International Endodontic Journal

doi:10.1111/j.1365-2591.2009.01639.x

Preliminary study of the presence and association of bacteria and archaea in teeth with apical periodontitis

Y. T. Jiang[1], W. W. Xia[1], C. L. Li[2], W. Jiang[1] & J. P. Liang[1]

[1]Department of Endodontics and Operative Dentistry, School of Stomatology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, Shanghai; and [2]Department of Periodontology, School of Stomatology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, Shanghai, China

Abstract

Jiang YT, Xia WW, Li CL, Jiang W, Liang JP. Preliminary study of the presence and association of bacteria and archaea in teeth with apical periodontitis. *International Endodontic Journal*, **42**, 1096–1103, 2009

Aim To investigate, by reverse transcription polymerase chain reaction (RT-PCR), the presence and association of bacteria and archaea in primary and secondary root canal infections.

Methodology A total of 77 root canal samples from 77 Chinese patients, 42 with necrotic pulp tissues (primary infection) and 35 with failed prior conventional root canal treatment (secondary infection), aseptically exposed at the first patient visit, were studied. Total RNA was isolated directly from each sample, and 16S rRNA gene-based RT-PCR assays were used to determine the presence of bacteria and archaea, respectively.

Results Bacteria were detected in 39/42 (93%) of root canal samples from teeth with primary infections, and archaea in 16/42 (38%). In the cases diagnosed as secondary root-infected canals, bacteria were detected in 30/35 (86%), whilst archaea were detected in 6/35 (17%) of cases. Amongst the canals, which were positive for bacteria, archaea were always found in combination with bacteria. The incidence of symptomatic cases positive for both bacteria and archaea (16/22, 73%) were significantly higher than those positive for bacteria alone (21/47, 45%) ($P < 0.05$).

Conclusions This study confirms the presence of archaea in root canal infections and further implicates them in an association with clinical symptoms. The nature of this association requires further study.

Keywords: archaea, bacteria, root canal infections, RT-PCR.

Received 24 August 2008; accepted 1 September 2009

Introduction

Chronic apical periodontitis is a condition describing a group of inflammatory diseases with a multitude of clinical features that afflicts humans (Baumgartner *et al.* 2006). Contemporary knowledge of the pathogenesis of apical periodontitis shows that primary infections are polymicrobial in character and dominated by anaerobic gram-negative bacteria (Sundqvist 1992, Gomes *et al.* 2004). Secondary infections may be caused by microorganisms that gain entry into the canal system after professional intervention and or as a result of coronal leakage before or after root filling (Siqueira 2002, Sakamoto *et al.* 2008). Besides bacteria, other infective factors such as *Candida* spp. (Sundqvist *et al.* 1998, Peciuliene *et al.* 2001), human cytomegalovirus and Epstein-Barr virus (Sabeti *et al.* 2003) have also been detected in infected root canals. More than 150 microbial species have been isolated and cultured from root canals (Sundqvist 1976, Molander *et al.*

Correspondence: Prof. Jing Ping Liang, Department of Endodontics and Operative Dentistry, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, Shanghai, China (Tel.: +86 21 6313 5412; fax: +86 21 6313 5412; e-mail: liangjp123@yahoo.com.cn).

1998, Baumgartner *et al.* 2004). These various microbes form a complex community of organisms that interact with each other and play an important role in the aetiology of apical periodontitis (Sedgley *et al.* 2008). Therefore, it seems unreasonable to reduce the suspected causative agents to a simple and specific (e.g. single species) aetiology. In order to have a more complete understanding of the role of microorganisms in root canal infections, the pathogenic theory should be evaluated from a microbial community perspective.

