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"Análise microbiológica de infecções endodônticas utilizando a associação das técnicas do" Multiple Displacement Amplification" (MDA) e da Hibridização DNA-DNA ("Checkerboard") "

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RESUMO

Resumo

A técnica do “Multiple Displacement Amplification” (MDA) vem sendo utilizado para amplificar uniformemente o genoma bacteriano presente em pequenas amostras, fornecendo grandes melhorias nas análises moleculares. O propósito desta pesquisa foi associar o MDA e a hibridização DNA-DNA (“checkerboard”) para examinar a microbiota de infecções endodônticas. Sessenta e seis amostras foram coletadas de infecções endodônticas. As amostras não amplificadas e aquelas amplificadas pelo MDA foram analisadas pelo “checkerboard” para a determinação dos níveis e proporções de 77 taxas bacterianas. Computaram-se a contagem, percentagem do total de DNA e percentagem de dentes colonizados para cada espécie em amostras amplificadas e não amplificadas. As diferenças significantes para cada espécie entre as amostras amplificadas e não amplificadas foram determinadas utilizando-se o teste de Wilcoxon e ajustado para comparações múltiplas. A quantidade média de DNA presente nas amostras clínicas variou de 6,80 (± 5,2) ng sem amplificação a 6,26 (± 1,73) μg após a utilização do MDA. Setenta das 77 sondas de DNA hibridizaram com uma ou mais das amostras não amplificadas, enquanto todas as sondas hibridizaram com no mínimo uma amostra após a amplificação. As espécies mais comumente detectadas no nível > 10^4 células bacterianas, nas amostras amplificadas e não amplificadas, foram *Prevotella tannerae* e *Acinetobacter baumannii* numa freqüência que variou de 89-100% das amostras. O número médio (± SEM) de espécies nas contagens >10^4 células bacterianas, nas amostras amplificadas, foi de 51,2 ± 2,2 e, nas não amplificadas, foi de 14,5 ± 1,7. A combinação do MDA e da hibridização DNA-DNA (“checkerboard”) demonstrou a presença de uma grande variedade de espécies bacterianas nas amostras

endodônticas demonstrando sua utilidade naqueles estudos que avaliam a microbiota presente nas infecções endodônticas.

Palavras chaves: Infecção endodôntica, bactérias, sondas de DNA, “Multiple Displacement Amplification” (MDA), hibridização DNA-DNA (“checkerboard”).

ABSTRACT

abstract

"THE USE OF MULTIPLE DISPLACEMENT AMPLIFICATION AND CHECKERBOARD DNA-DNA HYBRIDIZATION TO EXAMINE THE MICROBIOTA OF ENDODONTIC INFECTIONS".

Multiple Displacement Amplification (MDA) has been used to uniformly amplify bacterial genomes present in small samples, providing abundant targets for molecular analysis. The purpose of this investigation was to combine MDA and checkerboard DNA-DNA hybridization to examine the microbiota of endodontic infections. 66 samples were collected from teeth with endodontic infections. Non-amplified and MDA amplified samples were analyzed by checkerboard DNA-DNA hybridization for levels and proportions of 77 bacterial taxa. Counts, % DNA probe counts and % of teeth colonized for each species in amplified and non-amplified samples were computed. Significance of differences for each species between amplified and non- amplified samples was determined using the Wilcoxon signed ranks test and adjusted for multiple comparisons. The average amount of DNA present in clinical samples ranged from 6.80 (± 5.2) ng before to 6.26 (± 1.73) μg after MDA. 70 of the 77 DNA probes hybridized with one or more of the non-amplified samples, while all probes hybridized with at least one sample after amplification. Most commonly detected species at levels > 10^4 in both amplified and non-amplified samples were *Prevotella tannerae* and *Acinetobacter baumannii* at frequencies ranging from 89-100% of samples. The mean number (± SEM) of species at counts >10^4 in amplified samples was 51.2 ± 2.2 and in non-amplified samples was 14.5 ± 1.7. The combination of MDA and checkerboard DNA-DNA hybridization demonstrated the presence of wide range of bacterial species in endodontic samples and could facilitate studies evaluating the microbiota of endodontic infections.

Key words: Endodontic infection, bacteria, DNA probe, Multiple Displacement, Amplification, checkerboard DNA-DNA hybridization.

INTRODUÇÃO

Introdução

As alterações pulpares e perirradiculares são normalmente induzidas como resultado do envolvimento direto ou indireto das bactérias da cavidade oral (Kakehashi et al., 1965; Sundqvist, 1976; Fabricius et al., 1982).

Apesar de hoje se estimar que na cavidade oral existam mais de 700 espécies bacterianas (Paster et al., 2001), em condições normais os sistemas de canais radiculares (SCR) não apresentam uma microbiota residente. Entretanto, nos dentes cujo suprimento vascular encontra-se comprometido, o ambiente torna-se favorável à contaminação por patógenos oportunistas (Baumgartner & Falkler, 1991; Sundqvist, 1992).

No passado, os estudos relacionados à microbiota endodôntica, utilizavam técnicas de cultura pouco desenvolvidas, que favoreciam o crescimento de espécies aeróbias e ou facultativas (Hamp, 1957; Leavit et al., 1958). Espécies, como os *Streptococcus*, eram favorecidas enquanto outras espécies anaeróbias, como os *Bacteroides* (*Prevotella* e *Porphyromonas*), eram ignoradas. O *Staphylococcus aureus* e *Streptococcus* β hemolíticos eram isolados com menor freqüência (Seltzer & Farber, 1994).

