Universidade Federal de Minas Gerais
Faculdade de Odontologia

"Estudo das comunidades microbianas associadas às infecções endodônticas de dentes decíduos sintomáticos e assintomáticos pelas técnicas do Multiple-Displacement Amplification e Checkerboard DNA-DNA Hybridization"

WARLEY LUCIANO FONSECA TAVARES

Belo Horizonte
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Dissertação apresentada ao Programa de Pós-Graduação da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Mestre em Odontologia.

Área de Concentração – Endodontia

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Dedicatória

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RESUMO

RESUMO

O Mulltiple Displacement Amplification (MDA) tem sido utilizado para amplificação uniforme do genoma de espécies bacterianas em diferentes amostras da cavidade oral. O MDA é particularmente útil em pequenas amostras, visto que o mesmo gera uma quantidade de amostra de DNA abundante para a análise microbiana. O objetivo do presente estudo foi avaliar a microbiota de infecções endodônticas de dentes decíduos. Um total de 35 crianças, de 4 a 10 anos de idade, apresentando dentes com raízes intactas ou menos que 2/3 de rizólise foram envolvidas no estudo. Quarenta amostras foram coletadas e amplificadas pela técnica do MDA. As amostras amplificadas foram analisadas pela hibridização DNA-DNA (Checkerboard) para taxas de 83 espécies bacterianas. Foram computadas as porcentagens de dentes colonizados por cada uma das espécies em diferentes limiares nas amostras amplificadas. Os níveis das espécies bacterianas encontradas em diferentes condições clínicas foram analisados. A significância das diferenças entre as proporções de cada espécie foram determinadas para amostras de canais radiculares de dentes com ou sem câmara pulpar exposta à cavidade oral, fístula, edema, e dor. A significância das diferenças para cada espécie nos diferentes cenários clínicos foi analisada pelo teste Kruskall-Wallis. A quantidade de DNA (± DP) nas amostras antes da amplificação era 5.2 (± 4.7) ng. Após o MDA, as amostras continham, em média, 6.05 (± 2.3) µg de DNA. Oitenta das 83 sondas de DNA hibridizaram com uma ou mais amostras. As espécies bacterianas mais prevalentes em níveis > 10^4 células bacterianas foram *Actinomyces naeslundii* 1 e *Prevotella intermedia*, ambas presentes em 93.8% dos dentes analisados. O número médio de espécies (± DPM) detectadas por dente no nível de > 10^4 foi 20.19 (± 3.27). As espécies mais comumente encontradas neste nível foram *Actinomyces naeslundii 1* e *Prevotella intermédia.* Quando a média de sondas de DNA x 10^5 (± DPM) foi analisada, as espécies mais abundantes foram *A. naeslundii 1* (17.07±3.17)*, Prevotella nigrescens* (1.12 ± 0.55) e *P. intermedia* (1.01 ± 0.30). *Eikenella corrodens, Haemophilus aphrophilus,* e *Helicobacter pylori* não foram detectados em nenhuma das amostras. Em relação à análise da microbiota associada a diferentes sinais e sintomas clínicos, diferenças estatisticamente significantes foram detectadas em algumas situações. Vinte e sete amostras foram estatisticamente significantes ao serem encontradas em maiores

contagens em dentes abertos. *A. naeslundii* 1*, Veillonella parvula, Gemella morbillorum. Streptococcus oralis, Aggregatibacter actinomycetemcomitans* e *Neisseria mucosa* foram estatisticamente significantemente encontradas em maior número em dentes com exposição da câmara pulpar à cavidade oral. *P. intermedia*, *Neisseria mucosa*, *Streptococcus anginosus*, *Selenomonas noxia* e *Streptococcus sanguinis* foram detectados em contagens médias mais altas em dentes sem fístula. Não houve diferenças estatisticamente significantes na microbiota associada à presença ou ausência de edema. Dentes com dor apresentaram contagens elevadas de *Prevotella nigrescens* e *Prevotella oris.* A microbiota associada a canais radiculares de dentes decíduos demonstra ser mais complexa do que antes imaginado. Em conclusão, os resultados sugerem que espécies selecionadas estão associadas com os sinais e sintomas clínicos detectados em infecções endodônticas de dentes decíduos.

Palavras chaves: Bactérias, Dentes decíduos, Hibridização DNA-DNA (“checkerboard”), Infecção endodôntica, “Multiple Displacement Amplification” (MDA).

Abstract

ABSTRACT

"Microbial communities associated with asymptomatic and symptomatic deciduous root canal infections Analyzed by Multiple-Displacement Amplification and Checkerboard DNA-DNA Hybridization "

Multiple Displacement Amplification (MDA) has been used to uniformly amplify the genome from bacterial species in different types of oral samples. MDA is particularly useful in small samples, since it generates abundant target for microbial analysis. The aim of the present study was to combine MDA and Checkerboard DNA-DNA hybridization to evaluate the microbiota of endodontic infections in deciduous teeth A total of 35 children, 4 to 10 years old, having teeth with intact roots or less than 2/3 of physiological root resorption were involved in this study. Forty root canal samples were collected.and amplified. Amplified samples were analyzed by checkerboard DNA-DNA hybridization for levels of 83 bacterial taxa. . Percentages of teeth colonized by each species at different thresholds in amplified samples were computed. Levels of bacterial species present in different clinical conditions were analyzed. Significance of differences between mean proportions of each species were determined for root canal samples taken from teeth with (open tooth) or without (closed tooth) pulp chamber exposure to oral cavity, sinus tract, swelling, and pain. Significance of differences for each species in these clinical scenarios was sought with Kruskall-Wallis test. The mean amount of DNA (± SD) in the samples prior to amplification was 5.2 (± 4.7) ng. After MDA, samples contained, on average, 6.05 (± 2.3) µg of DNA. Eighty of 83 DNA probes hybridized with one or more samples. Most prevalent bacterial species at levels $> 10^4$ bacterial cells were *Actinomyces naeslundii* 1 and *Prevotella intermedia,* both present in 93.8%.of sampled teeth. The mean number of species (± SEM) detected per tooth at the $> 10^4$ level was 20.19 (± 3.27).The most commonly detected species at this level were *Actinomyces naeslundii 1* and *Prevotella intermedia* When mean DNA probe counts x 10^5 (± SEM) were analyzed, the most abundant species were *A. naeslundii 1* (17.07±3.17)*, Prevotella nigrescens* (1.12 ± 0.55) and *P. intermedia* (1.01

± 0.30). *Eikenella corrodens, Haemophilus aphrophilus,* and *Helicobacter pylori* were not detected in any of the samples. Upon the analysis of the microbiota associated with the different clinical signs and symptoms investigated, statistically significant differences could be detected in a few of them. Twenty seven species were statistically significantly increased in the open tooth group. *A. naeslundii 1, Veillonella parvula, Gemella morbillorum. Streptococcus oralis, Aggregatibacter actinomycetemcomitans* and *Neisseria mucosa* were statistically significant increased in teeth with pulp chamber exposure to oral cavity. *P. intermedia*, *Neisseria. mucosa*, *Streptococcus anginosus*, *Selenomonas noxia* and *Streptococcus sanguinis* were detected in higher mean counts in teeth without sinus tract. There were no statistically significant differences in the microbiota associated with presence or absence of swelling. Painful teeth presented increased counts of *Prevotella nigrescens* and *Prevotella oris.* The microbiota associated with root canals from deciduous teeth seems to be more complex than previously anticipated In conclusion, results suggest that selected species are associated with the clinical signs and symptoms detected in primary root canal infections.

Keywords: Bacteria, checkerboard DNA-DNA hybridization, Endodontic infection, Multiple Displacement Amplification, Primary teeth.

INTRODUÇÃO

INTRODUÇÃO

A manutenção dos dentes decíduos até a sua esfoliação é de fundamental importância para o crescimento e desenvolvimento normais dos arcos dentários, interferindo no posicionamento dos dentes permanentes, na postura da língua, dos lábios e das estruturas dos tecidos moles. Neste contexto, quando se faz necessário, o tratamento endodôntico destes dentes visa não somente preservar a homeostasia da cavidade oral, mas também evitar eventuais danos à formação do dente permanente subjacente (Pazelli et al., 2003; Bijoor & Kohli, 2005; Silva et al., 2006).

Os microrganismos presentes na cavidade oral são os principais agentes etiológicos das alterações pulpares e perirradiculares, podendo estar envolvidos, de forma direta ou indireta, no desenvolvimento destas patologias (Kakehashi et al., 1965; Sundqvist, 1976).

Ao longo dos anos, demonstrou-se o caráter polimicrobiano das infecções endodônticas na dentição permanente humana. Os estudos destas infecções utilizando cultura microbiológica encontraram um número médio de uma a trinta espécies bacterianas por canal radicular, com o número de células bacterianas variando de 10^2 a 10^8 UFC (Sundqvist, 1992, Lanna et al., 2001). Segundo Sundqvist (1992), parece também existir uma correlação entre o tamanho da lesão periapical e o número de espécies e células bacterianas presentes no sistema de canais radiculares (SCR) infectado.