Archaea, one of the three domains of life (Woese *et al.* 1990), have been isolated from the human oral cavity (Kulik *et al.* 2001), as well as the human gut (Miller & Wolin 1982) and vagina (Belay *et al.* 1990). Although they are now recognized as a component of human microbiota, none of the archaea domain has been established as a causative agent in human disease. However, they do share some characteristics with known pathogens that may reflect the potential to cause disease. Such characteristics include ample access to a host (i.e. opportunity) and capabilities for long-term colonization and coexistence with endogenous microbiota in a host (Miller & Wolin 1982, Belay *et al.* 1988, 1990, Kulik *et al.* 2001). Recently, there has been increasing interest in the relationship between archaea and periapical disease (Siqueira *et al.* 2005, Vianna *et al.* 2006, Vickerman *et al.* 2007), whereas there is little information on the association between bacteria and archaea in primary and secondary root canal infections.

The emergence of a variety of cultivation-independent molecular methods, based mainly on 16S rDNA sequences, has widened the scope of detectable microorganisms to include uncultivable organisms that might play significant roles, as yet undefined, in pathogenesis (Munson *et al.* 2002, Saito *et al.* 2006, Siqueira *et al.* 2007). Most of these studies focus on the detection of DNA, which may originate from dead cells, or even from free DNA, giving an erroneous account of current viable infection. As the ribosome-per-cell ratio is roughly proportional to the growth rate of bacteria (Wagner 1994), rRNA is regarded as an indicator of total bacterial activity. Hence, the purpose of this study was to detect the presence of metabolically active bacteria and archaea in untreated and treated root canals using 16S rRNA derived from isolated ribosomes by reverse transcription polymerase chain reaction (RT-PCR) (Williams *et al.* 2006), and to compare their presence with the incidence of clinical symptoms.

Materials and methods

Patient selection and clinical features

Seventy-seven teeth (one tooth per patient) were selected from patients who sought root canal treatment or retreatment at the Shanghai Ninth People's Hospital. Forty-two teeth presented with necrotic pulp tissues and 35 had been root filled >4 years previously and showed radiographic evidence of apical periodontitis. A detailed medical and dental history was obtained from each patient. Patients having received antibiotic treatment in the previous 3 months or having a systemic disease were excluded from the study. The Ethics Committee of Shanghai Jiao Tong University School of Medicine approved a protocol describing the specimen collection for this investigation, and all patients signed an informed consent form to participate in this study.

Patients were classified as symptomatic if they had a history of spontaneous pain, pain on percussion or pain upon palpation immediately prior to the consultation. The presence of swelling, lymphadenopathy or evidence of a sinus tract was considered symptomatic whether or not pain was present. Patients without the above criteria were considered asymptomatic. No teeth showed significant gingival recession or any of periodontal pockets deeper than 4 mm.

Sampling procedure

Samples from infected root canals were collected as previously described (Ng *et al.* 2003). After a two-stage access cavity preparation, which was made without the use of water spray but under manual irrigation with sterile saline solution and employing sterile burs, the teeth involved were individually isolated from the oral cavity with a previously disinfected rubber dam. Disinfection of the rubber dam and teeth was carried out using first 30% hydrogen peroxide and then 2.5% sodium hypochlorite. The solution was inactivated with 5% sodium thiosulphate to avoid interference with the bacteriological sampling. Aseptic techniques were used throughout endodontic therapy and sample acquisition. After initial entry into the pulp space, the patency of the root canal was established with minimal instrumentation and without the use of any chemically active irrigant. Pre-existing root filling material was removed using Gates Glidden drills and endodontic files without the use of chemical solvents. Irrigation with sterile saline solution was performed to remove any remaining

treatment materials prior to sample collection. In multi-rooted teeth, the criterion used to choose the canal to be microbiologically investigated was the presence of exudate or, in its absence, the canal associated with the periapical radiolucency. In each case, a single root canal associated with the criterion above was sampled in order to confine the microbial evaluation to a single ecological environment.

After minimal canal enlargement with sterile saline irrigant to allow access to the working length, dry, autoclaved paper points were placed in the canal space for 60 s. The samples were collected with as many paper points necessary to absorb all the fluid inside the canal and inserted to the full length of the canal as calculated from the preoperative radiograph. Afterwards, the paper points per root canal were pooled in a sterile tube containing 1 mL^{-1} Sample Protector (Takara, Dalian, China) and transported to the microbiology laboratory in dry ice, then stored at −80 °C for 4 weeks or less before extraction of total genomic RNA.

