DANIEL SAITO

CARACTERIZAÇÃO DAS COMUNIDADES BACTERIANAS ASSOCIADAS ÀS INFECÇÕES ENDODÔNTICAS: ABORDAGEM INDEPENDENTE DE CULTIVO

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Biologia Buco-Dental, Área de Concentração Microbiologia e Imunologia.

Orientador: Prof. Dr. Reginaldo Bruno Gonçalves

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Dedico este trabalho

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RESUMO

A presente tese teve como objetivo a caracterização das comunidades bacterianas associadas às infecções endodônticas pelo emprego de técnicas moleculares independentes de cultivo. Ao todo, foram analisadas amostras intra-radiculares provenientes de 34 elementos dentários associados a infecções endodônticas. A análise de bibliotecas clonais de DNA ribossomal 16S (16S rDNA) permitiu a identificação de 2 a 14 filotipos bacterianos (espécies) por elemento dentário (média= 9,6), perfazendo um total de 46 filotipos distintos. Dentre estes, 9% foram considerados previamente desconhecidos e classificados taxonomicamente como novos membros da ordem *Clostridiales*. Espécies reconhecidamente endodônticas dos gêneros *Bacteroides*, *Campylobacter*, *Eubacterium*, *Peptostreptococcus*, *Selenomonas*, *Treponema* e *Veillonella* foram detectadas, assim como representantes de gêneros menos freqüentemente descritos, como *Burkholderia, Filifactor* e *Megasphaera*. O emprego da técnica quantitativa de PCR em Tempo Real, possibilitou a detecção de *P. gingivalis*, *T. forsythia* e a coexistência de ambas em 24%, 56% e 18% dos pacientes avaliados, respectivamente. Nenhuma correlação significativa foi evidenciada entre os níveis de ambas as espécies, individualmente ou em conjunto, e a presença de sintomatologia dolorosa. O uso de T-RFLP na avaliação da estrutura das comunidades bacterianas revelou um total de 123 (endonuclease *Hha*I) e 122 (endonuclease *Msp*I) fragmentos de restrição terminais (T-RFs) distintos, com médias de 20,8 e 20,0 T-RFs por elemento dentário, respectivamente. Aproximadamente 50% dos fragmentos detectados apresentaram-se, no máximo, em 2 pacientes, indicando uma alta variabilidade na composição microbiana. As análises de clusterização e de estatística multivariada não revelaram diferenças significativas nas comunidades bacterianas entre os grupos de estudo assintomático, sensível ao toque e sintomático. De modo geral, os resultados obtidos reiteraram o conceito de que a microbiota associada às infecções endodônticas é essencialmente polimicrobiana, altamente variável entre indivíduos, e constituída predominantemente por bactérias anaeróbias Gram-positivas do filo *Firmicutes*. As espécies *P. gingivalis* e *T. forsythia*, embora relativamente prevalentes nas infecções endodônticas, não apresentaram correlação significativa com o desenvolvimento de sintomatologia dolorosa. Por fim, a ausência de agrupamentos de perfis bacterianos quanto aos parâmetros sintomatológicos sugere que a estrutura das comunidades bacterianas intra-radiculares não possui influência significativa no desenvolvimento da dor de origem endodôntica.

Palavras-chave: infecção endodôntica, 16S rDNA, biblioteca clonal, PCR em Tempo Real, T-RFLP.

ABSTRACT

The objective of the present study was to characterize the bacterial communities associated with endodontic infections by use of culture-independent molecular techniques. Overall, 34 intraradicular samples from teeth harboring endodontic infections were evaluated. 16S ribosomal DNA (16S rDNA) clone library analysis allowed the identification of 2 to 14 bacterial phylotypes (species) per tooth (mean= 9.6), with a total of 46 distinct phylotypes. Among the latter, 4 (9%) were considered previously unreported and further taxonomically classified as members of the order *Clostridiales*. Well-known endodontic representatives of *Campylobacter*, *Eubacterium*, *Peptostreptococcus*, *Selenomonas*, *Treponema* e *Veillonella* were detected, as well as members of less frequently reported genera, such as *Burkholderia, Filifactor* and *Megasphaera*. The application of the Real Time PCR technique permitted the detection of *P. gingivalis*, *T. forsythia* and a coexistence of both in 24%, 56% e 18% of the subjects, respectively. No significant correlations were evidenced among the levels of *P. gingivalis* and *T. forsythia*, individually or conjointly, and spontaneous endodontic pain. The use of T-RFLP in the analysis of bacterial community structures revealed a total of 123 (*Hha*I endonuclease) and 122 (*Msp*I endonuclease) distinct terminal restriction fragments (T-RFs), with 20.8 and 20.0 mean T-RFs per tooth, respectively. Approximately 50% of the detected fragments were exclusive to one or two patients, indicating a high inter-subject variability in the bacterial assemblages. Cluster and multivariate statistical analyses did not demonstrate significant differences in the bacterial community profiles among the asymptomatic, tender to percussion and symptomatic study groups. Taken together, the results of this study reiterate the concept that the microbiota associated with endodontic infections is essentially polymicrobial, highly variable among individuals, and predominantly composed of Gram-positive anaerobic bacteria from the phylum *Firmicutes*. The species *P. gingivalis* and *T. forsythia*, although relatively prevalent in root canal infections, did not present significant correlations with the development of symptomatic features. Lastly, the absence of clusters of bacterial profiles according to symptomatic parameters suggests that the intraradicular bacterial community structures, as a whole, do not bear significant influence on the development of pain of endodontic origin.

Keywords: bacteria, endodontic infection, 16S rDNA, clone library, Real Time PCR, T-RFLP.

SUMÁRIO

INTRODUÇÃO

O objetivo principal da terapia endodôntica é a eliminação dos microrganismos patogênicos e de seus subprodutos do interior dos canais radiculares, bem como a manutenção da condição asséptica pós-operatória (Bystrom & Sundqvist, 1981). Mesmo com os recentes avanços técnico-científicos da Odontologia moderna, a permanência de células bacterianas no interior dos canais é, ainda, um dos principais motivos de fracasso no tratamento endodôntico (Lin *et al.*, 1992). Nesse sentido, o conhecimento da microbiota associada às infecções endodônticas constitui requisito fundamental para o desenvolvimento de medidas terapêuticas mais efetivas.

Anteriormente à década de 1970, as dificuldades existentes com relação à cultura e à identificação de organismos anaeróbios mascarava a sua real importância na etiopatogenia das alterações pulpares e periapicais de origem infecciosa (Brown & Rudolf, 1957; Grossman, 1959; Melville & Birch, 1967). Com a subseqüente evolução das técnicas de cultivo microbiano em ambiente anaeróbio, o enfoque microbiológico na Endodontia sofreu grande mudança. A microbiota endodôntica, que antes era considerada basicamente composta por microrganismos aeróbios facultativos, demonstrou abrigar uma grande proporção de anaeróbios estritos (Gomes *et al.*, 2004; Sundqvist, 1992; Fabricius *et al.*, 1982; Sundqvist, 1976; Kantz & Henry, 1974). Atualmente, consideram-se as infecções endodônticas essencialmente polimicrobianas, e constituída predominantmente por bactérias anaeróbias dos gêneros *Actinomyces*, *Campylobacter*, *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, *Streptococcus*, *Veillonella*, dentre outras.

Embora a microbiota associada às infecções endodônticas encontre-se amplamente caracterizada por estudos de cultivo microbiano, estima-se hoje que aproximadamente 50% das bactérias orais não seja capaz de crescer sob condições laboratoriais (Paster *et al.*, 2001; Aas *et al.*, 2003). Uma vez que a microbiota endodôntica representa uma parcela restrita da microbiota presente na cavidade oral (Sunqvist, 1994), é razoável considerar que parte significativa da primeira encontra-se potencialmente subestimada pelos métodos de detecção tradicionais. Sob este novo paradigma, abordagens moleculares independentes de cultivo oferecem uma alternativa rápida e sensível, permitindo também a caracterização das frações fastidiosa e não-cultivável da microbiota endodôntica (Munson *et al.*, 2002; Rolph *et al.*, 2001).

A análise de bibliotecas clonais consiste na amplificação do gene ribossomal 16S (16S rDNA) pela Reação em Cadeia da Polimerase (PCR), ligação dos fragmentos amplificados em vetor plasmidial, clonagem em *Escherichia coli*, e seqüenciamento do DNA. As seqüências nucleotídicas são submetidas ao alinhamento múltiplo e comparadas juntamente com seqüências

de bancos de dados de DNA ribossomal, possibilitando a identificação filogenética das espécies bacterianas (filotipos) presentes na amostra. A técnica demonstra grande sensibilidade, gerando resultados bem mais informativos que a cultura microbiana (Kroes *et al.*, 1999; Rolph *et al.*, 1991), com excelente aplicabilidade nos mais diversos microambientes orais, como língua, palato, placa sub e supra-gengival, abscessos dento-alveolares, lesões de cancro e canais radiculares (Aas *et al.*, 2003; Paster *et al.*, 2002; Paster *et al.*, 2001; Munson *et al.*, 2002; Rolph *et al.*, 2001; Wade *et al.*, 1997).

As bactérias *Porphyromonas gingivalis* e *Tannerella forsythia* possuem fatores de virulência amplamente caracterizados (Inagaki *et al.*, 2006; Holt *et al.*, 1999), sendo reconhecidamente importantes na etiologia da doença periodontal (Socransky *et al.*, 2002; Socransky *et al.*, 1998). Estudos em modelos animais demonstraram que *P. gingivalis* e *T. forsythia* possuem atividades patogênicas sinergísticas, quando inoculadas em conjunto (Yoneda *et al.*, 2001; Takemoto *et al.*, 1997). Embora ambas já tenham sido relatadas em infecções endodônticas através da detecção estritamente qualitativa (Foschi *et al.*, 2005; Rôças *et al.*, 2001; Fouad *et al.*, 2002), a aplicação de técnicas quantitativas pode trazer informações adicionais sobre a importância patogênica destas espécies nesse ecossistema.

A PCR em Tempo Real (Real Time PCR) é uma variante da PCR convencional que oferece quantificação sensível e confiável de ácidos nucléicos (Heid *et al.*, 1996). A técnica baseia-se no monitoramento da intensidade de fluorescência oriunda da reação de PCR, a qual é diretamente proporcional aos níveis do DNA amplificado. Uma vez que os produtos da reação são detectados e quantificados diretamente no equipamento, a técnica dispensa o processamento posterior, minimizando potenciais erros de análise (Bustin, 2000). Estudos orais demonstraram que PCR em Tempo Real possui sensibilidade de detecção 41 vezes superior àquela obtida pela contagem de colônias anaeróbias (Martin *et al.*, 2002) e que, nos casos específicos de *P. gingivalis* e *T. forsythia*, fornece aumentos de 36 a 51% nas respectivas taxas de detecção (Verner *et al.*, 2006).

A análise de Polimorfismos de Comprimento de Fragmentos Terminais de Restrição (T-RFLP) é uma técnica baseada na amplificação de DNA, a qual tem sido empregada para se investigar a estrutura de comunidades microbianas complexas (Marsh, 2005; Blackwood *et al.*, 2003; Osborn *et al.*, 2000). Consiste na amplificação de 16S rDNA polimicrobiano por PCR, onde um dos *primers* é marcado por fluorescência na sua porção 5'. A subseqüente digestão com endonucleases tetraméricas produz fragmentos terminais de restrição, que são precisamente mensurados em seqüenciadores automatizados (Liu *et al.*, 1997). Uma vez que diferentes populações microbianas possuem sítios de restrição distintos, um perfil (fingerprint) genético é obtido para cada comunidade analisada. A técnica já foi empregada na comparação entre a

microbiota de pacientes saudáveis e periodontais (Sakamoto *et al*., 2003), na determinação de mudanças na estrutura de comunidades bacterianas sub-gengivais pós-tratamento (Sakamoto *et al*., 2004), no estudo da influência da qualidade de restaurações na composição da microbiota endodôntica (Hommez *et al*., 2004) e na análise comparativa entre as microbiotas intra-radicular e de abscessos dento-alveolares, em casos sintomáticos e assintomáticos (Sakamoto *et al*., 2006).

As técnicas moleculares supramencionadas abrigam, como característica comum, a possibilidade de detecção de bactérias sem a necessidade do crescimento laboratorial de microrganismos provenientes das amostras. Isso evita que os pesquisadores entrem em contato direto com patógenos humanos em potencial, garantindo maiores níveis de biossegurança. Ademais, uma vez que muitas das bactérias orais são reconhecidamente de crescimento lento, o uso das técnicas moleculares reduz significativamente o tempo experimental (Siqueira *et al*., 2002). Dentre as vantagens adicionais oferecidas pelas técnicas de detecção moleculares, destacam-se o processamento de amostras em larga escala e as altas reprodutibilidade, confiabilidade e sensibilidade (Marsh *et al*., 2005; Heid *et al*., 1996; Rolph *et al*., 2001; Bustin, 2000; Morrison *et al*., 1988).

PROPOSIÇÃO

A presente tese teve como objetivo o estudo da diversidade bacteriana em infecções endodônticas, por meio de técnicas moleculares independentes de cultivo laboratorial. Para tal, foram avaliados 34 elementos dentários portadores de infecções endodônticas, provenientes de diferentes pacientes. O estudo foi dividido em três fases, correspondentes às diferentes metodologias empregadas:

I. Caracterização da microbiota endodôntica, determinação da diversidade bacteriana e avaliação filogenética de filotipos (espécies) potencialmente novos, pela análise de bibliotecas clonais do gene ribossomal 16S (16S rDNA) em infecções endodônticas assintomáticas não-expostas à cavidade oral (n= 7),

II. Detecção quantitativa de *P. gingivalis* e *T. forsythia*, através da técnica de PCR em Tempo Real em infecções endodônticas assintomáticas (n= 10) e sintomáticas (n= 24), e determinação de possíveis correlações entre os níveis celulares detectados e a sintomatologia dolorosa de origem endodôntica,

III. Análise da estrutura de comunidades bacterianas associadas a infecções endodônticas, por meio da técnica de T-RFLP em infecções endodônticas assintomáticas (n= 7), sensíveis à percussão (n= 5) e sintomáticas (n= 13), e investigação de possíveis correlações entre a composição bacteriana, sensibilidade à percussão e dor espontânea de origem endodôntica.

