HHS Public Access

Author manuscript

J Endod. Author manuscript; available in PMC 2017 August 01.

Published in final edited form as:

J Endod. 2016 August ; 42(8): 1239–1245. doi:10.1016/j.joen.2016.05.014.

Microbial Ecosystem Analysis in Root Canal Infections Refractory to Endodontic Treatment

LCF Henriques[1], **LCN Brito**[1,2], **WLF Tavares**[1], **RP Teles**[3], **LQ Vieira**[4], **FRF Teles**[3], and **AP Ribeiro Sobrinho**[1]

[1]Departamento de Odontologia Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

[2]Faculdade de Odontologia, Universidade de Itaúna, Minas Gerais, Brasil

[3]Department of Periodontology, University of North Carolina at Chapel Hill School of Dentistry, Chapel Hill, North Carolina, USA

[4]Departamento de Bioquímica e Imunologia Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Abstract

Introduction—To combine Multiple Displacement Amplification (MDA) and checkerboard DNA–DNA hybridization to qualitatively and quantitatively evaluate the microbiota present in infections refractory to endodontic treatment.

Methods—The subjects of this study were 40 patients presenting periapical lesions refractory to endodontic treatment. Samples were taken by scraping or filing root canal walls with a #10 K-type hand file. Sample DNA was amplified by MDA, and the levels of 107 bacterial taxa were analyzed by checkerboard DNA–DNA hybridization. The taxa were divided into three distinct microbial populations, depending on their mean proportion in samples (% DNA probe counts ± SEM), as follows: dominant (≥3.0%), sub-dominant (>1.6 to 3.0%) and residual (≤1.6%) populations. The significance of differences was determined using the Mann-Whitney test.

Results—The taxa present with the highest mean proportions (constituting the dominant population) were *Corynebacterium diphtheriae* (8.03±0.98), *Porphyromonas gingivalis* (5.42±2.09), *Streptococcus sobrinus* (5.33±0.69), and *Stenotrophomonas maltophilia* (4.72±1.73). Among the sub-dominant population were *Eubacterium saphenum* (3.85±1.06), *Helicobacter pylori* (3.16±0.62), *Dialister pneumosintes* (3.12±1.1), *Clostridium difficile* (2.74±0.41), *Enterobacter agglomerans* (2.64±0.54), *Salmonella enterica* (2.51±0.52), *Mobiluncus mulieris* (2.44±0.6), and *Klebsiella oxytoca* (2.32±0.66). In the population of bacteria present at the lowest mean proportions (the residual population), *Bacteroides ureolyticus* (0.04±0.01), *Haemophilus influenzae* (0.04±0.02), and *Prevotella oris* (0.01±0.01) were found at the lowest mean proportions. *Enterococcus faecalis* was detected in the residual population (0.52±0.26).

*Corresponding author: Dr. Antônio Paulino Ribeiro Sobrinho - Departamento de Odontologia Restauradora, Faculdade de Odontologia, Universidade Federal de Minas Gerais, CEP 31270-901, Belo Horizonte, MG, Brazil. Phone: 55-31-3409-2470, Fax: 55-31-3409-2470, sobrinho.bhz@gmail.com.

Conclusion—The microbial climax community in teeth refractory to endodontic treatment not only harbor medically important species, but also contains distinct microbial consortia present with different population levels.

Keywords

checkerboard DNA–DNA hybridization; endodontic treatment failure; multiple displacement amplification; root-canal-treated teeth

Introduction

Failure of endodontic therapy is often caused by the persistence of microorganisms in root canal systems or by recontamination after inadequate coronal sealing. When treatment fails, bone resorption may occur as a consequence of periapical immune and inflammatory responses (1–4). When possible, endodontic retreatment can be performed to attempt to achieve healthy teeth.

Over the years, most studies have shown that the microbiota recovered from teeth refractory to endodontic treatment predominantly consisted of gram-positive bacteria, especially *Enterococcus faecalis* (2, 5, 6). Nevertheless, the high rate of detection of this species may have been influenced by significant limitations of microbial techniques, such as low sensitivity and an inability to detect fastidious and as-yet-uncultivated phylotypes, as such limitations may cause the bacterial diversity of diverse oral ecosystems to be underestimated (7).

Traditionally, the study of infectious diseases has focused on one or a small number of pathogens that cause a given infectious disease. The examination of complex mixtures of microorganisms has been hampered by the tradition of focusing on a small number of species thought to be pathogenic and by the lack of useful, rapid identification techniques (8). However, new concepts of biofilm infections are becoming established; microbial communities responsible for these infections are considered to be greater than the simple sums of their parts (9). In this regard, molecular assays have shown that the microbiota in teeth refractory to treatment are much more complex than was previously known (5). Mixed consortiums were recovered from those teeth, revealing that as-yet-uncultivated bacteria and taxa other than *E. faecalis* may participate in these infections (10).

