International Endodontic Journal

doi:10.1111/j.1365-2591.2010.01805.x

Microbiota of deciduous endodontic infections analysed by MDA and Checkerboard DNA–DNA hybridization

W. L. F. Tavares[1], L. C. Neves de Brito[1], R. P. Teles[2,3], M. L. A. Massara[1], A. P. Ribeiro Sobrinho[1], A. D. Haffajee[2,3], S. S. Socransky[2,3] & F. R. Teles[2,3]

[1]Federal University of Minas Gerais School of Dentistry, Belo Horizonte, Minas Gerais, Brazil; [2]Department of Periodontology, The Forsyth Institute, Boston, MA, USA; and [3]Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine

Abstract

Tavares WLF, Neves de Brito LC, Teles RP, Massara MLA, Ribeiro Sobrinho AP, Haffajee AD, Socransky SS, Teles FR. Microbiota of deciduous endodontic infections analysed by MDA and Checkerboard DNA–DNA hybridization. *International Endodontic Journal*, **44**, 225–235, 2011.

Aims To evaluate the microbiota of endodontic infections in deciduous teeth by Checkerboard DNA–DNA hybridization after uniform amplification of DNA in samples by multiple displacement amplification (MDA).

Methodology Forty samples from the root canal system of deciduous teeth exhibiting pulp necrosis with or without radiographically detectable periradicular/interradicular bone resorption were collected and 32 were analysed, with three individuals contributing two samples; these were MDA-amplified and analysed by Checkerboard DNA–DNA hybridization for levels of 83 bacterial taxa. Two outcome measures were used: the percentage of teeth colonized by each species and the mean proportion of each bacterial taxon present across all samples.

Results The mean amount of DNA in the samples prior to amplification was 5.2 (±4.7) ng and 6.1 (±2.3) μg after MDA. The mean number of species detected per sample was 19 (±4) (range: 3–66) to the nearest whole number. The most prevalent taxa were *Prevotella intermedia* (96.9%), *Neisseria mucosa* (65.6%), *Prevotella nigrescens* (56.2%) and *Tannerella forsythia* (56.2%). *Aggregatibacter (Haemophilus) aphrophilus* and *Helicobacter pylori* were not detected. *P. intermedia* (10%), *Prevotella tannerae* (7%) and *Prevotella nigrescens* (4.3%) presented the highest mean proportions of the target species averaged across the positive samples.

Conclusion Root canals of infected deciduous teeth had a diverse bacterial population. *Prevotella* sp. were commonly found with *P. intermedia*, *Prevotella tannerae* and *Prevotella nigrescens* amongst the most prominent species detected.

Keywords: bacteria, checkerboard DNA–DNA hybridization, deciduous, endodontic infection, multiple displacement amplification, primary teeth.

Received 7 December 2009; accepted 11 September 2010

Introduction

Necrosis of pulp tissue is usually initiated by caries and/or trauma (Raslan & Wetzel 2006). Necrosis of the pulp in primary teeth might lead to periapical disease and could potentially affect the permanent tooth germ. Pulp therapy in primary teeth with necrotic pulps aims at eradicating the endodontic infection and preventing this early loss. In this way, the health of the succeeding tooth germ can be preserved (Pazelli *et al.* 2003, Bijoor & Kohli 2005, da Silva *et al.* 2006). As most pulp pathoses are caused by microbial infections, knowledge of their diversity in the infected root canals of primary teeth should underpin the development of more efficacious endodontic therapies.

Several studies have investigated the microbiota associated with endodontic infections in adults (Sundqvist 1976, Baumgartner & Falkler 1991,

Correspondence: Antonio P. Ribeiro Sobrinho, Departamento de Odontologia Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, CEP 30.161-970, Belo Horizonte, MG, Brasil (Tel.: +55 31 34992470; e-mail: sobrinho.bhz@terra.com.br).

Siqueira *et al.* 2000, Lana *et al.* 2001, Rolph *et al.* 2001, Munson *et al.* 2002, de Souza *et al.* 2005, Brito *et al.* 2007, Sassone *et al.* 2007, 2008). In contrast, few studies have assessed the microbial composition of deciduous endodontic infections (da Silva *et al.* 2006, Ruviere *et al.* 2007, Cogulu *et al.* 2008), utilizing different detection methods and focusing on a variable number of bacterial taxa. Knowledge of the endodontic infections of primary teeth is therefore far from complete (Marsh & Largent 1967, Toyoshima *et al.* 1988, Pazelli *et al.* 2003, da Silva *et al.* 2006).

