doi: 10.1111/j.1365-2591.2006.01121.x

In vivo evaluation of microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue

M.E. Vianna[1, 2], H.P. Horz[2], B.P.F.A. Gomes[1] & G. Conrads[2]

[1]Endodontic Area, Department of Restorative Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil; and [2]Division of Oral Microbiology and Immunology, Department of Operative and Preventive Dentistry and Periodontology, and Department of Medical Microbiology, RWTH Aachen University Hospital, Aachen, Germany

Abstract

Vianna ME, Horz HP, Gomes BPFA, Conrads G. *In vivo* evaluation of microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue. *International Endodontic Journal*, **39**, 484–492, 2006.

Aim To determine *in vivo*, the degree of microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue when using two endodontic irrigating reagents, sodium hypochlorite (NaOCl) or chlorhexidine gel (CHX).

Methodology Thirty-two single rooted teeth with necrotic pulp were divided into two groups. One group ($n = 16$) was irrigated with 2.5% NaOCl, whilst the other group ($n = 16$) was irrigated with 2% CHX gel. Assessment of the bacterial load was accomplished by use of real-time quantitative-polymerase chain reaction (RTQ-PCR) directed against the small subunit ribosomal DNA using the SYBRGreen and TaqMan formats. Statistical analysis was performed using the Mann–Whitney test. For contrast, bacterial load was also determined by traditional culture techniques.

Results The bacterial load was reduced substantially in both groups (over 96%). However, using RTQ-PCR the bacterial load before and after chemo-mechanical preparation was greater when compared with evaluation using colony forming units (CFU). Furthermore, as measured by RTQ-PCR, the bacterial reduction in the NaOCl-group (SYBRGreen 99.99%; TaqMan: 99.63%) was significantly greater ($P < 0.01$) than in the CHX-group (SYBRGreen 96.62%; TaqMan: 96.60%). According to culture technique 75% of cases were free of bacteria after chemo-mechanical preparation in the NaOCl-group, whilst 50% of cases were bacteria free in the CHX-group.

Conclusion NaOCl has not only a higher capacity to kill microorganisms but is also more able to remove cells from the root canal.

Keywords: chlorhexidine gel, endodontic infection, irrigating solutions, microbial reduction, NaOCl.

Received 6 July 2005; accepted 13 January 2006

Introduction

One of the primary objectives in root canal treatment is to reduce the microbial population in the root canals of infected teeth. This is usually accomplished by mechanical preparation along with the use of irrigant solutions. Irrigating solutions should be able to dissolve organic tissue remnants, act as lubricants, and flush out debris from the prepared root canals (Harrison 1984). Since residual bacteria may be one of the causes of post-treatment disease (Nair *et al.* 1990), an endodontic irrigant should also exhibit powerful antimicrobial activity (Harrison 1984).

Sodium hypochlorite (NaOCl) is the most frequently used endodontic irrigating solution; however, it is known to have a cytotoxic effect (Tanomaru Filho *et al.*

Correspondence: G. Conrads, Division of Oral Microbiology and Immunology, Department of Operative and Preventive Dentistry & Periodontology, and Department of Medical Microbiology, RWTH Aachen University Hospital, Germany (e-mail: gconrads@ukaachen.de).

2002), and the more biocompatible chlorhexidine (CHX) has been recommended as an alternative. This is especially true in cases of teeth with open apices (Jeansonne & White 1994) or patients with an allergy to bleaching solutions (Tanomaru Filho *et al.* 2002). CHX is used mainly in liquid form in dentistry, but it has been investigated for use in the form of a gel as well (Ferraz *et al.* 2001, Gomes *et al.* 2001, Vivacqua-Gomes *et al.* 2002, Okino *et al.* 2004).