Esta tese foi elaborada no formato alternativo, conforme deliberado pela Comissão Central de Pós-Graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) (Anexo1). Assim, as metodologias acima mencionadas (fases) encontram-se apresentadas na forma de Capítulos. Os comprovantes de publicação, submissão a periódico, apresentação de trabalho em congresso sujeito a comissão julgadora e o certificado do Comitê de Ética em Pesquisa encontram-se nos Anexos.

CAPÍTULO 1

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Identification of bacteria in endodontic infections by sequence analysis of 16S rDNA clone libraries

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Abbreviations: CTAB (hexadecyltrimethylammonium bromide)

Footnote:

The GenBank accession numbers for the 16S rDNA sequences of Uncultured *Clostridiales* bacterium clone AG_D03, Uncultured *Clostridiaceae* bacterium clone AG_G04, Uncultured *Streptococcaceae* bacterium clone AF_F05 and Uncultured *Mogibacterium* sp. clone AF_H06 are AY821867, AY821868, AY821869, AY821870, respectively.

Summary

A significant proportion of oral bacteria are unable to undergo cultivation by existing techniques. In this regard, the microbiota from root canals still requires complementary characterization. The present study aimed at the identification of bacteria by the sequence analysis of 16S rDNA clone libraries from seven endodontically infected teeth. Samples were collected from the root canals, subjected to the polymerase chain reaction (PCR) with 16S rDNA universal primers, cloned and partially sequenced. Clones were clustered into groups of closely related sequences (phylotypes) and identification to the species-level was performed by comparative analysis with the GenBank, EMBL and DDBJ databases, according to a 98% minimum identity. All samples were positive for bacteria and the number of phylotypes detected per subject varied from 2 (patient AB) to 14 (patient AA). The majority of taxa (65.2%) belonged to the phylum *Firmicutes* of Gram-positive bacteria, followed by *Proteobacteria* (10.9%), *Spirochaetes* (4.3%), *Bacteroidetes* (6.5%), *Actinobacteria* (2.2%) and *Deferribacteres* (2.2%). A total of 46 distinct taxonomic units was identified. Four clones with low similarities with those previously deposited in the databases were sequenced to nearly full extent and were taxonomically classified as novel representatives of the order *Clostridiales*, including a new species of *Mogibacterium*. The identification of novel phylotypes associated with endodontic infections suggests that the endodontum may still harbor a relevant proportion of uncharacterized taxa.

Introduction

Although more than 150 species of bacteria have been identified in infected root canals, only a restricted number can be found simultaneously in the same tooth and a considerable variation of species is expected when analyzing distinct clinical conditions, individuals, or populations (Sundqvist, 1976; Molander *et al.*, 1998; Baumgartner *et al.*, 2004). Cultivation studies have shown a predominance of facultative and strict anaerobes in the endodontum, including representatives of *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella*, and *Streptococcus* (Sundqvist, 1992b; Le Goff *et al.*, 1997). Bacteria inside the canal are the major causes of periapical pathologies (Kakehashi *et al.*, 1965) and, if not adequately treated, can give rise to dentoalveolar abscess, a condition that has ability to initiate morbidity, life threatening illness (Walsh, 1997) and predispose to transient bacteremia during therapy (Savarrio *et al.*, 2005). Previous reports suggested that endodontic bacteria might be involved in extra-oral complications, such as chronic maxillary sinusitis (Melen *et al.*, 1986), orbital cellulitis (Ngeow, 1999), infective endocarditis (Bate *et al.*, 2000), rheumatoid arthritis (Breebaart

et al., 2002) and brain abscess (Henig *et al.*, 1978). In this regard, substantial understanding of the endodontic microbiota is an important requirement for both oral and medical microbiologists.

While it is common-knowledge that the development of efficient treatment strategies relies on the characterization of the endodontic microbial communities in entirety, cultivation-based techniques may cut down the range of detection, since a subset of oral inhabitants still cannot undergo cultivation (Paster *et al.*, 2001). In this new context, clone library analysis of ribosomal DNA, particularly the 16S rDNA, has become a trustworthy tool for determining bacterial diversity, often yielding more informative results when compared to cultivation alone (Kroes *et al.*, 1999; Rolph *et al.*, 2001). This broad-based cultivation-free approach has been employed in the investigation of polymicrobial human infections, such as periodontal disease, childhood caries, dentoalveolar abscesses, maxillary sinusitis and noma lesions (Wade *et al.*, 1997; Paster *et al.*, 2001; Paster *et al.*, 2002; Becker *et al.*, 2002; Hutter *et al.*, 2003; Paju *et al.*, 2003). Particularly in root canal infections, 16S rDNA sequence analysis has enabled detection of bacteria when culture had generated negative results and has permitted the identification of novel species in relatively small sets of samples (Rolph *et al.*, 2001; Munson *et al.*, 2002).

Here, we report the results of an investigation on the bacterial diversity of seven infected root canals by the analysis of 16S rDNA libraries, in an effort to contribute to the ongoing characterization of the root canal microbiota.

Methods

Subjects. Seven patients, 2 males and 5 females, ranging from 15 to 42 years old (mean 27.7 ± 8.4) were analyzed. Subjects had been referred for endodontic treatment at the Dental School of Piracicaba, and were selected for presence of pulpal necrosis and chronic periapical lesions by clinical and radiographic evaluation. In order to facilitate antisepsis of the operation field during sampling, only teeth with unexposed pulp chambers were included. These were represented by teeth with intact crowns or small, clinically acceptable restorations. Subjects with periodontal pockets >3mm, advanced bone-loss, acute abscesses, tooth fractures, sinus tracts or those who had undergone antibiotic therapy within two months prior to collection were not included. Written informed consent was obtained from all individuals and ethical approval was granted by the Ethical Committee for Human Subjects of the Dental School of Piracicaba, State University of Campinas.

Sample collection. Each patient was submitted to local anesthesia and the tooth was isolated with rubber-dam. Cleaning of the tooth crown was performed to eliminate food debris and dental plaque. Antisepsis of the crown and operation field was conducted with 1.0% NaOCl for 1 min,

followed by inactivation with 5% sodium thiosulfate. Coronal access cavity was gained by high-speed bur irrigated with sterile saline solution. As the pulp chamber was reached, a sterile #15 K-file was introduced at 3mm short of the root apex. After careful instrumentation, the active portion of the K-file was cut and placed in a test tube containing 1 mL of TE buffer (10 mM TrisHCl, 1 mM EDTA, 0.1 mM NaCl, pH 8.0). Three sterile #15 paper-points were consecutively introduced inside the canal for 20 sec each and placed in the same test tube. Samples were immediately transported to the laboratory and stored at – 20 ºC.

DNA extraction. Samples were thawed in water bath at 37 ºC for 10 min, vortexed for 30 sec and the paper points and K-files were removed from the tubes. Bacterial cells were pelleted by centrifugation at 20,000 *g* for 10 min and the supernatant was discarded. A DNA extraction protocol based on chloroform: isoamyl alcohol and hexadecyltrimethylammonium bromide (CTAB) was employed (Kuipers *et al.*, 1999; Smith *et al.*, 1989). DNA was re-suspended in TE buffer with 10 µg RNAse ml^{-1}, incubated in water bath at 37^{o}C for 30 min and stored at – 20 ºC until required.

16S rDNA amplification. PCR control tests for the 16S rDNA universal eubacterial primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg *et al.*, 1991) were performed with 21 bacteria strains, yielding positive amplification for all DNA tested, as determined by visualization on agarose gel electrophoresis (available as supplementary data in *JMM Online*). Polymicrobial 16S rDNA from the clinical samples was amplified by PCR in 25 µL mixtures, as previously described (Rodrigues *et al.*, 2003), except for the following modifications: 2 mM $MgCl_2$ and 1.5 U *Taq* DNA polymerase. PCR products were examined on 1.0% low melting point agarose-gel electrophoresis stained with ethidium bromide. Amplification products (about 1,500 bp) were purified with the GFX DNA Purification kit, according to the manufacturer's instructions (Amersham Biosciences).

Cloning of polymicrobial PCR products. 65.0 ng of each 16S rDNA PCR product was ligated to pMOS*Blue* vector and transformed in *Escherichia coli* DH10B cells, according to the manufacturer's instructions (Amersham Biosciences). Small-scale plasmid DNA preparations were conducted by an alkaline lysis protocol as described by Sambrook *et al.* (1989). Screening of recombinants was performed on 1.0% agarose-gel electrophoresis stained with ethidium bromide.

16S rDNA partial sequencing. Seventy recombinant clones per library were randomly selected for partial 16S rDNA sequencing reactions, performed in a DNA thermal cycler. Temperature profile for primers T7 (5'-TAATACGACTCACTATAGGG-3') and U19mer (5'-GTTTTCCCAGTCACGACG-3') included an initial step at 96 ºC for 2 min, followed by 30 cycles of 96 ºC for 30 sec, 50 ºC for 30 sec and 60 ºC for 4 min. Reactions were performed in 10 µL

mixtures containing 250 ng of template DNA, 1 µL of Big Dye ^{TM} Terminator Ready v.3.0 (Applied Biosystems), 0.5 µM of primer and 3 µL of sequencing buffer (200 mM TrisHCl pH 9.0, 5 mM $MgCl_2$).

16S rDNA sequence analysis. Sequences were automatically analyzed in ABI Prism 3100 Genetic Analyzer (Applied Biosystems – Hitachi) and grouped into clusters (phylotypes), according to a 99% minimum similarity (Kroes *et al.*, 1999; Hutter *et al.*, 2003). One representative of each phylotype was selected and submitted to the blastn algorithm (BLAST 2.0 - http://www.ncbi.nlm.nih.gov/BLAST), allowing comparison with sequences present in the GenBank, DDBL and EMBL databases. Only the highest-scored BLAST result was considered for phylotype identification, with a 98% minimum similarity (Stackebrandt & Goebel, 1994).

Phylogenetic analysis of novel phylotypes. Four clones with BLAST identities ≤97% were considered as representatives of novel phylotypes and were sequenced with additional primers 341-357f (5'-CCTACGGGAGGCAGCAG-3'), 357-341r (5'-CTGCTGCCTCCCGTAGG-3'), 685-704f (5'-GTAGSGGTGAAATSCGTAGA-3'), 704-685r (5'-TCTACGSATTTCACCSCTAC-3'), 1099-1114f (5'-GCAACGAGCGCAACCC-3') and 1114-1099r (5'-GGGTTGCGCTCGTTGC-3') (Lane, 1991). Contiguous sequences were assembled with the Phred/Phrap/Consed software package (www.phrap.org), followed by analysis with Chimera Check (RDP II - http://rdp.cme.msu.edu) to ensure inexistence of chimeric molecules. Novel phylotypes were taxonomically assigned with Naive Bayesian rRNA Classifier v.1.0 (RDP II) and submitted to phylogenetic analysis, along with close-related sequences from the order *Clostridiales* obtained by the Hierarchy Browser program (RDP II). All sequences were aligned with the ClustalW software (Thompson *et al.*, 1994) and visualized with Bioedit 7.0.4 (www.mbio.ncsu.edu/BioEdit). A phylogenetic tree was constructed with MEGA 2.1 (Kumar *et al.*, 2001), according to the calculation of a distance matrix (Jukes & Cantor, 1969) and tree reconstruction by the neighbor-joining method (Saitou & Nei, 1987). Bootstrap confidence values for branching nodes were inferred by the generation of 100 resampling trees.

Results and Discussion

The nucleotide sequence analysis of 16S rDNA clone libraries was used to investigate the bacterial diversity of seven endodontically infected teeth. All teeth evaluated were positive for the presence of bacteria. Overall, 46 taxonomic units (phylotypes) were detected (Table 1). In spite of the relative homogeneity of our study group, composed of asymptomatic teeth associated with unexposed necrotic pulps, chronic periapical lesions and no periodontal disease, a high variation in bacterial compositions could be observed: 33 (71.7%) phylotypes were subject-exclusive,

whereas only 13 (28.3%) could be detected in more than one patient. The number of phylotypes also ranged substantially among subjects: from 2 (patient AB) to 14 (patient AA) (mean 9.57 ±3.91). This variation is in accordance to both cultivation and molecular-based studies (Sundqvist, 1976; Jung *et al.*, 2000). Table 1 illustrates the percentage distribution of phylotypes within each subject and is intended to provide a quantitative view on the results. This type of analysis brings important contribution to our findings (Dewhirst *et al.*, 2000; Becker *et al.*, 2002; Munson *et al.*, 2002; Hutter *et al.*, 2003), but should be viewed with discretion, since multi-template PCR can be subjected to bias in template-to-product ratios (Suzuki & Giavannoni, 1996; Polz & Canavaugh, 1998).

In general, the results of this investigation reiterated data from cultivation and molecular-based investigations, with a predominance of anaerobic bacteria, especially from the phylum *Firmicutes* of Gram-positives (Sundqvist, 1992a; Munson *et al.*, 2002). Representatives of other phyla were found in much lower frequencies: *Proteobacteria* (10.9%), *Spirochaetes* (4.3%), *Bacteroidetes* (6.5%), *Actinobacteria* (2.2%) and *Deferribacteres* (2.2%); 9% of sequences could not be assigned at the phylum level. Common endodontic species, such as *Campylobacter gracilis*, *Eubacterium tardum*, *Peptostreptococcus anaerobius*, *Peptostreptococcus micros* and *Lachnospiraceae* sp. were positively identified with high percent identities (≥99%). Recently reported phylotypes were also detected: *Bacteroidales* oral clone MCE7_164 E2b, *Lachnospiraceae* oral clone MCE7_60 E1, *Lachnospiraceae* oral clone MCE9_173 E4, *Megasphaera* sp. oral clone MCE3_141 P1 (Munson *et al.*, 2002). *Dialister invisus*, a newly described oral Gram-negative coccobacillus (Downes *et al.*, 2003), was the most commonly found taxon (5 out of 7 subjects), followed by *Filifactor alocis* and *Eubacteriaceae* oral clone P2PB_46 P3 (4 out of 7 subjects). Accordingly, bacteria from the genus *Dialister* have been identified in oral infections with increasing frequencies (Contreras *et al.*, 2000; Munson *et al.*, 2002). *D. pneumosintes*, a species frequently associated with purulent infections, brain abscesses and bite-wounds (Goldstein *et al.*, 1984; Rousée *et al.*, 2002), has also been considered as putative pathogen in periodontal and endodontic infections (Ghayoumi *et al.*, 2002; Siqueira & Rôças, 2002) and could be detected in one subject.

