dentsply, Ballalgues, Switzerland) for teeth from the experimental group and Universal ProTaper NiTi files (Dentsply, Ballalgues, Switzerland) for teeth from the control group, in conjunction with 5.2% sodium hypochlorite for both groups as previously described (16). The samples were collected as follows. The pre-existing root canal filling was

removed without the use any solvent solution (experimental group). After patency negotiation, three paper points (#20) were introduced into the root canal, passing through the root apex (2 mm) for one minute. This procedure permitted extraction of RNA from the interstitial fluid around the root canal apex. After withdrawal, paper points were cut 4 mm from the tip and dropped into microcentrifuge tubes; samples were stored at -70°C. Root canals were sealed with vertically compacted thermoplasticized obturation in teeth from the control group. In teeth from the experimental group, root canals received endodontic dressing. Afterwards, coronal access was restored with eugenol-based cement.

Sample Preparation

Total RNA was extracted from each sample with TRIzol reagent (GIBCO/BRL Laboratories, Grand Island, NY, USA). Briefly, chloroform was added and the mixture was centrifuged at 12,000 x *g* at 4°C for 15 minutes; the aqueous phase was collected and RNA was precipitated by isopropanol. Samples were centrifuged at 12,000 x *g* at 4°C for 10 minutes. The RNA precipitate was washed once with 75% cold ethanol, dried, dissolved in RNase-free water and then incubated at 55°C for 10 minutes. The RNA was then stored at -70°C.

Real-time PCR

Complementary DNA was synthesized using 1 microgram of RNA through a reverse transcription reaction as described by Silva *et al.* (17). PCR was carried out under standard conditions as follows: a holding stage at 95°C (10 minutes);

a cycling stage of 40 cycles at 95°C (15 seconds) followed by 60°C (1 minute); and a melt curve stage at 95°C (15 seconds), 60°C (1 minute) and 95°C (15 seconds). The primer sequences used for quantitative PCR analysis of IFN-γ, TNF-α, IL-1β, IL-17A, IL-10 and MCP-1 mRNA expression are shown in Table I. Sequences were designed using PRIMEREXPRESS software (Applied Biosystems, Foster City, CA, USA) based on nucleotide sequences available in the GenBank database. The real-time PCR assay was performed using Step One Real-time PCR Systems (Applied Biosystems). A Syber-Green detection system (Applied Biosystems) was used to assay primer amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization. All samples were run in duplicates. Reactions were carried out in a volume of 25-μL and contained 1 μg of cDNA. Sequence Detection Software version v 2.0 (Applied Biosystems) was used to analyze data after amplification. Results were obtained as threshold cycle (Ct) values. Expression levels were then calculated using the comparative C(T) method (18).The values were calculated as the mean value of the duplicates for each patient, and the expression levels of mRNA in all samples were defined as the ratio of each specific primer to GAPDH expression.

Statistical Analysis

Data analysis was performed using SPSS for Windows (version 15.0; SPSS, Chicago, IL, USA). The Mann-Whitney test was used to determine statistical differences between the groups ($p < 0.05$).

Results

mRNA for Th1-associated cytokines IFN-γ and TNF-α was increased in cases of endodontic failure as compared to the control group. The expression of IL-1β, another type 1 cytokine, was not significantly different between groups ($p > 0.05$). Expression of IL-17A was significantly higher in cases of endodontic failure compared to control samples ($p < 0.05$). When compared to the internal control (GAPDH), mRNA expression of IL-10 was insignificant in both the experimental and control groups (data not shown). MCP-1 mRNA expression was significantly higher ($p < 0.05$) in lesions refractory to treatment as compared to the controls (Fig. 1).

Discussion

Several cytokines and chemokines are involved in the immune responses that take place during periapical lesions development (2, 11, 19, 20). In human (11, 15, 20, 21) or in animal models (19, 22, 23), inflammatory mediators have been detected in periapical lesions. Real-Time PCR has been widely used, allowing fast, accurate and sensitive mRNA quantification with a high throughput of samples. Quantitative PCR offers the opportunity to observe the amplification kinetics of a PCR in "real time" via accumulation and measurement of specific fluorescence signals with each cycle .However, several factors, such as phenol, ethanol, haemoglobin, heparin and even the reverse transcriptase, are known to inhibit PCR efficiency (24).To ensure PCR accuracy in our study, all samples were treated according to identical protocols and in parallel. In this study, we describe the mRNA gene expression levels of IFN-γ, TNF-α, IL-1β, IL-17A, IL-10 and MCP-1 in periapical lesions refractory to endodontic treatment.