Chlorhexidine targets cytoplasmatic (inner) membrane cells, thereby causing generalized membrane damage to the phospholipid bilayers. It affects membrane integrity and, depending on its concentration, causes a congealing of the cytoplasm. Despite the advantages of chlorhexidine, its activity is pH dependent and is greatly reduced in the presence of organic matter (McDonnell & Russell 1999). NaOCl antimicrobial activity, on the other hand, depends on the concentration of undissociated hypochlorous acid (HOCl), which exerts its germicidal effect by oxidative action on sulphydryl (-SH) groups of bacterial enzymes. As essential enzymes are inhibited, important metabolic reactions are disrupted, resulting in the death of the bacterial cells. When in contact with organic tissue HOCl acts as a solvent, releasing chlorine that, combined with the protein amino group, forms chloramines (Baumgartner & Cuenin 1992).

When tested *in vitro*, both substances exhibited a comparable potential to kill bacteria (Gomes *et al.* 2001, Vianna *et al.* 2004). But it remains unclear whether CHX is effective during chemo-mechanical preparation because of its inability to dissolve organic tissues (Okino *et al.* 2004).

Generally, the quantification of endodontic bacteria relies on traditional cultivation techniques, which are of great importance for studying microbial diseases. However, with the advent of molecular methods for the identification and characterization of microbial communities, it has become evident that the majority of microorganisms cannot be cultured with standard laboratory techniques (Amann *et al.* 1995, Pace 1997), consequently only an approximate quantification can be made by counting colony forming units (CFU). To provide a more accurate determination of the total microbial content in the root canal real-time quantitative-polymerase chain reaction (RTQ-PCR) with a broad-ranged primer pair directed against the bacterial 16S ribosomal RNA (rRNA) genes can be used. Principally, this technique is characterized by its wide dynamic range of quantification (7–8 $\log_{10}$ units), its high sensitivity (less than five copies of the template gene), and by its high technical precision (about 2% SD between replicate runs) (Ginzinger 2002).

The aim of the present study was to assess the total number of bacteria before and after the chemo-mechanical preparation of infected root canals *in vivo* using 2% chlorhexidine gel (CHX) or 2.5% sodium hypochlorite. Bacterial load was determined by both RTQ-PCR and CFU counting.

Materials and methods

Patient selection

Thirty-two patients requiring root canal treatment at the Piracicaba Dental School, SP, Brazil, who were otherwise healthy and who had not received antibiotic treatment during the previous 3 months, were selected. The age of the patients ranged from 19 to 63 years. All selected teeth were single rooted, asymptomatic, did not respond to sensitivity testing, had not received previous root canal treatment and had radiographic evidence of periapical bone loss ranging from 3 to 13 mm diameter. The teeth were restored with resin composite material and were free of caries at the time of endodontic treatment. The Human Volunteers Research and Ethics Committee of the Piracicaba Dental School approved a protocol describing the specimen collection for this investigation, and all patients signed an informed consent to participate.

Microbiological sampling

The teeth were isolated with a rubber dam. The crown and the surrounding rubber dam were disinfected with 30% H_2O_2 (v/v) for 30 s followed by 2.5% NaOCl for an additional 30 s. Subsequently, 5% sodium thiosulphate was used to inactivate the disinfectant agents (Möller 1966, Gomes *et al.* 2004). A swab sample was taken from the surface and streaked on blood agar plates to test for disinfection. An access cavity was prepared with sterile high-speed diamond burs under irrigation with sterile physiological saline. Before entering the pulp chamber, the access cavity was disinfected with the same protocol as above and the sterility again checked by taking a swab sample of the cavity surface and streaking onto blood agar plates. All subsequent procedures were performed aseptically. The samples (pre- and post-clinical procedures) were collected with four sterile paper points, which were consecutively

placed into each canal to the total length calculated from the pre-operative radiograph and then pooled in a sterile tube containing 1 mL reduced transport fluid (RTF) (Loesche *et al.* 1972). The samples were transported to the laboratory within 15 min for microbiological procedures. A 100 μL-portion of the samples were used for culture procedure and the other portion (900 μL) was frozen at −70 °C and transported on dry ice by an overnight delivery service to the Division of Oral Microbiology and Immunology, RWTH Aachen University Hospital, Aachen, Germany, for subsequent RTQ-PCR analysis. Post-treated samples were generated and processed accordingly.