The quantity of bacteria in the samples is an important factor in the checkerboard DNA-DNA hybridization technique because the level of detection for this assay is approximately 10^4 bacterial cells of a given species (11). Recently, to overcome these limitations, which could underestimate the presence of some taxa in the root canal microbial ecosystem because this ecosystem contains very few bacterial cells (12), researchers have used multiple-displacement amplification (MDA) before hybridizing samples. MDA has proved to be a simple and reliable method for amplifying sample DNA with minimal bias (13–17). The combined use of MDA and hybridization has contributed to the recognition that endodontic microbiota is far more complex than was previously thought (14, 16, 17).

The aim of the present study was to use MDA and checkerboard DNA–DNA hybridization together to qualitatively and quantitatively evaluate the microbiota of infections refractory to endodontic treatment and to determine dominant, sub-dominant and residual microbial populations in this ecosystem.

Materials and Methods

Human subjects

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

Sample collection

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

The selection and preparation of the teeth was performed as previously described (14, 16). In brief, the pre-existing root canal filling was removed using retreatment ProTaper NiTi files (Dentsply, Ballalgues, Switzerland) without the use of any solvent. Samples were taken by scraping or filing the root canal walls with a #10 K-type hand file (Maillefer, Ballaigues, Switzerland). The file was introduced into the canal at a level approximately 1 mm beneath the tooth apex. After the file was removed from the canal, the final 4 mm of the file was cut off the file using a sterile pair of surgical scissors and placed in a microcentrifuge tube containing 20 μl of alkaline lysis buffer (400 mM KOH, 100 mM dithiothreitol, 10 mM EDTA). After 10 min of incubation on ice, 20 μl of neutralization solution (400 mM HCl, 600 mM Tris-HCl, pH 0.6) was added. Samples were kept at 4°C until analysis.

Multiple displacement amplification (MDA) of root canal samples

Multiple displacement amplification was performed as previously described (14–17). The Illustra™ GenomiPhi™ V2 DNA Amplification Kit (GE Healthcare, USA) was used for whole genomic amplification following the manufacturer's instructions. In brief, 1 μl of each of the DNA templates (i.e., endodontic samples) was added to 9 μl of sample buffer (50 mM Tris-HCl pH 8.2, 0.5 mM EDTA containing random hexamer primers) in 200-μl microcentrifuge tubes (Stratagene, La Jolla, CA, USA). Templates in sample buffer were heat-denatured at 95°C for 3 minutes in a Perkin-Elmer Thermocycler and cooled to 4°C. One μl of phi 29 DNA polymerase mix containing additional random hexamers was mixed on ice with 9 μL of reaction buffer containing dNTPs. This mixture was then added to the denatured sample to make a final volume of 20 μl, and the samples were incubated at 30°C for 2 hours. Ten ng of lambda DNA (in a volume of 1 μl) was used as a control. The

amplification reaction was terminated by incubating the samples at 65°C for 10 min. The amplified material was either immediately used, stored short-term at 4°C, or stored long-term at −20°C.

The DNA content of the samples was measured prior to and after amplification using the PicoGreen™ dsDNA quantification assay (Invitrogen, Carlsbad, CA, USA). PicoGreen™ is a fluorescent nucleic acid stain that allows the quantification of as little as 25 pg/ml of double-stranded DNA in samples. The microbiological content of the amplified samples was then analyzed using checkerboard DNA-DNA hybridization.

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

The 107 reference strains used to prepare DNA probes are listed in Table 1. The growth conditions of the selected bacterial strains have been described previously (14–16, 18).

Preparation of the probes and standards for quantification

Checkerboard DNA-DNA hybridization was performed as previously described (11, 18). To prepare probes and standards, each species listed in Table 1 was grown on agar plates (except the two spirochetes, which were grown in broth) for 3–7 days. The cells were harvested and placed in 1.5 ml microcentrifuge tubes containing 1 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6). Cells were washed twice by centrifugation in TE buffer at 1300×g for 10 min. The cells were resuspended and lysed with either 10% SDS and proteinase K (20 mg/ml), for gram-negative strains, or with 150 μl of an enzyme mixture containing 15 mg/ml lysozyme (Sigma) and 5 mg/mL achromopeptidase (Sigma) in TE buffer (pH 8.0), for gram-positive strains. The pelleted cells were resuspended by 15 s of sonication and incubated at 37°C for 1 h. DNA was isolated and purified using the method of Smith *et al.* (19). The concentration of the purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm. The purity of the preparations was assessed using the ratio of the absorbances at 260 and 280 nm. Whole genomic DNA probes were prepared from each of the 107 test strains by labeling 1–3 μg of DNA with digoxigenin (Boehringer Mannheim, Indianapolis, IN, USA), using a random primer technique (20).