Nucleic acid isolation

The frozen paper point samples were thawed and dispersed by vortexing for 60 s. The Sample Protector contained glass beads 3 mm in diameter to facilitate mixing and homogenization of the sample prior to extraction. Then, the samples were centrifuged for 5 min at 12 000*g*, with the supernatant discarded and the pellet resuspended in 1 mL^{-1} TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The microbial RNA was extracted from the samples according to the manufacturer's protocol and reference method (Chomczynski & Sacchi 1987, 2006). Briefly, after incubating the homogenized samples for 5 min at room temperature to obtain complete dissociation of nucleoprotein complexes, 0.2 mL^{-1} of chloroform was added per 0.75 mL^{-1} of TRIzol Reagent in capped sample tubes. The tubes were shaken vigorously by hand for 15 s, incubated at room temperature for 15 min and centrifuged at 12 000*g* for 15 min at 5 °C. Following centrifugation, the mixture separated into a lower red, phenol–chloroform phase, an interphase and a colourless upper aqueous phase containing RNA. After transferring the aqueous phase to a clean tube, RNA was precipitated by adding 0.5 mL^{-1} isopropyl alcohol per 0.75 mL^{-1} of TRIzol Reagent used for the initial homogenization. Samples were then incubated at room temperature for 10 min and centrifuged at 12 000*g* for 10 min at 5 °C. The RNA precipitate, often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. After removing the supernatant, the RNA pellet was washed once by adding 1 mL^{-1} of 75% ethanol per 0.75 mL^{-1} of TRIzol Reagent used for the initial homogenization. Samples were mixed on a vortex and centrifuged at 7500*g* for 5 min at 5 °C. The RNA pellet was briefly dried and then reconstituted in RNAse free water.

DNAse treatments

Extracted crude RNA was treated enzymatically with DNAse to remove contaminant genomic DNA. For each reaction, 8 μL of extract was incubated for 30 min at 37 °C with DNAse (RQ1 RNAse free DNAse; Promega, Shanghai, China) in buffer plus inhibitors of RNAse (Recombinant RNasins Ribonuclease Inhibitor, Promega, Shanghai, China). After incubation, 1 μL of STOP DNAse was added to each tube and samples were incubated for 15 min at 70 °C to inactivate DNAse and to denature RNA. Samples were chilled on ice for 10 min. The absence of genomic DNA was confirmed by PCR performed with universal bacterial and archaeal primers (Yu & Morrison 2001, Lepp *et al.* 2004). The integrity and quantity of the purified RNA were examined by absorbance ratio A_{260}/A_{280} and RNA gel electrophoresis (Cury *et al.* 2008).

Reverse transcription of total RNAs

Complementary DNA (cDNA) synthesis was carried out with the Reverse Transcription System (Promega, Madison, WI, USA), and cDNA were quantified based on absorbance at 260 nm. The purified cDNA were checked on a 1.5% agarose gel and stored at −20 °C prior to amplification.

Universal bacterial primers and PCR conditions

The variable V3–V5 region of 16S rRNA was enzymatically amplified with primers located on conserved ends of the V3 and V5 region (Yu & Morrison 2001). The primers were as follows: primer 341f, 5′-CCTACGGGAGGCAGCAG-3′; primer 926r, 5′-CCGTCAATTCCTTTGAGTTT-3′. A combination of primer 341f and 926r was used to amplify the V3–V5 region of 16S rRNA in the different bacterial species, which correspond to positions 341–926 in *E. coli*. Each reaction mixture contained 2.5 μL of 10× PCR buffer [100 mmol L^{-1} Tris–HCI (pH 9), 15 mmol L^{-1} $MgCl_2$, 500 mmol L^{-1} KCI, 0.1% (w/v) gelatin, 1% (v/v) Triton X-100],