Clinical procedures

After accessing the pulp chamber and subsequent, pre-chemo-mechanical microbial sampling, the pulp chamber was thoroughly cleaned either with 2.5% NaOCl or with 2% CHX (Endogel, Itapetininga, SP, Brazil). The chlorhexidine gel consisted of a gel base (1% natrosol) and chlorhexidine gluconate at pH 7.0. Natrosol gel (hydroxyethyl cellulose) is a nonionic, highly inert and water-soluble agent (Ferraz *et al.* 2001). A K-file of size 10 or 15 (DYNA-FFDM, Bourges, France) was placed to the full length of the root canal calculated from the pre-operative radiograph. The coronal two-thirds of each canal was prepared initially using rotary files (GT® Rotary Files size 20, 0.10 taper and size 20, 0.08 taper; Dentsply Maillefer, Bailagues, Switzerland) at 350 rpm 4 mm shorter than the estimated length. Gates-Glidden burs sizes 5, 4, 3 and 2 (DYNA-FFDM) were used 2 mm shorter than the length prepared with the GT instrument. Working length (1 mm from the radiographic apex) was checked with a radiograph after inserting a file in the canal to the estimated working length confirmed by apical locator (Novapex, Forum Technologies, Rishon le-Zion, Israel). The apical preparation was performed using K-files ranging from size 35–45 followed by step back instrumentation, which ended after the use of three files larger than the last file used for the apical preparation.

The 32 patients were randomly divided into two groups. In one group the root canals were irrigated with 2.5% NaOCl, whilst a second group was treated with 2.0% CHX gel as the disinfectant; the working time for the chemo-mechanical procedure was established at 20 min for all cases. In the CHX-group, use of each instrument was followed by irrigation with a syringe (27-gauge needle) containing 1 mL of the gel and immediately after with 4 mL of physiological saline solution. The inactivation of CHX activity was accomplished with a rinse of 5 mL sterile solution containing 0.5% Tween 80% and 0.07% (w/v) lecithin over a 1 min period before the second (post-chemo-mechanical) sample was taken with four paper points in RTF. In the NaOCl-group use of each instrument was followed by irrigation of the canal with 5 mL of 2.5% NaOCl solution. The inactivation of NaOCl was accomplished with a rinse of 5 mL of sterile 0.5% sodium thiosulphate, for 1 min, before the post-chemo-mechanical preparation sample was taken. Finally, all teeth were filled using lateral compaction of gutta-percha cones (Dentsply-Herpo, Petrópolis, RJ, Brazil) with Endofill® sealer (Dentsply-Herpo, Petrópolis, RJ, Brazil), and the access cavities were restored with 2 mm of Cavit™ (3M Dental Products, St Paul, MN, USA) and Filtek™ Z250 (3M Dental Products).

Culture technique

Using 100 μL of the samples 10-fold serial dilutions were prepared. First, 50 μL of each dilution was inoculated on blood agar plates supplemented with 5% horse blood, 5 mg L^{-1} haemin and 1 mg L^{-1} menadione. Plates were incubated anaerobically (80% N_2, 10% H_2, 10% CO_2) at 37 °C for 7 days (Peters *et al.* 2002). Subsequently, 50 μL of each dilution was inoculated on BHI agar plates (Brain Heart Infusion agar, Oxoid, Basingstoke, UK), supplemented with 5% sheep blood and incubated aerobically (37 °C, air) for 24 and 48 h. After incubation, the total CFU were counted using a stereomicroscope at 16× magnification (Zeiss, Oberkoren, Germany).

Real-time quantitative-polymerase chain reaction

The deep-frozen pre- and post-chemo-mechanical preparation samples were thawed and dispersed by vortexing for 15 s. The DNA was extracted and purified with a Qiamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Using this extraction method, DNA from both Gram-positive and Gram-negative bacteria was retrieved with no apparent discrimination against either bacterial group (Vianna *et al.* 2005). The DNA concentration (A_{260}) and the purity (A_{260}/A_{280}) were calculated using Gene Quant II photometer (Pharmacia Biotech, Cambridge, UK).