Checkerboard DNA–DNA hybridization is a high-throughput molecular method that allows the identification and quantification of a wide range of bacterial species in multiple samples on a single nylon membrane. This technique has been employed in the study of the microbiota in saliva (Sachdeo *et al.* 2008), in supragingival plaque (Haffajee *et al.* 2008), in subgingival plaque (Haffajee *et al.* 2008, Teles *et al.* 2008), on oral soft tissue (Mager *et al.* 2003, Sachdeo *et al.* 2008), on dentures (Sachdeo *et al.* 2008), from dental implants (Gerber *et al.* 2006) and from root canals (Siqueira *et al.* 2000, Brito *et al.* 2007, Sassone *et al.* 2007, 2008).

The level of detection of the Checkerboard DNA–DNA hybridization technique is between 10^4 and 10^7 bacterial cells of a given species in each sample. The bacterial content of samples from endodontic disease may be below this detection level, so a DNA amplification step called multiple displacement amplification (MDA) can be used to enhance detection limits (Dean *et al.* 2002, Brito *et al.* 2007, Teles *et al.* 2007). MDA can amplify DNA present in oral biofilm samples with minimal bias (Dean *et al.* 2002, Hawkins *et al.* 2002, Yan *et al.* 2004). MDA enables the whole-genomic amplification of DNA targets (Dean *et al.* 2002). The template is replicated again and again by a 'hyper-branching' mechanism of strand displacement synthesis (Lizardi *et al.* 1998), with the polymerase laying down a new copy as it displaces previously made copies. Samples as small as 1 ng can be amplified 1000- to 10 000-fold (Mai *et al.* 2004). MDA uniformly amplifies the entire genomes (Hawkins *et al.* 2002) with minimal amplification bias (Hawkins *et al.* 2002, Nelson *et al.* 2002, Yan *et al.* 2004). This method allows the uniform amplification of the whole genomes present in a sample and has been effectively used as an aid in Checkerboard DNA–DNA hybridization (Brito *et al.* 2007, Teles *et al.* 2007). As bacterial genomes are uniformly amplified, the proportion that each taxon comprises the test species can be calculated.

The aim of the present study was to combine MDA and Checkerboard DNA–DNA hybridization to qualitatively and quantitatively evaluate the microbiota of endodontic infections in deciduous teeth.

Material and methods

Subject population and sample collection

Thirty-five subjects ranging in age from 4 to 10 years were recruited in the Department of Paediatric Dentistry, Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil. This study was approved by the Ethics Committee of the Federal University of Minas Gerais (ETIC132/07). Informed consent was obtained from the parents of the children enrolled in the study. The children had deciduous teeth exhibiting pulp necrosis, with or without radiographically detected periradicular/interradicular bone resorption. The selected teeth had intact roots or <2/3 of physiological root resorption. Selected teeth had clinical crowns that permitted effective rubber dam isolation. There was an absence of history of trauma associated with the selected teeth, periodontal involvement and previous root canal treatment. Thirty-one primary teeth were molars, and nine were single-rooted teeth. Samples from multi-rooted teeth were taken from the largest root canal always associated with the periapical lesion.

Forty selected teeth were isolated using a rubber dam. Complete asepsis was employed, using the methodology proposed by Moller (1966); hydrogen peroxide (30%) was applied on the isolated crown, followed by 5% iodine, which was inactivated by 5% sodium thiosulfate solution. The samples were taken by scraping or filing the root canal walls with a size 15 K-type hand file (Dentsply Maillefer, Ballaigues, Switzerland). The file was introduced into the canal up to the radiographically determined working length, which was taken as approximately 1 mm short of the tooth apex in cases with intact roots, or to the limit of physiologic root resorption. Where there was radiographic image interposition of the primary root and the permanent germ, the file was inserted up to the level of the cusp of the permanent germ.