Os canais radiculares infectados abrigam uma diversidade de espécies bacterianas, em sua maioria, oriundas da cavidade oral (Siqueira Jr et al., 2000). A microbiota endodôntica é predominantemente anaeróbia obrigatória, com elevada incidência de morfotipos gram-negativos, principalmente aquela localizada no segmento apical do SCR. Uma compreensão mais acurada dos microrganismos presentes nestas infecções se deu com o desenvolvimento das técnicas de coleta, transporte e cultivos dos isolados. Utilizando cultura microbiana, as espécies mais prevalentemente encontradas nestas infecções são o *Fusobacterium nucleatum, Eubacterium alactolyticum, Peptostreptococcus anaerobius*, *Bacteróides sp*, *Streptococcus sputigena* e actinomicetos (Sundqvist, 1994; Lana et al., 2001).

Nos anos recentes, os métodos moleculares vêm sendo utilizados, acrescentando informações significativas sobre a microbiologia das infecções orais (Socransky et al., 1994; Siqueira Jr. et al., 2000). Muitas espécies microbianas, que antes não eram identificadas, são hoje detectadas e confirmadas como integrantes desta microbiota (Paster et al., 2001). Como exemplo, a presença de espiroquetas nas doenças infecciosas orais foi considerada como um achado raro durante muitos anos. Atualmente, quatro espécies têm sido amplamente identificadas, inclusive nas infecções dos SCR: *Treponema dentícola, T. pectinovorum, T. socransckii e T. vicentii* (Dahle et al., 1996; Chan & McLaughlin, 2000; Siqueira *et al*., 2000 e 2001, 2005; Brito et al., 2007).

As técnicas baseadas na detecção do DNA permitem uma melhor descrição do ecossistema microbiano associado às infecções endodônticas, uma vez que podem detectar, inclusive, espécies não cultiváveis. Sabe-se hoje, que aproximadamente 50% das bactérias da cavidade oral permanecem ainda incultiváveis (Paster et al., 2001). Técnicas moleculares, tais como o PCR e o “Checkerboard” vêm sendo utilizadas em diversos estudos microbiológicos. Esta última técnica (“Checkerboard” - DNA-DNA Hybridization) permite analisar múltiplas amostras e múltiplas espécies bacterianas, simultaneamente (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 está em torno de 10^4 UFC, o que seria um complicador quando da utilização de amostras recuperadas de canais radiculares infectados devido ao pequeno número de células aí presentes Recentemente esta limitação foi superada, associando-se o “checkerboard” à técnica do ”Multiple Displacement Amplification” (MDA) (Teles et al., 2007; Brito et al., 2007). Esta última técnica permite uma amplificação uniforme do DNA genômico total: amostras com pequenas quantidades de DNA, como por exemplo, aquelas de apenas 1 ng, podem ser amplificadas 1000 vezes. A amplificação alcançada fornece material suficiente para que se realizem várias análises de uma mesma amostra, além de permitir que pequenas amostras possam ser avaliadas alcançando-se os níveis de detecção da técnica de hibridização DNA-DNA.

Utilizando-se esta associação de técnicas (MDA e “checkerboard”) em amostras recuperadas de 80 canais radiculares de pacientes atendidos na Faculdade de Odontologia da UFMG e processadas no Departamento de Periodontia do The Forsyth

Institute (Boston, USA), pela primeira vez, demonstrou-se uma elevada média de espécies microbianas em canais radiculares de dentes permanentes humanos infectados: aproximadamente 51 espécies. Estes resultados foram similares àqueles observados em amostras recuperadas de bolsas periodontais humanas (Brito et al., 2007).

Encontra-se bem estabelecido que a iniciação, formação e persistência das lesões periapicais estão associadas à presença de microrganismos nos canais radiculares e em suas ramificações. Devido ao seu aspecto difuso e organizacional, a completa eliminação da microbiota presente nos SCR infectados ainda é um desafio à pratica clinica (Soares, 2002).

A polpa de dentes decíduos reage às infecções bacterianas de maneira semelhante a dos dentes permanentes, ou seja, procura limitar a extensão do dano e iniciar a reparação tecidual Apesar disto, nem sempre os mecanismos de defesa e a tentativa de reparação nestes dentes são eficientes, podendo ocorrer reações inflamatórias irreversíveis, que culminam com a necrose pulpar (Raslan & Wetzel, 2006).

Algumas características morfológicas diferenciam dentes decíduos de dentes permanentes. Dentre elas podemos citar raízes mais finas, longas e curvas, bem como câmara pulpar ampla com assoalho fino e poroso, além de menor espessura dentinária em toda a sua extensão (Kramer, 1989; Toledo, 1996; Massara, 2002). Em conseqüência disto, os estímulos que atuam sobre a dentina, em particular os microrganismos, podem alcançar rapidamente a polpa e a região interradicular (Massara, 2002).

As superfícies radiculares internas e externas dos dentes decíduos são naturalmente irregulares pela exposição dos túbulos dentinários em decorrência da rizólize fisiológica ou patológica (Kramer, 1989; Godoy, 1999). Estas características anatômicas podem favorecer a instalação de uma microbiota específica nos canais radiculares com polpa necrótica, dificultando o tratamento destas infecções (Godoy, 1999). Somando-se a estas características morfológicas, não é grande o número de estudos que se ativeram sobre o perfil microbiano das infecções endodônticas em dentes decíduos portadores de necrose pulpar e lesão periapical.

Utilizando-se cultura microbiológica, os estudos têm demonstrado que as infecções endodônticas nestes dentes são polimicrobianas, com o predomínio de microrganismos anaeróbios, de forma similar ao observado nos dentes permanentes (Brook *et al*., 1981; Toyoshima *et al*., 1988; Brook 1991; Sato *et al*., 1993; Godoy, 1999; Faria, 2001; Pazelli *et al,* 2003; Silva et al., 2006). Muito recentemente, achados

correspondentes a estes vêm sendo alcançados utilizando-se técnicas de biologia molecular (Ruviére et al., 2007; Cogulu et al., 2008).

Apesar dos avanços recentes na caracterização dos agentes etiológicos envolvidos nas infecções endodônticas, muitas informações ainda se fazem necessárias com respeito à microbiota presente em dentes decíduos. Dentre elas, destaca-se a associação entre espécies específicas presentes no SCR e sinais e sintomas clínicos. Ao se estudar estas infecções a mesma limitação observada nos dentes humanos permanentes persiste em relação ao tamanho da amostra clínica. O sucesso demonstrado em vencer esta limitação, em recente trabalho do nosso grupo de pesquisa (Brito et al., 2007), levou à execução deste estudo que, por meio da associação das técnicas do MDA (Multiple Displacement Amplification) e de Hibridização DNA-DNA, procurou caracterizar as populações microbianas prevalentes nas infecções endodônticas em dentes decíduos, correlacionando estes achados aos dados clínicos presentes.

ARTIGO CIENTÍFICO

ARTIGO CIENTÍFICO

ARTIGO :

“MICROBIAL COMMUNITIES ASSOCIATED WITH ASYMPTOMATIC AND SYMPTOMATIC DECIDUOUS ROOT CANAL INFECTIONS ANALYZED BY MULTIPLE-DISPLACEMENT AMPLIFICATION AND CHECKERBOARD DNA-DNA HYBRIDIZATION “

ABSTRACT

Multiple Displacement Amplification (MDA) has been used to uniformly amplify the genome from bacterial species in different types of oral samples. MDA is particularly useful in small samples, since it generates abundant target for microbial analysis. The aim of the present study was to combine MDA and Checkerboard DNA-DNA hybridization to evaluate the microbiota of endodontic infections in deciduous teeth A total of 35 children, 4 to 10 years old, having teeth with intact roots or less than 2/3 of physiological root resorption were involved in this study. Forty root canal samples were collected.and amplified. Amplified samples were analyzed by checkerboard DNA-DNA hybridization for levels of 83 bacterial taxa. . Percentages of teeth colonized by each species at different thresholds in amplified samples were computed. Levels of bacterial species present in different clinical conditions were analyzed. Significance of differences between mean proportions of each species were determined for root canal samples taken from teeth with (open tooth) or without (closed tooth) pulp chamber exposure to oral cavity, sinus tract, swelling, and pain. Significance of differences for each species in these clinical scenarios was sought with Kruskall-Wallis test. The mean amount of DNA (± SD) in the samples prior to amplification was 5.2 (± 4.7) ng. After MDA, samples contained, on average, 6.05 (± 2.3) µg of DNA. Eighty of 83 DNA probes hybridized with one or more samples. Most prevalent bacterial species at levels > 10^4 bacterial cells were *Actinomyces naeslundii* 1 and *Prevotella intermedia,* both present in 93.8%.of sampled teeth. The mean number of species (± SEM) detected per tooth at the > 10^4 level was 20.19 (± 3.27).The most commonly detected species at this level were *Actinomyces naeslundii 1* and *Prevotella intermedia* When mean DNA probe counts x 10^5 (± SEM) were analyzed, the most abundant species were *A. naeslundii 1* (17.07±3.17)*, Prevotella nigrescens* (1.12 ± 0.55) and *P. intermedia* (1.01 ± 0.30). *Eikenella corrodens, Haemophilus aphrophilus,* and *Helicobacter pylori* were not detected in any of the samples. Upon the analysis of the microbiota associated with the different clinical signs and symptoms investigated, statistically significant differences could be detected in a few of them. Twenty seven species were statistically significantly increased in the open tooth group. *A. naeslundii 1, Veillonella parvula, Gemella morbillorum. Streptococcus oralis, Aggregatibacter actinomycetemcomitans* and *Neisseria mucosa* were statistically significant increased in teeth with pulp chamber

exposure to oral cavity. *P. intermedia*, *Neisseria. mucosa*, *Streptococcus anginosus*, *Selenomonas noxia* and *Streptococcus sanguinis* were detected in higher mean counts in teeth without sinus tract. There were no statistically significant differences in the microbiota associated with presence or absence of swelling. Painful teeth presented increased counts of *Prevotella nigrescens* and *Prevotella oris.* The microbiota associated with root canals from deciduous teeth seems to be more complex than previously anticipated In conclusion, results suggest that selected species are associated with the clinical signs and symptoms detected in primary root canal infections.