For RTQ-PCR primers and a probe that were designed from highly conserved regions of the 16S rDNA were used (Nadkarni *et al.* 2002); forward

primer EuF: 5′-TCCTACGGGAGGCAGCAGT-3′, reverse primer EuR: 5′-GGACTACCAGGGTATCTAATCCTGTT-3′, amplifying 466 bp from the 16S-region 331–797 (according to *Escherichia coli* position). As the TaqMan probe, EuTaq: 6-FAM-5′-CGTATTACCGCGGCTGCTG-GCAC-3′-TAMRA was used.

Amplification and detection of DNA by RTQ-PCR was performed with the aid of the ABI-PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using optical grade 96-well plates. In each run, four negative controls (nuclease free water as template), and series dilutions of DNA from *Prevotella nigrescens* as standard (as described bellow) were included. All samples were analysed twice using two different assay chemistries (i.e. by use of SYBR®Green PCR Master Mix, and TaqMan® PCR Master Mix; Applied Biosystems). Samples were run in duplicate in a total volume of 25 μL. Final reactions contained 100 nmol L^{-1} of each primer, and 2 μL of template DNA (approximately 50 ng of template DNA). The temperature profiles were as follows: (i) SYBRGreen: denaturation 94 °C for 10 min; 40 cycles: 94 °C for 1 min, stringent annealing at 60 °C for 1 min, and elongation at 72 °C for 1 : 30 min; followed by a final elongation at 72 °C for 5 min. Melting curve analysis was performed to assess reaction specificity. (ii) Taq-Man: denaturation 94 °C for 10 min; 40 cycles: 94 °C for 1 min, stringent annealing at 60 °C for 1 min and 45 s.

Data acquisition and subsequent analysis was performed using the ABI-PRISM 7000 SDS software (Applied Biosystems). The amount of initial template DNA was calculated by determining the threshold cycle (Ct), the number of PCR cycles required for the fluorescence to exceed a threshold value significantly higher than the background fluorescence. A threshold value of 0.2 was assumed which was approximately 10 times the background fluorescence, defined as the mean fluorescence values of the first six to 15 PCR cycles.

DNA extracted from *P. nigrescens* ATCC 33563 was used to establish the standard curve based on a series of 10-fold dilution. Standards were prepared for each run to test for variation amongst different runs. Using the PCR profiles outlined above, quantification in both TaqMan and the SYBRGreen format was possible down to 100 rRNA gene copy numbers.

Data were tested for normality using the GMC program (USP, Ribeirão Preto, SP, Brazil) and found to be nonparametric. Statistical analysis of the data was performed using the Mann–Whitney test (BioEstat program, CNpQ, 2000; Brasília, DF, Brazil), with significance level at $P < 0.01$.

Results

The disinfection of the rubber dam, the crown and its surrounding was tested before and after entry into the pulp chamber by taking swab samples, which were plated on blood agar plates. In all 32 cases, no microbial growth was observed in either sample. After chemo-mechanical preparation all root canals were dry (i.e. without exudate), and, as all cases were asymptomatic, it was possible to fill the canal and restore the teeth at the same appointment. Bacterial quantification by real-time PCR before and after chemo-mechanical preparation of the root canals was performed by the SYBRGreen and the TaqMan detection format.

Since there exists a wide range of rRNA-operon numbers amongst different bacterial taxa from 1 to ≥15 (Farrelly *et al.* 1995, Nadkarni *et al.* 2002), a precise calculation of cell numbers of a multi-species population is impossible. The bacterial DNA levels quantified by RTQ-PCR therefore were converted to theoretical rRNA gene copy numbers, since the ratio between rRNA genes and cells is unknown. The calculation of gene copy numbers was based on *P. nigrescens* as the representative standard bacterium, because *Prevotella* species are known to possess a maximum of only two RNA-operons.