Phylotypes corresponding to recently proposed pathogens of periodontal disease were identified, corroborating molecular data from Paster *et al.* (2001) and Hutter *et al.* (2003). Among those, *Treponema socranskii* has already been detected in the endodontum, being one of the most common root canal treponemes (Baumgartner *et al.*, 2003), while Uncultured *Eubacterium* clone PUS9.170 has also been found in dentoalveolar abscesses (Wade *et al.*, 1997). *Pseudoramibacter alactolyticus* and *Filifactor alocis* were positively detected with high percent

matches (99%) and have proven to be frequent inhabitants of root-filled, refractory cases (Siqueira & Rôças, 2004).

Forty-two (91.3%) clones were identified to the species-level and four (8.7%) corresponded to sequences with no resemblance to any other previously deposited in the databases (Figure 1), according to the established 98% nucleotide identity threshold. This parameter is in accordance to a previously proposed species definition criterion, based on DNA-DNA re-association assays (Stackebrandt & Goebel, 1994) and lays within the range of values employed by similar studies, which vary from 98% (Sakamoto *et al.*, 2000; Rolph *et al.*, 2001; Munson *et al.*, 2002) to 99% (Kroes *et al.*, 1999; Drancourt *et al.*, 2000; Hutter *et al.*, 2003). Applying a 97% threshold value did not bring modifications to our findings, whereas a 99% value resulted in the detection of 15 potentially novel species, as opposed to only 4. The application of such a stringent condition was nonetheless rejected, since it could generate redundancies in the results, due to the intragenomic heterogeneities in 16S ribosomal RNA operons (Coenye & Vandamme, 2003; Acinas *et al.*, 2004).

Among the novel phylotypes detected (Figure 1), Uncultured *Mogibacterium* sp. clone AF_H06 (AY821870) was the only taxonomically assigned to the genus level. *Mogibacterium* is a newly proposed genus of bacteria originally isolated from periodontal pockets and infected root canals, represented by anaerobic Gram-positive bacilli (Nakazawa *et al.*, 2000). Representatives of this genus were shown to be frequent in endodontic infections, as observed by Rolph *et al.* (2001), who identified clones closely related to *M. neglectum*, *M. vescum* and *M. diversum*, with 97% nucleotide identities. The epidemiological importance of this group of bacteria in endodontic infections is still to be investigated.

Interestingly, some important endodontic species, such as and *Fusobacterium nucleatum* and the black-pigmented anaerobes *Porphyromonas gingivalis*, *Porphyromonas endodontalis* and *Prevotella intermedia* could not be detected. Lack of primer specificity was readily discounted, since we had successfully tested our universal primers with *F. nucleatum* ATCC 10953 and many species within the phylum *Bacteroidetes*, including *P. gingivalis* ATCC 33277, *P. endodontalis* ATCC and *P. intermedia* ATCC 25611 (data in *JMM Online*). Similar root canals studies were also not able to find any *P. gingivalis* phylotypes (Rolph *et al.*, 2001; Munson *et al.*, 2002). Fusobacteria have been frequently encountered in root canals by cultivation (Sundqvist, 1992a) and PCR assays (Fouad *et al.*, 2002), but its prevalence seems to be relatively low in 16S rDNA-based studies. Accordingly, Munson *et al.* (2002) identified one *Fusobacterium* clone out of 624 sequenced, whereas Rolph *et al.* (2001) detected *F. nucleatum* solely in the refractory subset of cases. The absence of some bacteria species in the present study can be attributed to intrinsic technique limitations, or to the small sample-set investigated.

In conclusion, the results from this study are in accordance to those from similar research, revealing a predominance of anaerobic species from the phylum *Firmicutes* of Gram-positive bacteria in infected root canals, particularly from the class *Clostridia*. The identification of uncultured clones, originally encountered in the endodontum, saliva and subgingival plaque demonstrates, over again, that the endodontic and periodontal microbial communities may share a relevant proportion of bacteria, despite their established anatomical interrelationships (Kerekes & Olsen, 1990; Rupf *et al*., 2000). The identification of novel phylotypes adds to the concept that the endodontum may still harbor a relevant proportion of uncharacterized taxa.

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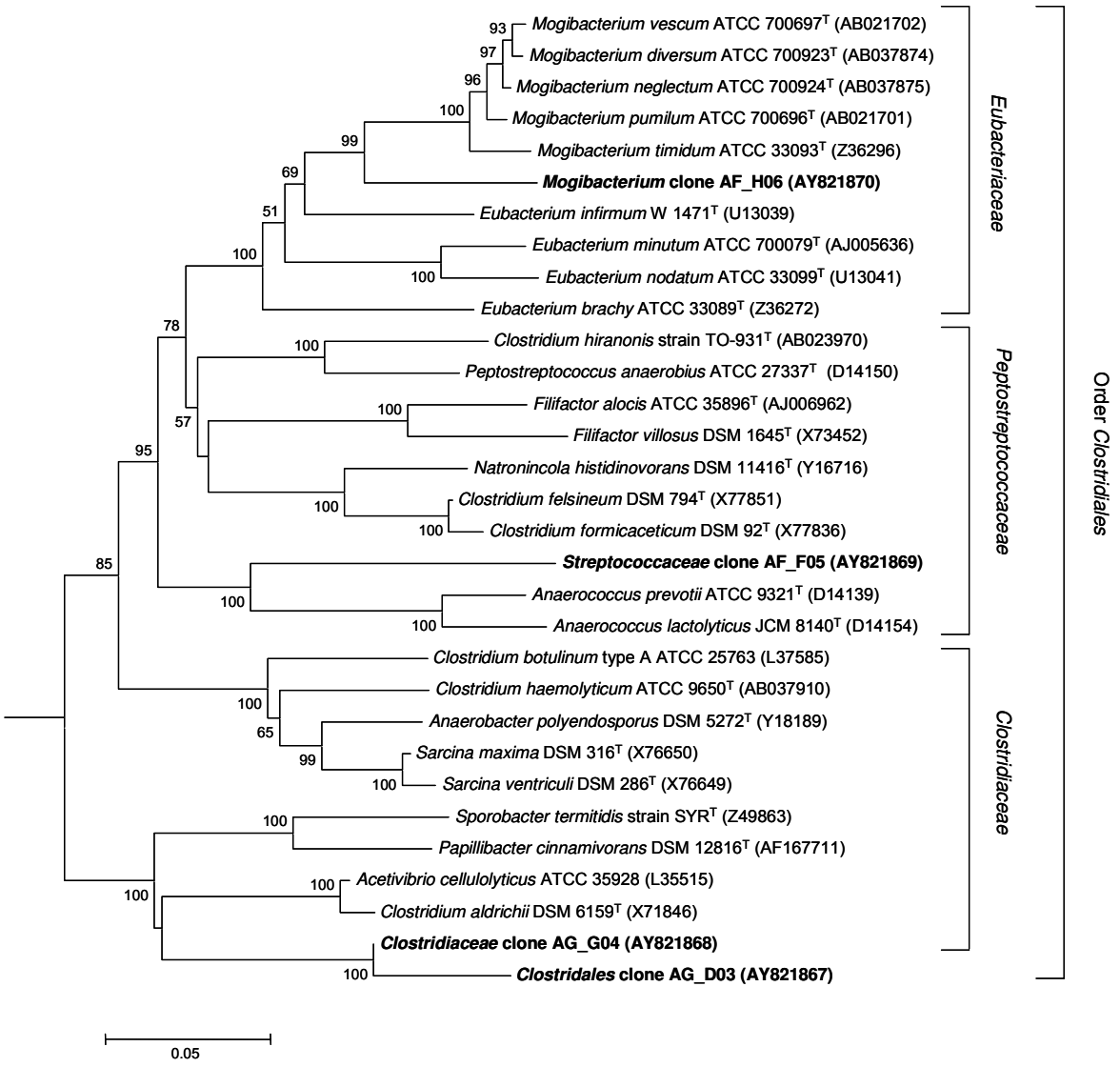

Figure 1. 16S rDNA phylogenetic inter-relationships of four novel phylotypes from unexposed endodontic infections, indicated in bold type, and reference strains from the order *Clostridiales* (acession numbers in parenthesis). Distance matrix calculated by the Jukes and Cantor method (1969). Phylogenetic tree constructed by the neighbor-joining method of Saitou & Nei (1987) and rooted for *Escherichia coli* K12 16S rDNA. Bootstrap confidence values generated over 100 tree replications (values above 50% are shown next to the branching nodes). Scale bar represents the number of nucleotide substitutions per site.

Table 1. Highest-scored BLAST search results from 46 taxonomic units detected in unexposed root canal infections by the sequence analysis of 16S rDNA clonal libraries and percentage distribution of phylotypes per subject.

BLAST search result	Identity (%)	Phylotypes distribution per subject (%)						
		AA	AB	AC	AD	AE	AF	AG
Bacteroidales oral clone MCE7_120 E3	98					4.2		
Bacteroidales oral clone MCE7_164 E2b	99				2.9			
Bacteroides-like sp. oral clone X083	98	1.4						
Burkholderia fungorum strain NW-2	100			7.4				
Burkholderia phenazinium	99				5.7			
Burkholderia sp. CI6	98					4.2		
Campylobacter gracilis	99	1.4						
Catabacter hongkongensis	100				2.9			
Clostridiales oral clone MCE3_9 E1	93						9.1	15.0
Clostridiales oral clone P4PB_122 P3	92							5.0
Desulfobulbus sp. oral clone R004	98					20.8		
Dialister invisus	100	28.8			2.9	4.2	3.0	10.0
Dialister pneumosintes	99							5.0
Eubacteriaceae oral clone P2PB_46 P3	99				20.0	33.3	6.1	15.0
Eubacterium sp. oral clone BU061	99	2.7						
Eubacterium sp. oral clone CK047	99					4.2		
Eubacterium sp. oral clone DA014	94						6.1	
Eubacterium sp. oral clone FX028	98					4.2		
Eubacterium tardum SC87K	99	23.3			11.4		12.1	
Eubacterium timidum	99						27.3	10.0
Filifactor alocis	99	1.4		22.2		12.5		20.0
Filifactor sp. oral clone BP1-58	99	1.4						
Filifactor sp. oral clone BP1-88	99			22.2				
Flexistipes sp. E3_33 oral isolate	99				2.9			
Lachnospiraceae bacterium oral clone BP1-14	99				5.7			
Lachnospiraceae oral clone MCE7_60 E1	99				2.9			
Lachnospiraceae oral clone MCE9_173 E4	99				34.3	16.7		
Lactobacillus panis DAF 1	98		12.5					
Megasphaera sp. oral clone BB166	98			7.4				5.0
Megasphaera sp. oral clone MCE3_141 P1	99	11.0		3.7				
Mogibacterium neglectum	93						3.0	
Peptostreptococcus micros ATCC 33270	100						3.0	
Peptostreptococcus anaerobius clone LK54	99	1.4		14.8				
Peptostreptococcus sp. oral clone CK035	99							15.0
Pseudoramibacter alactolyticus strain 23263T	99	15.1			8.6			
Selenomonas sp. oral clone JS031	98			3.7				
Selenomonas-like sp. oral strain FNA3	98			7.4				
Treponema socranskii subsp. 04	99						3.0	
Treponema sp. 6:H:D15A-4	98	4.1						
Uncultured *actinobacterium* clone APe2_64	98						12.1	
Uncultured *bacterium* BH017	99						6.1	
Uncultured *bacterium* inhufec A-37	99						6.1	
Uncultured *bacterium* W090	99	2.7					3.0	
Uncultured *Eubacterium* clone PUS9.170	99	2.7		11.1				
Unidentified oral bacterium RP55-6	98	2.7						
Veillonella sp. ADV 360.00	99		87.5					
Total number of phylotypes		14	2	9	11	9	13	9

CAPÍTULO 2

Artigo submetido ao periódico *Oral Microbiology and Immunology*
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Real Time PCR quantitative detection of *Porphyromonas gingivalis* and *Tannerella forsythia* in primary endodontic infections

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Running title: Real Time PCR of endodontic bacteria

Abstract

Sensitive detection of bacteria is an essential requisite for understanding the etiology of root canal infections and consequent periapical lesions. *Porphyromonas gingivalis* and *Tannerella forsythia* are oral bacteria capable of triggering intensified immunological responses. Quantitative detection of these bacterial species in root canal infections could bring important insight into their clinical relevance.

A Real Time PCR assay was applied to determine the levels of *P. gingivalis* and *T. forsythia* in 34 cases of primary endodontic infections. Total genomic DNA was extracted from intraradicular samples, followed by species-specific DNA amplification with primers targeting the *bspA* surface antigen gene of *T. forsythia* and the Arg-gingipain (*rgp*) gene of *P. gingivalis.* Relative bacterial levels were determined according to standard curves adjusted to genome copy numbers, followed by numerical correction with the absolute mass of DNA from each sample. Overall, *P. gingivalis*, *T. forsythia* and a coexistance of both species were encountered in 24%, 56% and 18% of the subjects. Adjusted genome copy levels ranged from zero to 1.26×10^4, and from zero to 1.28×10^5, respectively. No significant associations among the abundances of the target bacteria, or the combination of both, and the presence of pain of endodontic origin could be observed.

T. forsythia was highly prevalent and numerous in the study subjects, whereas *P. gingivalis* was moderately frequent and less abundant. Despite previous reports associating *T. forsythia* and *P. gingivalis* with specific clinical parameters, they did not display any correlations with endodontic symptoms.

Introduction

Root canal bacteria are the primary etiological agents of pulpal and periapical diseases and their persistence after instrumentation is still among the major causes of failure in the endodontic therapy. Whilst the oral cavity is currently estimated to harbor more than 500 bacterial species (Paster *et al*. 2001), only a restricted parcel is able to thrive inside the root canal. De facto, the microbial structure commonly observed in endodontic infections is only a stationary sequel of a highly dynamic process of bacterial succession, guided by ecological interactions played upon microbial elements and environmental factors established inside the canal (Figdor and Sunqvist 2007). Over the years, it has become generally accepted that primary endodontic infections are, in essence, a mixed composition of facultative and strictly anaerobic bacteria and, in this aspect, combinations of bacteria are expected to arise (Sundqvist 1992). Truly, it has been shown that particular associations of endodontic species can observed in the intraradicular habitat, either in asymptomatic (Peters *et al*. 2002), or in symptomatic teeth (Gomes *et al*. 2004). Specific associations of residual bacteria have also shown to be more frequent in cases of persistent periapical lesions in monkeys, when compared to the monoinfected or aseptic counterparts (Fabricius *et al*. 2006).