After removal from the canal, the final 2 mm of the file was removed using a sterile pair of surgical scissors and dropped into a microcentrifuge tube containing 20 μL of alkaline lysis buffer (400 mmol L^{-1} KOH, 100 mmol L^{-1} DTT, 10 mmol L^{-1} EDTA). After 10 min of incubation on ice, 20 μL of neutralization solution (400 mmol L^{-1} HCl, 600 mmol L^{-1} Tris–HCl,

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 the 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]Type strains.
[c]ATCC strains 43718 and 29523.
[d]ATCC strains 11827 and 11828.

pH = 0.6) was added, and the sample was kept at −20 °C until MDA was performed. Amongst the teeth sampled, three were symptomatic cases, 15 exhibited exudation, 22 were associated with sinus tracts and 26 radiographically detectable periapical radiolucencies.

Multiple displacement amplification (MDA) of root canal samples

MDA was performed as described by Teles *et al.* (2007). Genomiphi™ (Amersham Biosciences, Arlington Heights, IL, USA) was used for whole-genomic amplification as described by the manufacturer. In brief, 1 μL of each of the DNA templates (i.e. endodontic samples) was added to 9 μL of sample buffer (50 mmol L^{-1} Tris–HCl pH 8.2, 0.5 mmol L^{-1} EDTA containing random hexamer primers) in 200 μL microcentrifuge tubes (Stratagene, La Jolla, CA, USA). Templates in sample buffer were heat denatured at 95 °C for 3 min in a Perkin-Elmer Thermocycler and cooled to 4 °C. One microlitre of phi 29 DNA polymerase mix (the replicative polymerase from the phage phi29 bacterial virus – Blanco & Salas 1984, Meijer *et al.* 2001) including additional random hexamers was mixed on ice with 9 μL of reaction buffer containing dNTPs. The mixture was then added to the denatured sample to make a final volume of 20 μL and incubated at 30 °C for 16–18 h. Ten nanograms of Lambda DNA (contained in 1 μL) was used as a control (Phage Lambda is an *Escherichia coli* bacteriophage, the DNA of which is a DNA template to monitor proper enzyme activity, thus it is a DNA source for positive controls). The amplification reaction was terminated by incubation of the samples at 65 °C for 10 min. The amplified material was either immediately used, stored short term at 4 °C or at −20 °C for longer storage.

The DNA content of the samples was measured prior to and after amplification using the Picogreen™ dsDNA quantification assay (Invitrogen, Carlsbad, CA, USA). Picogreen™ is a fluorescent nucleic acid stain that allows the quantification of as little as 25 pg mL^{-1} of double-stranded DNA in samples.

Checkerboard DNA–DNA hybridization

Preparation of probes and standards for quantification.
Checkerboard DNA–DNA hybridization was performed as previously described (Socransky *et al.* 1994, 2004). For the preparation of probes and standards, each of the species listed in Table 1 was grown on the surface of blood agar plates (except the two spirochaetes, which were grown in broth) for 3–7 days. The cells were harvested and placed in 1.5-mL microcentrifuge tubes containing 1 mL of TE buffer (10 mmol L^{-1} Tris–HCl, 0.1 mmol L^{-1} EDTA, pH = 7.6). Cells were washed twice by centrifugation in TE buffer at 1300 *g* for 10 min. The cells were resuspended and lysed with either 10% SDS and Proteinase K (20 mg mL^{-1}) for Gram-negative strains or in 150 μL of an enzyme mixture containing 15 mg mL^{-1} lysozyme (Sigma) and 5 mg mL^{-1} achromopeptidase (Sigma, St Louis, MO, USA) in TE buffer (pH = 8.0) for Gram-positive strains. The pelleted cells were resuspended by 15 s of sonication and incubated at 37 °C for 1 h. DNA was isolated and purified using the method of Smith *et al.* (1989). The concentration of the purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm. The purity of the preparations was assessed by the ratio of the absorbances at 260 and 280 nm. Whole-genomic DNA probes were prepared from each of the 83 test strains by labelling 1–3 μg DNA with digoxigenin (Boehringer Mannheim, Indianapolis, IN, USA) using a random primer technique (Feinberg & Vogelstein 1983).