Key words: Primary teeth , Deciduous, Endodontic infection, bacteria, Multiple Displacement, Amplification, checkerboard DNA-DNA hybridization.

INTRODUCTION

Necrosis of the pulp tissue is caused by caries and/or trauma. (Raslan & Wetzel, 2006).Necrosis of the pulp in primary teeth might lead to periapical disease with possibility of involving the permanent tooth germ. Pulpal therapy in primary teeth with necrotic pulp aims at eradicating the endodontic infection and preventing the early loss of the dental element. This way, the succeeding tooth germ health can be preserved without risking dental functions (Pazelli et al., 2003; Bijoor & Kohli, 2005; Silva et al., 2006). Since most pulpal pathologies are infectious in nature, the knowledge of the primary teeth root canal microbial composition is paramount for the development of efficient endodontic therapies.

Over the years, the pathogenesis of pulp and periradicular infection has been attributed to the presence of bacteria and their by-products. (Kakehashi et al., 1965; Sundqvist, 1976). The composition of the endodontic microbiota was first described as predominantly aerobic and facultative (Farber & Seltzer, 1998). However, the improvement of anaerobic culturing techniques allowed considerable progress in the understanding of the etiopathogenesis of endodontic infections in primary and permanent teeth. It has been demonstrated that the majority of these infections are polymicrobial, with high prevalence of obligate anaerobic bacteria. (Baumgartner et al., 1991; Lana et al., 2001; Pazelli et al.,2003; Gomes et al., 2004; Silva et al., 2006).

Several studies investigated the microbiota associated with endodontic infections in adults (Baumgartner et al., 1991, Sunqvist et al., 1992; Lana et al., 2001; Gomes et al., 2004). The majority of these studies used molecular methods (Siqueira et al., 2000; Souza et al., 2005; Brito et al., 2007; Sassone et al., 2007; Sassone et al ., 2008). However, few publications studied the microbial composition of infected root canals in primary teeth (Pazelli et al., 2003; Silva et al., 2006; Ruviere et al., 2007), in the presence or absence of clinical symptoms (Cogulu et al., 2008), and most of them employed culture-based techniques. These techniques have demonstrated limitations for the study of complex biofilms in the oral cavity, such as the difficulty in the detection of fastidious anaerobic microorganisms and moderate sensitivity and specificity (Siqueira & Roças ,2005). Thus, there is a gap in knowledge regarding the microbial composition of infected root canals in primary teeth (Marsh & Largent, 1967; Toyoshima et al., 1988; Sato et al., 1993; Pazelli et al., 2003; Silva et al., 2006).

Recently, studies using molecular biology tools have led to a better understanding of the oral microbiota. These techniques allowed the identification of difficult to grow

bacterial species as well as uncultivated and unrecognized phylotypes. They have provided a more cost-effective, specific, and sensitive method to evaluate the microbiological profiles of oral pathologies, including endodontic infections (Socransky et al., 1998; Jung et al., 2000; Siqueira et al., 2000; Siqueira et al., 2001;De Souza et al., 2005) . Checkerboard DNA-DNA Hybridization is a high-throughput molecular method that allows the identification and quantification of a wide range of bacterial species present in multiple samples on a single nylon membrane. This technique was first described by Socransky et al. (1994), and has been used to investigate complex microbial ecosystems (Socransky et al., 2004). The checkerboard DNA-DNA hybridization technique has been employed in the study of the microbiota present in saliva (Sachdeo et al., 2008), supragingival bacterial plaque (Haffajee et al., 2008), subgingival bacterial plaque (Haffajee et al., 2008; Teles et al., 2008), oral soft tissue samples (Mager et al., 2003; Sachdeo et al., 2008), dentures (Sachdeo et al., 2008), dental implants (Gerber et al., 2006) and root canals (Siqueira et al., 2000; Brito et al.,2007; Sassone et al.,2007).

The quantity of bacteria in the samples is an important factor in the checkerboard DNA-DNA hybridization technique. Tipically, level of detection is between 10^4 and 10^7 bacterial cells of a given species in each sample. The bacterial content of samples from endodontic pathologies may be below this level of detection without a DNA amplification step. Multiple Displacement Amplification (MDA) might be performed to overcome this limitation. MDA is a reliable method for amplification of DNA present in oral biofilm samples with minimal bias. This method allows the uniform amplification of the whole genomes present in a sample and has been efficiently used as an aid in Checkerboard DNA-DNA Hybridization(Teles et al., 2007; Brito et al., 2007).

The aim of the present study was to combine MDA and checkerboard DNA-DNA hybridization to quantitatively and qualitatively assess the taxa present in the endodontic infections of primary teeth, and to analyze the correlation of this taxa with the following clinical parameters; exposure of pulp chamber to the oral cavity, swelling, sinus tract, and pain.

MATERIAL AND METHODS

Subject population and sample collection

Thirty five subjects ranging in age from 4 to10 years were recruited in the Department of Pediatric Dentistry, Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil. The subjects had primary teeth with endodontic infections, with or without radiographically detected periradicular/interadicular bone rarefaction. The teeth had intact roots or less than 2/3 of physiological root resorption. The selection of the teeth was based on clinical crown conditions that permitted effective placement of rubber dam isolation. The reason for the primary teeth infection was caries that was detected in almost all cases. However, causes of pulp necrosis are sometimes difficult to determine clinically. Additionally, there was no history of trauma associated with the selected teeth, no periodontal pockets and no previous intervention in the root canals. Thirty one teeth were primary molars and nine teeth were primary single-rooted. In the case of multi-rooted teeth, the sample was taken from the largest root canal.

After obtaining informed consent from the children's parents, the 40 selected teeth were isolated using a rubber dam. Complete asepsis was employed, using the methodology proposed by Möller (1966); Hydrogen peroxide (30%) 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 #15 K-type hand file (Maillefer, Ballaigues, Switzerland). The file was introduced into the canal up to the working length, which was obtained by pre-operatory radiographic exam, following a previously described protocol (Garcia-Godoy, 1987; Massara & Toledo, 2005). Briefly, in the cases without radiographic image interposition of the primary root and the permanent germ, the working length was 1 mm short of the tooth apex. In the cases where there was radiographic image interposition of the primary root and the permanent germ, the file was inserted up to the level of the cuspid of the permanent germ.

After removal from the canal, the file was cut off below the handle and dropped into an Eppendorf microcentrifuge tube (Eppendorf –manufacturer info) containing 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 MDA was performed. Prior

to sampling, the presence of swelling, pain, and sinus tract were recorded. It was also taken into account whether or not the tooth had pulp chamber exposure to oral cavity (open/closed teeth) at the time of the sampling.

Multiple displacement amplification (MDA) of root canal samples

The procedure was the same as described by Teles et al (2007). 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 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 L13cysteine, 5 µg/ml thiamine pyrophosphate and 0.5% bovine serum. All strains were grown at 35ºC under anaerobic conditions (80% N2, 10% CO2, 10% H2).

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 83 test strains by labeling 1-3 µg of DNA with digoxigenin (Boehringer Mannheim, Indianapolis, IN) using a random primer technique (17).

A human DNA probe was also prepared in order to compare the presence of human and bacterial cells in the root canal of primary infected teeth.

Checkerboard DNA-DNA hybridization

Checkerboard DNA-DNA hybridization was performed as previously described (26,54,55).In brief, following amplification and quantification, amplified samples were boiled for 10 min. 5 µl (approximately 1500ng of DNA) of the amplified sample were placed in an Eppendorf tube containing 1 ml of TE buffer prior to 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 one probe to detect *Streptococcus mutans*, and a second membrane that employed 42 probes to species thought to be implicated in endodontic infections. Sensitivity and 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 83 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

Eight samples failed to yield dependable amplification product. These samples were eliminated from the study. Microbiological data were available for 32 MDA amplified root canal samples. The microbial data were expressed in 2 ways: counts (levels), and prevalence (% of teeth colonized at levels > 10^4) of 83 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. They are not actual counts of the original sample, but the “DNA equivalents” after amplification. Significance of differences between mean counts of each species in the presence or absence of swelling, pain, sinus tract, and pulp chamber exposure to oral cavity were sought using the Kruskal-Wallis test . This analysis was not 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 the amplification averaged 5.2 (±4.7) ng and 6.05 (± 2.3) μg after amplification, an approximately 1000 fold amplification.