The calculated rRNA gene copy numbers, differed considerably amongst patient samples (Table 1). The initial bacterial load in the NaOCl-group ranged from 3.2×10^3 to 1.2×10^8 copy numbers (median: 2.8×10^6) according to the SYBRGreen assay, and from 3.3×10^4 to 8.7×10^7 copy numbers (median: 7.6×10^6) according to the TaqMan assay. Comparable results were obtained in the CHX-group. Here, the initial bacterial load ranged from 6.9×10^4 to 6.7×10^7 copy numbers (median: 2.3×10^6) according to the SYBRGreen assay, and from 4.6×10^4 to 6.7×10^7 copy numbers (median: 3.0×10^6) according to the TaqMan format. After chemo-mechanical preparation of the infected root canals the gene copy numbers declined in all cases. However, the residual bacterial load was still measurable by RTQ-PCR except for one case (sample H3, Table 1). In the NaOCl-group the post-treated amount of gene copy numbers ranged from below the detection limit ($<10^2$) to 2.1×10^4 (median 2.0×10^2) according to SYBRGreen assay, and from 4.2×10^3 to 6.4×10^5 (median 1.6×10^4) according to the TaqMan assay. In the CHX-group the

Table 1 Bacterial load (expressed as rRNA copy numbers) and percentage reduction determined for root canal samples of 32 teeth with periapical lesions, before and after chemo-mechanical preparation with either NaOCl (H1–H16) or CHX (C1–C16) as irrigating substance[1]

NaOCl-group							CHX-group						
	SYBRGreen			TaqMan				SYBRGreen			TaqMan		
Sample	Before	After	% Reduction	Before	After	% Reduction	Sample	Before	After	% Reduction	Before	After	% Reduction
H1	1.9×10^7	2.0×10^2	99.99	3.2×10^7	6.4×10^5	98.00	C1	6.9×10^4	6.3×10^4	8.69	4.6×10^4	4.1×10^4	10.86
H2	3.2×10^3	1.0×10^2	96.87	4.3×10^4	1.2×10^4	72.09	C2	1.6×10^5	7.8×10^3	95.12	3.6×10^5	1.1×10^4	96.94
H3	1.9×10^5	<100	100	3.7×10^5	2.5×10^4	93.24	C3	4.7×10^5	3.8×10^5	19.14	8.9×10^5	4.8×10^5	46.06
H4	1.5×10^7	2.0×10^2	99.99	1.1×10^7	7.6×10^3	99.93	C4	2.0×10^5	7.0×10^4	65.00	2.4×10^5	2.2×10^4	90.83
H5	3.0×10^6	2.0×10^2	99.99	4.3×10^6	4.2×10^3	99.90	C5	1.3×10^7	5.3×10^4	99.59	8.4×10^6	2.8×10^4	99.66
H6	2.0×10^6	1.4×10^4	99.30	3.1×10^6	1.6×10^4	99.48	C6	1.6×10^6	2.8×10^4	98.25	2.4×10^6	3.4×10^5	85.83
H7	2.2×10^7	2.2×10^3	99.99	1.1×10^7	1.2×10^4	99.89	C7	3.0×10^6	4.3×10^4	98.56	3.7×10^6	1.1×10^3	99.97
H8	5.3×10^7	1.0×10^2	99.99	3.6×10^7	1.7×10^4	99.95	C8	6.3×10^5	1.5×10^4	97.61	1.0×10^6	1.0×10^4	99.00
H9	7.1×10^7	2.1×10^4	99.97	5.9×10^7	3.6×10^4	99.93	C9	4.3×10^7	4.7×10^4	99.89	6.7×10^7	5.1×10^4	99.92
H10	1.2×10^8	1.6×10^4	99.98	8.7×10^7	3.1×10^5	99.64	C10	1.8×10^5	6.1×10^4	66.11	4.2×10^5	4.4×10^4	89.52
H11	3.3×10^4	3.0×10^2	99.09	3.3×10^4	1.7×10^4	48.48	C11	1.4×10^7	5.6×10^5	96.00	2.1×10^7	1.2×10^6	94.28
H12	5.8×10^5	2.0×10^2	99.96	1.0×10^6	1.7×10^4	98.30	C12	1.2×10^6	6.6×10^4	94.50	1.8×10^6	5.4×10^4	97.00
H13	1.9×10^6	1.0×10^2	99.99	2.4×10^6	1.2×10^4	99.30	C13	4.7×10^6	1.3×10^5	97.23	9.1×10^6	3.4×10^5	96.26
H14	2.5×10^6	3.0×10^2	99.98	2.1×10^6	8.1×10^3	99.61	C14	3.0×10^6	6.0×10^4	98.00	4.4×10^6	1.1×10^5	97.50
H15	1.9×10^7	1.0×10^2	99.99	1.7×10^7	1.0×10^4	99.94	C15	1.7×10^7	1.5×10^5	99.11	1.8×10^7	4.4×10^5	97.55
H16	2.2×10^6	1.8×10^4	99.18	3.5×10^7	2.2×10^4	99.93	C16	6.7×10^7	4.2×10^6	93.73	6.3×10^7	9.6×10^6	84.76
Median	2.8×10^6	2.0×10^2	99.99 (a)	7.6×10^6	1.6×10^4	99.63 (A)	Median	2.3×10^6	6.2×10^4	96.62 (b)	3.0×10^6	5.3×10^4	96.60(B)