Com o desenvolvimento das técnicas de coleta, transporte e cultivos dos isolados pôde-se observar que a verdadeira infecção endodôntica é constituída, em sua maioria, por espécies anaeróbias estritas (Bergenholtz, 1974; Sundqvist, 1976; Gomes, 1996, Lana et al., 2001). A diversidade das infecções endodônticas tem sido estabelecida com uma média de 3 a 12 espécies por canal (Sundqvist, 1992; Gomes et

al., 1994; Lana et al., 2001), dentre elas, as espécies mais prevalentes são pertencentes aos gêneros: *Peptostreptococcus*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Eubacterium*, *Actinomyces* e *Streptococcus* (Sundqvist, 1994). Acreditava-se que o baixo número de espécies encontradas nas infecções endodônticas comparado ao grande número de espécies recuperadas nas bolsas periodontais, se devia às pressões seletivas que ocorrem nos SCR infectados (Sundqvist, 1992).

Nos anos recentes, com o advento das técnicas de biologia molecular, houve uma melhora significativa na sensibilidade, especificidade e custo benefício das análises microbiológicas associadas a cavidade oral (Socransky et al., 1998; Siqueira et al., 2000a, 2001a, 2002, 2005; Roças et al., 2001; Fouad et al., 2002; Baumgartner, 2004; Kawada et al., 2004; Siqueira & Roças, 2004; Socransky et al., 2004; Foshi et al., 2005; Dahlén & Leonhardt, 2006; Sakamoto et al., 2006; Seol et al., 2006). Muitas espécies microbianas, que antes acreditavam não estarem presentes nestas infecções, são hoje detectadas e confirmadas como integrantes desta microbiota (Paster et al., 2001). Como exemplo de tal fato, a presença de espiroquetas nas infecções endodônticas, antes raramente detectadas, têm sido frequentemente identificadas: *Treponema dentícola*, *T. pectinovorum*, *T. socransckii* e *T. vicentii* (Dahle et al., 1996; Chan & McLaughlin, 2000; Siqueira et al., 2000b, 2001b; Roças et al., 2001, 2003; Dahle et al., 2003).

Das mais de 700 espécies microbianas que habitam a cavidade oral humana, 50% destas permanecem ainda hoje não cultivadas (Paster et al., 2001, 2002). Bactérias orais têm sido implicadas em doenças como a endocardite bacteriana (Berbari et al., 1997), as osteomielites em crianças (Dodman et al., 2000), as doenças respiratórias (Scannapieco, 1999) e as doenças cardíacas (Beck et al., 1996). Conseqüentemente, o

conhecimento da microbiota presente nestas infecções, bem como sua taxonomia, se faz necessário para o seu diagnóstico e tratamento adequados.

A habilidade dos microrganismos em se implantar ou não em certos sítios do hospedeiro dependerá, além de outros fatores, de seu número, virulência e da resistência do hospedeiro. Raros são os estudos que quantificaram a microbiota dos SCR infectados, associando as populações bacterianas dominantes às condições clínicas específicas. Isto se deveu à falta de sensibilidade dos métodos de coleta e cultivo, em sítios, por exemplo, como o endodôntico, onde se recuperam amostras extremamente pequenas (Zavistok et al., 1980).

As técnicas de identificação moleculares têm permitido uma avaliação mais completa da microbiota associada às infecções orais (Dahlén & Leonhardt, 2006; Haffajee et al., 2006; Haffajee & Socransky, 2006; Teles et al., 2006). O uso das técnicas baseadas na detecção do DNA permite uma melhor descrição do ecossistema microbiano associado às infecções endodônticas, uma vez que detectam, inclusive, espécies fastidiosas e /ou não cultiváveis (Fouad et al., 2002; Siqueira & Roças, 2004a, 2004b, 2006; Siqueira et al., 2005; Sakamoto et al., 2006; Seol et al., 2006).

Técnicas moleculares, tais como a reação de cadeia da polimerase (PCR) e a hibridização DNA-DNA (“checkerboard”), vêm sendo utilizadas na detecção dos microrganismos presentes nos SCR infectados (Gatti et al., 2000; Siqueira et al., 2000a, 2000b, 2001a, 2001b; Fouad et al., 2002; Siqueira et al., 2002; Siqueira & Roças, 2004a, 2004b, 2006; Roças & Siqueira, 2006). Esta última técnica (“Checkerboard”) permite analisar várias amostras e espécies bacterianas, simultaneamente, em uma simples membrana de nylon (Socransky et al., 1994). Nenhum outro método apresenta o mesmo custo-benefício. Por sua vez, sua eficiência depende da viabilidade do DNA

para que as sondas sejam preparadas, padronizadas e para que as amostras sejam analisadas. Além do mais, o tamanho da amostra bacteriana pode ser um problema, uma vez que seu nível de detecção necessita de amostras que contenham mais de 10^4 UFC.

Mais recentemente, tentando aperfeiçoar a técnica do “checkerboard”, outra técnica, o ”Multiple Displacement Amplification” (MDA), foi preconizada para amplificar o DNA bacteriano previamente à análise pela Hibridização DNA-DNA (Teles et al., 2007). O MDA é um método que pode gerar uma grande quantidade de DNA a partir de pequenas amostras. Esta técnica utiliza a enzima Ø29 DNA polimerase e primers randomizados para amplificar o DNA genômico total. O uso desta enzima assegura um baixo erro de replicação, pois conserva uma cópia acurada da seqüência do DNA original (Esteban et al., 1993), além de eliminar a necessidade dos passos advindos do método de purificação do DNA, que poderiam levar a sua contaminação (Dean et al., 2002; Hosono et al., 2003).

Amostras com pequenas quantidades de DNA, como por exemplo, aquelas de apenas 1 ng, podem ser amplificadas 1000 vezes por esta técnica (MDA). Diferente do PCR, que utiliza sequências específicas, esta técnica permite a amplificação do DNA da amostra de um modo uniforme, com poucos desvios de amplificação. Através da eliminação da ciclagem térmica, o MDA evita artefatos de seqüência que poderiam favorecer a amplificação de uma seqüência sobre outra. A amplificação alcançada pode fornecer material suficiente para que se realizem várias análises de uma mesma amostra (Dean et al., 2002).