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

The membrane with fixed DNA was placed in a Miniblotter 45 apparatus (Immunetics) with the lanes of DNA at 90° to the channels of the device. A 30 × 45 'Checkerboard' pattern was produced. Each channel was used as an individual hybridization chamber for separate DNA probes. Bound probes were detected by anti-digoxigenin antibody conjugated with alkaline phosphatase and a chemifluorescent substrate. Signal intensities of the endodontic samples and the stan-

dards (containing 10^5 and 10^6 cells of each species) on the same membrane were measured using a Storm FluorImager (Molecular Dynamics, Sunnyvale, CA, USA). Signals were converted to absolute counts by comparison with standards on the membrane (Socransky *et al.* 2004). Failure to detect a signal was recorded as zero.

Two membranes were run for each sample: one containing the 'standard' 40 DNA probes used to examine periodontal samples as well as a probe to detect *Streptococcus mutans.* A second membrane employed 42 probes to species thought to be implicated in endodontic infections. Sensitivity and specificity tests were performed for all probes before performing the Checkerboard DNA–DNA hybridization analysis of the root canal samples. The protocol to validate the specificity of these 83 probes was similar to that described by Socransky *et al.* (2004). If cross-reactions were observed, the cross-reacting probes were discarded and new probes were constructed and validated (Socransky *et al.* 2004).

Data analysis

The microbial data were expressed in two ways. The prevalence of each species, reflected by presence/absence data, indicated the proportion of samples in which the species were detected at $>10^4$ cells in MDA-amplified samples. Since the sample DNA was amplified, absolute numbers could not be determined. Thus, proportions that each species comprised the total DNA probe count were computed for each sample. Proportion data were expressed as percentage of total DNA probe count for each species and averaged across samples.

Results

Quantification of DNA before and after MDA of endodontic samples

DNA from each root canal sample was amplified using MDA. Eight samples failed to provide good-quality amplified DNA. The amount of DNA [± standard deviation (SD)] present in the 32 remaining samples before the amplification averaged 5.2 (±4.7, SD) ng and 6.1 (±2.3) μg after amplification, an approximately 1000-fold amplification. Amongst the 32 samples included in this study, five were from single-rooted teeth and 27 from multi-rooted teeth. Twelve of the samples were from females (n = 12), and the mean age (±SD) was 6.8 years (±1.9) (range: 4–12 years). Of the eight samples excluded because of absence of amplification, two were from single-rooted teeth and six from multi-rooted teeth. Five of the samples were from females (n = 5), and the mean age (±SD) was 6.4 years (±1.8) (range: 4–9 years).

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 equivalent to $>10^4$ bacterial cells (in 5 μL of amplified sample) was 19 (±4) (range: 3–66). *Aggregatibacter (Haemophilus) aphrophilus* and *Helicobacter pylori* were not detected in any of the samples.

Figure 1 demonstrates the mean percentage of samples exhibiting counts of each of the 81 bacterial species detected at the level of $>10^4$ bacterial cells. The most prevalent bacterial species was *Prevotella intermedia*, detected in 96.9% of the sampled teeth. It was followed by *Neisseria mucosa* (65.2%), *Prevotella nigrescens* (56.2%), *Tannerella forsythia* (56.2%), *Prevotella denticola* (53.1%) and *Fusobacterium nucleatum* ss *vincentii* (50.0%). The least prevalent species detected at the $>10^4$ bacterial cells level were *Enterococcus faecalis* (3.2%) and *Eikenella corrodens* (3.1%).

Figure 2 presents the mean proportions (percentage of DNA probe counts) of the target species averaged across the positive samples. *P. intermedia* showed the highest mean proportions (10.0%) followed by *Prevotella tannerae* (7.0%) and *P. nigrescens* (4.3%), whilst *Campylobacter ureolyticus* (0.05%), *Legionella pneumophilia* (0.04%), and *E. faecalis* (0.03%) showed the lowest mean proportions.