Associations of *P. gingivalis* and *T. forsythia* are directly involved with risk factors of periodontal disease (Grossi *et al*. 1995) and, together with *Treponema denticola*, constitute the 'red-complex', a group consisting of Gram negative anaerobic bacteria implicated with adult and refractory periodontal disease (Socransky *et al*. 2002, 1998). *P. gingivalis* is probably the most widely investigated oral pathogen and possesses a plethora of pathogenic properties including fimbriae, proteinases, lipoteichoic acids, exopolisaccharides, and hemin-binding proteins (Holt *et al.* 1999). *T. forsythia* harbors a multi-functional surface and secreted protein responsible for many pathogenic activities, including bacterial coaggregation, fibronectin and fibrinogen binding, epitelial cell attachment and invasion, and proinflamatory cytokine and chemokine induction (Inagaki *et al*. 2006). Interbacterial binding and growth induction by *P. gingivalis* and *T. forsythia* cells have been observed *in vitro* (Yoneda *et al*. 2005, Yao *et al*. 1996). This synergistic interaction is also evidenced in the *in vivo* scenario, where coinoculation of both species can trigger heightened pathogenicity, with abscess formation and sepsis (Yoneda *et al.* 2001, Takemoto *et al.* 1997).

Previous reports have shown positive correlations between endodontic bacterial load and size of periradicular lesion and periapical abscesses (Figdor and Sundqvist 2007). In this sense, the application of quantitative techniques can provide a more comprehensive view on the clinical relevance of particular species of bacteria (Gomes *et al*. 2004, Jung *et al*. 2001). A substantial number of microbiological examinations on root canal infections has relied on cultivation and,

more recently, on strictly qualitative molecular identification techniques such as the conventional Polimerase Chain Reaction (PCR). While cultivation provides invaluable information on growth and phenotypic properties of viable cells, it still lacks the necessary sensitivity for the very fastidious or as-yet uncultivable bacterial species (Rolph *et al*. 2001). Likewise, end-point PCR, a highly sensitive molecular technique broadly used for microbial identification, does not offer reliable quantitative capabilities for today's standards (Bustin *et al*. 2000, Ferre 1992).

The Real Time PCR technology provides precise fluorescence-based quantification of target DNA levels by a cycle-to-cycle monitoring of amplification products (Heid *et al*. 1996). Since fluorescence acquisition is done at the initial log-linear phase of DNA amplification, it circumvents common quantification biases inherent to end-point PCR analysis (Ferre *et al*. 1992). Also, as reaction products are directly detected inside the test tube, there is no the need for post processing, increasing automation and minimizing potential analysis errors (Bustin 2000). SYBR Green I is an intercalating dye that binds to the minor groove of double-stranded DNA. It can be applied with virtually any set of PCR primers, offering improved versatility and simplicity of use (Bustin 2000). It has been proved to be a sensitive, rapid and accurate reporter molecule for nucleic acid quantification, with specificity and robustness comparable to those of other available chemistries (Maeda *et al*. 2003, Morrison *et al*. 1998).

In the present study, a SYBR Green I Real Time PCR assay was used to determine the relative levels of *P. gingivalis* and *T. forsythia* in 34 cases of primary endodontic infections, in an effort to bring light into their clinical relevance.

Material and Methods

Subjects. Working approval was granted by the Ethical Committee for Human Subjects of the Dental School of Piracicaba. All subjects had been referred for endodontic treatment at the Dental School of Piracicaba, and were selected for the presence of pulpal necrosis by clinical and radiographic evaluations. One tooth per subject was included in the study. Overall, thirty four patients, 14 males and 20 females, ranging from 15 to 61 years old (mean 34.24 ± SD 13.73) were analyzed. Subjects who had undergone antibiotic therapy within two months prior to collection were not included. According to the observed clinical parameters, patients were classified in two categories for further correlation with the detected bacterial levels: symptomatic (n= 24) and asymptomatic (n= 10). Symptomatic cases were considered those harboring teeth associated with tenderness to percussion, sensibility to palpation, spontaneous pain, or a combination of those features. All other cases were considered asymptomatic.

Sample collection. Samples were collected as described elsewhere (Saito *et al.* 2006). Briefly, the teeth to be sampled were isolated with rubber dam, cleaned and opened using a sterile procedure. Intraradicular samples were obtained by the introduction of a sterile #15 K-file and 4 consecutive sterile paper points inside the root canal. The file and the paper points were placed in a test tube containing 1 mL of TE buffer (10 mM TrisHCl, 1 mM EDTA, 0.1 mM NaCl, pH 8.0) and immediately transported to the laboratory.

Bacterial strains. The following reference strains were used as controls for conventional PCR and Real Time PCR assays: *Aggregatibacter actinomycetemcomitans* ATCC 29522, *Bacteroides fragilis* ATCC 25285, *Bacteroides merdae* M-36, *Bacteroides vulgatus* ATCC 8482, *Escherichia coli* ATCC 12795*, Porphyromonas assacharolytica* ATCC 25260, *Porphyromonas circumdentaria* ATCC 51356, *Porphyromonas endodontalis* ATCC 35406, *Porphyromonas gingivalis* ATCC 33277, *Porphyromonas levii* ATCC 29147, *Porphyromonas salivosa* NCTC 11632, *Prevotella oulora* ATCC 43324, *Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* NCTC 9336, *Pseudomonas aeruginosa* ATCC 10145, *Streptococcus salivarius* ATCC 25975*, Streptococcus sanguinis* ATCC 10556, *Streptococcus sobrinus* ATCC 27607, *Streptococcus mutans* ATCC 25175 and *Tannerella forsythia* ATCC 43037.

DNA extraction. DNA from clinical samples and reference bacteria was extracted as described elsewhere (Saito *et al.* 2006), ressupended in 30 µL of TE buffer and immediately stored at – 20 ºC until further required.

Preparation of standard DNA: Standard DNA solutions corresponding to 10^7 genome copies of *P. gingivalis* and *T. forsythia* were prepared based on their respective genome sizes (2.34 Mb for *P. gingivalis* (Nelson *et al.* 2003) and 3.41 Mb for *T. forsythia* (Tanner & Izard 2000), and considering the mean weight of a nucleotide pair to be 1.023×10^{-12} ng (Dolezel *et al.* 2003). These solutions were subsequently used to obtain serial 10-fold dilutions down to 10^1 genome copies per reaction.

Design of PCR primers. Primers targeting the arginin-specific cystein-proteinase (Arg-gingipain or *rgp*) gene of *P. gingivalis* were previously published (Morillo *et al.* 2004) (table 1). Primers for the surface antigen *bspA* gene of *T. forsythia* were obtained based on the complete gene sequence acquired from the GenBank database (accession number AF054892). The Primer3 software was used for the design of candidate oligonucleotide sequences (http://frodo.wi.mit.edu). A final set of primers was chosen according to the lowest potential to form secondary structures, as determined by analysis with the Netprimer software (http://www.premierbiosoft.com/netprimer) (table 1).

Validation of markers by end-point PCR. Conventional PCR pilots were performed in total volumes of 25 µL with the following conditions: 1x PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM primers, 1.5 U Platinum *Taq* DNA polymerase (Invitrogen). The temperature profiles were: a) for *P. gingivalis* (*rgp* gene): initial denaturation at 96°C for 3 min; 36 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1min; final extension at 72°C for 10 min; b) for *T. forsythia* (*bspA* gene): initial denaturation at 96°C for 3 min; 36 cycles of denaturation at 94°C for 1 min, annealing at 46°C for 1 min and extension at 72°C for 1min; final extension at 72°C for 10 min. Supplementary PCR assays with universal 16S rDNA primers (table 1) were performed to assess the presence of bacteria in the clinical samples, according to the conditions described elsewhere (Saito *et al*. 2006).

Optimization of the Real Time PCR assay. Reactions were conducted in a Roche LightCycler 1.0 System under the LightCycler 4.24 Run Software (Roche Applied Science). Standard DNA corresponding to 10^3 genome copies of *P. gingivalis* and *T. forsythia* were used as templates. Optimization was carried out with various $MgCl_2$ and primer concentrations, as stated by the manufacturer's instructions (Roche). Final reaction mixtures were performed in total volumes of 10 µL containing 2.0 µL DNA, 1.0 µL LightCycler FastStart DNA Master SYBR Green I (Roche), 4mM $MgCl_2$ and 0.5 µM of each primer. The temperature profiles were: a) for *P. gingivalis* (*rgp* gene): hot-start denaturation at 95°C for 10 min and 48 cycles of denaturation at 94°C for 10 s, annealing at 60°C for 7 s, extension at 72°C for 7 s and fluorescence acquisition at 78°C for 3 s; b) for *T. forsythia* (*bspA* gene): hot-start denaturation at 95°C for 10 min and 60 cycles of denaturation at 95°C for 10 s, annealing at 46°C for 7 s, extension at 72°C for 7 s and fluorescence acquisition at 83°C for 3 s.

Real Time PCR amplification of standard DNA. Amplification profiles for *P. gingivalis* and *T. forsythia* were generated from duplicate reactions using 10-fold dilutions of standard DNA (figure 1). The computer-assisted second derivative maximum algorithm was used for crossing point (CP) inference of each DNA dilution. Standard curves were generated by linear regression analysis, and used as a basis for further quantification of target DNA from the clinical samples (figure 1).

Real Time PCR amplification of DNA from clinical samples. Prior to quantification of target bacteria, the total ammount of DNA from each clinical sample was assessed by spectrophotometry at 260nm. Real Time PCR amplification assays were conducted in duplicates, and as described for standard DNA. Genome copy levels were inferred based on the previously obtained standard curves. In order to minimize potential variations in sample volume, absolute genome copy levels were normalized by numerical division by the total ammount of DNA measured from each sample. 3.0 µL of each Real Time PCR product was submited to 1.0%

agarose gel electrophoresis and stained with ethidium bromide to verify reaction efficiency and specificity.

Melting curve analysis. Reaction specificities were further verified by melting curve analysis (figure 2). The following temperature profiles were used: a) for *P. gingivalis*: a progressive temperature increase from 70°C to 95°C at a 0.1°C/s transition rate, with continuous fluorescence acquisition; b) for *T. forsythia*: a progressive temperature increase from 70°C to 95°C at a 0.1°C/s transition rate, with continuous fluorescence acquisition. To minimize potential primer-dimer interferences, fluorescence acquisition temperatures were adjusted to approximately 4°C below the melting point of each amplification product.

Sequencing of selected PCR products. Representative electrophoretic gel bands were selected to ascertain Real Time PCR amplicon identities from the clinical samples. DNA was purified with the GFX DNA Purification kit, as stated by the manufacturer (Amersham Biosciences). DNA sequencing reactions were performed in 10 µL mixtures containing approximatelly 100 ng template DNA, 1 µL Big Dye Terminator Ready version 3.0 (Applied Biosystems), 0.5 mM primer and 3 µL sequencing buffer (200 mM Tris/ HCl pH 9.0, 5 mM $MgCl_2$). Nucleotide sequences were visualized and edited with the Bioedit 7.0.5.3 software (http://www.mbio.ncsu.edu/BioEdit) and submitted to the BLASTN algorithm (BLAST 2.0; http://www.ncbi.nlm.nih.gov/blast) for comparison with sequences from the GenBank database. A 98% nucleotide identity threshold was used for positive identification to species level (Saito *et al.* 2006).

Statistical analysis. The R (www.r-project.org) and BioEstat (http://www.mamiraua.org.br/) softwares were used for statistical analysis. Data exploration with the Lilliefors normality test indicated that data were non-homogeneous even after log transformation. The null hypothesis that the target species were not associated with endodontic symptoms was evaluated with the Mann-Whitney non-parametrical test, with a significance level $\alpha = 0.05$. In order to assess the correlation between the coexistence of the target bacteria and the clinical parameters, the integrated levels of *P. gingivalis* and *T. forsythia* were used in the cases where both appeared concurrently; in all other cases (i.e. exclusive presence of either species or absence of both), the integrated levels were assigned as null. Lastly, potential interbacterial associations were assessed with the Fisher's exact test.

Target species	Orientation	Position	Oligonucleotide sequence (5' – 3')	Amplicon size	Reference
P. gingivalis (*rgp* gene)	forward	1308	CCTACGTGTACGGACAGAGCTATA	72 bp	Morillo *et al.* 2004
	reverse	1379	AGGATCGCTCAGCGTAGCATT		
T. forsythia (*bspA* gene)	forward	1911	TCACTATTGTGTCTCGCTG	133 bp	this study

	reverse	2043	TCTCTCCGATTGTGGTTA		
Total bacteria (16S *rRNA* gene)	forward (fD1)	8	AGAGTTTGATCCTGGCTCAG	1534 bp	Weisburg *et al.* 1991
	reverse (rD1)	1541	AAGGAGGTGATCCAGCC		

Table 1. PCR primers used in this study.

Results

Real Time PCR amplification of standard DNA. No evidence of non-specific or cross-reaction products was observed for the *bspA* and *rgp* markers, as determined by conventional end-point PCR (data not shown). Melting curve analysis of Real Time PCR amplicons showed well-depicted peaks for both markers (figure 2). The minimum number of cycles required to amplify the full range of serially diluted DNA ranged from 23 to 43 for *rgp*, and from 24 to 54 for *bspA* (figure 1). The application of an amplification efficiency formula (Pfaffl 2001) revealed values of 2.0 and 1.6 for *P. gingivalis* and *T. forsythia*, provided that a 2.0 value corresponds to an optimum efficiency. Linear regression equations used for inference of genome copy levels of the target species in the clinical samples are shown in figure 1, along with the respective r^2 values.

Real Time PCR amplification of DNA from clinical samples. All samples were positive for the presence of bacteria, as observed by conventional end-point PCR with 16S rDNA universal primers (data not shown). Overall, Real Time PCR detected *P. gingivalis*, *T. forsythia* and a coexistence of both species in 8/34 (24%), 19/34 (56%) and 6/34 (18%) of the subjects, respectively. Normalized genome copy levels in the clinical samples ranged from zero to 1.26×10^4, for *P. gingivalis*, and from zero to 1.28×10^5, for *T. forsythia* (mean 4.86×10^2 and 7.71×10^3 genome copies per sample, respectively).

Statistical analysis. No statistical difference between the symptomatic and asymptomatic groups was observed with the Mann-Whitney test, based on the normalized genome copy levels of either species [($p= 0.07$ (*P. gingivalis*) and $p= 0.46$ (*T. forsythia*)] or the combination of both ($p= 0.13$). According to the Fisher's exact test, no statistical significant conclusions could be drawn in regard to potential interbacterial associations among the target bacteria ($p= 0.11$).