0.2 mmol deoxynucleotide triphosphate, 1 U of HotStarTaq DNA polymerase (Qiagen, Hamburg, Germany), 0.25 mmol of each forward and reverse primer, 50 ng template cDNA and enough sterile MilliQ water to bring the final volume to 50 μL. PCR amplification was performed using the Techne thermocycler (Biometra, Göttingen, Germany). Amplification consisted of 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, and extension for 1 min at 72 °C. The first cycle was preceded by an initial template denaturation step of 4 min at 94 °C, and the last cycle was followed by a final extension step of 7 min at 72 °C. PCR products were separated by electrophoresis in 1.5% agarose gels in 1× TAE buffer (40 mmol L^{-1} Tris acetate, 20 mmol L^{-1} sodium acetate, 1 mmol L^{-1} EDTA, pH 8.0) and visualized under UV light, following an ethidium bromide staining, the positive samples were recorded.

Universal archaeal primers and PCR conditions

Fragments of 16S rRNA from samples were PCR amplified by using broad-range archaeal primers SDArch0333aS15 (5′-TCCAGGCCCTACGGG-3′) and SDArch0958aA19 (5′-YCCGGCGTTGAMTCCAATT-3′) (Lepp *et al.* 2004). Each reaction mixture contained 2.5 μL of 10× PCR buffer [100 mmol L^{-1} Tris–HCl (pH 9), 15 mmol L^{-1} $MgCl_2$, 500 mmol L^{-1} KCl, 0.1% (w/v) gelatin, 1% (v/v) Triton X-100], 0.2 mmol deoxynucleotide triphosphate, 1 U of HotStarTaq DNA polymerase (Qiagen), 0.25 mmol of each forward and reverse primer, 50 ng template cDNA and enough sterile MilliQ water to bring the final volume to 50 μL. Archaeal 16S rRNA genes were amplified under the following cycle conditions: 35 cycles of 94 °C (30 s), 58 °C (30 s) and 72 °C (30 s) followed by a 3-min extension at 72 °C. PCR products were separated by electrophoresis in 1.5% agarose gels in 1× TAE buffer and visualized under UV light, following an ethidium bromide staining, the positive samples were recorded.

Statistical analysis

Chi-squared analysis was used to determine a statistically significant difference between the prevalence of bacteria and archaea, and statistical correlation of clinical symptoms with the prevalence of bacteria alone or both bacteria and archaea. Statistical analysis was performed with SAS Software (version 6.12, SAS Institute, Cary, NC, USA). Significance level was set at $P < 0.05$.

Results

A total of 77 samples, 42 teeth with primary endodontic infection and 35 with secondary endodontic infection (i.e. failed treatment), were subjected to RT-PCR with universal bacterial primers and archaeal primers. Table 1 shows the distribution of bacteria and archaea in different root canal infections. In all subjects, the prevalence was 88.14% and 28.5%, respectively. The positive rate of bacteria was 92.9% in primary apical periodontitis (39/42) and 85.7% in secondary apical periodontitis (30/35). Archaea were detected in 38.1% (16/42) of canals with necrotic pulps and 17.1% (6/35) in treated canals.

Of the 69 root canals positive for bacteria, 37 (53.6%) were from patients with symptoms. Archaea were always found in combination with bacteria, and the symptomatic cases positive for both bacteria and archaea were significantly higher than those positive for bacteria alone (Table 2) ($P < 0.05$).

Discussion

Historically, conventional culture methods have been used to detect bacteria in infected root canals, thus only allowing detection of bacteria capable of dividing (Sundqvist 1994, Le Goff *et al.* 1997). However, over

Table 1 Prevalence of bacteria and archaea found in 77 root canals[a]

Group	Bacteria (%)	Archaea (%)	*P**
Primary root canal infection (*n* = 42)	39 (92.9)	16 (38.1)	
Secondary root canal infection (*n* = 35)	30 (85.7)	6 (17.1)	
Total (*n* = 77)	69 (88.1)	22 (28.5)	0.001

[a]Data are number and (percentage) of subjects.
*Percentage of archaea is obviously lower than that of bacteria ($P < 0.01$).