Sample preparation and microbial analysis

Following amplification and quantification, the amplified endodontic samples were boiled for 10 min. Approximately 1500 ng of DNA (5 μL) from 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 with ultraviolet light (Stratalinker 1800, La Jolla, CA, USA) followed by baking at 120°C for 20 min. The Minislot device permitted the deposition of 28 different samples in individual lanes on a single membrane, as well as two control lanes containing standards for quantification; 1 and 10 ng of DNA from each bacterial species tested, equivalent to 10^5 and 10^6 cells, respectively, were used as standards for quantification.

Checkerboard DNA-DNA hybridization was performed as described previously by Socransky *et al.* (11, 18). The membrane containing 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 using an anti-digoxigenin antibody conjugated with alkaline phosphatase, together with a chemifluorescent substrate. Signal intensities of the endodontic samples and the standards (containing 10^5 and 10^6 cells of each species) on the same membrane were measured using a Storm FluorImager (Molecular Dynamics, Sunnyvale, CA, USA). Signals were converted to absolute counts by comparison with the standards on the same membrane (18). Failure to detect a signal was recorded as a count of zero.

Three membranes were run for each sample: one containing a standard set of 40 DNA probes that are routinely used to examine periodontal samples, as well as a probe to detect *Streptococcus mutans*. A second membrane contained 42 probes for additional species thought to be implicated in endodontic infections. A third membrane was used to assess levels of medically important microbial taxa. Sensitivity and specificity tests were performed for all probes prior to the checkerboard DNA-DNA hybridization analysis, using a protocol similar to that described by Socransky *et al.* (18).

Data analysis

Because the sample DNA was amplified, the absolute quantity of a given bacterial species in a sample could not be determined. Thus, the relative proportions of the total DNA probe count for each species were computed for each sample and then averaged across subjects in each group separately.

The significance of differences between the proportions of test species in samples from selected subjects was assessed using the Mann-Whitney test.

Results

Quantification of DNA before and after MDA of endodontic samples

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

Microbial species in root canal samples refractory to endodontic treatment

To better analyze the microbial consortium that colonize teeth refractory to endodontic treatment, we divided the taxa into three distinct microbial populations, depending on their mean proportion (% of DNA probe counts ± SEMs), as follows: high (≥3.0%), middle (>1.6 to 3.0%) and low mean proportions (≤1.6%), corresponding to dominant, sub-dominant and residual populations. These mean proportions of the target 107 test species are presented in Fig. 1. The dominant taxa were *Corynebacterium diphtheriae* (8.03±0.98), *Porphyromonas gingivalis* (5.42±2.09), *Streptococcus sobrinus* (5.33±0.69), and *Stenotrophomonas*

maltophilia (4.72±1.73). Among the sub-dominant populations were *Eubacterium saphenum* (3.85±1.06), *Helicobacter pylori* (3.16±0.62), *Dialister pneumosintes* (3.12±1.1), *Clostridium difficile* (2.74±0.41), *Enterobacter agglomerans* (2.64±0.54), *Salmonella enterica* (2.51±0.52), *Mobiluncus mulieris* (2.44±0.6), and *Klebsiella oxytoca* (2.32±0.66). In the residual population, *Bacteroides ureolyticus* (0.04±0.01), *Haemophilus influenzae* (0.04±0.02), and *Prevotella oris* (0.01±0.01), was found with the lowest mean proportions. *Enterococcus faecalis* (0.52±0.26) was among the species detected. However, *E. faecalis* was detected at a high mean proportion (10.1%) in only one sample site from among 40 teeth analyzed (Table 2). *Candida albicans*, *Escherichia coli E. coli*, and *Lactobacillus acidophilus* were not detected in any sample (Fig. 1).

The mean counts (>105±SEM) of the 107 test species in amplified root canal samples taken from 40 teeth is presented in Figure 2. The species are ordered according to mean counts. *C. diphtheriae* exhibited the highest mean counts (6.47X105±1.12.), followed by *Streptococcus sobrinus, P. gingivalis, Clostridium difficile* and *Eubacterium saphenum*, while *Candida glabrata* exhibited the lowest mean counts (0.001X105±0.001), followed by *P. oris* and *Candida tropicalis.* Twenty species were not detected in any of the samples.

Discussion

Multiple microbial species are needed to produce apical periodontitis, including in teeth refractory to treatment (10, 21). In a developing ecosystem, pioneer organisms colonize a location first and alter the habitat, making it suitable for colonization by other species (8). However, the population levels and the distinct microbial species in such climax communities are regulated by multifactorial processes, such as changes in the physical or chemical properties of the region or changes in the host (22). In this regard, all microorganisms that act as pathogens must be present at high levels to play a role in the biofilm community as well as in the modulation of the host immune response.