Microbial species in root canal samples

The mean number of species (± standard error of the mean [SEM]) detected in the amplified samples at a threshold of an equivalent to >10^4 bacterial cells was 20.19 (± 3.27). Using this threshold, the tooth that harbored the greatest number of species had 66 bacterial species and the tooth that exhibited fewer species had 3 species. If a threshold of >10^5 bacterial cells was used, then 1.97(± 0.49) species were detected. In the tooth that harbored the greatest number of species in this threshold, 12 species could be found. Twelve teeth did not show any bacterial species at the >10^5 level. *Eikenella corrodens, Haemophilus aphrophilus and Helicobacter pillory* were not detected in any of the samples.

Figure 1 demonstrates the mean percentage of teeth exhibiting counts of each of the 83 bacterial species at the level of > 10^4 bacterial cells. The most prevalent bacterial species in this category were *Actinomyces naeslundii 1* and *Prevotella intermedia*, both present in 93.8% of the sampled teeth. They were followed by *Neisseria mucosa* (68.8%), *Prevotella nigrescens* (65.6%), *Tannerella forsythia* (62.5%), *Prevotella denticola* (53.1%) and *Fusobacterium nucleatum ss vincentii* (43.8%). The least prevalent species at the > 10^4 bacterial cells level were *Lactobacillus acidophilus, Campylobacter gracilis, Veillonella dispar* and *Enterococcus faecalis* (3.1%).

When the same analysis was performed to compute the percentage of teeth harboring species at the >10^5 bacterial cells, the most abundant species were *A naeslundii 1* (62.5%), *P. intermedia* (18.8%) and *Prevotella tannerae* (18.8%).

Figure 2 demonstrates the mean counts (x 10^5 ± SEM) of the 83 bacterial species analyzed in the amplified samples. *A. naeslundii 1* (17.07 ± 3.17) was the specie detected in the highest counts. It was not included in the figures for "aesthetic" reasons. Since counts were beyond the values for all the other bacterial species, the inclusion of

this species in the figures would compromise the appreciation of the levels of all the other species. *A. naeslundii 1* was followed by *Prevotella nigrescens* (1.12 ± 0.55), and *P. intermedia* (1.01 ± 0.30). *Treponema socranskii* (0.01 ± 0.0), *C. gracilis* (0.01 ± 0.01), *L. acidophilus* (0.01 ± 0.01), *Staphylococcus epidermidis* (0.01 ± 0.01), *Campylobacter ureolyticus,* (0.01 ± 0.0), *V. dispar*(0.01 ± 0.01), and *E. faecalis* (0.01 ± 0.01) showed the lowest mean counts detected.

Figure 3 presents the mean counts (x 10^5 ± SEM) of the test species in detected in amplified root canal samples taken from 5 teeth that had pulp chamber exposure to oral cavity (open) at the time of the sampling and 27 teeth that were closed, without pulp chamber exposure to oral cavity at that time. Counts are presented in descending order of mean counts in samples from open teeth. *A. naeslundii 1*, *P. tannerae*, *P. nigrescens* and *P intermedia* exhibited the highest mean counts. For the reasons mentioned above, *A. naeslundii* 1 was not included in the figure. Twenty seven species were statistically significantly increased in the open tooth group. They included *A. naeslundii 1, Veilonella. parvula, Gemella morbillorum. Streptococcus oralis, Aggregatibacter actinomycetemcomitans* and *Neisseria mucosa*. *Tannerella forsythia* was statistically significantly increased in samples from closed teeth. Facultative anaerobes such as *S. mutans*, *S. mitis*, *Streptococcus oralis*, *Streptococcus gordonii*, *Selenomonas sputigena*, *Streptococcus salivarius*, *Lactobacillus casei*, *Streptococcus vestibularis*, *Streptococcus parasanguinis*, *S. warneri* and *S epidermidis* were detected in higher mean counts in teeth with pulp chamber exposure to oral cavity ($p<0.05$).

Figure 4 presents the mean counts(x 10^5 ± SEM) of the species present in amplified samples from 15 teeth with swelling and 17 teeth without swelling. *A. naeslundii 1, P. tannerae , P nigrescens*, *P intermedia*, *P micra and P. denticola* exhibited increased counts in teeth without swelling, despite not being statistically different. There was a trend in significance ($p = 0.048$) for the increased presence of *Streptococcus anginosus* in teeth without swelling. Overall, there were no statistically significant differences between the two groups.

The mean counts (x 10^5 ± SEM) of species detected in 23 teeth with sinus tract and 9 teeth without sinus tract is presented in Figure 5. Species such as P. intermedia, , *N. mucosa*, *S. anginosus*, *S. noxia* and *S. sanguinis* were detected in statistically significant higher mean counts in teeth without sinus tract ($p<0.05$).

Figure 6 demonstrates the mean counts of bacterial species present in 29 teeth without pain and 3 teeth with pain. The counts of *P. nigrescens* and *P oris* were statistically significantly increased in teeth that exhibited pain ($p<0.05$).

DISCUSSION

The purpose of the present investigation was to examine the microbial composition of root canal infection from necrotic deciduous teeth. This study also aimed at assessing the bacterial species associated with clinical signs and symptoms. Although many studies have evaluated the microbial composition of root canal infections in permanent teeth (Baumgartner et al., 1991, Sunqvist et al., 1992; Lana et al., 2001; Gomes et al., 2004; Siqueira et al., 2000; Souza et al., 2005; Brito et al., 2007; Sassone et al., 2007; Sassone et al ., 2008), there are few reports on the topic regarding primary teeth (Marsh & Largent, 1967; Toyoshima et al., 1988; Sato et al., 1993;Pazelli et al., 2003; Silva et al., 2006; Ruviere et al., 2007). Furthermore, this investigation increased the range of bacterial species examined in root canal samples. A previous study by Brito et al, (2007), analyzed the levels of 77 bacterial species in endodontic samples. In the present study, the levels of 83 bacterial taxa were assessed. Bacterial species that were present in the root canal system at very low numbers could be detected due to the MDA amplification step performed prior to the Checkerboard DNA-DNA Hybridization analysis. Such species would not have been detected otherwise, unless a method more sensitive than Checkerboard DNA-DNA Hybridization was used. Direct detection might be feasible using PCR-based methods, however, these techniques analyze much fewer taxa and samples at a time. Besides, when the quantification issue is taken into account with the use of Real Time PCR these limitations become even more evident, not to mention the increased cost and complexity of the procedure.

On average, 20.19 species per tooth were detected in the amplified samples, much more than the numbers reported by others employing techniques such as culture (Lana et al., 2001;Gomes et al., 2004), PCR (Cogulu et al., 2008), and RT-PCR (Horz et al., 2005). It is, tough, still a smaller figure than the average of 51.2 species reported previously (Brito et al., 2007). The tooth that harbored the greatest number of species at levels > 10^4 bacterial cells had 66 species and the tooth that exhibited fewer species had 3 species. It is noteworthy that in only in twelve teeth bacteria were detected at the >10^5 level, suggesting that root canal infection harbored several species but in low number over time.

It has been established that the bacterial diversity in any environment is severely underestimated when assessed by means of culture-based techniques (Papapanou,

2002). Studies using culture-independent molecular methods have shown that 700 species can be recovered from oral biofilms (Paster, 2001), half of which has been cultivated to date. Checkerboard DNA-DNA hybridization enables the quantitative analysis of a large number of samples for large numbers of species, and does provide a major benefit for oral microbial ecology (Socransky&Hafajee, 2005). Nevertheless, some bacterial species may be present in the infected root canal system in low counts, below the level of detection of the checkerboard DNA-DNA hybridization technique. In that way the role of these pathogens would be underestimated in the endodontic complex microbial ecosystem. To overcome this limitation, the MDA technique seems to be a useful tool, providing a simple and reliable method to amplify the sample DNA with minimal bias (Teles et al., 2007, Brito et al., 2007). In the present study, DNA from the root canal samples was amplified using this method. The amount of DNA in the samples ranged from 5.2 (±4.7) ng before to 6.21 (±2.35) µg after MDA, an approximately 1000-fold amplification from the original sample. Since the checkerboard DNA-DNA hybridization is able to detect 10^4 bacterial cells, it is possible that 10 cells of a given specie could be detected. . The use of MDA for the analysis of endodontic samples has enabled a greater appreciation of the complexity of the root canal microbiota (Teles et al., 2007; Brito et al., 2007). Due to the very limited amount of bacterial cells in non-amplified samples, theses samples were not analyzed. Even though the number of bacterial cells involved in endodontic infections seems to be small, other studies that focused on the analysis of the endodontic microbiota using checkerboard DNA-DNA hybridization (Sassone et al 2007, 2008) did not employ an amplification step. One of the possible reasons for their ability to detect several bacterial taxa at levels x 10^5 is that the sample collection was performed using files and paper point. These two sampling tools appear to have different abilities to collect cells, as paper points are more absorbent and might become saturated before it reaches the apex, where the true potential pathogens are located (Teles et al., 2008).

The sample collection is an important step in microbial investigations. Most studies related to root canal infection employed Kerr or Hedstroem files followed by two to four paper points to collect the samples (Siqueira et al., 2000; Pazelli et al., 2003; Souza et al., 2005, Silva et al., 2006; Sassone et al., 2007, Ruviére et al., 2007, Cogulu et al., 2008). That method of harvesting may not be specific for the microbiota in the apical third, since the entire content of the canal could be collected. To certify that only the apical portion of the canal was sampled in this study, a K file was inserted in

reaming motions in working length and only the two final mm were cut off. That difference in sampling may justify the prevalence of streptococci in the previous studies that used the file plus the paper points (Pazelli et al., 2003; Silva et al., 2006; Ruviére et al., 2007). The facultative anaerobic species, i.e streptococci, may be present in higher counts in the coronal third of the canal, where the availability of a saccharolytic nutrition and a high tension of oxygen would enable their prevalence. In the present study this genera was identified in higher counts only in opened teeth, suggesting that their presence was associated with the changes in ecology due to pulp chamber exposition to oral cavity.