Discussion

Real Time PCR is a highly sensitive technique that brings unprecedented contribution to pathogen quantification in mixed oral infections (Boutaga *et al.* 2006). Previous reports have shown that it can harbor 41-fold greater sensitivities than colony counting for oral anaerobic bacteria, offering detection increases of up to 36% and 51%, when considering the particular cases of *T. forsythia* and *P. gingivalis* (Verner *et al*. 2006, Martin *et al*. 2002). While such observations might be, on one hand, a direct consequence of the elevated cultivation demands of these fastidious species, on the other, they reflect the truly high accuracy and detection capabilities of the Real Time PCR technique. Indeed, it has been demonstrated that the technique can successfully differentiate a blank sample from another containing only 1 copy of target nucleic acid (Morrison *et al*. 1998).

In this study, a SYBR Green I – based Real Time PCR assay was applied to investigate the relative levels of the oral pathogens *P. gingivalis* and *T. forsythia* in primary endodontic infections. Because SYBR Green I will bind to any double-stranded DNA, a careful and throughout optimization step should be performed to avoid simultaneous quantification of potential non-specific products. We have taken preventive technical measures to minimize such biases, including preliminary *in silico* examination of primers, visualization of gel bands and analysis of melting curves. The evidence of well-defined unique peaks on melting curve analysis assured satisfactory reaction specificities for both markers, with no evidences of primer-dimer formation. As additional precautions, real time-monitoring of products was conducted by fluorescence acquisition at temperatures where double-stranded DNA was presumably composed only of specific products (Morrison *et al*. 1998) and representative bands from clinical samples were selected for DNA sequencing confirmation of amplicons.

The crossing-point (CP) is, by definition, the cycle number at which the fluorescence signal is acquired. It can be infered by a user-defined method, also known as the fit point, or by a computer-assisted algorithm, such as the second derivative. In this method, the CP corresponds to the cycle where the second derivative of the fluorescence intensity curve reaches its first maximum value and which, in turn, coincides with the beginning of the log-linear phase of the amplification curve (Luu-The *et al*. 2005). In our study, the second-derivative method was chosen due to its higher consistency and adequacy in the detection of low levels of target DNA, when compared to the fit point method (Luu-The *et al*. 2005).

Black-pigmented bacteria are among the most predominant organisms in oral anaerobic infections, commonly found in gingivitis, periodontitis, endodontic infections and dentoalveolar abscesses (van Winkelhoff *et al*. 1985). *P. gingivalis*, a Gram-negative rod, is probably the most

pathogenic and widely studied black-pigmented bacteria (Holt et al. 1999). Detection frequencies of *P. gingivalis* in root canal infections varies considerably. By the use of 16S rDNA PCR methodologies, Siqueira *et al*. (2004) and Fouad *et al*. (2002) were able to detect it in 4% of the samples, while Foschi *et al*. (2005) revealed the species in 13% and of the study cases. Higher prevalences were observed by Gomes *et al*. (2005) (38%) and Siqueira *et al*. (2002) (41%), also by PCR approaches. In this study, *P. gingivalis* was detected in 24% of the subjects, corroborating the results of a DNA-DNA hybridization assay of Siqueira *et al.* (2002) (28%) and an oligonucleotide probing study of Jung *et al*. (2001) (27%).

T. forsythia, formerly *Bacteroides forsythus*, is a Gram-negative anaerobic rod originally recovered from subjects with progressive advancing periodontitis, and afterwards isolated from various forms of periodontal disease, root canal infections and perimplantitis (Tanner and Izard 2006). The prevalence of *T. forsythia* in the root canal environment can also fluctuate considerably. While Rôças *et al*. (2001), Gomes *et al*. (2006), Fouad *et al*. (2002) and Jung *et al*. (2001) found the species in 26%, 24%, 21% and 18% of primary endodontic infections by PCR-based methodologies, Foschi *et al*. (2005) and Siqueira *et al*. (2004) were able to detect it only in 7% and 4% of the cases by conventional and nested 16S rDNA PCR, respectively. In the present study, *T. forsythia* was observed in 56% of the test subjects, an estimate close to those obtained by Siqueira and Rôças (2003) with nested PCR (52%) and by Gonçalves and Mouton (1999) with immunocapture-PCR (54%).

Relative disparities in the prevalence of intraradicular bacteria are usually expected when comparing the results from different authors. This might be due to a combination of factors, such as case selection criteria, sampling and DNA extraction methods, geographical origin of subjects, or detection techniques (Baumgartner *et al*. 2004, Siqueira *et al*. 2002). For instance, Munson *et al*. (2002) were unable to detect any clones of *T. forsythia* by 16S rDNA sequencing analysis, but revealed the presence of the species in one subject by cultivation. Similar high-throughput clone library studies were unable to detect either *T. forsythia* or *P. gingivalis* in primary or refractory endodontic infections (Saito *et al*. 2006, Rolph *et al*. 2001), indicating that detection of these particular species of bacteria could be dependent on the technique employed.

According to the corrected genome copy levels, *P. gingivalis* was not significantly associated with pain of endodontic origin, confirming the results of other molecular investigations (Foschi *et al*. 2005, Rôças *et al.* 2001 and Jung *et al*. 2000). Nonetheless, Hashioka *et al*. (1992) found representatives of *Porphyromonas spp.* to be implicated, as a group, with subacute endodontic symptoms in a cultivation study. We found no significant correlation between the detected levels of *T. forsythia* and endodontic symptoms, in agreement with the observations obtained by non-quantitative molecular investigations (Foschi *et al*. 2005, Siqueira *et al*. 2003,

Rôças *et al*. 2001 and Jung *et al*. 2000). A nested-PCR study conducted by Gomes *et al.* (2006), however, detected significant association between *T. forsythia* and tenderness to percussion in a larger sample set containing primary and treated cases of endodontic infections. Lastly, when considering *P. gingivalis* and *T. forsythia* as a complex, no indications of their involvement with clinical symptoms could be statistically evidenced in the present study.

In conclusion, the application of a Real Time PCR methodology has shown that *T. forsythia* can be highly prevalent and numerous in endodontic infections, whereas *P. gingivalis* is only moderately frequent and less abundant, displaying 16-fold lower average levels than those observed for *T. forsythia*. Our results reveal that *P. gingivalis* and *T. forsythia* do not play a primary role in the development of endodontic pain, either individually or in conjunction.

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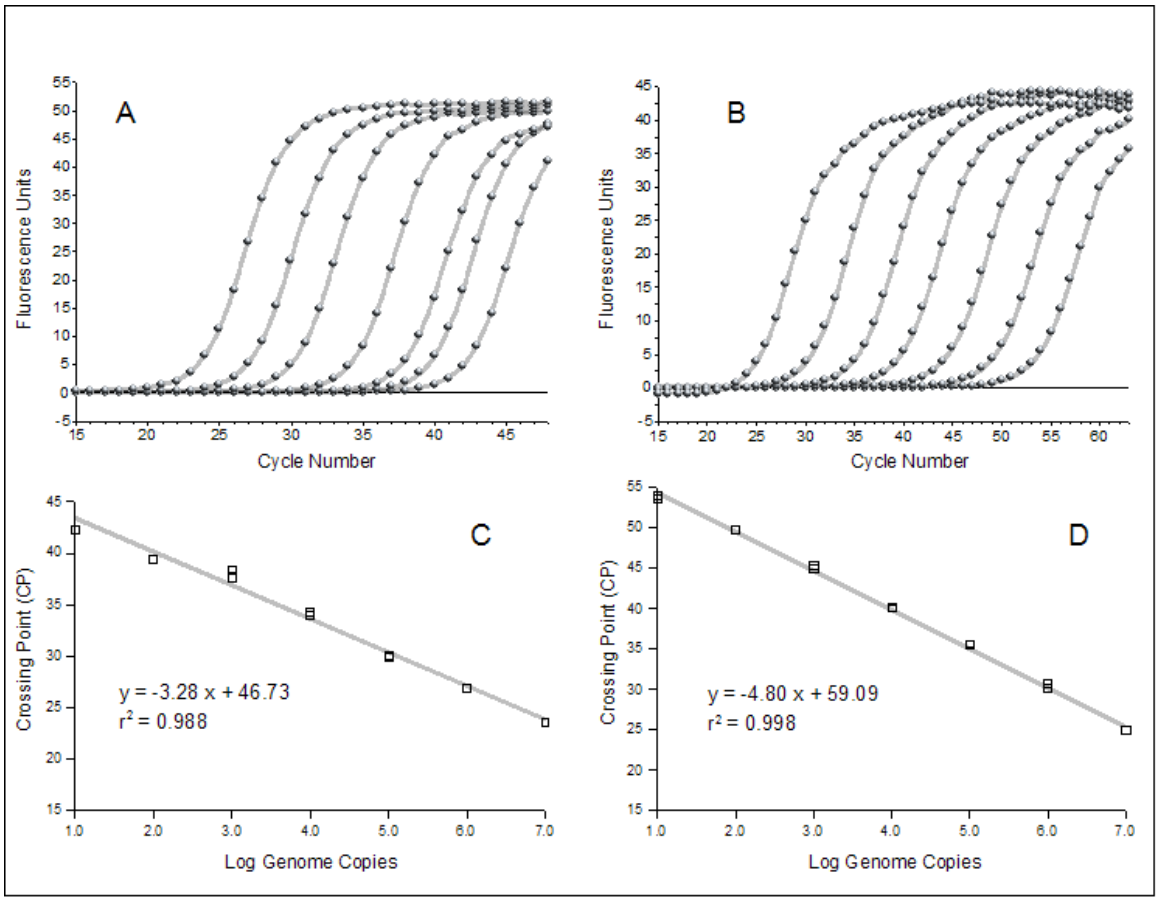

Figure 1. Amplification curves for *P. gingivalis* (A) and *T. forsythia* (B). Reactions performed with 10-fold serial dilutions of standard DNA ranging from 10^7 to 10^1 genome copies per reaction (left to right). Respective log-based standards curves (C and D) were determined by regression analysis using the crossing point (CP) values from duplicate reactions.

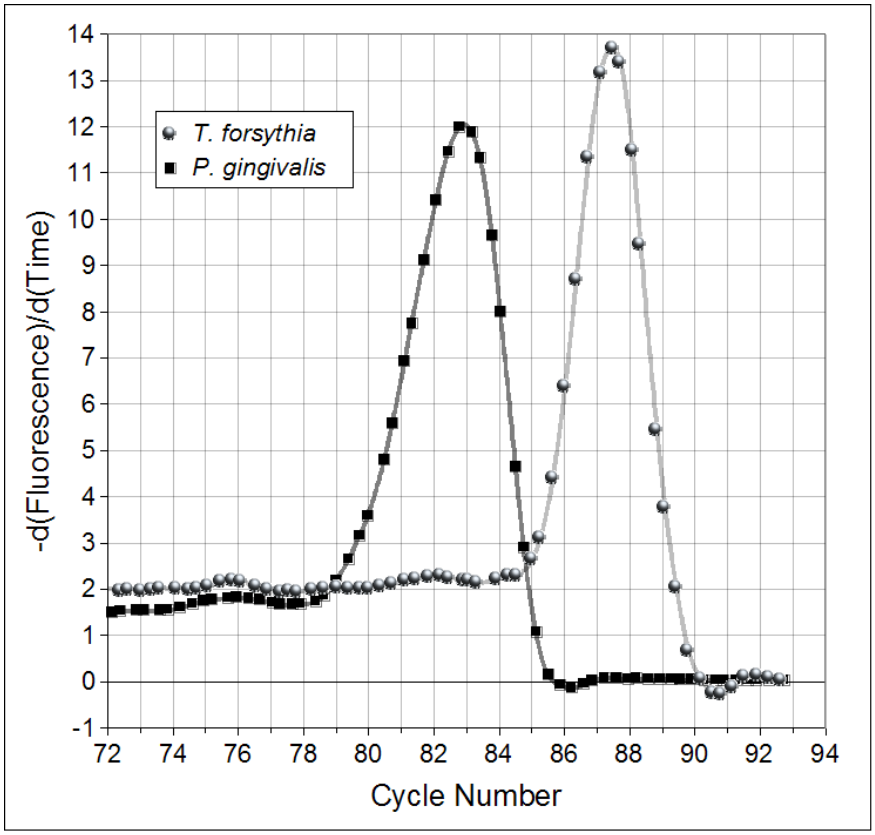

Figure 2. Melting profiles of Real Time PCR amplified standard DNA. The expected melting temperatures for *P. gingivalis* (*rgp*) and *T. forsythia* (*bspA*) were 82.8 °C and 87.5 °C, respectively.

CAPÍTULO 3

Trabalho apresentado no 24º. Congresso Brasileiro de Microbiologia (Out/2007) (Anexo 4). Artigo em vias de submissão ao periódico *Journal of Clinical Microbiology*

Intraradicular bacterial composition by Terminal Restriction Length Polymorphism (T-RFLP) analysis

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Running title: Intraradicular bacteria by T-RFLP

Introduction

Under physiologic conditions, the dental pulp is free of any residing microorganisms. Nonetheless, physical or chemical trauma or caries infection can eventually involve the pulp tissue, leading to necrosis and subsequent endodontic infection. The necrotic pulp provides a selective habitat for the establishment of a complex microbiota, predominantly composed of strictly anaerobic bacterial species (Sundqvist 1992a). Bacteria residing in the endodontic milieu are able to engender destructive inflammatory responses in the perirradicular tissues, which in turn can give rise to periapical lesions (Stashenko *et al.* 1992) and important systemic complications (Bate *et al.* 2000, Breebaart *et al.* 2002). Elimination of bacteria from inside the canal is, therefore, a crucial goal in endodontic therapy.

Albeit substantial efforts have been taken towards the throughout characterization of the endodontic microbiota (Saito *et al.* 2006, Gomes *et al.* 2004, Sundqvist 1992b, Munson *et al.* 2002, Rolph *et al.* 2001), the intraradicular ecosystem still harbors plenty microbial complexity to warrant further investigations by contemporary methodologies. Previous reports have demonstrated that particular bacterial species can be responsible for the development of endodontic related signs and symptoms such as spontaneous and pre-operative pain, swelling, odor, tenderness to percussion and purulent exudate (Foschi *et al.* 2005, Gomes *et al.* 2004, Jacinto *et al.* 2003, Peters *et al.* 2002, Fouad *et al.* 2002, Hashioka *et al.* 1992). In light of these observations, it is reasonable to assume that the root canal bacterial communities can also encompass, as a whole, an important role in the development of clinical features. Beyond doubt, the application of microbial ecology tools that allow for a broader view on community structure could bring important information to the study of the root canal microbiota.