[1]DNA from *Prevotella nigrescens* was used to establish the standard curve for calculating the gene copy numbers. The linear scope of detection ranged from 10^2 to 10^8.
Different letters (a,b) and (A,B) indicate a significance difference (Mann-Whitney test, $P < 0.01$); lower-case letters indicate differences using SYBRGreen; and capital letters indicate differences using TaqMan.

detection values ranged from 7.8×10^3 to 4.2×10^6 (median 6.2×10^4) according to the SYBRGreen assay, and from 1.1×10^3 to 9.6×10^6 (median: 5.3×10^4) according to the TaqMan assay. Irrespective of the variation of the initial individual bacterial load, the decline of measurable rRNA gene copy numbers was largely consistent within groups. Whilst the median of bacterial reduction in the NaOCl-group was 99.99% (SYBRGreen) and 99.63% (TaqMan), respectively, the median of bacterial reduction in the CHX-group was lower (SYBRGreen 96.62%; TaqMan: 96.60%). Using the nonparametric Mann–Whitney test it was found that the reduction of the total bacterial load was significantly different between the NaOCl-group and the CHX-group ($P < 0.01$), irrespective of the detection system used (i.e. SYBRGreen and TaqMan).

By culture analysis, microorganisms were detected in all initial (pre-treatment) samples with a mean value of 2.4×10^5 CFU (5.0×10^4 median) in the NaOCl-group and a mean value of 2.1×10^5 CFU (8.8×10^4 median) in the CHX-group (Table 2). In contrast CFU counts in the post-chemo-mechanical preparation samples declined drastically with a mean value of 87 CFU (0 median) in the NaOCl-group and a mean value of 4.8×10^2 CFU (10 median) in the CHX-group. The bacterial reduction was similar in both groups ranging from 97.89 to 100%. However, in the NaOCl-group 75% (12 cases) were free of bacteria after chemo-mechanical preparation according to culture technique, whilst only 50% (eight cases) were bacteria free in the CHX-group.

Discussion

Assessment of microbial load

In the present study, the degree of bacterial reduction after applying NaOCl and CHX as irrigating substances was assessed and two different quantification strategies compared. For evaluating chemo-mechanical preparation, the bacterial load, pre- and post-preparation has been determined by culture in many studies. The mean number of CFU in the initial samples has been reported to range from 6.5×10^3 to 1×10^5 (Byström & Sundqvist 1981, 1985, Sjögren *et al.* 1991, Ørstavik *et al.* 1991, Peters *et al.* 2002), and the number of bacteria after chemo-mechanical preparation from 0 to 10^3 (Byström & Sundqvist 1981, Sjögren *et al.* 1991, 1997, Peters *et al.* 2002). These values are in accordance with the cell numbers that were determined by culture analysis in the present study.