Significant levels of TNF-α mRNA were found in periapical lesions of teeth refractory to endodontic treatment as compared to the periapical area of healthy teeth. As a potent immunologic mediator of acute and chronic inflammatory responses, TNF-α has the capability to increase bone resorption (25). Our findings are in accordance with previous reports that demonstrated higher levels of TNF-α in root canal exudates and periapical lesions in human cases of chronic apical periodontitis (21, 26).

IL-1 has been demonstrated to play a pivotal role in several chronic diseases, and it is known to be one of the most active stimulators of osteoclastic

bone resorption (27,28). It was shown, in an animal model, that anti-IL-1α antisera were able to significantly neutralize bone resorption activity in periapical lesions (22). Moreover, in humans, IL-1β levels were reduced in periapical lesions after root canal treatment (21). In this study, IL-1β mRNA expression was not significantly increased in refractory lesions when compared to healthy teeth. In periapical inflammation, it would be predictable that Th1 CD4+ T cells would up-regulate IL-1 and other pro-inflammatory cytokines (6, 29). It is possible that we did not see increased expression of IL-β mRNA in lesions due the timing of tissue collection. The significantly higher pro-inflammatory cytokine expression observed in refractory lesions (IFN-γ, TNF-α and IL-17A) might upregulate IL-1β expression at a later or different stage of periapical lesion development. In this regard, it was shown that IL-1α is detected in higher levels during reabsortive active phase than in chronic phase of lesion development (6, 30).

$CD4^{+}Th17^{+}$ cells are predominantly localized in inflamed tissues (31) and produce pro-inflammatory cytokines, including IL-17 A/F heterodimer, TNF-α, IL-22 and IL-26, all of which promote inflammation (32). IL-17 primarily acts on stromal endothelial and epithelial cells as well as on a subset of monocytes to induce the secretion of pro-inflammatory mediators. IL-17 is associated with both the reabsortive active phase and chronic phase of periapical lesions, (12). Our results are in accordance with the work of Xiong *et al.* (12), demonstrating the presence of IL-17 in chronic lesions. A positive correlation between IFN-γ and IL-17 has been demonstrated in cultures from periapical lesions, suggesting that both cytokines are important for the exacerbation of

inflammation (11). In this study, both IFN-γ and IL-17A cytokine mRNA expression levels were significantly higher in periapical lesions refractory to endodontic treatment. As many as half of all IL-17^{+} cells are also IFN-γ^{+} in humans (11,33). Although it is not well established if these cells represent a stable phenotype or a transitional phase from Th17 to Th1 or *vice versa*, both cytokines are present in periapical lesions refractory to endodontic treatment.

Increased expression of MCP-1 has been associated with the greater recruitment of cells to inflammatory sites (15, 19, 20). We found that MCP-1 mRNA expression was increased in lesions refractory to endodontic treatment as compared to control teeth. This result is in accordance with previous data that demonstrated higher expression of MCP-1 in periapical granulomas and cysts when compared to healthy pulp tissues (20).

The importance of IL-10 in controlling the degree and duration of inflammatory reactions has been observed in several chronic inflammatory and autoimmune pathologies (34). Using knockout murine models, Sasaki *et al.* (35) demonstrated that the development of periapical lesions was not modified in the absence of IFN-γ and IL-12. On the other hand, IL-10 and IL-6 knockout animals had significantly greater infection-stimulated bone resorption compared with wild-type mice (23, 36). Conversely, in this study, a very low expression of IL-10 was observed in both subject groups. It is important to point out that control group was comprised by teeth with vital pulp, presenting periapical healthy tissue, in which detection of IL-10 was not expected. On the other hand, in periapical interstitial fluid from teeth with necrotic pulp, IL-10 m-RNA expression was found in appreciable levels (data not shown). Nevertheless, a possible

reason to explain the lower expression of IL-10 observed in cases of endodontic failure could be a cross-immune regulation by the prevalent expression of type-1 upon type-2 cytokines, as demonstrated by others (1, 6, 11, 19, 37).

In summary, the results of the present study show that mRNA expression of TNF-α, IFN-γ, IL-17A and MCP-1 might be involved in the maintenance of the periapical immune response in cases of endodontic failure. Alternatively, they reflect an inflammatory process that has not been resolved due to infection maintenance. Despite the fact that refractory endodontic failure represents chronic periapical lesions, it was observed that pro-inflammatory cytokine profile significantly prevail in those subjects.