Community structure analysis is a microbial characterization approach that relies on two fundamental variables: species richness (the number of species in the community) and species evenness (the quantity of each species) (Liu *et al.* 1997). Assessment of these variables is impaired in traditional culture-based techniques, since cultivation can shift the original microbial composition by imposing additional selective pressures (Liu *et al.* 1997). In addition, approximately 50% of the oral bacteria cannot undergo cultivation (Paster *et al.* 2001, Aas *et al.* 2003) and, in this sense, community structure studies should be preferably conducted by molecular techniques capable of accounting the very fastidious and non-culturable taxa.

Terminal Restriction Length Polymorphism (T-RFLP) offers high-throughput quantitative analysis of community structure and community dynamics in complex environments (Blackwood *et al.* 2003, Osborn *et al.* 2000). In brief, the technique employs PCR targeting the bacterial 16S rRNA gene, in which one of the markers is fluorescently labeled at its 5' end. The PCR product is

digested with an endonuclease with a 4-base pair recognition site, and the terminal restriction fragment is measured by an automated DNA sequencer (Marsh 2005, Marsh *et al.* 1999, Liu *et al.* 1997). Since different bacterial populations have distinct restriction recognition sites in the 16S rRNA gene, a genetic fingerprint of bacterial composition is obtained for each polymicrobial sample. T-RFLP has been successfully applied for comparing the bacterial composition of saliva from healthy and periodontal patients (Sakamoto *et al.* 2003), assessing shifts in microbial profiles after periodontal treatment (Sakamoto *et al.* 2004), investigating the effect of coronal restoration quality on endodontic bacterial composition (Hommez *et al.* 2004), and for comparing the bacterial communities of intraradicular samples and pus aspirates from symptomatic and asymptomatic teeth (Sakamoto *et al.* 2006).

In this study, the T-RFLP technique was applied to assess the intraradicular bacterial composition associated with asymptomatic, tender, and symptomatic endodontic infections, and to contrast the community structures associated with these three clinical categories.

Material and Methods

Subjects. Twenty five patients, 12 males and 13 females, ranging from 15 to 61 years old (mean 32.4± 13.7) were analyzed. Working approval was granted by the Ethical Committee for Human Subjects of the Piracicaba Dental School, State University of Campinas and written consent was required from all participants. Subjects harbored teeth with endodontic infections, and had been previously referred for endodontic treatment at the Piracicaba Dental School. Inclusion criteria included single or multi-rooted teeth with pulpal necrosis, with no distinction to the cause of endodontic involvement (caries or trauma). Teeth were evaluated for the presence of pulpal necrosis by visual inspection, probing, percussion, and radiographic evaluation. Subjects who had undergone antibiotic therapy within two months prior to collection or those who had been previously subjected to root canal treatment were not included. Specimens were classified in three study groups: asymptomatic (Group I, n= 7), tender (Group II, n= 5) and symptomatic (Group III, n= 13). Group I was composed of asymptomatic teeth, Group II of teeth tender to vertical percussion, and Group III of teeth harboring spontaneous pain with or without associated swelling or purulent exudate.

Sample collection. Each patient was submitted to local anesthesia and the tooth was isolated with a rubber dam. The crown was cleaned to eliminate debris and dental plaque. Antisepsis of the crown and operation field was conducted with 2.5% sodium hypochlorite for 1 min, followed by inactivation with 5% sodium thiosulfate (Ng *et al.* 2003). Coronal access cavity was gained by high-speed bur irrigated with sterile saline solution. Intraradicular samples were obtained by the

introduction of a sterile #15 K-file followed by 4 consecutive sterile paper points inside the root canal (Saito *et al.* 2006). The file and the paper points were placed in a test tube containing 1 mL of TE buffer (10 mM TrisHCl, 1 mM EDTA, 0.1 mM NaCl, pH 8.0) and immediately transported to the laboratory.

DNA extraction. The DNA from the clinical samples and reference bacteria were extracted as described elsewhere (Saito *et al.* 2006) and ressupended in 30 □L of TE buffer, being immediately stored at – 20 ºC until further analysis.

Primer set selection. The Primer Sequence Prevalence Analysis program (MICA3 - http://mica.ibest.uidaho.edu/primer.php) was used to verify the range of detection of all possible combinations of the primers 27F, 63F, 1389R, 1392R and 1492R against the 16S rDNA Ribosomal Database Project II Release 9.37, with 1 mismatch allowed within 10 bases from the 5' end of either primer. The primer set 63F (5'- CAGGCCTAACACATGCAAGTC -3') and 1389R (5'- ACGGGCGGTGTGTACAAG -3') revealed the highest number of positive matches, and therefore was chosen for subsequent T-RFLP analyses. The 63F primer was labelled with 6'-carboxyfluorescein (6-FAM) at its 5' end to allow fluorescent detection of the terminal restriction fragment.

Amplification of DNA. 100 µL reactions were performed with 60 ng DNA, 1x PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.4 µM primers (IDT Technologies) and 1.0 U *Taq* DNA polimerase (Invitrogen Co.), according to the following temperature profile: initial denaturation at 94 °C for 3 min; 25 cycles of denaturation at 94 °C for 1 min, annealing at 56°C for 1 min and extension at 72 °C for 2min; final extension at 72 °C for 10 min. 3 µL aliquots of each PCR product was submitted to 1.0% agarose gel electrophoresis and stained with ethidium bromide. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), as stated by the manufacturer.

Endonuclease restriction of amplified DNA. 200 ng of each purified product were restricted with *Hha*I (GCG'C) and *Msp*I (C'CGG) endonucleases (New England Biolabs Inc.) in separate 15 µL reactions as instructed by the manufacturer. Aliquots of the original PCR products were left undigested and used as negative controls for T-RFLP analysis.

Generation of T-RFLP profiles: 2 uL of each restricted PCR product and the respective negative control were mixed with 7 uL deionized formamide and 1 uL MM1000 ROX size standard (Bioventures Inc.), denatured at 95 °C and immediately placed on ice. Duplicate mixtures were injected for 30 s into an ABI 3100 Sequence Analyzer (Applied Biosystems - Hitachi). T-RLFP profiles were generated by the Local Southern Method in GeneScan 3.7 Software (Applied Biosystems), using a 5 fluorescence units threshold.

Filtering and binning of T-RFLP profiles. T-RFLP data from duplicates were exported as spreadsheets, filtered by the IBEST analysis tools (Abdo *et al.* 2006) using a cutoff value of 6 standard deviations of total peak area, and binned (aligned) by the T-Align software (Smith *et al.* 2005) with a 1.0 base width. T-Align was also used to generate consensus profiles based on the average fluorescence intensities of corresponding peaks from the duplicates. Peaks that appeared exclusively in one of the duplicates were not included in the final consensus profiles. Peak height data were transformed into relative peak heights, as a ways to level up total fluorescence among samples after the removal of analytical noise.

Data analysis. Total, unique, single and double T-RFs were determined for each endonuclease with the EstimateS 8.0 software (Colwell 2006). The hypothesis that the T-RF richness among study groups was not statistically different was assessed with the T test with a significance $\alpha = 0.05$. A Bray-Curtis dissimilarity matrix from the combined (concatenated) *Hha*I and *Msp*I relative peak height data sets was obtained with the Vegan package for R (www.r-project.org) and used for cluster analysis with the MEGA 4 software (Tamura *et al.* 2007) according to the Unweighted Pair Group Method with Arithmetic mean (UPGMA). Principal Coordinates Analysis (PCoA) was accomplished based on the Bray-Curtis dissimilarity matrix from the previous step with the Canoco 4.5 software (Biometris). A quantitative display of relative peak heights from the consensus profiles of all study subjects was obtained with the Cluster 3.0 and TreeView 1.1 softwares for microarray data analysis (Eisen *et al.* 1998). T-RFs present in more than 30% of the subjects were predicted by the TAP-T-RFLP program (Marsh *et al.* 2000) based on a 16S rDNA datafile from most oral bacterial species (TRFMA; Nakano *et al.* 2006), adopting an error interval of 1 base pair. When no particular taxa could be predicted with this datafile, the 16S rDNA Ribosomal Database Project II Release 9.47 (http://rdp.cme.msu.edu) containing 16S rDNA sequences from 63,638 bacteria was used instead.

Results

All 25 samples were positive for bacteria, as evidenced by PCR with primers FAM-63F and 1389R. T-RFLP analysis with *Hha*I and *Msp*I endonucleases revealed a total of 520 *Hha*I terminal restriction fragments (T-RFs$^{(H)}$) and 501 *Msp*I terminal restriction fragments (T-RFs$^{(M)}$) in 25 cases of endodontic infections, with 123 unique T-RFs$^{(H)}$ and 122 unique T-RFs$^{(M)}$. T-RF richness per subject varied from 8 to 34 for *Hha*I, and from 8 to 32 for *Msp*I analyses (table 1). No statistical significant differences of T-RF richness among the study groups was observed with either endonuclease, according to the T test (groups I and II: *Hha*I p= 0.12, *Msp*I p= 0.57; groups I and III: *Hha*I p= 0.81, *Msp*I p= 0.40; groups II and III: *Hha*I p= 0.19, *Msp*I p= 0.96).

UPGMA analysis of combined *Hha*I and *Msp*I profiles exhibited no evident tendency for clustering according to the predefined study groups (figure 1). Similarly, PCoA multivariate analysis did not reveal any distinct grouping of samples in broad, or as a function of the study groups (figure 2). The quantitative display (heatmap) of relative peak heights obtained with both endonucleases, along with TAP-T-RFLP prediction of the fragments found in more than 30% of the subjects, is displayed in figure 4.

*Hha*I and *Msp*I endonucleases presented, in general, very similar resolving powers (table 1). Nonetheless, when comparing the results among study groups, slightly different behaviors could be observed: *Msp*I revealed 11 (15%) more unique T-RFs than *Hha*I in Group I, while *Hha*I revealed 8 (13%) more unique T-RFs than *Msp*I in Group II (table 1). The application of the T test demonstrated that such differences in enzyme efficiencies were not statistically significant, either within study groups (Group I p= 0.48, Group II p=0.26, Group II p= 0.76), or when considering the whole sample set (p= 0.67). With the exception of some highly predominant taxa, the observed bacterial assemblages were markedly variable. This is more easily conceived by the fact that 60/123 (48.8%) T-RF$^{(H)}$s and 63/122 (51.6%) T-RF$^{(M)}$s were detected in no more than 2 subjects (single plus double T-RFs) (table 1). In other words, when considering presence/absence data, half of the terminal fragments were very rare in our sample set and, therefore, could have accounted for most of the inter-subject variability observed. Highly predominant T-RFs that could be detected in more than half of the subjects included *Hha*I terminal restriction fragments T-RF$^{(H)}$59 (*Pedobacter* sp. oral clone AV100, *Flavobacterium*-like sp. oral clone AZ123), T-RF$^{(H)}$62 (*Tannerella forsythia*, *Campylobacter* spp.), T-RF$^{(H)}$64 (*Bacteroides* spp.), T-RF$^{(H)}$343 (*Selenomonas* sp. oral clone EZ011), T-RF$^{(H)}$514 (*Heliobacterium* spp., Uncultured *Chloroflexi* bacterium) and T-RF$^{(H)}$556 (*Veillonella* spp.), and *Msp*I terminal restriction fragments T-RF$^{(M)}$56 (*Capnocytophaga* spp., *Dialister* sp. oral clone BS095), T-RF$^{(M)}$183 (Uncultured bacterium), T-RF$^{(M)}$245 (*Fusobacterium* spp., *Leptotrichia* spp., *Mogibacterium* spp.), T-RF$^{(M)}$258 (*Selenomas* sp. oral clone JI021), T-RF$^{(M)}$266 (*Peptococcus*-like sp. oral clone I070, *Actinomyces* spp.), T-RF$^{(M)}$267 (*Selenomonas* spp., *Veillonella* spp., *Actinomyces* spp.) and T-RF$^{(M)}$464 (*Peptostreptococcus* sp. oral clone CK035).

Discussion

The intraradicular microbial communities have been extensively investigated by culture-based (Sundqvist 1992b, Gomes *et al.* 2004, Jacinto *et al.* 2003) and molecular-based techniques, including conventional PCR (Fouad *et al.* 2002, Conrads *et al.* 1997), Real Time PCR (Vianna *et al.* 2006), DNA-DNA checkerboard hybridization (Siqueira *et al.*), DGGE (Siqueira *et al.*

2004), and 16S rDNA clone libraries (Saito *et al.* 2006, Munson *et al.* 2002, Rolph *et al.* 2001). Terminal Restriction Length Polymorphism (T-RFLP) analysis brings important contribution to the study of the endodontic microbiota, allowing for rapid and efficient determination of community structure, also offering the possibility of taxonomical interpretation of terminal fragments (Marsh *et al.* 2000).

We have chosen primers 63F and 1389R for PCR amplification due to their higher range of detection when compared to other commonly used primers, as verified by computer-assisted preliminary evaluation (data not shown). Selection of this primer set was also favoured by the fact that the great majority of published 16S rDNA sequences are derived from the oligonucleotides 27F and 1492R; hence, th e use of primers that lay internally to such residues may facilitate the *in silico* prediction of T-RFs (Osborn *et al.* 2000). The election of *Hha*I and *Msp*I as our restriction enzymes was founded on their improved efficiency in discriminating T-RFs on the basis of taxonomical information, when compared to other tetrameric endonucleases (Moyer at al. 1996).

An average of 20.8 T-RFs$^{(H)}$ and 20.0 T-RFs$^{(M)}$ per subject was obtained, indicating that, for the analysis of endodontic bacterial communities, both enzymes presented comparable restriction efficiencies. In regard to the taxonomical interpretation of the most prevalent fragments, however, *Hha*I has proved to be more proficient in resolving T-RFs related to *Bacteroides* spp. and *Veillonella* spp., while *Msp*I was more capable of discriminating T-RFs representing *Campylobacter* spp. (figure 4). It is important to emphasize, however, that the number of terminal fragments identified in the present study does not reflect the actual diversity of the microbiota under investigation. In fact, fingerprinting techniques do not encompass sufficient sensitivity to detect the rare taxa, but still compose powerful and efficient tools for comparing biodiversity levels among complex ecosystems (Danovaro *et al.* 2007).