Table 2 Correlation of clinical symptoms with prevalence of bacteria alone or in combination with archaea[a]

	Symptomatic (%)	Asymptomatic (%)	*P**
Bacteria + archaea (*n* = 22)	16 (72.7)	6	0.03
Bacteria alone (*n* = 47)	21 (44.7)	26	

[a]Data are number and (percentage) of subjects.
*Symptomatic cases positive for both bacteria and archaea were significantly higher than those positive for bacteria alone ($P < 0.05$).

the past few years it has been demonstrated that nonculturable bacteria make up an undetermined proportion of the microbial population in the infected root canal system (Siqueira & Rôças 2003, 2005, Saito *et al.* 2006). Moreover, several reports have found that bacteria become less culturable under starvation conditions, and these viable but nonculturable (VBNC) bacteria demonstrate metabolic activity (Mason *et al.* 1986, Kaprelyants *et al.* 1993, Oliver 1995), maintain their pathogenic features and resume division when favourable environmental conditions are restored (Lleò *et al.* 2001). The VBNC state might be a survival strategy that persists in the root canal. Because the nonculturable microorganisms could play a part in the perpetuation of periapical disease, it becomes mandatory, for proper study of infected root canals, to develop and apply methods capable of detecting such bacterial forms (Siqueira & Rôças 2003, 2005).

Amongst the various molecular methods, PCR has proven useful for detecting target microorganisms in endodontic samples (Siqueira & Rôças 2003). Conventional PCR assays, however, detect only the presence or absence of genomic DNA of microorganisms present in the root canal space and cannot distinguish between viable and nonviable microorganisms. Recent research demonstrates that PCR-detectable DNA from dead bacteria might persist after cell death (Young *et al.* 2007). Ribosomes can be used as markers for bacterial activity because the number of ribosomes (and their rRNA) per cell maybe roughly proportional to the growth activity of bacteria in pure culture (Wagner 1994). For successful isolation of intact RNA, it is important to avoid the death of bacteria and enzymatic degradation of RNA during the handling and processing of samples. In this study, Sample Protector (an aqueous tissue storage reagent) was used to overcome these problems by simply adding the reagent directly to the root canal samples and providing immediate RNA stabilization prior to RNA isolation. Isolation of high-quality RNA is another important step for the downstream processes. Any extracted RNA must be devoid of contaminants such as salt, protein, solvents and genomic DNA. The extracted RNA was 'quality controlled' using gel electrophoresis, PCR and optical density measurements. Gel electrophoresis and 'no-RT' control during RT-PCR were used to check for genomic DNA contamination. Optical density was used to assay the RNA yield and to check for contamination by salt, solvent, protein, etc.

Although the total number of viable cells present in a population can be determined by using 4′,6-diamidino-2-phenylindole (DAPI) or acridine orange staining or by establishing the presence of an intact cytoplasmic membrane [(BacLight®, Molecular Probes, Inc, Eugene, OR, USA) or propidium iodide] (Oliver 2005), detection of mRNA by RT-PCR is regarded as the most appropriate method of evaluating the specific RNA against a large background of procaryotic and eucaryotic cells present in root canal samples. This study reports the application of ribosome isolation and subsequent RT-PCR, leading to the identification of the metabolic portion of root canal microbial communities. Such data should provide a more realistic basis for discussion about the correlation between clinical symptoms and viable microbial species.

Archaea are microorganisms distinct from bacteria and eukaryotes (Woese *et al.* 1990). They can be found in most ecosystems and are often prevalent in extreme environments. RT-PCR of the present study indicated that both bacteria and archaea can be detected in primary and secondary root canal infections, supporting the notion of the poly microbial nature of infected root canal systems. The prevalence of archaea in infected root canals in a Chinese population sample was 28.5%, which is in agreement with other surveys of endodontic infections (Vianna *et al.* 2006). Despite their abundant and ubiquitous association with humans, animals and plants, no pathogenic archaea have so far been described.