However, the prevalence of some oral pathogens could have often been under-estimated by culture-based techniques as those approaches may fail to grow certain bacteria, especially fastidious anaerobic microorganisms such as spirochetes or *Tannerella forsythia*

Table 2 Bacterial load (expressed as CFU) and percentage reduction determined for root canal samples of 32 teeth with periapical lesions, before and after chemo-mechanical preparation with either NaOCl (H1–H16) or CHX (C1–C16) as irrigating substance

NaOCl-group				CHX-group			
Sample	Before	After	% Reduction	Sample	Before	After	% Reduction
H1	5.4×10^5	20	99.9	C1	1.0×10^4	20	99.8
H2	4.0×10^2	0	100	C2	4.4×10^5	0	100
H3	1.2×10^3	0	100	C3	1.0×10^6	5.8×10^3	99.42
H4	1.1×10^5	0	99.95	C4	2.5×10^4	0	100
H5	6.2×10^5	0	100	C5	7.6×10^5	4×10^2	99.34
H6	9.6×10^4	0	100	C6	9.6×10^4	0	100
H7	5.0×10^4	2.4×10^2	99.78	C7	4.4×10^4	4.6×10^2	99.04
H8	5.0×10^4	0	100	C8	8.1×10^4	20	99.97
H9	9.8×10^5	4.6×10^2	100	C9	3.7×10^5	6.8×10^2	99.81
H10	9.0×10^4	6.8×10^2	99.22	C10	7.6×10^3	1.6×10^2	97.89
H11	1.0×10^4	0	100	C11	2.6×10^6	0	100
H12	3.0×10^3	0	100	C12	2.5×10^4	0	100
H13	9.0×10^4	0	100	C13	6.0×10^4	0	100
H14	8.8×10^5	0	100	C14	3.0×10^4	0	100
H15	1.0×10^3	0	100	C15	1.5×10^5	0	100
H16	5.0×10^5	0	100	C16	1.1×10^5	2.4×10^2	99.79
Mean	2.4×10^5	8.7×10^1	99.93	Mean	2.1×10^5	4.8×10^2	99.69
Median	5.0×10^4	0	100	Median	8.8×10^4	10	99.98

No significance difference was found amongst the percentage of reduction from NaOCl-group and CHX-group (Mann–Whitney test, $P = 0.2$).

(Vianna *et al.* 2005). The culture-independent PCR methodology constitutes a valuable tool for characterizing the endodontic microflora (Siqueira & Rocas 2003). The particular value of this approach is that also uncultivable bacterial species are included in the measurement leading to a more accurate quantification. In the present study, a RTQ-PCR assay was used that had been demonstrated to detect a wide range of bacterial species in the oral cavity, and has been thoroughly evaluated with respect to sensitivity and reproducibility (Martin *et al.* 2002, Nadkarni *et al.* 2002, Kuboniwa *et al.* 2004). Hence, as root canal infections are not caused by a pre-defined group of cultivable pathogens but might stem from a mixed population of cultivable and uncultivable bacteria one can expect the endodontic bacterial load to be higher when measured by RTQ-PCR. In fact, the present data clearly suggest this, except for samples C2 and C3 before chemo-mechanical preparation.

However, owing to the different nature of the two methodologies (CFU counting and RTQ-PCR) results obtained by either strategy cannot be directly compared and require detailed interpretation. Since the clonal origin of a single CFU is not necessarily one single cell (e.g. for streptococci) the CFU based analysis can lead to an underestimation of the actual cell number. Conversely, the number of 16S rRNA gene copies measured by RTQ-PCR can exceed the actual cell number since the different members of a multi-species population might possess a wide range of rRNA-operon numbers (ranging from 1 to ≥15) (Farrelly *et al.* 1995). In addition, RTQ-PCR detects also DNA from nonviable or lysed cells increasing the discrepancy between data derived by RTQ-PCR and CFU counting. As no quantification strategy allows an exact enumeration of the microbial cells present in the root canal the question is how meaningful are RTQ-PCR derived data for root canal treatment. There are several lines of evidence that suggest that the RTQ-PCR might provide a clinically interesting measure for determining the microbial load in endodontics.