MDA provided a more than 1000-fold amplification of the DNA present in the sample. However, not all the DNA amplified was of bacterial origin. In case human DNA was present, it would be amplified too. In fact, it is likely that human DNA was abundant in the sample, since these were deciduous teeth undergoing not only infection- which elicits an inflammatory immune response- but also physiological root resorption. The content of human DNA present in the amplified samples could be appreciated with the use of a human DNA probe (data not shown).

Recently, studies that had focused on necrotic root canal microbiota have suggested that some species were more apt than others at colonizing these sites (Fabricius et al., 1982 ; Sunqvist, 1992; Baugartner et al., 1992; Lana et al., 2001). Sensitive and accurate techniques support this premise and demonstrate that root canal microbiota is more complex than previously known. (Siqueira et al.,2000; Sakamoto et al., 2006; Brito et al., 2007). The presence, levels and prevalence of 83 bacterial species were investigated in 32 infected root canals. Among them, only *E.corrodens, H. aphrophilus* and *H. pillory* were not detected in any of the samples.

Root canal infections are mixed infections, with a great predominance of obligate and facultative anaerobic bacteria (Sundqvist 1992; Lana et al., 2001). In this study, the most prevalent bacterial species were *A. naeslundii* and *P. intermedia*, present in 93.8% of the samples. They were followed by others obligate anaerobic bacteria, as *N. mucosa*, *T. forsythia, P. nigrescens*, *P. denticola*, *F. nucleatum* ss *vincentii*, also very prevalent in the root canals examined. Using checkerboard technique, Sassone et al. (2007) showed that more than 70% of primary root canal samples were colonized by *N. mucosa* and *F. nucleatum* ss *vincentii,* which is in accordance with the present study. However, *E. faecalis was* the least prevalent species in this study, and were found in high percentage of the teeth by those authors

Several studies have focused on the composition of subgingival plaque suggesting a role for a specific number of microorganisms in the initiation of periodontal infections (Moore & Moore, 1994; Haffajee & Socransky, 1994). In root canal infections, researchers have shown a temporal change of the bacterial community (Fabricius et al., 1982; Tani-Ishi et al., 1994) and, significant differences have been observed in the composition of pathogenic microbiota present in symptomatic and asymptomatic cases (Gomes et al., 1996; Siqueira et al., 2004, Sakamoto et al., 2006). Many factors can influence the growth and development of these microorganisms in root canals, such as nutrient availability, low oxygen tension, bacteria interaction, as well as disintegrated pulp tissue and tissue fluids that are essential nutrient sources (Sundqvist, 1992, Fidgor & Sundvist, 2007). These ecological factors might be responsible for the presence of few species at counts x 10^5. *A naeslundii* 1, *P. intermedia*, *P. tannerae*, species that were present at that level in a high percentage of teeth, encompasses the dominant microbiota in these infections.

The clinical signs and symptoms evaluated in the present study were crown conditions (exposition or not of pulp chamber to oral cavity), presence or absence of sinus tract and swelling, along with history of spontaneous pain. When microbial ecologists examine microorganisms present in a particular habitat they attempt to analyze the effects of the microorganisms on their environment and the influence of the habitat on its residents (Socransky & Haffajee, 2005). Among several factors, oxygen balance might influence the dynamic growth and microbial colonization of the infected root canal system (Fabricius et al., 1982; Tani Ishi et al., 1992; Fidgor & Sundqvist, 2007). Given these differences in habitat, one could expect major differences in the composition of the microbiota (Ximenes-Fyvie et al., 2000). The major differences, however, were in counts of many of the species examined. Interestingly, the highest mean counts of anaerobic species were observed in samples from closed teeth, predominantly *A. naeslundii* 1, *P. tannerae*, *P. nigrescens* and *P. intermedia*. Not surprisingly, facultative anaerobes were detected in higher mean counts in open teeth, despite the presence of several anaerobes in those teeth. These findings were in accordance with previous studies performed in permanent teeth (Lana et al., 2001; Gomes et al., 2004). *T. forsythia* was found in 62.5% of the samples in the present study, and was statistically significant ($p<0,05$) increased in samples from closed teeth. This periodontal pathogen, member of the “red complex”, was detected in amplified and non-amplified samples in root canal infections of Brazilian adult subjects (Brito et al.,

2007). Using checkerboard DNA-DNA hybridization, *T. forsythia* was the most prevalent species in endodontic samples (39.3%) (Siqueira et al., 2000). Recently, evaluating the microbiota of primary endodontic infections by checkerboard DNA-DNA hybridization, Sassone et al (2007) found this species in 65.2% of the samples. In this study, *P.tannerae, P.nigrescens, P. denticola, F.nucleatum ss vincentii, P.endodontalis*, and *T. denticola,* were also present in higher counts in closed teeth, although not statistically significant. However, the interactions between bacterial species in a biofilm and between bacterial species and the nonbacterial habitat are dynamic. They reflect a back and forth interplay between host and colonizing species.

The other signals evaluated in this study were the presence of sinus tract and swelling. As sinus tract could contribute to microbial succession, providing nutrients that confer an ecologic advantage to the species or altering concentration of substrates, it was expected that this clinical signal could be related to differences in the root canal community. Statistically significant differences were observed regarding the mean counts ($x\ 10^5 \pm$ SEM) of species. *P. intermedia*, *N. mucosa*, *S. anginosus*, *S. noxia* and *S. sanguinis* were detected in higher mean counts in teeth without sinus tract. Using checkerboard hybridization, Sassone et al (2008) found the similar results concerning *S. anginosus* in samples from teeth without a sinus tract. On the other hand, there were no statistically significant differences in the microbiota whether swelling was present or not, despite a trend in significance for the increased presence of *S. anginosus* in teeth without swelling. Critical to correlate clinical signals with the influence of a given microbiota is the recognition that these relationships are not one-way (Socransky & Haffajee, 2005). The host may influence the microbiota, but in turn the microbiota influences the host.

Significant differences can be observed in the composition of pathogenic microbiota present in symptomatic and asymptomatic cases, suggesting that the structure of the bacterial community might determine the installation of specific symptoms (Gomes et al., 1996; Jacinto et al., 2005; Siqueira et al., 2004; Sakamoto et al, 2006; Yoshida et al, 1987, Chu et al., 2005). The current study showed statistically significant increased counts of black pigmented species such as *P. nigrescens* and non-pigmented ones, such as *P. oris* in teeth that exhibited pain. These findings were in accordance with previous studies that have been performed in permanent teeth (Gomes & Pinheiro, 2004; Yoshida et al., 1987; Gomes et al., 1994, 1996). In primary teeth, Cogulu et al. (2008) did not detect *P. intermedia/nigrescens* pain association, and the

authors speculated that this outcome may be due to anatomic differences between deciduous and permanent tooth root canals.

The frequent detection of potential endodontic pathogens in primary and permanent root canal infections has important ecological and clinical aspects. The microbial communities in deciduous root canal infections are similar to that observed in permanent teeth, what encourages the employment of similar therapies. Meticulous reduction or elimination of bacterial infection may be crucial in achieving the goal of successful root canal treatment. Even though the clinical signs and symptoms observed in this study were correlated with particular microbial aspects, more studies are needed, analyzing an even larger number of samples. This way it will be possible to reach a better understanding of the pathogenesis of deciduous root canal infections, as well as to design more specific endodontic therapies.

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FIGURES

Figure 1 – Bar chart of the mean prevalence (%of teeth colonized by counts of >10 ^{4} ± SEM) of individual species in primary teeth root canal samples. The prevalence of each species was computed for each tooth and then averaged across teeth. The data are ordered in descending order of prevalence in the amplified samples. *ss*, subsp.