However, amongst 700 different bacterial species that have been identified from dental plaque and oral cavity (Paster *et al.* 2001, Aas *et al.* 2005), only a relatively small and select group of bacteria are detected in the root canal, and appear to have the properties necessary to invade tubules and survive within the intratubular environment (Love & Jenkinson 2002). Furthermore, the infected root canal is a unique environment, unlike other infectious oral diseases. Apical periodontitis is caused by infection of the root canal space, normally devoid of microbes in a healthy state (Nair 2004). Hence, there is good reason to assume that archaea share some characteristics with known pathogens that may reflect the potential to cause apical disease. Such characteristics include ability to colonize the human host and evasion of host defenses. These virulence factors have recently been demonstrated in other medical fields (Cavicchioli *et al.* 2003, Eckburg *et al.* 2003).

In the present study, in which the percentage of archaea in patients with apical periodontitis was obviously lower than that of bacteria (88.1%; $P < 0.01$), it is noteworthy that archaea were always found in combination with bacteria, and there was a

statistically significant difference between the percentage of symptomatic cases positive for both archaea and bacteria and bacteria alone ($P < 0.05$). More recently, it has been demonstrated that bacteria may co-operate for invasion of dentinal tubules (Love & Jenkinson 2002). With this in mind, the potential symbiotic relationship between archaea and bacteria may fulfil a similar role in endodontic infections.

Methanogens might be the only archaea in the human body (Vianna *et al.* 2006, Vickerman *et al.* 2007). They are strict anaerobes characterized by the ability to produce methane from H_2/CO_2 and, in some cases, from formate, acetate or methanol. Hydrogen is the waste end product of the metabolism of microorganisms in anoxic environments. Maintaining a low hydrogen concentration is important because the anaerobic fermentative process becomes increasingly unfavourable as the partial pressure of hydrogen increases, which affects microbial growth. The methanogens depend on the hydrogen and carbon dioxide produced by other species; in return some of these other species grow better in the presence of the methanogens because of the altered patterns of redox balance associated with reduced partial pressure of hydrogen due to interspecies hydrogen transfer (Lovley 1985, Bonch-Osmolovskaya & Stetter 1991, Conrad 1999). It can be deduced from the mechanism of 'interspecies hydrogen transfer' that methanogens may play an important role in increasing activity of some species of microorganisms in the root canal system and contribute to local apical tissue damage.

The microbial community in the root canal system is thought to undergo ecological succession as different species combinations emerge at different levels (Sundqvist & Figdor 2003). It has been suggested that metabolic competition for hydrogen with sulphate-reducing bacteria (Vianna *et al.* 2006), such as the *Desulfovibrio* or treponemal species (Lepp *et al.* 2004), might inhibit the coexistence of these bacteria with methanogenic archaea. This might explain in part the presence of archaea in some, but not all, cases of endodontic infections. An understanding of the interactions between archaea, bacteria and other members of the root canal microbiota may help elucidate the bacterial physiological and pathological functions underlying periapical disease activity.

Conclusion

This study showed archaea to be present in root canals but always with bacteria. Their combined presence was associated with a significantly higher prevalence of clinical symptoms compared with the sole presence of bacteria.

Acknowledgements

This work was supported by the Science and Technology Commission of Shanghai (08DZ2271100), Shanghai Leading Academic Discipline Project (S30206) and the National Natural Science Foundation of China (30801291).

References

Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE (2005) Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology* **43**, 5721–32.

Baumgartner JC, Siqueira JF Jr, Xia T, Róças IN (2004) Geographical differences in bacteria detected in endodontic infections using polymerase chain reaction. *Journal of Endodontics* **30**, 141–4.

Baumgartner JC, Hutter JW, Siqueira JF Jr (2006) Endodontic microbiology and treatment of infections. In: Cohen S, Hargreaves KM eds. *Pathways of the Pulp*, 9th edn. St Louis, MO: CV Mosby/Elsevier, pp. 580–607.

Belay N, Johnson R, Rajagopal BS, de Macario EC, Daniels L (1988) Methanogenic bacteria from human dental plaque. *Applied and Environmental Microbiology* **54**, 600–3.

Belay N, Mukhopadhyay B, Conway de Macario E, Galask R, Daniels L (1990) Methanogenic bacteria in human vaginal samples. *Journal of Clinical Microbiology* **28**, 1666–8.