We are aware that T-RF prediction based on fragment length information may, at times, lack sufficient precision for single taxa inference (i.e. one T-RF may correspond to more than one taxonomic unit) (Nakano *et al.* 2006, Marsh 2005). Nonetheless, using a restricted database composed of 16S rDNA sequences from oral bacteria (TRFMA) reduced substantially the number of taxa correspondent to a particular fragment length and, consequently, increased the precision of our taxonomic inference. In reality, *in silico* prediction of the most predominant fragments revealed the presence of strict anaerobic bacteria from the genera *Actinomyces*, *Bacteroides*, *Capnocytophaga*, *Eubacterium*, *Fusobacterium*, *Petptococcus*, *Peptostreptococcus*, *Selenomonas* and *Veillonella* (figure 4), which have been extensively reported as common inhabitants of the root canal microenviroment (Saito *et al.* 2006, Gomes *et al.* 2004, Jacinto *et al.* 2003, Rolph *et al.* 2001, Sundqvist 1992b).

Terminal fragment T-RF$^{(M)}$56 was one of the most prevalent in our study subjects (17/25), and was predicted as *Tannerella forsythia*, a Gram-negative filament-shaped anaerobic bacteria which bears a multi-functional protein responsible for a number of pathogenic properties (Holt *et al.* 2000), and has been considered as a putative periodontal pathogen due to its high prevalence in disease patients (Paster *et al.* 2001). A recent study by Gomes *et al.* (2006) revealed a significant association between *T. forsythia* and tenderness to percussion in primary and secondary endodontic infections. In our investigation, *T. forsythia* was evenly distributed among our study groups and did not display any evident trend towards symptoms. Other highly prevalent terminal fragments present in more than 17/25 (68%) of the subjects included T-RF$^{(M)}$245 (*Fusobacterium* spp., *Leptotrichia* spp., *Microbacterium* sp. oral strain C24KA), T-RF$^{(M)}$258 (*Selenomonas* sp. oral clone JI021), T-RF$^{(H)}$343 (*Selenomonas* sp. oral clone EZ011) and T-RF$^{(H)}$556 (*Veillonella* spp.).

Fragments predicted as *Porphyromonas*-like sp. oral clone DA064 (T-RF$^{(H)}$64) and representatives of *Bacteroides* spp. (T-RF$^{(M)}$58), were detected in 14/25 and 8/25 of the patients, respectively. *Porphyromonas* spp. and *Bacteroides* spp. are putative pathogenic bacteria in endodontic infections, significantly implicated with odor, spontaneous pain and tenderness to percussion (Gomes *et al.* 2004, Jacinto *et al.* 2003, Hashioka *et al.* 1992). Likewise, T-RFs corresponding to *D. pneumosintes* (T-RF$^{(M)}$271) and *Dialister* oral clones BS095 (T-RF$^{(H)}$558 and T-RF$^{(M)}$52), BS016 and MCE7_134 (T-RF$^{(M)}$271) were detected in more than 30% of the cases (figure 4). *Dialister* is a genus composed of anaerobic Gram-negative bacilli and has been progressively reported in both primary (Jacinto *et al.* 2007, Saito *et al.* 2006, Munson *et al.* 2002) and refractory (Rolph *et al.* 2001) endodontic infections by 16S rDNA analysis. Our results accord with previous evidence (Siqueira and Rôças 2002), in the sense that representatives of this genus can be highly frequent in the endodontic milieu.

Restriction fragments TRF$^{(H)}$433 and TRF$^{(H)}$460 were both detected in 11 subjects, out of which 10 either tender or symptomatic. TRF$^{(H)}$433 was presumed as *Campylobacter* sp. oral clones BB120 and HB035, phylotypes originally reported in a case of refractory periodontitis (Paster *et al.* 2001) and on the hard palate of healthy subjects (Aas *et al.* 2005). TRF$^{(H)}$460 was predicted as *Bergeyella* sp. oral clone AK152, a phylotype first reported by B. J. Paster and coworkers in an unpublished work on subgingival plaque (GenBank accession number AY008691), and shares a 99.7% nucleotide identity with *Bergeyella* sp. clone AF14, a recently detected phylotype from the amniotic fluid and subgingival plaque of a patient with preterm birth (Han *et al.* 2006). *Bergeyella zoohelcum*, the genus type species, is found in the oral cavity of dogs and cats and is frequently associated with bite wounds in humans (Reina and Borrell 1992,

Shuckla *et al.* 2004). Taking these evidences together, a pathological role for *Bergeyella* sp. in endodontic infections is conceivable.

In the present study, the weight of the intraradicular bacterial assemblages in the development of tenderness to percussion and spontaneous pain was investigated. Overall, we did not find any significant differences in T-RF richness or community structures among asymptomatic, tender and symptomatic endodontic infections. These observations were coherently achieved by univariate and multivariate statistical analyses and by hierarchical clustering. In this regard, our results discord from those of Sakamoto *et al.* (2006) and Siqueira *et al.* (2004), who detected differences in the bacterial composition of asymptomatic and symptomatic endodontic infections by T-RFLP and DGGE methodologies. However, both authors used specimens from different disease sites (root canals for asymptomatic infections and pus aspirates for symptomatic infections) and Sakamoto *et al.* (2006) used three additional restriction enzymes for T-RFLP analysis, possibly contributing to the observed variations in the bacterial profiles. In our investigation, samples were recovered from a single disease site (root canals). In this context, the inexistence of detectable differences in community structures among the study groups could also be a direct consequence of the more concise sampling strategy adopted herein.

Conclusions

T-RFLP analysis indicated that the bacterial composition of root canal infections is highly variable among individuals, with approximately 50% of the fragments present in no more than 2 of the subjects. Nonetheless, certain taxa displayed ubiquitous presence, namely *Tannerella forsythia*, *Selenomonas* sp. oral clones JI021 and EZ011, and *Veillonella* spp. The inexistence of statistically significant differences in T-RF richness among study groups, along with the lack of clearly visible clusters on hierarchical and multivariate analyses, supports the hypothesis that the intraradicular bacterial community assemblages, in entirety, do not play a role in the development of endodontic symptoms.

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Table 1. Terminal restriction fragments identified in 25 cases of endodontic infections (identified as S1 – S25).

	*Hha*I	*Msp*I
Group I. Asymptomatic (n= 7)		
S2	21	31
S8	8	21
S11	22	15
S15	18	13
S18	26	20
S19	18	27
S22	23	26
Total Group I	136	153
Distinct	73	84
Single*	42	51
Double**	14	15
Mean	19.4	21.9
Group II. Tender (n= 5)		
S5	28	12
S10	27	31
S14	25	25
S17	21	9
S25	21	19
Total Group II	122	96
Distinct	70	62
Single*	38	30
Double**	20	20
Mean	24.4	19.2
Group III. Symptomatic (n= 13)		
S1	11	8
S3	14	16
S4	27	25
S6	17	13
S7	18	23
S9	13	16
S12	21	20
S13	15	22
S16	34	32
S20	26	23
S21	21	20
S23	26	16
S24	19	18
Total Group III	262	252
Distinct	95	93
Single*	40	43
Double**	16	19
Mean	20.1	19.4
Overall Total	520	501
Overall Distinct	123	122
Overall Single*	35	42
Overall Double**	25	21
Overall Mean	20.8	20.0

* Exclusive to one subject; ** Exclusive to two subjects.

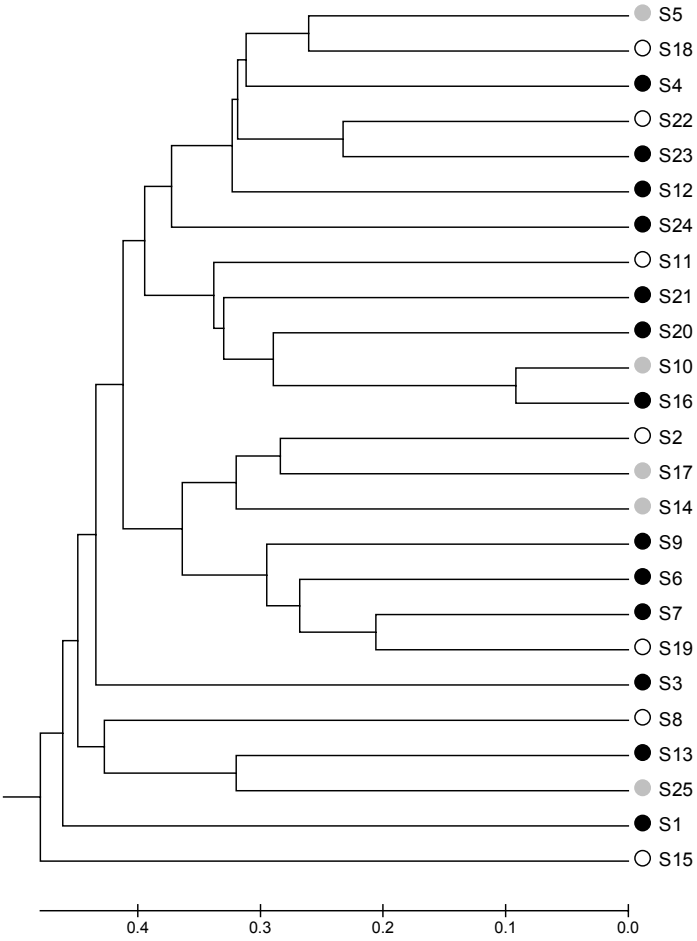

Figure 1. UPGMA dendogram of T-RFLP profiles from 25 cases of endodontic infections. A combined *Hha*I and *Msp*I data set was used to obtain a peak height sensitive Bray-Curtis dissimilarity matrix.

Legend:

Asymptomatic (○)
Tender (•)
Symptomatic (●)

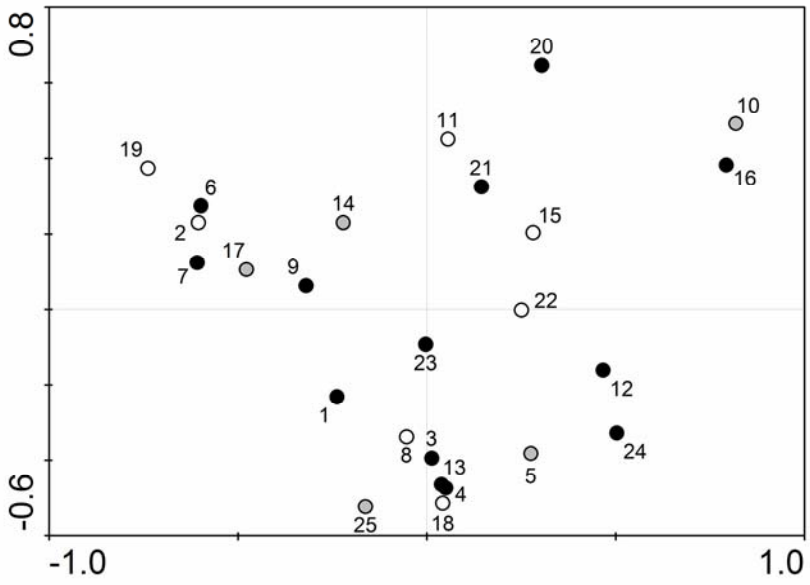

Figure 3. Principal Coordinates Analysis plot (1st and 2nd axes) of T-RFLP profiles obtained from 25 cases of endodontic infections. A combined *Hha*I and *Msp*I data set was used to obtain a peak height sensitive Bray-Curtis dissimilarity matrix.

Legend:

Asymptomatic (○)
Tender (•)
Symptomatic (●)

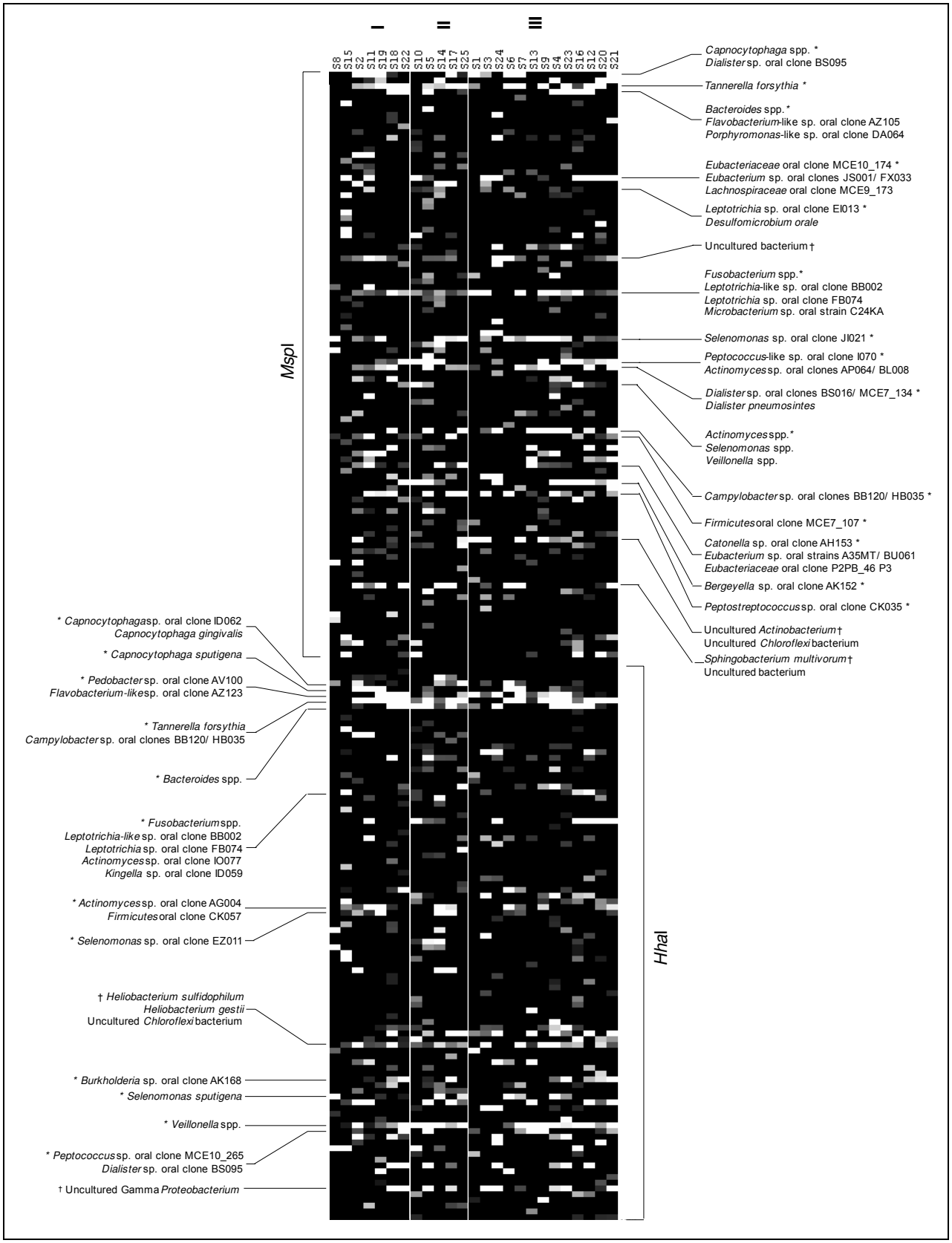

Figure 4. Quantitative display (heatmap) of relative T-RF peak heights obtained with *Hha*I and *Msp*I endonucleases from 25 cases of endodontic infections. TAP-T-RFLP predicted peaks are shown. Subjects are divided according to the observed clinical parameters: I (asymptomatic), II (tender), III (symptomatic).