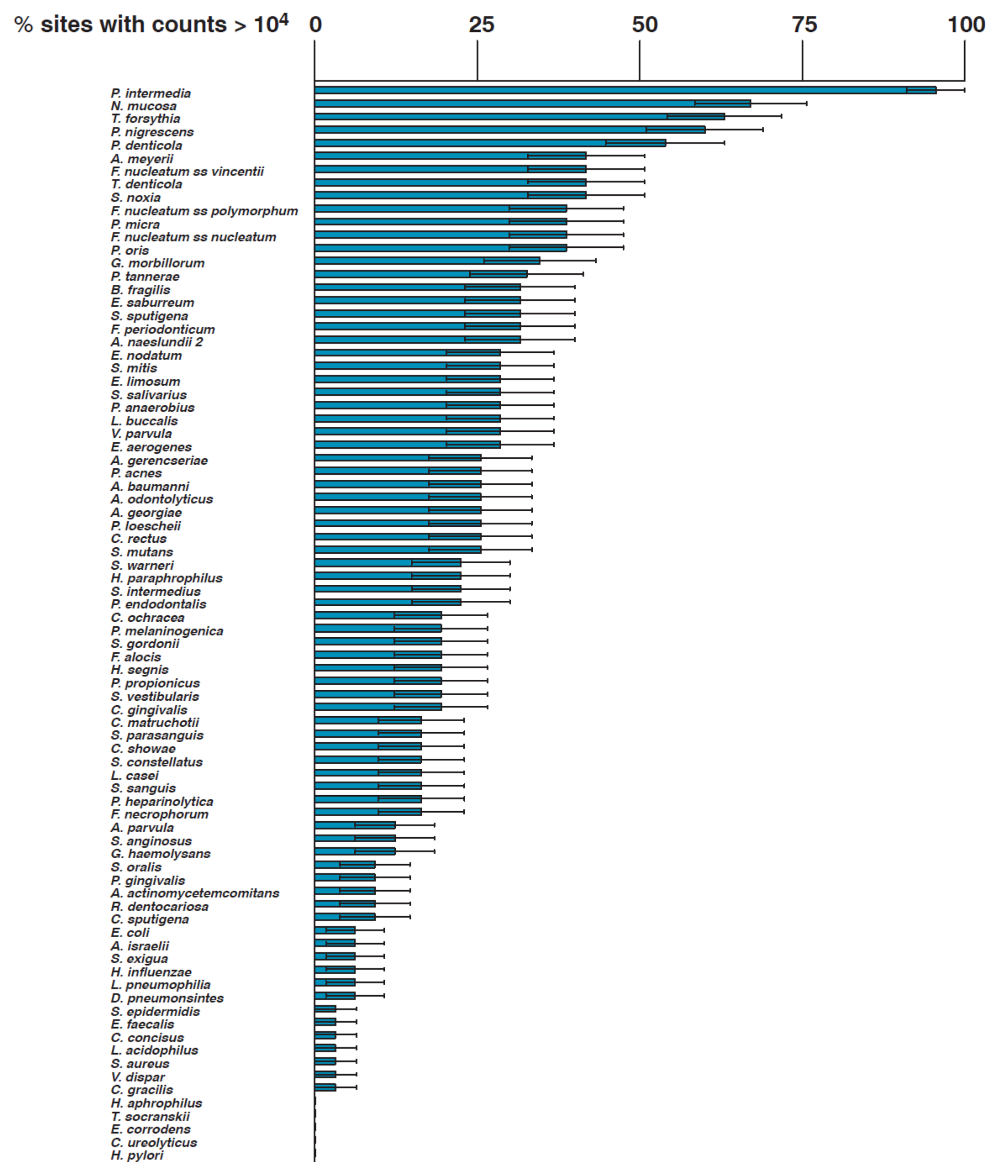

Figure 2 - Bar chart of the mean counts (x 10^5 ± SEM) of the 83 test species in primary teeth root canal samples. The counts for each species were averaged across

teeth and presented in descending order of mean counts detected in the samples. *ss,* subsp. *Actinomyces naeslundii* 1 was not included in the figures for “aesthetic” reasons. Since its counts were beyond the values for all the other bacterial species, the inclusion of this species in the picture would compromise the appreciation of the levels of all the other species.

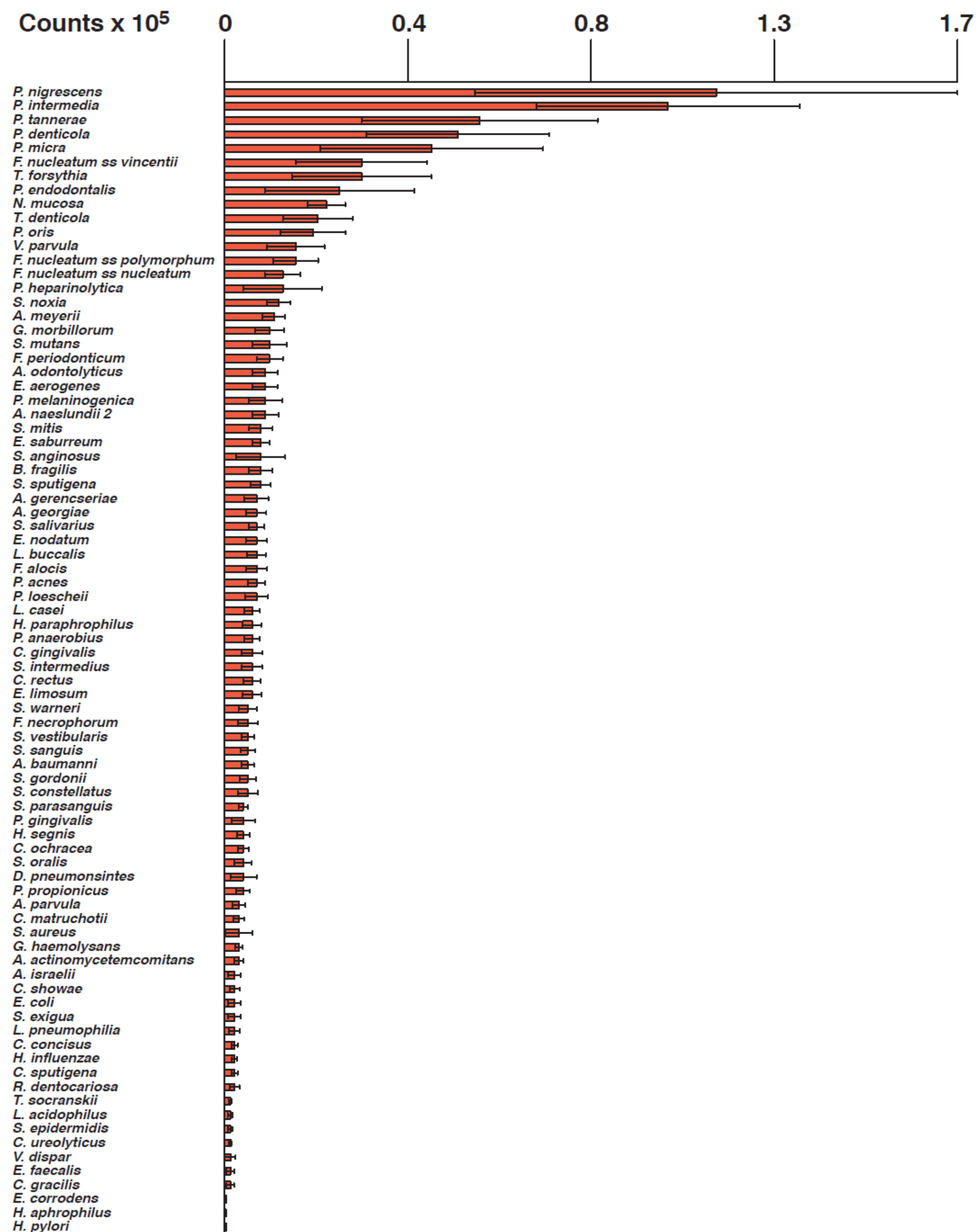

Figure 3 - Profiles of the mean counts (x $10^5 \pm$ SEM) of the 83 test species harvested from 27 closed teeth and 5 open teeth. The counts of each species were computed for each tooth and then averaged across teeth for the two clinical crown conditions. The species were ordered according to descending order of

mean counts. Significance of differences in species counts between closed and opened teeth was determined using Kruskal-Wallis. * P<0.05 ; ** P<0.01; *** P<0.001.