Bonch-Osmolovskaya EA, Stetter KO (1991) Interspecies hydrogen transfer in cocultures of thermophilic archaea. *Systematic and Applied Microbiology* **14**, 205–8.

Cavicchioli R, Curmi PM, Saunders N, Thomas T (2003) Pathogenic archaea: do they exist? *BioEssays* **25**, 1119–28.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162**, 156–9.

Chomczynski P, Sacchi N (2006) The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. *Nature Protocols* **1**, 581–5.

Conrad R (1999) Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. *FEMS Microbiology Ecology* **28**, 193–202.

Cury JA, Seils J, Koo H (2008) Isolation and purification of total RNA from *Streptococcus mutans* in suspension cultures and biofilms. *Brazilian Oral Research* **22**, 216–22.

Eckburg PB, Lepp PW, Relman DA (2003) Archaea and their potential role in human disease. *Infection and Immunity* **71**, 591–6.

Gomes BP, Pinheiro ET, Gadê-Neto CR *et al.* (2004) Microbiological examination of infected dental root canals. *Oral Microbiology and Immunology* **19**, 71–6.

Kaprelyants AS, Gottschal JC, Kell DB (1993) Dormancy in non-sporulating bacteria. *FEMS Microbiology Reviews* **104**, 271–86.

Kulik EM, Sandmeier H, Hinni K, Meyer J (2001) Identification of archaeal rDNA from subgingival dental plaque by PCR amplification and sequence analysis. *FEMS Microbiology Letters* **196**, 129–33.

Le Goff A, Bunetel L, Mouton C, Bonnaure-Mallet M (1997) Evaluation of root canal bacteria and their antimicrobial susceptibility in teeth with necrotic pulp. *Oral Microbiology and Immunology* **12**, 318–22.

Lepp PW, Brinig MM, Ouverney CC, Palm K, Armitage GC, Relman DA (2004) *Methanogenic Archaea* and human periodontal disease. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 6176–81.

Lleò MM, Bonato B, Tafi MC, Signoretto C, Boaretti M, Canepari P (2001) Resuscitation rate in different enterococcal species in the viable but non-culturable state. *Journal of Applied Microbiology* **91**, 1095–102.

Love RM, Jenkinson HF (2002) Invasion of dentinal tubules by oral bacteria. *Critical Reviews in Oral Biology and Medicine* **13**, 171–83.

Lovley DR (1985) Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Applied and Environmental Microbiology* **49**, 1530–1.

Mason CA, Hamer G, Bryers JD (1986) The death and lysis of microorganisms in environmental processes. *FEMS Microbiology Reviews* **39**, 373–401.

Miller TL, Wolin MJ (1982) Enumeration of *Methanobrevibacter smithii* in human feces. *Archives of Microbiology* **131**, 14–8.

Molander A, Reit C, Dahlén G, Kvist T (1998) Microbiological status of root-filled teeth with apical periodontitis. *International Endodontic Journal* **31**, 1–7.

Munson MA, Pitt-Ford T, Chong B, Weightman A, Wade WG (2002) Molecular and cultural analysis of the microflora associated with endodontic infections. *Journal of Dental Research* **81**, 761–6.

Nair PN (2004) Pathogenesis of apical periodontitis and the causes of endodontic failures. *Critical Reviews in Oral Biology and Medicine* **15**, 348–81.

Ng YL, Spratt D, Sriskantharajah S, Gulabivala K (2003) Evaluation of protocols for field decontamination before bacterial sampling of root canals for contemporary microbiology techniques. *Journal of Endodontics* **29**, 317–20.

Oliver JD (1995) The viable but non-culturable state in the human pathogen, *Vibrio vulnificus*. *FEMS Microbiology Letters* **133**, 203–8.

Oliver JD (2005) The viable but nonculturable state in bacteria. *The Journal of Microbiology* **43**, 93–100. Review.

Paster BJ, Boches SK, Galvin JL *et al.* (2001) Bacterial diversity in human subgingival plaque. *Journal of Bacteriology* **183**, 3770–83.

Peciuliene V, Reynaud AH, Balciuniene I, Haapasalo M (2001) Isolation of yeasts and enteric bacteria in root-filled teeth with chronic apical periodontitis. *International Endodontic Journal* **34**, 429–34.