A literatura mostra que a utilização do MDA era restrita a amplificação do DNA genômico humano (Bergen et al., 2005; Tzvetkov et a., 2005). Muito pouco se sabia a respeito de sua utilização em amostras bacterianas, menos ainda em infecções orais.

Para ser aplicável ao estudo da microbiologia das infecções orais tornou-se imperativo que o MDA funcionasse preferencialmente em amostras recém recuperadas, mais do que em DNA extraído e purificado. As pesquisas, dentre elas esta, desenvolvidas no The Forsyth Institute (Boston, USA), demonstraram a efetividade desta técnica como ferramenta importante nos estudos das infecções microbianas.

Neste estudo, associou-se o MDA à técnica do “checkerboard” para se avaliar o perfil microbiológico das pequenas amostras obtidas dos canais radiculares infectados de pacientes atendidos na clínica da Disciplina Optativa de Endodontia da FO-UFMG. O grande número de microrganismos detectados nestas amostras, quando amplificadas pelo MDA, abre perspectivas para que novas pesquisas sejam realizadas na área, e possamos, no futuro, compreender mais acuradamente o papel das diferentes espécies microbianas nas infecções endodônticas, o que poderá levar ao desenvolvimento de novas estratégias terapêuticas.

OBJETIVOS

Objetivos

2.1 - Objetivo geral

Avaliar a microbiota dos Sistemas de Canais Radiculares infectados de pacientes atendidos na Clínica da Disciplina Optativa de Endodontia da Faculdade de Odontologia da UFMG, utilizando a associação das técnicas “Multiple Displacement Amplification” (MDA) e a hibridização DNA-DNA (“checkerboard”).

2.2 - Objetivos específicos:

- Identificar os microrganismos prevalentes nas infecções endodônticas;
- Determinar o número médio de espécies presentes nestas infecções;
- Investigar a aplicabilidade do MDA em amplificar a quantidade de DNA recuperado de amostras coletadas de infecções endodônticas;
- Avaliar quantitativamente e qualitavamente as taxas presentes nestas infecções associando o MDA e a hibridização DNA-DNA.

TRABALHO CIENTÍFICO

Trabalho Científico

TRABALHO 1 - “THE USE OF MULTIPLE DISPLACEMENT AMPLIFICATION AND CHECKERBOARD DNA-DNA HYBRIDIZATION TO EXAMINE THE MICROBIOTA OF ENDODONTIC INFECTIONS”.

The use of multiple displacement amplification and checkerboard DNA-DNA hybridization to examine the microbiota of endodontic infections

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Running Title: Microbiota of Endodontic Infections

Key words: Endodontic infection, bacteria, DNA probe, multiple displacement amplification, checkerboard DNA-DNA hybridization

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ABSTRACT:

Introduction: Multiple Displacement Amplification (MDA) has been used to uniformly amplify bacterial genomes present in small samples, providing abundant targets for molecular analysis. The purpose of this investigation was to combine MDA and checkerboard DNA-DNA hybridization to examine the microbiota of endodontic infections.

Methods: 66 samples were collected from teeth with endodontic infections. Non-amplified and MDA amplified samples were analyzed by checkerboard DNA-DNA hybridization for levels and proportions of 77 bacterial taxa. Counts, % DNA probe counts and % of teeth colonized for each species in amplified and non-amplified samples were computed. Significance of differences for each species between amplified and non- amplified samples was sought using the Wilcoxon signed ranks test and adjusted for multiple comparisons.

Results: The average amount of DNA present in clinical samples ranged from 6.80 (± 5.2) ng before to 6.26 (± 1.73) µg after MDA. 70 of the 77 DNA probes hybridized with one or more of the non-amplified samples, while all probes hybridized with at least one sample after amplification. Most commonly detected species at levels > 10^4 in both amplified and non-amplified samples were *Prevotella tannerae* and *Acinetobacter baumannii* at frequencies ranging from 89-100% of samples. The mean number (± SEM) of species at counts >10^4 in amplified samples was 51.2 ± 2.2 and in non-amplified samples was 14.5 ± 1.7.

Conclusions: The combination of MDA and checkerboard DNA-DNA hybridization demonstrated the presence of wide range of bacterial species in endodontic samples and could facilitate studies evaluating the microbiota of endodontic infections.

INTRODUCTION

The microbiology of endodontic infections has been studied for many years (4; 47; 58). However, the association between specific microorganisms found in root canals and the symptoms of endodontic infections is poorly understood. Early studies of the endodontic microbiota indicated a predominance of aerobic and facultative bacterial species (16). This conclusion was questioned by the development of anaerobic culturing techniques which clarified the etiopathogenesis of endodontic infections by demonstrating the common occurrence of obligate anaerobic bacteria (4; 23; 30). Nevertheless, culture-based techniques have limitations such as the difficulty in detecting fastidious anaerobic microorganisms and moderate sensitivity and specificity (43).

Recently, molecular biology techniques have provided a more cost-effective, specific and sensitive method to evaluate the microbiological profile of oral pathologies, including endodontic and periodontal infections (37; 38; 43; 52-54). This technologys permits the detection of microbial species that are difficult to grow as well as uncultivated and unrecognized phylotypes (34) which would lead to a better understanding of the oral microbiota including endodontic infections (19; 35; 49; 56)

Checkerboard DNA-DNA hybridization is a high throughput method to analyze large numbers of DNA samples using large numbers of DNA probes on a single nylon membrane (55). The quantity of bacteria in the samples is an important factor in the checkerboard DNA-DNA hybridization technique, since the level of detection is about 10^4 bacterial cells of a given species. Samples from endodontic pathologies often contain very few bacterial cells and may be below the level of detection of the checkerboard method without a DNA amplification step. To overcome these limitations,

the present study used multiple displacement amplification (MDA) before hybridizing the samples. MDA allows uniform amplification of the whole genome of DNA targets (3; 12; 33; 63; 64), increasing the amount of DNA obtained from the endodontic bacterial samples. Furthermore, MDA provides enough amplified DNA to perform multiple analyses of the same sample using different DNA probe sets. The aim of this study was to combine MDA and checkerboard DNA-DNA hybridization to quantitatively and qualitatively assess the taxa present in root canals during endodontic infections.