Studies that have attempted to analyze root canal microbial ecosystem have been limited by the bacterial cells present in this ecosystem (12). As a consequence, the inability to detect some taxa and the difficulties with counting other taxa could lead to their possible roles in the endodontic microbial ecosystem being underestimated. The high sensitivity of the combination of MDA and checkerboard DNA-DNA hybridization overcomes this limitation, as has been demonstrated previously (14–17). As MDA amplified the total DNA in the samples approximately 1000-fold, it is likely that even a number of bacterial cells below the level of detection of the checkerboard DNA–DNA hybridization technique alone were detected by this approach. In this study, we assessed 40 patients presenting periapical lesions refractory to endodontic treatment using probes for 107 different microbial taxa. Based on the mean proportions (% of DNA probe counts ± SEM) of each taxa, we divided the microbial population into species with high (≥3.0%), middle (>1.6 to 3.0%) and low (≤ 1.6%) mean proportions (Fig. 1).

C. diphtheria, P. gingivalis, S. sobrinus, and *S. maltophilia* were among the taxa present with the highest mean proportions in these infections. Notably, *C. diphtheria* has already been recovered from periodontal disease sites (23) and root canal infections (14). *C. diphtheriae*

belongs to the family Mycobacteriaceae and is gram-positive and aerobic; it causes the disease diphtheria, which primarily affects the upper respiratory tract. The most commons site of infection is the pharynx and the tonsils, but the bacteria can also invade the nasal tissues, larynx and skin. However, it is important to note that toxin production occurs only when the bacillus is itself infected by a specific virus (bacteriophage) carrying the genetic information for the toxin (*tox* gene) (24). Only toxigenic strains can cause severe disease (25). Black-pigmented anaerobic rods, such as *P. gingivalis*, are members of the "red complex" (8) and are involved in the etiology and perpetuation of endodontic infections. They have been detected in permanent and deciduous teeth (14, 16, 17), as well as in teeth refractory to endodontic treatment (10).

S. sobrinus is an anaerobic, gram-positive bacteria that is normally found in the oral cavities. It is difficult for *S. sobrinus,* like most human pathogens, to grow and survive outside an animal host. In addition to living in acidic environments, this organism also produces lactic acid as a byproduct of the anaerobic metabolism of glucose, and this ability to produce lactic acid is one of the main reasons why this organism is considered a human pathogen. Recently, comparing the microbiota of endodontic infections in necrotic pulp from HIV-positive and HIV-negative subjects, *S. sobrinus* was found to be among the most prevalent taxa in HIV-positive subjects (17).

One goal of this investigation was to extend our previous studies that found a greater number of bacterial species in root canal samples than had other studies; in our previous studies, species present in low numbers were detected by MDA (14, 16, 17). Moreover, over the past decade we have emphasized that root canal infections may be a source of medically important species (14). In this regard, the present study found *S. maltophilia* to be among the taxa present at the highest mean proportion in refractory endodontic infections, as has been previously described in primarily endodontic infections (17). *Stenotrophomonas* infections have been associated with high morbidity and mortality in severely immunocompromised and debilitated individuals (26). Risk factors associated with *Stenotrophomonas* infection include admission to an intensive care unit, HIV infection, malignancy, neutropenia, central venous catheters, recent surgery, trauma, and previous therapy with broad-spectrum antibiotics (26–28). The majority of *S. maltophilia* strains are characterized by their resistance to many currently available broad-spectrum antimicrobial agents, including those of the carbapenem class (29); because of this broad antimicrobial resistance, it is often difficult to treat patients infected with *S. maltophilia*.

The residual population (≤1.6%) comprised a large proportion of the bacterial species present in teeth refractory to root canal treatment, similar to findings from previous studies of indigenous gastrointestinal microbiota (30). Despite the low relative proportions of bacteria in the residual population, the relevance of population should not be underestimated. Even members present in low numbers may provide the community with advantageous properties and thus serve as key species within complex communities (31). Moreover, disturbances in local factors, such as nutrients sources, and competitive processes may result in a shift in the microbial composition, allowing a residual population to become dominant (9). In agreement with this hypothesis, previous findings from PCR-DGGE and PCR studies have found high interindividual variability in the bacterial community profiles

of cases of treatment failure (10, 21), revealing while a high proportion of such populations may be found in one individual, a low proportion may be found in another (21, 32).