Sabeti M, Simon JH, Slots J (2003) Cytomegalovirus and Epstein-Barr virus are associated with symptomatic periapical pathosis. *Oral Microbiology and Immunology* **18**, 327–8.

Saito D, Leonardo Rde T, Rodrigues JL, Tsai SM, Höfling JF, Gonçalves RB (2006) Identification of bacteria in endodontic infections by sequence analysis of 16S rDNA clone libraries. *Journal of Medical Microbiology* **55**, 101–7.

Sakamoto M, Siqueira JF Jr, Rôças IN, Benno Y (2008) Molecular analysis of the root canal microbiota associated with endodontic treatment failures. *Oral Microbiology and Immunology* **23**, 275–81.

Sedgley CM, Lee EH, Martin MJ, Flannagan SE (2008) Antibiotic resistance gene transfer between *Streptococcus gordonii* and *Enterococcus faecalis* in root canals of teeth ex vivo. *Journal of Endodontics* **34**, 570–4.

Siqueira JF Jr (2002) Endodontic infections: concepts, paradigms and perspectives. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **94**, 281–93.

Siqueira JF Jr, Rôças IN (2003) PCR methodology as a valuable tool for identification of endodontic pathogens. *Journal of Dentistry* **31**, 333–9.

Siqueira JF Jr, Rôças IN (2005) Exploiting molecular methods to explore endodontic infections. Part 2. Redefining the endodontic microbiota. *Journal of Endodontics* **31**, 488–98.

Siqueira JF Jr, Rôças IN, Baumgartner JC, Xia T (2005) Searching for *Archaea* in infections of endodontic origin. *Journal of Endodontics* **31**, 719–22.

Siqueira JF Jr, Rôças IN, Paiva SS, Magalhães KM, Guimarães-Pinto T (2007) Cultivable bacteria in infected root canals as identified by 16S rRNA gene sequencing. *Oral Microbiology and Immunology* **22**, 266–71.

Sundqvist G (1976) *Bacteriological Studies of Necrotic Dental Pulps*. (PhD Dissertation). Sweden: University of Umeå.

Sundqvist G (1992) Associations between microbial species in dental root canal infections. *Oral Microbiology and Immunology* **7**, 257–62.

Sundqvist G (1994) Taxonomy, ecology, and pathogenicity of the root canal flora. *Oral Surgery, Oral Medicine, and Oral Pathology* **78**, 522–30.

Sundqvist G, Figdor D (2003) Life as an endodontic pathogen: ecological differences between the untreated and root filled root canals. *Endodontic Topics* **6**, 3–28.

Sundqvist G, Fidgor D, Persson S, Sjögren U (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative retreatment. *Oral Surgery, Oral Medicine, and Oral Pathology* **85**, 86–93.

Vianna ME, Conrads G, Gomes BP, Horz HP (2006) Identification and quantification of *Archaea* involved in primary endodontic infections. *Journal of Clinical Microbiology* **44**, 1274–82.

Vickerman MM, Brossard KA, Funk DB, Jesionowski AM, Gill SR (2007) Phylogenetic analysis of bacterial and archaeal species in symptomatic and asymptomatic endodontic infections. *Journal of Medical Microbiology* **56**, 110–8.

Wagner R (1994) The regulation of ribosomal RNA synthesis and bacterial cell growth. *Archives of Microbiology* **161**, 100–6.

Williams JM, Trope M, Caplan DJ, Shugars DC (2006) Detection and quantitation of *E. faecalis* by real-time PCR (qPCR), reverse transcription-PCR (RT-PCR), and cultivation during endodontic treatment. *Journal of Endodontics* **32**, 715–21.

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains *Archaea*, *Bacteria*, and *Eucarya*. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4576–9.

Young G, Turner S, Davies JK, Sundqvist G, Figdor D (2007) Bacterial DNA persists for extended periods after cell death. *Journal of Endodontics* **33**, 1417–20.

Yu Z, Morrison M (2001) Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* **70**, 4800–6.