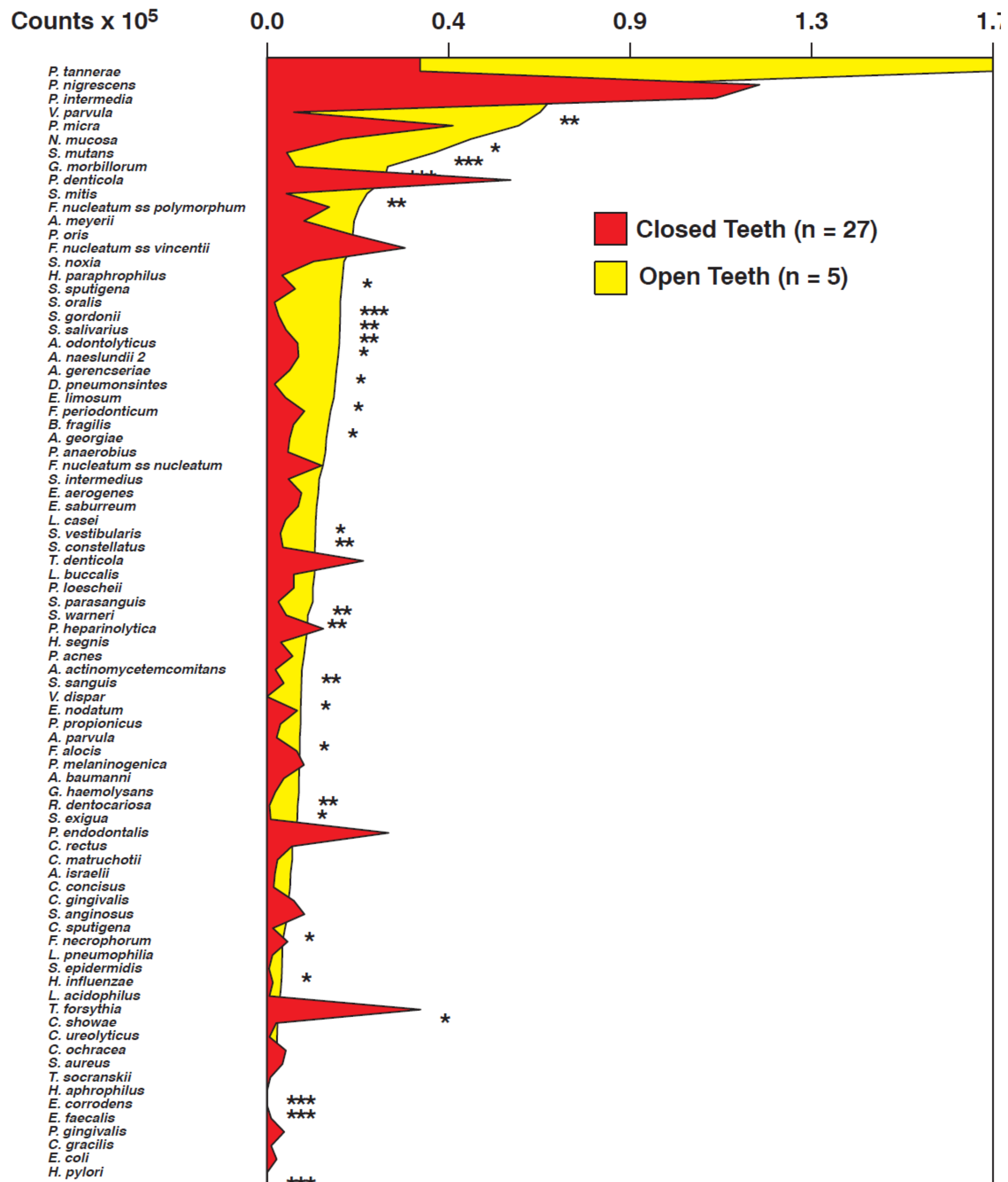

Figure 4 - Profiles of the mean counts (x 10^5 ± SEM) of the 83 test species harvested from 15 subjects with swelling and 17 subjects without swelling. The counts of each species were computed for each tooth and then averaged across subjects for the two clinical conditions. The species were ordered according to descending order of mean counts. Significance of differences in species counts

between the two clinical conditions was determined using Kruskal-Wallis. * P<0.05

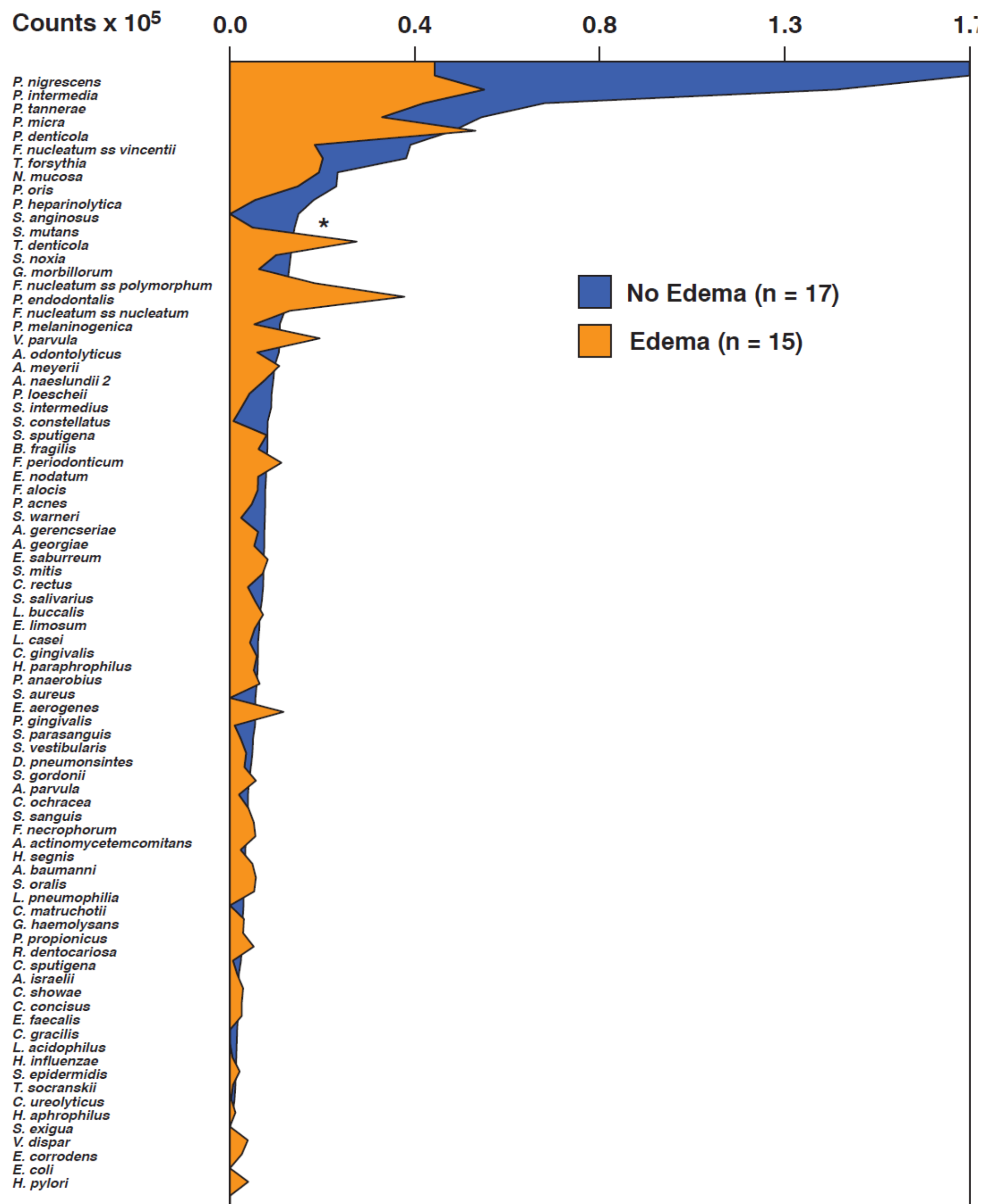

Figure 5 – Profiles of the mean counts (x 10^5 ± SEM) of the 83 test species harvested from 23 subjects with sinus tract and 9 subjects without sinus tract. The counts of each species were computed for each tooth then averaged across teeth for the two clinical conditions. The species were ordered according to descending

order of mean counts. Significance of differences in species counts between the two clinical conditions was determined using Kruskal-Wallis. * $P<0.05$.

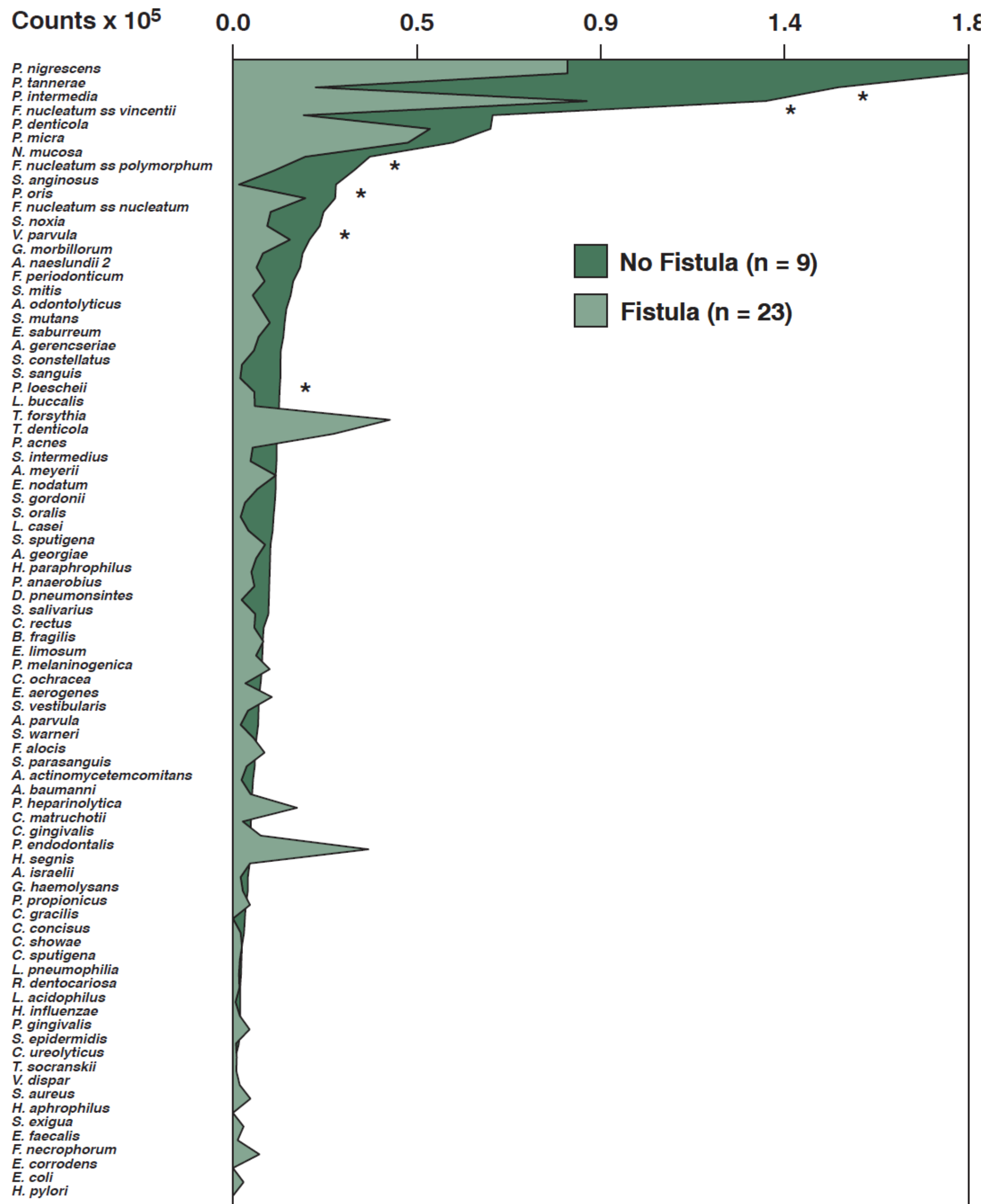

Figure 6 - Profiles of the mean counts (x $10^5 \pm$ SEM) of the 83 test species harvested from 3 subjects with painful sensation and 29 subjects without pain. The counts of each species were computed for each tooth and then averaged across teeth for the two clinical conditions. The species were ordered according to descending order of mean counts. Significance of differences in species counts

between the two clinical conditions was determined using Kruskal-Wallis. * $P<0.05$.