Over the past two decades, root canal treatment failure has been attributed to the presence of *E. faecalis* (2, 33, 34). In this study, *E. faecalis* was detected at a low mean proportion (0.52±0.26; Fig. 1). Recently, Murad *et al.* (6) found a high prevalence of *E. faecalis* (28%) by checkerboard DNA-DNA hybridization without prior amplification of sample DNA. Notably, it was observed in this study that among the 40 samples previous amplified by MDA, *E. faecalis* was absent in only one sample (97.5% prevalence). *E. faecalis* was present with the highest mean proportion at only one sample site, however (Table 2). Conversely, combining culture methods with a culture-independent approach, Anderson *et al.* (35) found *E. faecalis* in no more than 2 cases and only found this bacterium using culture methods. Hence, our findings are in agreement with previous reports (5, 10) that have questioned the relevance of *E. faecalis* in endodontic treatment failure, because this bacterial species survives and persists at treated root canal sites with a high prevalence but accounts for only a low mean proportion of the bacteria usually found at such sites.

The microbial climax community in teeth refractory to endodontic treatment is much more complex than previously thought. These microbial communities not only harbor medically important species but also contain distinct microbial consortia present with different population levels.

Acknowledgments

This work was supported in part by the Krakow Harvard/Forsyth Endowed Endodontic Research Fund (to LCNB and FRFT) and the National Institutes of Health / National Institute of Dental and Craniofacial Research (R01-DE024767 to FRFT).

This work was also supported in part by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors wish to thank the postgraduate program at the School of Dentistry of UFMG. LCNB, APRS and LQV are CNPq fellows.

References

1. Stashenko P, Teles R, D'Souza R. Periapical inflammatory responses and their modulation. Crit Rev Oral Biol Med. 1998; 9:498–52. [PubMed: 9825224]
2. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1998; 85:86–93. [PubMed: 9474621]
3. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. Crit Rev Oral Biol Med. 2004; 15:348–81. [PubMed: 15574679]
4. Henriques LC, de Brito LC, Tavares WL, Vieira LQ, Ribeiro Sobrinho AP. Cytokine analysis in lesions refractory to endodontic treatment. J Endod. 2011; 37:1659–62. [PubMed: 22099900]
5. Siqueira JF Jr, Rocas IN. Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004; 97:85–94. [PubMed: 14716262]
6. Murad CF, Sassone LM, Faveri M, Hirata R Jr, Figueiredo L, Feres M. Microbial diversity in persistent root canal infections investigated by checkerboard DNA-DNA hybridization. J Endod. 2014; 40:899–906. [PubMed: 24935532]
7. Paster BJ, Olsen I, Aas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontol 2000. 2006; 42:80–7. [PubMed: 16930307]