MATERIAL AND METHODS

Subject population and sample collection

Sixty six subjects ranging in age from 11-81 years were recruited in the Department of Endodontics, Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil. The subjects had teeth with endodontic infections, with or without radiographically detected periradicular lesions.

The selection of teeth was based on clinical crown conditions that permitted effective placement of rubber dam isolation in teeth with pulp necrosis. The reason for the primary infection was caries; that was detected in almost all cases, although causes of pulp necrosis are sometimes difficult to be determined clinically. Additionally, there was no history of trauma associated to the selected teeth. All sampled teeth had never been treated before and were asymptomatic, without acute abscess.

57 teeth were molars, 6 teeth were premolars, while 3 teeth were single-rooted. In the case of multi-rooted teeth, the sample was taken from the largest root canal.

After obtaining informed consent, the 66 selected teeth were isolated using a rubber dam. Complete asepsis was employed, using the methodology proposed by Möller (32). 30% hydrogen peroxide was applied on the isolated crown, followed by 5% iodine that was inactivated by 5% sodium thiosulfate solution. The samples were taken by scraping or filing the root canal walls with a #10 K-type hand file (Maillefer, Ballaigues, Switzerland). The file was introduced into the canal to a level approximately 1 mm short of the tooth apex. After removal from the canal, the file was cut off below the handle and dropped into an Eppendorf microcentrifuge tube containing a solution of 20 µl of alkaline lysis buffer (400 mM KOH, 100 mM DTT, 10 mM EDTA). After 10 min of incubation on ice, 20 µl of neutralization solution (400 mM HCl, 600 mM Tris HCl, pH=0.6) was added, and the sample was kept at 4˚C until multiple displacement amplification was performed.

For comparison, a second set of samples was taken from 46 of the 66 root canals. In that set of samples, the files were placed into an Eppendorf microcentrifuge tube containing 100 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6). 100 µl of 0.5 M NaOH were added to the sample and it was maintained at 4˚C until checkerboard DNA-DNA hybridization was performed.

Multiple displacement amplification of root canal samples

The collected samples were amplified using the Genomiphi ™ DNA amplification kit (Amersham Biosciences, Arlington Heighs, IL). 1 µl of each sample was added to 9 µl of sample buffer (50 mM Tris-HCl pH 8.2, 0.5 mM EDTA) containing random hexamer primers) in individual 200 µl microcentrifuge tubes (Stratagene, La Jolla, CA). This solution was then heat-denatured at 95˚C for 3 min in a Perkin-Elmer Thermocycler and cooled to 4˚C. The reaction mixture was prepared on

ice by combining 9 µl of reaction buffer containing dNTPs, and 1 µl of enzyme mix, containing Φ29 DNA polymerase, for each sample. The reaction mixture was added to each denatured sample to make a final volume of 20 µl and incubated at 30˚C for 16-18 hr. 10 ng of λ DNA (contained in 1 µl) were used as an amplification control. The amplification reaction was terminated by heating the samples at 65˚C for 10 min. The amplified material was stored short-term at 4˚C or at -20˚C for longer storage. The DNA content of the amplified samples was measured using the Picogreen™ dsDNA quantification assay (Invitrogen, Carlsbad, CA). The microbiological content of the amplified samples was analyzed using checkerboard DNA-DNA hybridization.

Bacterial strains and growth conditions

The 77 reference strains used for the preparation of DNA probes are listed in Table 1. The majority of strains were grown on Trypticase soy agar supplemented with 5% defibrinated sheep blood (Baltimore Biological Laboratories (BBL), Cockeysville, MD) with some exceptions. *Tannerella forsythia* was grown on Trypticase soy agar supplemented with 5% sheep blood and 10 µg/ml N-acetylmuramic acid (Sigma Chemical Co., St. Louis, MO). *Porphyromonas gingivalis* was grown on Trypticase soy agar supplemented with 5% sheep blood, 0.3 µg/ml menadione (Sigma) and 5 µg/ml hemin (Sigma). *Eubacterium* and *Neisseria* species were grown on Fastidious Anaerobic Agar (BBL) with 5% defibrinated sheep blood. *Treponema denticola* and *Treponema socranskii* were grown in Mycoplasma broth (Difco Laboratories, Detroit, MI) supplemented with 1 mg/ml glucose, 400 µg/ml niacinamide, 150 µg/ml spermine tetrahydrochloride, 20 µg/ml Na isobutyrate, 1 mg/ml L-cysteine, 5 µg/ml thiamine pyrophosphate and 0.5% bovine serum. All strains were grown at 35ºC under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2).

DNA isolation and preparation of DNA probes

Bacterial strains were grown anaerobically on the surface of blood agar plates (except the two spirochetes, which were grown in broth) for 3-7 days. The cells were harvested and placed in 1.5 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6). Cells were washed twice by centrifugation in TE buffer at 1300 x g for 10 min. The cells were resuspended and lysed with either 10% SDS and Proteinase K (20 mg/ml) for gram-negative strains or in 150 µl of an enzyme mixture containing 15 mg/ml lysosyme (Sigma) and 5 mg/ml achromopeptidase (Sigma) in TE buffer (pH 8.0) for gram-positive strains. The pelleted cells were resuspended by 15 s of sonication and incubated at 37ºC for 1 h. DNA was isolated and purified using the method of Smith et al. (51). The concentration of the purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm. The purity of the preparations was assessed by the ratio of the absorbance at 260 nm and 280 nm. Whole genomic DNA probes were prepared from each of the 77 test strains by labeling 1-3 µg of DNA with digoxigenin (Boehringer Mannheim, Indianapolis, IN) using a random primer technique (17).