Discussion

The purpose of the present investigation was to evaluate the microbiota of endodontic infections in deciduous teeth. The bacterial diversity in any environment is underestimated when assessed by culture-based techniques (Munson *et al.* 2002, Papapanou 2002). Infected root canals yielded a maximum of 10–12 species when assessed by culture methods (Sundqvist 1976, Gomes *et al.* 2004), whilst this range raises to 42–51 species in studies using culture-independent molecular methods (Siqueira *et al.* 2000, Brito *et al.* 2007). Conventional PCR and quantitative PCR (qPCR) are often used for direct detection of taxa in root canal samples. However, the number of samples and taxa that can be analysed is limited by the costs and

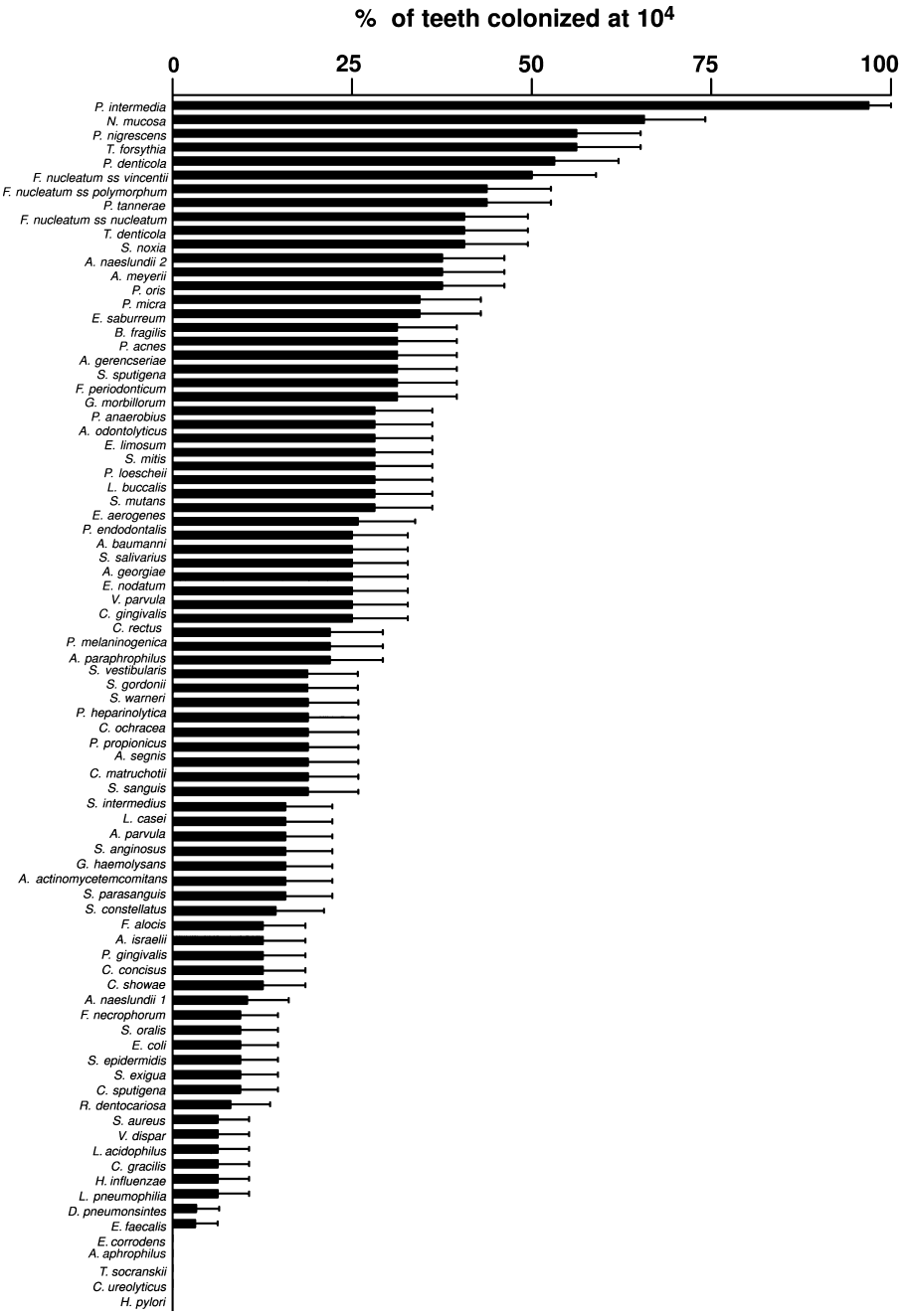

Figure 1 Mean prevalence (% of teeth colonized by counts of $>10^4$) of individual species in root canal samples from primary teeth. The data are ordered in descending order of prevalence in the amplified samples.

complexity of the procedure, particularly when using qPCR.