8. Socransky SS, Haffajee AD. Periodontal microbial ecology. Periodontol 2000. 2005; 38:135–87. [PubMed: 15853940]
9. Siqueira JF Jr, Rocas IN. Community as the unit of pathogenicity: an emerging concept as to the microbial pathogenesis of apical periodontitis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009; 107:870–8. [PubMed: 19464660]
10. Sakamoto M, Siqueira JF Jr, Rocas IN, Benno Y. Molecular analysis of the root canal microbiota associated with endodontic treatment failures. Oral Microbiol Immunol. 2008; 23:275–81. [PubMed: 18582326]
11. Socransky SS, Smith C, Martin L, Paster BJ, Dewhirst FE, Levin AE. "Checkerboard" DNA-DNA hybridization. Biotechniques. 1994; 17:788–92. [PubMed: 7833043]
12. Zavistoski J, Dzink J, Onderdonk A, Bartlett J. Quantitative bacteriology of endodontic infections. Oral Surg Oral Med Oral Pathol. 1980; 49:171–4. [PubMed: 6928290]
13. Nelson JR, Cai YC, Giesler TL, Farchaus JW, Sundaram ST, Ortiz-Rivera M, et al. TempliPhi, phi29 DNA polymerase based rolling circle amplification of templates for DNA sequencing. Biotechniques. 2002; (Suppl):44–7.
14. Brito LC, Teles FR, Teles RP, Franca EC, Ribeiro-Sobrinho AP, Haffajee AD, et al. Use of multiple-displacement amplification and checkerboard DNA-DNA hybridization to examine the microbiota of endodontic infections. J Clin Microbiol. 2007; 45:3039–49. [PubMed: 17634304]
15. Teles F, Haffajee AD, Socransky SS. Multiple displacement amplification as an aid in checkerboard DNA-DNA hybridization. Oral Microbiol Immunol. 2007; 22:118–25. [PubMed: 17311635]
16. Tavares WL, Neves de Brito LC, Teles RP, Massara ML, Ribeiro Sobrinho AP, Haffajee AD, et al. Microbiota of deciduous endodontic infections analysed by MDA and Checkerboard DNA-DNA hybridization. Int Endod J. 2011; 44:225–35. [PubMed: 21083570]
17. Brito LC, Sobrinho AP, Teles RP, Socransky SS, Haffajee AD, Vieira LQ, et al. Microbiologic profile of endodontic infections from HIV– and HIV+ patients using multiple-displacement amplification and checkerboard DNA-DNA hybridization. Oral Dis. 2012; 18:558–67. [PubMed: 22335194]
18. Socransky SS, Haffajee AD, Smith C, Martin L, Haffajee JA, Uzel NG, et al. Use of checkerboard DNA-DNA hybridization to study complex microbial ecosystems. Oral Microbiol Immunol. 2004; 19:352–62. [PubMed: 15491460]
19. Smith GL, Socransky SS, Smith CM. Rapid method for the purification of DNA from subgingival microorganisms. Oral Microbiol Immunol. 1989; 4:47–51. [PubMed: 2628868]
20. Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem. 1983; 132:6–13. [PubMed: 6312838]
21. Rocas IN, Siqueira JF Jr, Aboim MC, Rosado AS. Denaturing gradient gel electrophoresis analysis of bacterial communities associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004; 98:741–9. [PubMed: 15583550]
22. Savage DC. Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol. 1977; 31:107–33. [PubMed: 334036]
23. Beveridge TJ, Goldner M. Statistical relationship between the presence of human subgingival anaerobic diptheroids and periodontal disease. J Dent Res. 1973; 52:451–3. [PubMed: 4205147]
24. Oram M, Woolston JE, Jacobson AD, Holmes RK, Oram DM. Bacteriophage-based vectors for site-specific insertion of DNA in the chromosome of Corynebacteria. Gene. 2007; 391:53–62. [PubMed: 17275217]
25. Holmes RK. Biology and molecular epidemiology of diphtheria toxin and the tox gene. J Infect Dis. 2000; 181(Suppl 1):S156–67. [PubMed: 10657208]
26. Paez JI, Tengan FM, Barone AA, Levin AS, Costa SF. Factors associated with mortality in patients with bloodstream infection and pneumonia due to Stenotrophomonas maltophilia. Eur J Clin Microbiol Infect Dis. 2008; 27:901–6. [PubMed: 18483755]
27. Kwa AL, Low JG, Lim TP, Leow PC, Kurup A, Tam VH. Independent predictors for mortality in patients with positive Stenotrophomonas maltophilia cultures. Ann Acad Med Singapore. 2008; 37:826–30. [PubMed: 19037515]

28. Falagas ME, Kastoris AC, Vouloumanou EK, Rafailidis PI, Kapaskelis AM, Dimopoulos G. Attributable mortality of Stenotrophomonas maltophilia infections: a systematic review of the literature. Future Microbiol. 2009; 4:1103–9. [PubMed: 19895214]

29. Denton M, Kerr KG. Microbiological and clinical aspects of infection associated with Stenotrophomonas maltophilia. Clin Microbiol Rev. 1998; 11:57–80. [PubMed: 9457429]

30. Berg RD. The indigenous gastrointestinal microflora. Trends Microbiol. 1996; 4:430–5. [PubMed: 8950812]

31. Sogin ML, Morrison HG, Huber JA, Mark Welch D, Huse SM, Neal PR, et al. Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc Natl Acad Sci U S A. 2006; 103:12115–20. [PubMed: 16880384]

32. Siqueira JF Jr, Rocas IN, Rosado AS. Application of denaturing gradient gel electrophoresis (DGGE) to the analysis of endodontic infections. J Endod. 2005; 31:775–82. [PubMed: 16249718]

33. Hancock HH 3rd, Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001; 91:579–86. [PubMed: 11346739]

34. Gomes BP, Pinheiro ET, Jacinto RC, Zaia AA, Ferraz CC, Souza-Filho FJ. Microbial analysis of canals of root-filled teeth with periapical lesions using polymerase chain reaction. J Endod. 2008; 34:537–40. [PubMed: 18436030]

35. Anderson AC, Hellwig E, Vespermann R, Wittmer A, Schmid M, Karygianni L, et al. Comprehensive analysis of secondary dental root canal infections: a combination of culture and culture-independent approaches reveals new insights. PLoS One. 2012; 7:e49576. [PubMed: 23152922]