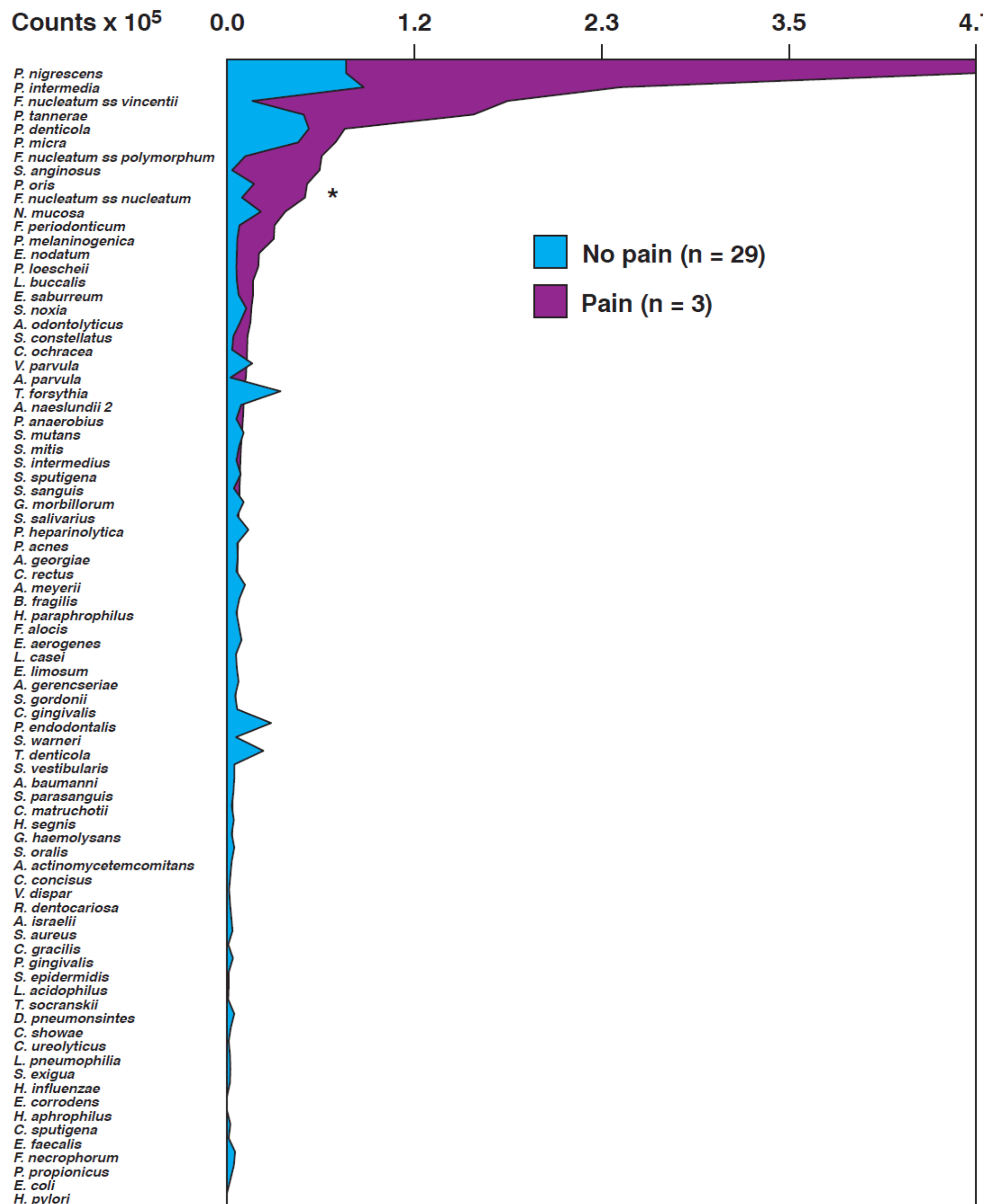

Table 1 – Strains of bacterial species used to prepare DNA probes and standards

Strain[a]	Strain[a]
Acinetobacter baumannii (19606)[b]	*Lactobacillus casei* (393)[b]
Actinomyces georgiae (49285)[b]	*Legionella pneumophila* (33153)[b]
Actinomyces gerencseriae (23860)[b]	*Leptotrichia buccalis* (14201)[b]
Actinomyces israelii (12102)[b]	*Neisseria mucosa* (19696)[b]
Actinomyces meyeri (35568)[b]	*Peptostreptococcus anaerobius* (27337)[b]
Actinomyces naeslundii I (12104)[b]	*Parvimonas micra* (33270)[b]
Actinomyces naeslundii II (43146)	*Porphyromonas endodontalis* (35406)[b]
Actinomyces odontolyticus (17929)[b]	*Porphyromonas gingivals* (33277)[b]
Aggregatibacter (Actinobacillus) actinomycetemcomitans[c]	*Prevotella denticola* (35308)[b]
Aggregatibacter (Actinobacillus) actinomycetemcomitans[c]	*Prevotella nigrescens* (33563)[b]
Aggregatibacter (Haemophilus) aphrophilus (33389)[b]	*Prevotella heparinolytica* (35895)[b]
Aggregatibacter (Haemophilus) paraphrophilus (29242)[b]	*Prevotella intermedia* (25611)[b]
Aggregatibacter (Haemophilus) segnis (33393)[b]	*Prevotella loescheii* (15930)[b]
Atopobium parvulum (33793)[b]	*Prevotella melaninogenica* (25845)[b]
Bacteroides fragilis (25285)[b]	*Prevotella oris* (33573)[b]
Campylobacter concisus (33237)[b]	*Prevotella tannerae* (51259)[b]
Campylobacter ureolyticus (33387)[b]	*Propionibacterium propionicum* (14157)[b]
Campylobacter gracilis (33236)[b]	*Propionibacterium acnes* I[d]
Campylobacter rectus (33238)[b]	*Propionibacterium acnes* II[d]
Campylobacter showae (51146)[b]	*Rothia dentocariosa* (17931)[b]
Capnocytophaga gingivals (33624)[b]	*Selenomonas noxia* (43541)[b]
Capnocytophaga ochracea (33596)[b]	*Selenomonas sputigena* (35185)[b]
Capnocytophaga sputigena (33612)[b]	*Slackia exigua* (700122)[b]
Corynebacterium matruchotii (14266)[b]	*Staphylococcus aureus* (33591)
Dialister pneumosintes (GBA27)	*Staphylococcus epidermidis* (14990)[b]
Eikenella corrodens (23834)[b]	*Staphylococcus warneri* (27836)[b]
Enterococcus faecalis (29212)	*Streptococcus anginosus* (33397)[b]
Enterococcus aerogenes (13048)[b]	*Streptococcus constellatus* (27823)[b]
Escherichia coli (10799)	*Streptococcus gordonii* (10558)[b]
Eubacterium limosum (8486)[b]	*Streptococcus intermedius* (27335)[b]
Eubacterium nodatum (33099)[b]	*Streptococcus mitis* (49456)[b]
Eubacterium saburreum (33271)[b]	*Streptococcus mutans* (25175)[b]
Filifactor alocis (35896)[b]	*Streptococcus oralis* (35037)[b]
Fusobacterium necrophorum (25286)[b]	*Streptococcus parasanguinis* (15912)[b]
Fusobacterium nucleatum ss nucleatum (25586)[b]	*Streptococcus salivarius* (27945)
Fusobacterium nucleatum ss polymorphum (10953)[b]	*Streptococcus sanguinis* (10556)[b]
Fusobacterium nucleatum ss vincentii (49256)[b]	*Streptococcus vestibularis* (49124)[b]
Fusobacterium periodonticum (33693)[b]	*Tannerella forsythia* (43037)[b]
Gemella haemolysans (10379)[b]	*Treponema denticola* (B1)
Gemella morbillorum (27824)[b]	*Treponema socranskii* (S1)
Haemophilus influenza (33533)[b]	*Veillonella dispar* (17748)[b]
Helicobacter pylori (43504)[b]	*Veillonella parvula* (10790)[b]
Lactobacillus acidophilus (4356)[b]	

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

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