Checkerboard DNA-DNA hybridization

Checkerboard DNA-DNA hybridization was performed as previously described (26; 54; 55). In brief, following amplification and quantification, amplified samples and non-amplified samples were boiled for 10 min. 3 µl (approximately 900 ng of DNA) of the amplified sample were placed in an Eppendorf tube containing 1 ml of TE buffer prior to boiling. The non-amplified samples were neutralized by adding 800 µl of 5M ammonium acetate after boiling. Then, the samples were placed into the extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA), concentrated onto a nylon

membrane (Boehringer Mannheim) by vacuum and fixed onto the membrane by crosslinking using ultraviolet light (Stratalinker 1800, La Jolla, CA) followed by baking at 120˚C for 20 min. The Minislot device permitted the deposition of 28 different samples in individual lanes on a single membrane, as well as two control lanes containing 10^5 and 10^6 cells of each bacterial species tested. The membrane with fixed DNA was placed in a Miniblotter 45 apparatus (Immunetics) with the lanes of DNA at 90˚ to the channels of the device. A 30 x 45 "checkerboard" pattern was produced. Each channel was used as an individual hybridization chamber for separate DNA probes. Bound probes were detected by anti-digoxigenin antibody conjugated with alkaline phosphatase and a chemifluorescent substrate. Signal intensities of the endodontic samples and the standards (containing 1ng and 10 ng of each bacterial species) on the same membrane were measured using a Storm FluorImager (Molecular Dynamics, Sunnyvale, CA). The values were then converted to absolute counts using linear regression. Failure to detect a signal was recorded as zero.

Two membranes were run for each sample, one containing the "standard" 40 DNA probes used to examine periodontal samples and a second membrane that employed 37 probes to species thought to be important in endodontic samples. Specificity tests were performed for all probes before performing the checkerboard DNA-DNA hybridization with the root canal samples. The protocol to validate the specificity of these 37 probes was similar to the one used for the original set of 40 probes. The probes were tested against purified DNA from all other species, as described by Socransky et al. (54). If cross-reactions were observed, those probes were discarded and new probes constructed and validated.

Data analysis

Microbiological data were available for 46 non-amplified and 66 MDA amplified root canal samples, taken from 66 subjects. The microbial data were expressed in 3 ways: counts (levels), proportions (% DNA probe counts), and prevalence (% of teeth colonized at levels > 10^4) of 77 bacterial species. Count data were expressed as counts x10^5 in each sample and averaged across subjects. The amplified counts that were presented reflect the "number" of organisms detected after MDA amplification of the sample compared with non-amplified standards. They are not actual counts of the original sample, but the "DNA equivalents" after amplification.

Significance of differences between non-amplified and amplified samples for each species was sought using the Wilcoxon signed ranks test. Adjustments were made for multiple comparisons as described by Socransky et al. (53).

In a similar fashion, mean proportions of each species were determined for root canal samples taken from teeth with or without radiographically detected periradicular lesions. Significance of differences between groups was determined using the Mann-Whitney test and adjusted for multiple comparisons.

RESULTS

Quantification of DNA after MDA of endodontic samples

DNA from the root canal samples was amplified using MDA. The amount of DNA present in the samples before amplification averaged 6.80 (± 5.2) ng and 6.26 (± 1.73) µg after amplification, an approximately 1000-fold amplification. Amplified samples provided far better signals than those observed using non-amplified samples (Fig. 1).

Microbial species in root canal samples

The mean number of species (±SEM) detected in amplified and non-amplified root canal samples at a threshold of > 10^4 was 51.2 ± 2.2 and 14.5 ± 1.7 respectively. If a detection threshold of > 10^5 was employed then 11.3 ± 1.4 and 0.8 ± 0.2 species were detected in the amplified and non-amplified samples respectively. Fig. 2 presents the mean counts (x 10^5, ± SEM) of the 77 test species in amplified and non-amplified root canal samples taken from 46 teeth. The species were ordered according to mean counts. In non-amplified samples, *Prevotella tannerae* exhibited the highest mean counts (0.91 ± 0.25 x 10^5), followed by *Acinetobacter baumannii* and *Prevotella oris* , while *Streptococcus mitis* exhibited the lowest mean counts at (0.01 ± 0.001 x 10^5), followed by *Streptococcus salivarius* and *Actinobacillus actinomycetemcomitans.* 7 species were not detected in any of the non- amplified samples. In amplified samples, *P. tannerae* exhibited the highest mean counts x 10^5, 3.32 ± 0.69, followed by *P. oris* and *Streptococcus mutans*, while *Campylobacter concisus* exhibited the lowest mean counts (0.15 ± 0.02 x 10^5), followed by *Leptotrichia buccalis* and *S. salivarius* .

The mean proportions (% DNA probe counts, ± SEM) of the 77 test species in non-amplified and amplified root canal samples are presented in Fig. 3. In non-amplified samples, *P. tannerae* and *Acinetobacter baumannii* were detected in the highest mean proportions, 11.20 (± 1.48) and 11.14 (± 1.88), while *Escherichia coli* (0.05 ± 0.03) showed the lowest detected mean proportions, followed by *Actinomyces odontolyticus* In amplified samples, *P. tannerae* was detected in the highest mean proportions (5.33 ± 0.64) followed by *P. oris* and *S. mutans*, and *Streptococcus*

salivarus in the lowest mean proportions (0.28 ± 0.03), followed by *L. buccalis* and *Lactobacillus oris*.

Fig.4 presents the mean % of sampled sites exhibiting counts of the 77 test species at levels > 10^4, in non-amplified and amplified samples. *P. tannerae* and *A. baumanni* were detected in all amplified and > 90% of non-amplified samples. Other species that were frequently detected included *Prevotella heparinolytica* in both types of samples, *Actinomyces meyeri, Streptococcus parasanguinis, Atopobium rimae* and *Porphyromonas endodontalis* in MDA-amplified samples. *Prevotella oris, Selenomonas sputigena, Haemophilus aphrophilus* and *Mogibacterium timidum* were detected in > 50% of non-amplified samples.