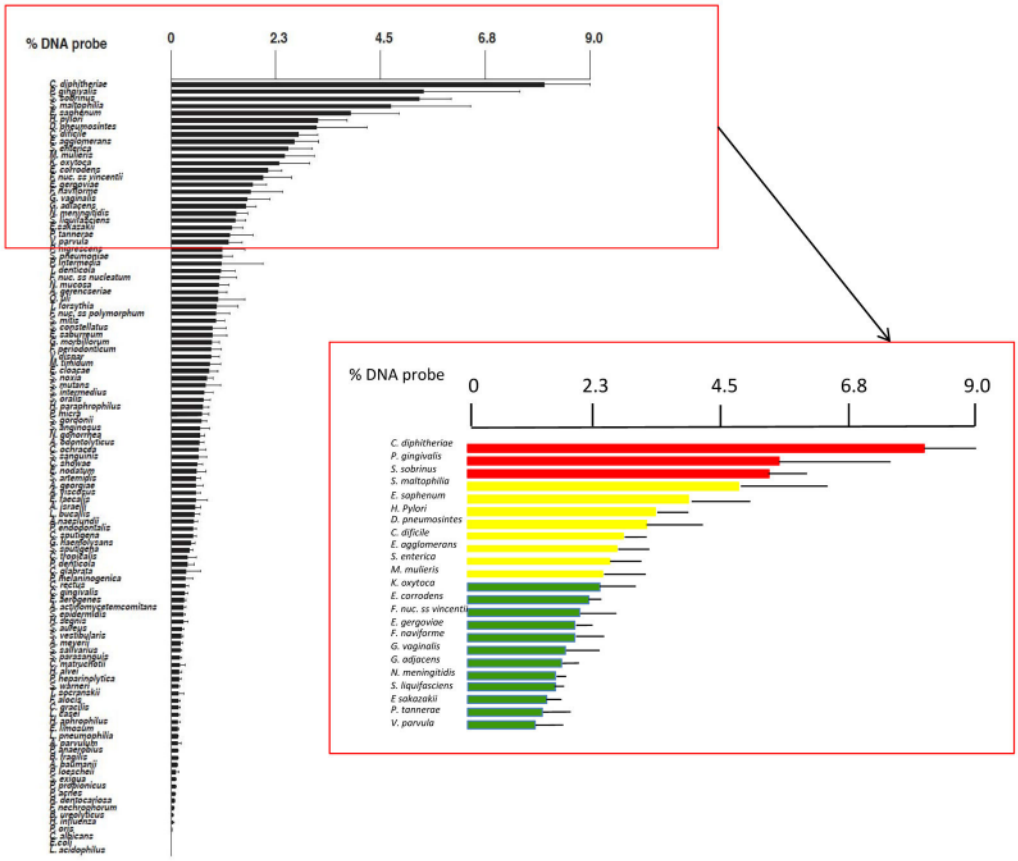

Figure 1.

The percentage of the mean proportion of DNA probe counts (±SEM) for 107 microbial species in MDA-amplified root canal samples collected from 40 patients presenting periapical lesions refractory to endodontic treatment. Microbial populations were divided according to their mean proportions, as dominant (≥3.0%; red bars), sub-dominant (>1.6 to 3.0%; yellow bars) and residual (≤ 1.6%; green bars) populations. The percentage of the total DNA probe count was computed for each species for each sample and averaged across samples. The significance of differences was determined using the Mann-Whitney test.

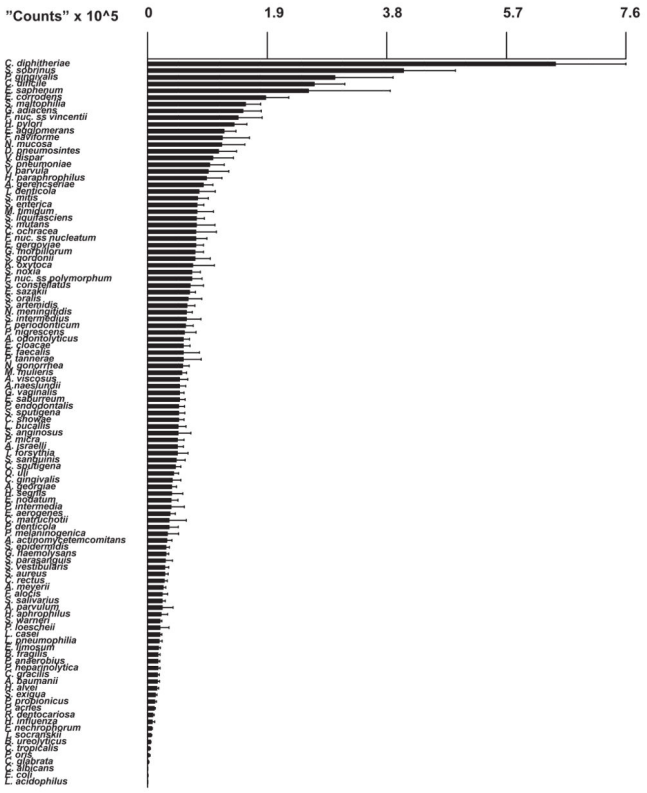

Figure 2.

Bar chart of the mean counts (X105±SEM) of the 107 test species in amplified root canal samples (n=40). The counts for each species were averaged across subjects and are presented in descending order of mean counts detected in the amplified samples. *ss*, subsp.