Checkerboard DNA–DNA hybridization enables the quantitative analysis of multiple taxa in large numbers of samples (Socransky & Haffajee 2005). However, individual bacterial species may be present in the infected root canal system in numbers below the level of detection of the Checkerboard DNA–DNA hybridization technique. The lack of detection of such taxa would underestimate their possible role in the

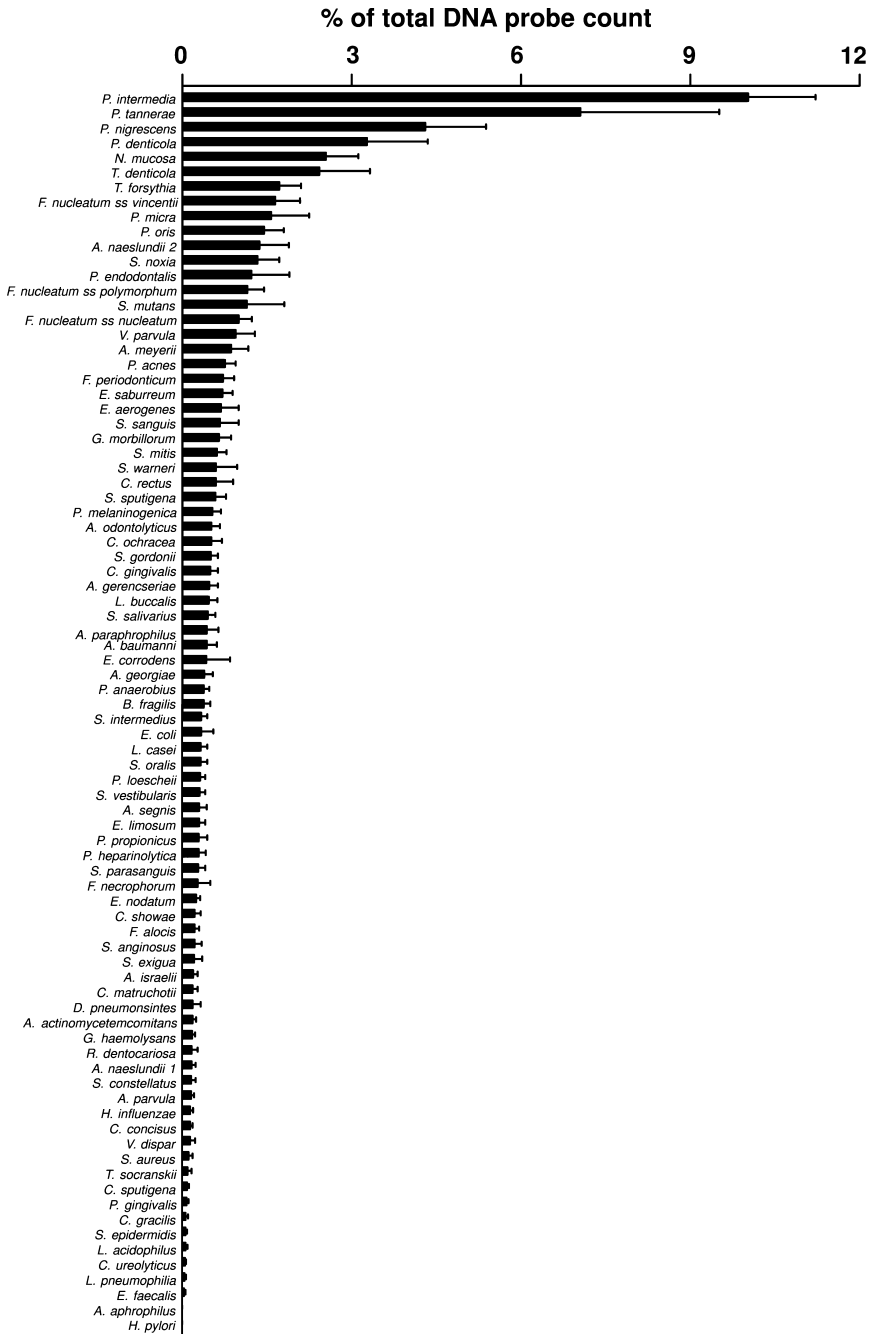

Figure 2 Quantitative analysis of Microbiota of 32 deciduous endodontic infections analysed by multiple displacement amplification (MDA) and Checkerboard DNA–DNA hybridization represented by the percentage of mean proportion DNA probe counts (±SEM) of test species. The percentage of the DNA probe count was computed for each species for each sample and averaged across samples. The data are ordered in descending order of mean percentages of DNA probe counts detected in amplified samples.