Figs.5 and 6 demonstrate the mean % DNA probe counts of the 77 test species in non-amplified and amplified root canal samples, respectively, taken from teeth with or without a radiographically apparent periapical lesion. There were no significant differences between clinical groups after adjusting for multiple comparisons for either the non-amplified or amplified samples.

DISCUSSION

There were 2 major goals of the present investigation. The first was to increase the range of species examined in root canal samples. The second was to use MDA amplification to detect species that might be present in low numbers in these samples. In order to achieve the first goal, DNA probes to 77 bacterial species were employed in a checkerboard hybridization format. The species included the “standard” 40 DNA probes used to examine periodontal samples and the remaining 37 species were selected

because of their potential importance in endodontic samples. Previous studies have employed somewhat lower numbers of DNA probes, typically to less than 50 bacterial species (11; 20; 49; 50; 56). The use of larger numbers of DNA probes and the amplification of the target sample led to the detection of a far wider range of taxa in sampled sites than previously recognized. Indeed, an average of 51.2 species were detected in the MDA-amplified samples at counts $>10^4$, a higher figure than the 3 to 8 species found in samples evaluated in other studies (30; 49).The limited amount of bacterial species found in root canals in previous investigations might be due to sampling and/or technical limitations. The number of species found in the present study (50 species) was actually predicted by Tronstad & Sunde (61).

One unique aspect of this study was the use of MDA to amplify whole genomic DNA of the samples, generating highly accurate copies of the entire genomes of the mixture of species (60). As expected, the 77 species evaluated were detected in higher mean counts in the MDA-amplified samples than in non-amplified samples, suggesting that this technology may be useful for endodontic samples that contain only small numbers of cells. The MDA technique enables the amplification of DNA templates present at low levels. Therefore, bacterial species that could not be detected in non-amplified samples could be detected after amplification accounting, in part, for the differences in proportions between non-amplified and amplified endodontic samples.

The wide range of species detected in the root canal samples in the present investigation was expected. The use of molecular techniques to examine the composition of biofilm samples from different surfaces in the oral cavity has indicated that these biofilms are composed of a much wider spectrum of species than was originally described using cultural techniques. Indeed, suggestions of 700-1000 species

that can colonize oral biofilms have been put forth (1; 34). The direct connection of the root canal to a source of mixed microorganisms, such as a dental carious lesion or perhaps the periodontal pocket environment, might foster the entry of a diverse range of bacterial species that could colonize at low levels for prolonged periods of time. The use of MDA amplification provides the ability to detect such organisms. The typical amplification of the sample in this investigation was about 1000-fold. Since checkerboard DNA-DNA hybridization can detect species at levels of 10^4, it is theoretically possible that 10 cells of a species in a sample could have been detected in this study. Other techniques such as PCR also have the ability to detect bacterial species when present in low numbers for the study of endodontic samples (18; 19; 45). The major differences between the use of PCR and MDA-amplification for the detection of bacterial species is that MDA uniformly amplifies a genome or mixtures of genomes (12; 60), while PCR is limited to selected species and requires specific primers for their amplification. In addition, there may be some concern about amplification bias when using PCR techniques. It has been demonstrated that PCR amplification can result in bias that varies from 10^2 to 10^6, while MDA bias has been estimated to be less than 3-fold (12).

The combination of the two techniques - the checkerboard DNA-DNA hybridization and the MDA- was recently tested and validated by Teles et al. (60). In that study, the authors assessed the amplification bias (calculated to be 3.28) and the suitability of the MDA technique in providing DNA probes and standards. The authors also demonstrated the potential of the MDA technique in amplifying clinical bacterial samples. Additionally, the authors analyzed amplified and non-amplified clinical samples in parallel for the presence of 40 bacterial species. Both types of samples

provided comparable signals, demonstrating to be unlikely that MDA would introduce significant bias in the checkerboard technique.

Sampling of biofilms whether in root canals or periodontal pockets, carious lesions or the oral soft tissues presents numerous difficulties and is never perfect. In the present investigation, for example, we could not directly compare the data from amplified and non-amplified samples from the same teeth since the samples were taken separately and did not necessarily represent the same "pool" of microorganisms. Further, the sample may represent only a small portion of the microbes present in the sampled root canal. Additionally, samples might vary in size and one has to account for those variations. In order to achieve that, the authors presented the microbial data also in proportions (%DNA probe count). This type of data presentation was included because absolute counts do not always provide a complete picture of an ecosystem. A species may be present at, for example, 10^5 in one sample and make up only 1% of that sample. Proportions provide an additional means of viewing data in a complex ecosystem.

A second issue in sampling is the sampling technique *per se.* In previous studies about the endodontic microbiota, samples were taken using files and paper points (49) or paper points alone (5). In the present investigation, only files were employed. Due to their increased rigidity in comparison to paper points, files could collect bacterial samples in narrow canals far from the crown and near to the apex. Additionally, paper points might collect samples from all other parts of the root canal but the area of interest. In most instances, paper points do not collect the samples present in the terminus of the root canals where the environment had the lowest reduction-oxidation potential (58). A third issue in sampling, particularly when one has the ability to detect

very low number of organisms, is the possibility of contamination from sources outside the root canal. In the current investigation, the authors employed the asepsis methodology proposed by Möller (32). In a study by Lana et al. (30), the authors used the same methodology and confirmed the aseptic status of the crown by culture, but even meticulous precautions are not always sufficient to prevent contamination of the samples.

In spite of the potential limitations of sampling, there were a number of interesting observations resulting from the new combined methodology for bacterial enumeration.