Table 1

Bacterial strains [a]	*Legionella pneumophila (33152)*
Acinetobacter baumannii (19606)	*Leptotrichia buccalis (14201)*
Actinomyces georgiae (49285)	*Mobiluncus mulieris (35243)*
Actinomyces gerencseriae (23860)	*Mogibacterium timidum (33093)*
Actinomyces israelii (12102)	*Neisseria gonorrhea (21823)*
Actinomyces meyeri (35568)	*Neisseria meningitidis (13077)*
Actinomyces naeslundii (12104	*Neisseria mucosa (19696)*
Actinomyces odontolyticus (17929)	*Olsenella uli (49627)*
Actinomyces viscosus (43146)	*Peptostreptococcus anaerobius (27337)*
Aggregatibacter actinomycetemcomitans [b]	*Parvimonas micra (33270)*
Atopobium parvulum (33793	*Porphyromonas endodontalis (35406)*
Bacteroides fragilis (25285	*Porphyromonas gingivalis (33277)*
Bacteroides ureolyticus (33387)	*Prevotella denticola (35308)*
Campylobacter gracilis (33236)	*Prevotella heparinolytica (35895)*
Campylobacter rectus (33238)	*Prevotella intermedia (25611)*
Campylobacter showae (51146)	*Prevotella loescheii (15930)*
Capnocytophaga gingivalis (33624	*Prevotella melaninogenica (25845)*
Capnocytophaga ochracea (33596)	*Prevotella nigrescens (33563)*
Capnocytophaga sputigena (33612)	*Prevotella oris (33573)*
Clostridium difficile (9689)	*Prevotella tannerae (51259)*
Corynebacterium diphtheriae (13812)	*Propionibacterium acnes* [c]
Corynebacterium matruchotii (14266)	*Propionibacterium propionicum (14157)*
Dialister pneumosintes (GBA27)	*Rothia dentocariosa (17931)*
Eikenella corrodens (23834)	*Salmonella enterica (27870)*
Enterococcus faecalis (10100)	*Selenomonas artemidis (43528)*
Enterobacter aerogenes (13048)	*Selenomonas noxia (43541)*
Enterobacter agglomerans (27155)	*Selenomonas sputigena (35185)*
Enterobacter cloacae (10699)	*Serratia liquifasciens (11367)*
Enterobacter gergoviae (33028)	*Slackia exigua (700122)*
Enterobacter sakazakii (12868)	*Staphylococcus aureus (14458)*
Escherichia coli (10798)	*Staphylococcus epidermidis (14990)*
Eubacterium limosum (8486)	*Staphylococcus warneri (27836)*
Eubacterium nodatum (33099)	*Stenotrophomonas maltophilia (13637)*
Eubacterium saburreum (33271)	*Streptococcus anginosus (33397)*
Eubacterium saphenum (49989)	*Streptococcus constellatus (27823)*
Filifactor alocis (35896)	*Streptococcus gordonii (10558)*
Fusobacterium naviforme (25832)	*Streptococcus intermedius (27335)*
Fusobacterium necrophorum (25286)	*Streptococcus mitis (49456)*

Fusobacterium nucleatum ss. nucleatum (25586)	*Streptococcus mutans (25175)*
Fusobacterium nucleatum ss. polymorphum (10953)	*Streptococcus oralis (35037)*
Fusobacterium nucleatum ss. vincentii (49256)	*Streptococcus parasanguinis (15912)*
Fusobacterium periodonticum (33693)	*Streptococcus pneumoniae (49619)*
Gardnerella vaginalis (49145)	*Streptococcus salivarius (27945)*
Gemella hemolysans (10379)	*Streptococcus sanguinis (10556*
Gemella morbillorum (27824)	*Streptococcus sobrinus (33478*
Granulicatella adiacens (49175)	*Streptococcus vestibularis (49124)*
Haemophilus aphrophilus (33389)	*Tannerella forsythia (43037)*
Haemophilus influenzae (33533)	*Treponema denticola (B1)*
Haemophilus paraphrophilus (29242)	*Treponema socranskii (S1)*
Haemophilus segnis (33393)	*Veillonella dispar (17748)*
Hafnia alvei (13337)	*Veillonella parvula (10790)*
Helicobacter pylori (43504)	**Fungal strains** [a]
Klebsiella oxytoca (12833)	*Candida albicans (10231)*
Lactobacillus acidophilus (4356)	*Candida glabrata (90030)*
Lactobacillus casei (393)	*Candida tropicalis (750)*

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

[b] ATCC strains 43718 and 29523

[c] ATCC strains 11827 and 11828

TABLE 2

The *E. faecalis* proportions (DNA probe counts) per sample

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