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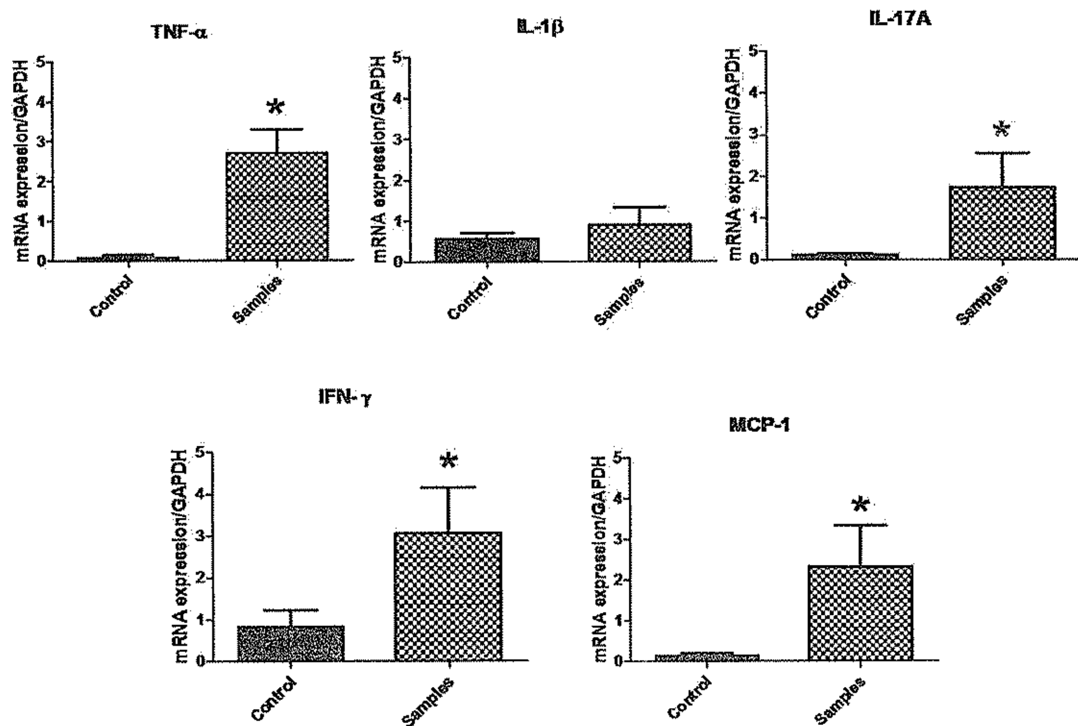

FIGURE 1. Expression of IFN-γ, TNF- ,MCP-1, , IL-1, IL10- and IL-17 in periapical tissues refractory to endodontic treatments. Levels of expression were determined by real-time PCR and quantified by comparison with the internal control (GAPDH). Bars represent the mean values of samples recovered from 20 patients; lines represent the standard error of the mean. * indicates $p < 0.05$ by the Wilcoxon test.

TABLE 1. Primer sequences

Gene	Sense and antisense	Mt* (°C)	bp*
GAPDH	5'-GCA CCA CCA ACT GCT TAG CA- 3' 5'-TGG CAG TGA TGG CAT GGA GGA- 3'	80	96
TNF-α	5'-TTC TGG CTC AAA AAG AGA ATT G- 3' 5'-TGG TGG TCT TGT TGC TTA AGG- 3'	76	73
IL-1β	5'-TGG CAG AAA GGG AAC AGA A- 3' 5'-ACA ACA GGA AAG TCC AGG CTA- 3'	73	59
IL-17A	5'-CAA TGACCT GGA ATT ACC CAA- 3' 5'-TGA AGG CAT GTG AAA TCG AGA- 3'	70	52
IFN-γ	5'-GAA CTG TCG CCA GCA GCT AAA- 3' 5'-TGC AGG CAG GAC AAC CAT TA- 3'	80	95
CCL2	5'-AAG ACC ATT GTG GCC AAG GA- 3' 5'-CGG AGT TTG GGT TTG CTT GT- 3'	80	93
IL-10	5'-GGT TGC CAA GCC TTG TCT GA- 3' 5'-TCC CCC AGG GAG TTC ACA T- 3'	81	107

*Mt: melting temperature; bp: base pairs of amplicon size.