The wide range of bacterial species detected has already been alluded to. The nature of the species that were frequently detected included many species in the new battery of DNA probes employed in the current investigation. Among the species detected in the highest mean counts were species that form black-pigmented colonies such as *P. tannerae* and non-pigmented *Prevotella* species such as *P. oris*. *P. tannerae* was previously reported as an uncultivable organism (15) and its frequency of occurrence in endodontic infections was not appreciated until Xia et al. (62) detected it in 60% of the endodontic samples using PCR amplification of the 16S rRNA sequence. When multiplex PCR was used to detect black-pigmented bacterial species in samples from endodontic infections, *P. tannerae* was found in only 5% of samples, possibly due to incomplete optimization of the multiplex technique (38). Other black-pigmented bacterial species were also detected in relatively high mean counts and proportions in amplified samples including *P. endodontalis, Prevotella loescheii, Prevotella nigrescens, Prevotella intermedia, P. gingivalis* and *Prevotella melaninogenica*. *P. endodontalis* has been isolated using cultural techniques from

infected root canals (24; 57) and its prevalence was demonstrated to be even higher when examined by molecular techniques (19; 22; 40; 48).

Periodontal pathogens of the red complex (52), *T. forsythia*, *P. gingivalis* and *T. denticola* were detected in both MDA-amplified and non-amplified samples. In the present study, *T. denticola* was detected in higher proportions than *T. forsythia* and *P. gingivalis* in the amplified samples, a finding similar to that of Haffajee et al. (25) in their examination of subgingival plaque samples from Brazilian subjects with periodontitis. Other investigators evaluated the occurrence of these 3 species in endodontic infections using PCR techniques and checkerboard DNA-DNA hybridization (36; 49). Using PCR, Roças et al. (36) found that *T. denticola* was the most prevalent of the 3 species (44%) while Siqueira et al. (49) using checkerboard DNA-DNA hybridization, found that *T. forsythia* was the most prevalent (39.3%) In other investigations (18;40) *T. denticola* was detected in 56% and 79% of samples from endodontically involved teeth. The fastidious growth requirements of *T. denticola* and *T. forsythia* have led to an underestimation of their prevalence in cultural studies of endodontic infections. However, based on their frequent detection using molecular techniques, they might be considered potential endodontic pathogens (40). Of interest is the recent demonstration that *T. denticola* was highly pathogenic in mono-infections of the dental pulp in a mouse model system (18).

In addition to the detection of the periodontal pathogens of the red complex, the present investigation indicated that many members of the “orange” complex were also present in endodontic infections. *Fusobacterium nucleatum*, a member of the orange complex, has commonly been isolated from root canal infections (4; 30; 45; 58). In the current study, the *F. nucleatum* subspecies were present in relatively high

proportions and levels, with *Fusobacterium nucleatum ss polymorphum* being detected somewhat more frequently than the other subspecies. *F. nucleatum* has been considered to be a "bridging" species in dental plaque due to its ability to co-aggregate with many other species (29). Evidence suggests that *F. nucleatum* not only facilitates the survival of obligate anaerobic bacteria in oxygen environments (6), but also enhances the colonization of members of the "red" complex species via direct binding (31; 39).

Recent studies using molecular identification approaches have indicated the high prevalence of species that had infrequently been isolated in culture (5; 8;13; 28; 41; 42; 44; 46). In the current study, the average proportions of fastidious bacteria, such as *T. denticola, T. socranskii, Filifactor alocis*, and *Dialister pneumosintes* ranged from 0.94 and 2.6 % of the total DNA probe counts. Some of the findings of the current investigation were different from those reported in the literature. For example, the frequent detection of *A. baumannii* in endodontic infections was in contrast with the lower proportions detected in the study of Siqueira et al. (50) who also employed the checkerboard DNA-DNA hybridization technique to detect bacterial species in samples from acute bacterial abscesses. In a study investigating the presence of respiratory pathogens in dental plaque of hospitalized patients with chronic lung diseases, Didilescu et al. (14) found high prevalence of *A.baumannii* in hospitalized patients (85.3%) and low prevalence (38.7%) in healthy controls. In the last decade, nosocomial infections caused by multidrug-resistant *A. baumannii* have been reported (2; 7; 9; 27). Initial concern about carbapenem-resistant *A. baumannii* (CRAB) began when the first nosocomial outbreak occurred in the United States in 1991 (21). Since then, CRAB infections and hospital wide outbreaks have been reported from many other countries

(2; 9; 10; 59). It remains to be established whether the oral cavity of systemically healthy individuals may be a reservoir for *A.baumannii*. Besides, further studies are needed to clarify if this species is an important part of the endodontic microbiota, and if it plays a significant role in endodontic infections.

While others have found specific bacterial communities to be associated with asymptomatic or symptomatic endodontic infections (18; 22; 41), the current study found no significant differences between the endodontic microbiota in the presence or absence of periradicular lesions irrespective of whether the samples were amplified or not.

At this time, the root canal “selective environment paradigm” seems to be related more to the limitations of the microbiological techniques used than the physical or nutritional constraints of the root canal. The recognition of greater microbial complexity of root canal infections parallels the greater complexity found in subgingival plaque and other oral samples revealed using molecular techniques. The real complexity of root canal infections will be better understood when even more comprehensive microbiological assessment is performed.

FIGURE LEGENDS

Fig.1. Checkerboard DNA-DNA hybridization membrane showing the hybridization of 40 of the 77 DNA probes to endodontic samples. Standards containing 10^5 and 10^6 cells of each test species are shown in the bottom lanes of the membrane. Signals indicate the detection of each species in pairs of non-amplified (n) or amplified (a).

Fig.2. Bilateral bar chart of the mean counts (x 10^5, ± SEM) of the 77 test species in non-amplified (n= 46) and amplified (n=66) root canal samples. The counts for each species were averaged across subjects and presented in descending order of mean count.

Fig.3. Bilateral bar chart of the mean % DNA probe count (± SEM) of 77 bacterial species in non-amplified (n=46) and amplified (n=66) root canal samples. % DNA probe count was computed for each species at each sample site and averaged across subjects.