endodontic microbial ecosystem. MDA provides a simple and reliable method to amplify the sample DNA with minimal bias (Hawkins *et al.* 2002, Nelson *et al.* 2002, Yan *et al.* 2004, Brito *et al.* 2007, Teles *et al.* 2007). The characteristics of the enzyme used in MDA and the reaction *per se* contribute to minimizing

the bias. The Φ29 DNA polymerase has a very low error rate of one in 10^6–10^7 nucleotides in its intrinsic enzymatic activity (Esteban *et al.* 1993) and during amplification (Nelson *et al.* 2002), in contrast to 3×10^4 for Taq DNA polymerase (Eckert & Kunkel 1991). Thus, the accumulation of mutations following a 10 000-fold amplification is just three per 10^6 nucleotides (Nelson *et al.* 2002). PCR-based amplification results in bias that varies from 10^2 to 10^6, whilst MDA bias for human genomic DNA has been estimated to be less than threefold (Dean *et al.* 2002). Additionally, the MDA is an isothermal reaction, in contrast to PCR-based techniques, which require multiple cycles at different temperatures and thus can be biased by differences in % GC content of different genomes (Teles *et al.* 2007). In the present study, MDA amplification of DNA from the root canal samples led to an approximately 1000-fold amplification. As the amplified sample was obtained from an aliquot of the original sample and the detection limit of the Checkerboard DNA–DNA hybridization is 10^4 bacterial cells, it was possible that as few as 10 cells of a given species were present in the sample prior to amplification and which could be detected after amplification.

On average, 19 species were detected per amplified sample, substantially more than previously detected by culture (Lana *et al.* 2001, Gomes *et al.* 2004) and by clonal analysis (Jacinto *et al.* 2007) in adults. This figure is lower than the average of 51.2 species recently reported in endodontic infections in adults, using a similar methodology to the one employed in the present study (Brito *et al.* 2007). Ruviere *et al.* (2007) used whole-genomic probes to detect 34 bacterial taxa in root canal samples from deciduous teeth. The authors found, on average 7.47 bacterial species (range: 0–10) in teeth exhibiting irreversible pulpitis and 11.45 species (range: 0–27) in teeth with pulp necrosis and chronic apical periodontitis. It suggests that the difference in numbers may be because of the primary teeth harbouring a less complex microbiota or that infection in adults may have been present longer, allowing time for greater diversity to develop.

Studies focusing on root canal infections often employ 'K'- or 'H'-type files followed by two to four paper points to collect the samples (Siqueira *et al.* 2000, Pazelli *et al.* 2003, de Souza *et al.* 2005, da Silva *et al.* 2006, Ruviere *et al.* 2007, Sassone *et al.* 2007, 2008, Cogulu *et al.* 2008). This method may not target the microbiota in the apical third of the root canal, because the entire content of the canal could be collected. To ensure that the apical portion was the main area sampled in this study, a K file was inserted using a reaming motion to the working length and only the apical 2 mm was sectioned. The difference in sampling may account for the prevalence of streptococci in the studies using paper points (Pazelli *et al.* 2003, da Silva *et al.* 2006, Ruviere *et al.* 2007). The facultative anaerobic species, such as the *Streptococcus* species, may be present in higher counts in the coronal third of the canal, where conditions may favour their growth. In the present study, this genus was identified in higher counts only in teeth with pulp chamber exposure. Differences in sampling might also explain the lower bacterial cell numbers found in comparison with 10^5–10^7 cells per sample reported by other authors using H files associated with paper points to collect samples analysed by Checkerboard DNA–DNA hybridization (Sassone *et al.* 2007, 2008), paper points and real-time PCR (Blome *et al.* 2008), and paper points and culture techniques (Vianna *et al.* 2008) to study endodontic infection in adults.