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4- CONSIDERAÇÕES FINAIS

O sucesso do tratamento endodôntico depende de vários fatores : seleção do caso clínico, correto diagnóstico e criteriosa execução da técnica de preparo mecânico-químico e obturação tridimensional do SCR. Falhas no tratamento endodôntico podem ocorrer em casos de acidentes e iatrogenias, também nos casos em que há a persistência ou a reintrodução de microrganismos no sistema de canais radiculares. Quando o tratamento endodôntico não alcança o sucesso almejado, indica-se o retratamento.

Os estudos que se ativeram às populações microbianas que habitam os SCR infectados, ao longo dos anos, demonstraram que a metodologia utilizada interfere enormemente nos resultados alcançados. Assim, inicialmente acreditava-se que a microbiota presente em dentes com lesões refratárias ao tratamento endodôntico seria significativamente menor do que a recuperada em infecções endodônticas primárias (Sundqvist et al. 1998, Siqueira & Roças 2004). A microbiota alí presente constituía-se principalmente de bactérias Gram-positivas (Molander *et al.* 1998, Sundqvist *et al.* 1998, Pinheiro *et al.* 2003, Siqueira & Roças 2004), com o predomínio do *Enterococcus faecalis* (Molander *et al.*, 1998). Hoje, sabe-se que tais resultados foram influenciados por limitações nas técnicas de coleta e cultivo, que apresentavam baixa sensibilidade e inabilidade na detecção de microrganismos fastidiosos e incultiváveis (Paster *et al.* 2006). Técnicas de biologia molecular, como a hibridização DNA-DNA (checkerboard), procuram reverter essa limitação,

demonstrando ser essa microbiota um pouco mais complexa (Siqueira *et al.* 2000, Brito *et al.* 2007, Vianna *et al.* 2008,Tavares *et al.* 2011).

Neste estudo, avaliou-se a microbiota dos SCR de dentes com lesões refratárias ao tratamento endodôntico, associando-se o MDA à hibridização DNA-DNA, pois em trabalhos anteriores (Brito *et al.* 2007, Tavares *et al.* 2011), demonstrou-se a pertinência da associação dessas técnicas na análise da pequena amostra tradicionalmente recuperada das infecções endodônticas. Ao amplificar em 1000 vezes a amostra inicial, o MDA possibilita a observação pelo "checkerboard", que exige um conteúdo mínimo de 10^4 células. Os estudos, analisando a microbiota endodôntica (Brito *et al.*, 2007, Tavares *et al.*, 2011), demonstraram que ela pode albergar todos os microrganismos residentes na cavidade bucal, contrariando o dogma de que esse ambiente seria seletivo para uma pequena amostra de microrganismos (Sundqvist, 1976, Sundqvist, 1992, Lana *et al.*, 2001).

Tradicionalmente, ao se caracterizar a microbiota do trato gastro-intestinal, dividem-se as populações microbianas em grupos, de acordo com a sua proporção naquele sítio (Savage, 1977). Neste estudo, baseado nessa caracterização, optamos por dividir as populações microbianas em dominante (>4%), sub-dominante (>2 a 4%) e residual (< 2%), considerando-se a proporção média de cada espécie (% de DNA das sondas ± SEM) nas 40 amostras clínicas avaliadas, o que nos permitiu visualizar como as espécies microbianas se distribuem na comunidade clímax. Tornou-se evidente que um número restrito, apenas seis se encontravam presentes na população dominante (*C.diphtheriae, S.constellatus, P.gingivalis, G.adjacens, P.denticola,*

e *E. nodatum).* A grande maioria se enquadrava no grupo residual. Há vários estudos sobre as interações microbianas que ocorrem nos SCR infectados, enfatizando fatores ecológicos como: potencial redox, temperatura, viabilidade de nutrientes e interações bacterianas. Sugerem que eles influenciariam a dinâmica de crescimento e colonização microbiana nos SCR infectados (De Sanctis *et al.*, 1991, Sundquivst 1992,1994, Chu Frederick *et al.*, 2005, Socransky & Haffajee 2005), e que a estrutura da comunidade microbiana pode determinar a sintomatologia clínica (Yoshida *et al.*, 1987, Siqueira *et al.*, 2004, Chu Frederick *et al.,* 2005). Contudo, utilizando diferentes métodos de identificação, esses estudos não retrataram a real distribuição microbiana na comunidade clímax, limitando-se a aferir a presença dessa ou daquela espécie no sítio em análise. Impossível era determinar se todos os membros daquela comunidade se encontravam em igualdade populacional e, consequentemente se desempenhavam papéis de relevância no biofilme e na indução de resposta no hospedeiro. As contribuições do presente estudo seriam: demonstrar que a infecção endodôntica em dentes com lesões refratárias é mista e complexa, possibilitar a análise da infecção quanto à sua prevalência populacional no sítio endodôntico, não apenas a sua detecção.