Fig.4. Bilateral bar chart of the mean prevalence (% of teeth colonized by counts > 10^4 ± SEM) of individual species in non-amplified (n=46) and amplified (n=66) root canal samples. The prevalence of each species was computed for each subject and then averaged across subjects. The data are ordered in descending order of prevalence.

Fig.5. Bi-lateral bar chart of the mean % DNA probe counts (± SEM) of 77 bacterial species in non-amplified root canal samples taken from 20 teeth without a radiographically detected periapical lesion and 26 teeth with a periapical lesion. The proportion of each species was averaged across subjects in the 2 clinical groups

separately. Significance of differences between groups was determined using the Mann-Whitney test and adjusted for multiple comparisons.

Fig.6. Bi-lateral bar chart of the mean % DNA probe counts (± SEM) of 77 bacterial species in MDA-amplified root canal samples taken from 36 teeth without a radiographically detected periapical lesion and 30 teeth with a periapical lesion. Averaging and statistical testing were as described in Fig.5.

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Table 1: Strains of bacterial species used to prepare DNA probes and standards.

Acinetobacter baumannii (19606)	*Haemophilus segnis* (33393)
Actinobacillus actinomycetemcomitans * *Actinobacillus actinomycetemcomitans*	*Lactobacillus oris* (49062)
Actinomyces georgiae (49285)	*Leptotrichia buccalis* (14201)
Actinomyces gerencseriae (23860)	*Neisseria mucosa* (19696)
Actinomyces israelii (12102)	*Peptostreptococcus anaerobius* (27337)
Actinomyces meyeri (35568)	*Peptostreptococcus micros* (33270)
Actinomyces naeslundii I (12104)	*Porphyromonas endodontalis* (35406)
Actinomyces naeslundii II (27044)	*Porphyromonas gingivalis* (33277)
Actinomyces odontolyticus (17929)	*Prevotella heparinolytica* (35895)
Atopobium parvulum (33793)	*Prevotella intermedia* (25611)
Atopobium rimae (49626)	*Prevotella loescheii* (15930)
Campylobacter concisus (33237)	*Prevotella melaninogenica* (25845)
Campylobacter ureolyticus (33387)	*Prevotella nigrescens* (33563)
Campylobacter gracilis (33236)	*Prevotella oris* (33573)
Campylobacter rectus (33238)	*Prevotella tannerae* (51259)
Campylobacter showae (51146)	*Propionibacterium propionicum* (14157)
Capnocytophaga gingivalis (33624)	*Propionibacterium acnes I* ** *Propionibacterium acnes II*
Capnocytophaga ochracea (33596)	*Rothia dentocariosa* (17931)
Corynebacterium matruchotii (14266)	*Selenomonas artemidis* (43528)
Dialister pneumosintes (GBA27)	*Selenomonas noxia* (43541)
Eikenella corrodens (23834)	*Selenomonas sputigena* (35185)
Enterococcus faecalis (29212)	*Staphylococcus epidermidis* (14990)
Escherichia coli (10799)	*Streptococcus anginosus* (33397)
Eubacterium brachy (33089)	*Streptococcus constellatus* (27823)
Eubacterium limosum (8486)	*Streptococcus gordonii* (10558)
Eubacterium nodatum (33099)	*Streptococcus intermedius* (27335)
Eubacterium saburreum (33271)	*Streptococcus mitis* (49456)
Mogibacterium timidum (33093)	*Streptococcus mutans* (25175)
Filifactor alocis (35896)	*Streptococcus oralis* (35037)
Fusobacterium naviforme (25832)	*Streptococcus parasanguinis* (15912)
Fusobacterium necrophorum (25386)	*Streptococcus salivarus* (27945)
Fusobacterium nucleatum ss nucleatum (25586)	*Streptococcus sanguinis* (10556)
Fusobacterium nucleatum ss polymorphum (10953)	*Streptococcus vestibularis* (49124)
Fusobacterium nucleatum ss vincentii (49256)	*Tannerella forsythia* (3037)

Fusobacterium periodonticum (33693)	*Treponema denticola* (B1)
Gemella haemolysans (10379)	*Treponema socranskii* (S1)
Gemella morbillorum (27824)	*Veillonella dispar* (17748)
Haemophilus aphrophilus (33389)	*Veillonella parvula* (10790)
Haemophilus paraphrophilus (29242)	

All strains were obtained from the American Type Culture Collection (ATCC) except *Treponema denticola* B1 and *Treponema socranskii* S1, which were obtained from The Forsyth Institute *ATCC strains 43718 and 29523; **ATCC strains 11827 and 11828.

CONCLUSÕES

Conclusões

Em resumo, podemos dizer que:

1- Ao avaliar as amostras de sistemas de canais radiculares humanos infectados, as espécies bacterianas mais prevalentes, tanto nas amostras amplificadas anteriormente pelo MDA, quanto nas não amplificadas, foram a *Prevotella tannerae*, seguida da *Acinetobacter baumannii*, numa freqüência que variou de 89-100% das amostras.

2- A média de espécies encontradas por canal a um nível superior a 10^4 UFC, quando as amostras foram amplificadas, foi de 51,2 ± 2,2, e quando não amplificadas, foi de 14,5 ± 1,7.

Concluímos que:

1- A técnica do "Multiple Displacement Amplification" (MDA) se mostrou eficaz na amplificação da pequena amostra recuperada dos SCR infectados;

2- A combinação do MDA e do "checkerboard" permitiu uma maior sensibilidade na detecção da presença microbiana nestas infecções;

3- A grande quantidade de espécies bacterianas detectadas neste estudo relacionou-se ao grande número de sondas bacterianas utilizadas;

4- Foi possível reconhecer que a maior complexidade da microbiota das infecções endodônticas, detectada neste estudo, assemelha-se à complexidade da microbiota presente nas bolsas periodontais e em outros sítios da cavidade oral.

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