* Fragment predicted with the TRFMA Oral database

† Fragment predicted with the RDP Release 9.47 database

CONSIDERAÇÕES GERAIS

Na presente tese, a microbiota endodôntica foi avaliada por meio de três técnicas independentes de cultivo laboratorial: análise de bibliotecas clonais 16S rDNA (Fase I), PCR em Tempo Real (Fase II) e T-RFLP (Fase III). As técnicas foram aplicadas em amostras microbiológicas intra-radiculares de naturezas diversas: a análise de bibliotecas clonais foi realizada em amostras assintomáticas não-expostas ao meio oral; PCR em Tempo Real foi aplicada em amostras assintomáticas e sintomáticas; e T-RFLP foi utilizada em amostras assintomáticas, sensíveis à percussão e sintomáticas. Devido aos detalhes técnicos inerentes às diferentes metodologias e grupos de estudo utilizados, o universo amostral das três fases variou consideravelmente: 7 amostras para a Fase I, 34 para a Fase II e 25 para a Fase III.

Em geral, a análise de bibliotecas clonais demonstrou uma predominância de espécies anaeróbias, especialmente as Gram-positivas do filo *Firmicutes*. Apesar da relativa homogeneidade deste grupo de estudo, uma alta variação na composição bacteriana pôde ser observada entre os pacientes avaliados, constatada principalmente pela alta predominância de filotipos restritos a apenas um dos pacientes (77 %). Esta variabilidade na estrutura das comunidades bacterianas intra-radiculares também foi observada pelo emprego da técnica de T-RFLP em um grupo amostral mais heterogêneo (amostras assintomáticas, sensíveis e sintomáticas), ao se constatar que 50% dos fragmentos terminais de restrição (T-RFs) apresentaram-se exclusivos a, no máximo, 2 pacientes.

A técnica de T-RFLP foi aplicada na determinação da riqueza e abundância de componentes bacterianos da microbiota intra-radicular, revelando um total de 122 (enzima *Hha*I) e 123 (enzima *Msp*I) fragmentos terminais de restrição (T-RFs) distintos. Se considerarmos que cada fragmento corresponde a, no mínimo, uma unidade taxonômica distinta (Marsh, 2005), pode-se afirmar que esta técnica permitiu a detecção de um mínimo de 123 filotipos bacterianos distintos. Este valor é significativamente maior que aquele obtido pela análise clonal (46 filotipos). Tal diferença foi também constatada quando se compararam as médias de unidades taxonômicas detectadas pelos dois estudos: 9,6 pela análise clonal, contra 20,4 por T-RFLP. Os diferentes tamanhos dos universos amostrais (7 *versus* 25) podem ter contribuído para a constatação destas diferenças. No entanto, como os *primers* 16S rDNA utilizados nas duas técnicas não foram os mesmos, parte das diferenças observadas pode ser explicada também por possíveis diferenças nas abrangências de detecção destes marcadores.

Uma parcela dos gêneros bacterianos identificados (preditos) pela análise em T-RFLP foi corroborada pela análise clonal como, por exemplo, *Bacteroides*, *Burkholderia*, *Campylobacter*, *Dialister*, *Eubacterium*, *Peptostreptococcus*, *Selenomonas* e *Veillonella*. Algumas exceções foram

constatadas, como *Actinomyces*, *Bergeyella*, *Capnocytophaga*, *Mogibacterium*, *Peptococcus*, identificados somente por T-RFLP e *Catabacter*, *Desulfobulbus*, *Filifactor*, *Lactobacillus*, *Megasphaera* e *Treponema*, detectados somente pela análise clonal. Visto que as duas técnicas possuem sensibilidades e resoluções taxonômicas distintas (Danovaro *et al.*, 2007) e devido ao fato de diferentes marcadores universais haverem sido empregados, os resultados obtidos pelas duas técnicas devem ser, preferencialmente, considerados em conjunto, e não contrastados.

O emprego de *primers* espécie-específicos direcionados aos fatores de patogenicidade *rgp* e *bspA* em PCR em Tempo Real permitiu a detecção de *P. gingivalis* e *T. forsythia* em 24% e 56% dos pacientes, respectivamente. Entretanto, nenhuma destas espécies foi observada pela análise clonal, enquanto que apenas *T. forsythia* pôde ser predita por T-RFLP. Diferenças significativas são, em realidade, esperadas quando se empregam diferentes técnicas de detecção em amostras intra-radiculares (Gomes *et al.*, 2005; Siqueira *et al.*, 2002; Rolph *et al.*, 2001). Realmente, a técnica de PCR em Tempo Real possui sensibilidade de detecção muito superior àquelas obtidas pelo cultivo ou pela PCR convencional, sendo capaz de diferenciar uma amostra negativa de outra contendo apenas 1 cópia do DNA-alvo (Morrison *et al.*, 1988). É possível, contudo, que o uso de primers espécie-específicos tenha contribuído substancialmente na detecção de *P. gingivalis* e *T. forsythia*, visto que os *primers* 16S rDNA universais, comumente utilizados em estudos de detecção de bactérias orais, não englobam todos os grupos bacterianos (Horz *et al.*, 2005).

Levando-se em consideração os resultados das três metodologias aplicadas nesta tese, pode-se afirmar que as técnicas moleculares independentes de cultivo demonstraram alta aplicabilidade no estudo das comunidades bacterianas associadas às infecções endodônticas. Em termos gerais, a análise de bibliotecas clonais de 16S rDNA possibilitou a detecção de novos filotipos da ordem *Clostridiales*, a PCR em Tempo Real demonstrou alta sensibilidade na detecção espécie-específica de dois importantes patógenos orais, enquanto que T-RFLP permitiu a caracterização global das estruturas bacterianas de forma abrangente e eficiente. Apesar de diferentes conjuntos de *primers* 16S rDNA haverem sido utilizados, notou-se uma relativa concordância entre as técnicas de análise de bibliotecas clonais e de T-RFLP, na medida em que demonstraram a existência de uma alta variabilidade na composição das comunidades bacterianas intra-radiculares e a detecção de unidades taxonômicas reconhecidamente endodônticas.

CONCLUSÕES

Com base nos resultados obtidos, pode-se concluir que:

1) A microbiota associada às infecções endodônticas é essencialmente polimicrobiana e composta, predominantemente, por bactérias anaeróbias Gram-positivas do filo *Firmicutes*.

2) Existe uma alta variabilidade na composição das comunidades bacterianas intra-radiculares entre diferentes indivíduos, tanto em relação à riqueza de espécies, como em relação aos tipos taxonômicos presentes.

3) Muito embora *P. gingivalis* e *T. forsythia* encontrem-se em consideráveis prevalências nas amostras intra-radiculares, os seus respectivos níveis celulares não apresentam correlação significativa com o desenvolvimento de sintomatologia dolorosa. O mesmo foi observado com respeito aos níveis celulares integrados (coexistência) de ambas as espécies.

4) A ausência de agrupamentos definidos de perfis bacterianos, segundo os parâmetros sintomatológicos, sugere que a estrutura das comunidades bacterianas intra-radiculares, em termos globais, não possui influência significativa no desenvolvimento da dor ou da sensibilidade de origem endodôntica.

Em suma, os resultados evidenciam que as técnicas moleculares permitem uma visão globalizada da composição microbiana que dificilmente seria obtida por técnicas tradicionais e que, portanto, possuem alta aplicabilidade na caracterização das comunidades bacterianas associadas às infecções endodônticas. A alta diversidade na estrutura das comunidades observadas e a detecção de filotipos nunca relatados em estudos anteriores sugerem que a microbiota endodôntica ainda requer caracterização adicional por técnicas contemporâneas.

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ANEXO 1

INFORMAÇÃO CCPG/OO2/06[6]

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG n° 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2° desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

Profa. Dra. Teresa Dib Zambon Atvars
Presidente
Comissão Central de Pós-Graduação

ANEXO 2

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Identification of bacteria in endodontic infections by sequence analysis of 16S rDNA clone libraries

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A significant proportion of oral bacteria are unable to undergo cultivation by existing techniques. In this regard, the microbiota from root canals still requires complementary characterization. The present study aimed at the identification of bacteria by sequence analysis of 16S rDNA clone libraries from seven endodontically infected teeth. Samples were collected from the root canals, subjected to the PCR with universal 16S rDNA primers, cloned and partially sequenced. Clones were clustered into groups of closely related sequences (phylotypes) and identification to the species level was performed by comparative analysis with the GenBank, EMBL and DDBJ databases, according to a 98 % minimum identity. All samples were positive for bacteria and the number of phylotypes detected per subject varied from two to 14. The majority of taxa (65·2 %) belonged to the phylum *Firmicutes* of the Gram-positive bacteria, followed by *Proteobacteria* (10·9 %), *Spirochaetes* (4·3 %), *Bacteroidetes* (6·5 %), *Actinobacteria* (2·2 %) and *Deferribacteres* (2·2 %). A total of 46 distinct taxonomic units was identified. Four clones with low similarity to sequences previously deposited in the databases were sequenced to nearly full extent and were classified taxonomically as novel representatives of the order *Clostridiales*, including a putative novel species of *Mogibacterium*. The identification of novel phylotypes associated with endodontic infections suggests that the endodontium may still harbour a relevant proportion of uncharacterized taxa.

INTRODUCTION

Although more than 150 species of bacteria have been identified in infected root canals, only a restricted number can be found simultaneously in the same tooth and a considerable variation of species is expected when analysing distinct clinical conditions, individuals or populations (Sundqvist, 1976; Molander *et al.*, 1998; Baumgartner *et al.*, 2004). Cultivation studies have shown a predominance of facultative and strict anaerobes in the endodontium, including representatives of *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella* and *Streptococcus* (Sundqvist, 1992b; Le Goff *et al.*, 1997). Bacteria inside the canal are the major cause of periapical pathologies (Kakehashi *et al.*, 1965) and, if not adequately treated, can give rise to dentoalveolar abscess, a condition that has ability to initiate morbidity, life-threatening illness (Walsh, 1997), and to predispose to transient bacteraemia during therapy (Savarrio *et al.*, 2005). Previous reports suggested that endodontic bacteria might be involved in extra-oral complications, such as chronic maxillary sinusitis (Melen *et al.*, 1986), orbital cellulitis (Ngeow, 1999), infective endocarditis (Bate *et al.*, 2000), rheumatoid arthritis (Breebaart *et al.*, 2002) and brain abscess (Henig *et al.*, 1978). In this regard, substantial understanding of the endodontic microbiota is an important requirement for both oral and medical microbiologists.

While it is common knowledge that the development of efficient treatment strategies relies on the characterization of the endodontic microbial communities in their entirety, cultivation-based techniques may cut down the range of detection, since a subset of oral inhabitants still cannot

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of uncultured *Clostridiales* bacterium clone AG_D03, uncultured *Clostridiaceae* bacterium clone AG_G04, uncultured *Streptococcaceae* bacterium clone AF_F05 and uncultured *Mogibacterium* sp. clone AF_H06 are AY821867, AY821868, AY821869 and AY821870, respectively.

PCR results with reference bacteria are available as supplementary material in JMM Online.

ANEXO 3

ORAL MICROBIOLOGY AND IMMUNOLOGY

Submission Confirmation

Thank you for submitting your manuscript to *Oral Microbiology and Immunology*.

Manuscript ID:	OMI-2007-0151
Title:	Real Time PCR quantitative detection of *Porphyromonas gingivalis* and *Tannerella* i primary endodontic infections
Authors:	Saito, Daniel Saito, Cristiane Coutinho, Luiz Cannavan, Fabiana Tsai, Siu Höfling, José Goncalves, Reginaldo
Date Submitted:	09-Oct-2007

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CERTIFICADO

Certificamos que **Análise Estrutural da Microbiota de Infecções Endodônticas por Análise de Polimorfismos de Comprimento de Fragmentos Terminais Restringidos (T-Rflp)** participou na qualidade de Pôster, sendo apresentado por **Daniel Saito**, tendo como co-autores Reginaldo Bruno Gonçalves; Terence L. Marsh; José Francisco Hofling no "**24º CONGRESSO BRASILEIRO DE MICROBIOLOGIA**", no período de 03 a 06 de Outubro de 2007, em Brasília/DF.

Brasília, 06 de Outubro de 2007.

Marina Baquerizo Martinez
Presidente SBM

Mariza Landgraf
Vice - Presidente SBM

SBM Sociedade Brasileira de Microbiologia

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CERTIFICADO

Certificamos que o Projeto de pesquisa intitulado "Detecção e identificação molecular de bactérias em canais radiculares infectados", sob o protocolo nº **108/2002**, do Pesquisador ***Daniel Saito***, sob a responsabilidade do Prof. Dr. ***Reginaldo Bruno Gonçalves*** e ***Renato de Toledo Leonardo***, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa – FOP.

Piracicaba, 06 de novembro de 2002

We certify that the research project with title "Molecular detection and identification of bacteria in infected root canals", protocol nº **108/2002**, by Researcher ***Daniel Saito***, responsibility by Prof. Dr. **Reginaldo Bruno Gonçalves** and **Renato de Toledo Leonardo**, is in agreement with the Resolution 196/96 from National Committee of Health/Health Department (BR) and was approved by the Ethical Committee in Research at the Piracicaba Dentistry School/UNICAMP (State University of Campinas).

Piracicaba, SP, Brazil, November 06 2002

Prof. Dr. Pedro Luiz Rosalen
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Antonio Bento Alves de Moraes
Coordenador
CEP/FOP/UNICAMP