Examinando-se a microbiota dominante, pode-se inferir quais microrganismos modulavam o processo patológico naquele dado momento. Entretanto, a presença de organismos sub-dominantes e residuais não pode ser subestimada, pois as mudanças ecológicas que ocorrem no ambiente com o tempo alterarão o perfil dessas populações.

Durante as últimas décadas, os casos clínicos de insucesso no tratamento endodôntico foram atribuídos principalmente à presença do *E. faecalis* (Molander *et al.,* 1998, Sundqvist *et al.,* 1998, Hancock *et al.* 2001, Pinheiro *et al.*, 2003, Gomes *et al.,* 2008). A essa espécie imputou-se quase toda a responsabilidade. Ela se destacou entre os poucos microrganismos que ali eram recuperados. Utilizando-se, entretanto, 107 espécies, dentre tradicionais periodontopatógenos, microrganismos prevalentes nas infecções endodônticas, patógenos de interesse médico e leveduras, observou-se que o papel do *E. faecalis* nessas infecções é questionável e deve ser revisto, como sugeriram outros autores (Rôças *et al.,* 2004, Sakamoto *et al.*, 2008). Apesar de detectado em 34 das 40 amostras analisadas, sua proporção variou de 0.55 ± 0.27 (%de DNA das sondas ± SEM), estando presente, em altas proporções, em apenas uma das amostras.

A relação entre infecção dos sistemas de canais radiculares e respostas imuno-inflamatórias periapicais já está bem comprovada na literatura (Kakehashi *et al.*, 1965, Sundqvist, 1976, Moller *et al.*, 1981, Fabricius *et al.*, 1982, Teles *et al.*, 1997, Kawashima & Stashenko, 1999, Silva *et al.*, 2005, Colic *et al.*, 2009b). Com a infecção dos SCR, células imuno-competentes migram para o tecido periodontal adjacente ao ápice do dente em questão, no intuito de prevenir a disseminação microbiana. Os estudos sobre as respostas imunoperiapicais em humanos utilizavam a remoção cirúrgica do granuloma ou cisto, quando então, realizavam-se as análises dos espécimes clínicos. No presente estudo propusemos uma nova metodologia, que se mostrou eficaz, permitindo-nos coletar o fluido intersticial perirradicular, de forma conservadora,

evitando-se a cirurgia paraendodôntica. Utilizando-se o PCR em tempo real, demonstrou-se que prevaleceu um perfil de citocinas pró-inflamatórias nas lesões refratárias ao tratamento endodôntico. Este resultado, quando confrontado aos achados microbiológicos obtidos nos mesmos sítios, se torna perfeitamente compreensível, pois ambas as coletas ocorreram no mesmo momento.

Utilizando metodologias inovadoras, este trabalho demonstrou, de maneira inequívoca, a pertinência em se analisar a infecção endodôntica sob o ponto de vista da quantificação populacional para se entender o papel de uma dada espécie microbiana na comunidade clímax, tanto na dinâmica do biofilme quanto na resposta imune arquitetada no paciente. Finalmente, espera-se que este estudo contribua para uma compreensão mais bem fundamentada dos aspectos microbianos e imunológicos que permeiam as infecções de dentes com lesões refratárias ao tratamento endodôntico.

5- CONCLUSÕES

- A microbiota recuperada de dentes portadores de infecções refratárias ao tratamento endodôntico demonstrou ser mais complexa do que anteriormente demonstrado, utilizando-se a associação das técnicas MDA/ Checkerboard e de um maior número de sondas microbianas.

- Observou-se, na determinação das proporções médias de cada espécie microbiana, que um pequeno grupo se encontrava em níveis mais elevados (*C. diphtheria, S. constellatus, P. gingivalis, G. adjacens* , e *P. denticola*) quando comparado ao das demais espécies avaliadas.

- Apesar da maioria dos estudos vincularem a presença *E. faecalis* ao insucesso do tratamento endodôntico, seu papel nessas infecções deve ser revisto.

- As respostas imuno-periapicais à infecção presente nos dentes com lesões refratárias ao tratamento endodôntico apresentou um perfil pró-inflamatório.

6 - REFERÊNCIAS (INTRODUÇÃO E DISCUSSÃO)

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