Root canal infections are polymicrobial and predominated by obligate and facultative anaerobes (Sundqvist 1992, Lana *et al.* 2001). In this study, the most prevalent bacterial species was *P. intermedia*, present in 96.9% of the samples. It was followed by other obligate anaerobes, such as *P. nigrescens*, *T. forsythia*, *P. denticola*, *F. nucleatum* ss *vincenti*, as well as *N. mucosa*, a facultative organism. These findings were in contrast with the results reported by Ruviere *et al.* (2007), where *Campylobacter rectus*, *Treponema denticola* and *Gemella morbillorum* were the most prevalent taxa, and Cogulu *et al.* (2008), who found that *T. denticola* and *Porphyromonas gingivalis* were the most prevalent species. The sampling procedures and methods for bacterial detection employed in those studies may account for those differences. Sassone *et al.* (2007, 2008) showed that more than 70% of root canal samples in permanent teeth were colonized by *N. mucosa* and *F. nucleatum* ss *vincentii*, which is in accord with the present study, even though Sassone *et al.* (2007, 2008) performed those studies on samples from adult teeth. However, *E. faecalis*, which was detected infrequently in this study, was found in high prevalence by those authors. *E. faecalis* has been frequently found in association with secondary endodontic infection in adults (Pirani *et al.* 2008, Vianna *et al.* 2008). Cogulu *et al.* (2008) reported the high prevalence of this species in primary infection of permanent teeth of children. Conceivably, the anatomy of permanent teeth favours colonization by this taxon. It is possible that it

is a transient species introduced by food or human contact, since *E. faecalis* detection is a rare event at the advancing front of dentinal lesions (Zehnder & Guggenheim 2009).

The combination of MDA and Checkerboard DNA–DNA hybridization permitted a better appreciation of the microbiota associated with root canal infection in deciduous teeth. The present study confirmed the polymicrobial nature of these infections and that they comprised a large number of facultative and anaerobic species. The data show that deciduous endodontic infections are highly diverse, presenting, on average, 19 taxa per sample (range 3–66 taxa). The data also suggest that *Prevotella* sp. may play an important role in the microbiota of endodontic infections in primary teeth. *P. intermedia*, *P. nigrescens* and *P. denticola* were highly prevalent in the samples analysed. *P. intermedia*, *P. tannerae* and *P. nigrescens* represented the highest proportions of the total DNA probe count. However, more studies analysing a larger number of samples using standardized sampling methods are needed to foster a better understanding of the pathogenesis of deciduous apical periodontitis, as well as aid the design of more efficient endodontic treatments.

Conclusions

Root canals of infected deciduous teeth present wide bacterial diversity. *Prevotella* sp. were the most prevalent amongst the samples, and *P. intermedia*, *Prevotella tannerae* and *Prevotella nigrescens* gave the highest proportion of hybridization of the total DNA probe counts.

Acknowledgements

Thanks are due to Department of Periodontology of The Forsyth Institute and the postgraduate program at the School of Dentistry of UFMG. This study was supported by FAPEMIG, CAPES, CNPq and in part by grants T32-DE-07327 (F.T.) and DE12108 and DE14242 from the National Institute of Dental and Craniofacial Research.

Glossary

Phi DNA polymerase mix

Phi (i.e. the Greek letter Φ) 29 is a bacteriophage (a bacterial virus) often found in *Bacillus subtilis*. Phi 29 DNA polymerase is the replicative polymerase from the phage phi29 (Φ29) (Blanco & Salas 1984, Meijer *et al.* 2001).

In the Genomiphi kit used in this study, the enzyme was contained in a Phi DNA polymerase mix, along with random hexamers (i.e. six nucleotide-long random primers).

Lambda DNA used as a control

Phage Lambda (i.e. Greek letter λ) is an *Escherichia coli* bacteriophage. Its DNA ('Lambda DNA') is often used for a wide range of applications in molecular biology, including activity and specificity assays of restriction enzymes, preparation of DNA molecular weight standards and cloning. It is provided with the Genomiphi kit and used as a DNA template to monitor proper enzyme activity. Hence, it is a DNA source for positive controls.

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