First, as stated above, noncultivable bacterial species are included in the RTQ-PCR approach. Secondly, the number of rRNA operons present within species plays a central role in their capacity to multiply (Klappenbach *et al.* 2000). For example, high numbers of rRNA gene copies confer a selective advantage permitting microbes to respond quickly in environments characterized by fluctuations and resource discontinuity (Stevenson & Schmidt 2004). Therefore, the amount of rRNA gene copies, rather than the actual cell number, might reflect the potential of the residual microbes to recover and to re-initiate root canal infections. Lastly, the major objective of the root canal treatment is not only to kill bacteria, but also to remove cell debris (Martin 1991). Any remaining in the root canal can be a risk factor because they serve as substrate for viable microbes present in dentine tubules and might induce an inflammatory host response in the surrounding tissues. For example, released endotoxin (LPS) from Gram-negative bacteria as well as other bacterial degradation products can cause an inflammatory reaction and periapical bone resorption (Pitts *et al.* 1982, Henderson *et al.* 1996, Leonardo *et al.* 2004). Thus, DNA from lysed or nonviable cells measured by RTQ-PCR could serve as a marker for remaining cell debris in the root canal.

In summary, RTQ-PCR derived data might complement CFU counts with respect to the overall cleanliness of the root canal. However, detection of living bacteria using culture techniques might still be the most valuable parameter to estimate the risk for continued infection.

SYBRGreen versus TaqMan analysis

In the present study, both the SYBRGreen and TaqMan assay chemistry was used, the latter format being more specific but also more cost-intensive. Both detection formats led, with a few exceptions (samples C6 and C7), to similar results, which accords with a comparison previously performed by Maeda *et al.* (2003). In the NaOCl-group, however, after chemo-mechanical preparation, a trend towards lower gene copy numbers was observed when using the SYBRGreen-format. This might largely be due to the SYBRGreen-specific effect on impairing PCR efficiency (Nath *et al.* 2000), which becomes more relevant with low template concentrations. This means that the TaqMan-based approach is more accurate, especially when low target samples are used. Irrespective of this difference, the SYBRGreen-based data showed sufficient sensitivity with respect to the microbial reduction in the root canal, making this approach an economical alternative to the TaqMan format.

Comparison of NaOCl with CHX

Sodium hypochlorite is the most commonly employed root canal irrigant, but no general agreement exists regarding the optimal concentration to be used, which might range from 0.5% to 5.25%. Within this range its

antimicrobial activity increases proportionally (Vianna *et al.* 2004); however, its toxicity also increases. As a trade-off between possible toxic side effects and antimicrobial activity, a medium strength concentration was used.

Nontoxicity to periapical tissues is one of the requirements of an ideal irrigating substance. Others are antimicrobial activity, water solubility and the capacity to dissolve organic matter (Kuruvilla & Kamath 1998, Okino *et al.* 2004). Except for its toxicity NaOCl meets those properties, whilst CHX is less toxic but not effective in dissolving organic matter.

Both substances (2% CHX-gel and 2.5% NaOCl) were successful in reducing the number of bacteria in most cases. However, NaOCl was superior when assessed by both, culture technique and RTQ-PCR.

Final considerations

It is clear that mechanical preparation reduces considerably the number of microorganisms; the addition of an irrigating solution promotes the disinfection (Byström & Sundqvist 1985). Although residual bacteria might be one of the major factors responsible for post-treatment disease, remaining bacterial structures (e.g. proteins, carbohydrates, and lipids) are also a risk factor either as substrates for microbes present in the dentinal tubules or as potential modulins stimulating inflammation in the apical portion of the root canal. The present study suggests that in comparison with CHX, NaOCl has not only a higher capacity to kill endodontic pathogens *in vivo*, but also to support cell removal. Whilst the culture technique is principally sufficient to evaluate the antimicrobial potential of disinfectants, RTQ-PCR based analysis also provides a measure for cell debris clearance.

Acknowledgements

This work was supported by grants of the following organizations: CAPES (BEX 3410/04–8), FAPESP (02/13980–9), and the START program of the Faculty of Medicine, RWTH Aachen, Aachen, Germany. We would like to thank Ilse Seyfarth, Vreni Merriam, and Diane M. Citron for various forms of assistance.

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