Reduction in the Cultivable Bacterial Populations in Infected Root Canals by a Chlorhexidine-based Antimicrobial Protocol

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Abstract

The present clinical study was conducted to assess the bacterial reduction after chemomechanical preparation using 0.12% chlorhexidine digluconate solution as an irrigant and the additive antibacterial effect of intracanal dressing with calcium hydroxide ($Ca(OH)_2$) associated with 0.12% chlorhexidine digluconate gel. According to stringent inclusion/exclusion criteria, 13 teeth with primary intraradicular infections and chronic apical periodontitis were selected and followed in the study. Bacterial samples were taken at the baseline (before treatment) (S1), after chemomechanical preparation using chlorhexidine (CHX) as an irrigant (S2), and after a 7-day dressing with $Ca(OH)_2$/CHX paste (S3). Cultivable bacteria recovered from infected root canals at the three stages were counted and identified by means of 16S ribosomal RNA gene sequencing analysis. At S1, all canals were positive for bacteria, with the mean number of 3.5 taxa per canal (range, 2–9). At S2, 7 cases (53.8%) still harbored cultivable bacteria, with a mean number of 1.7 taxon per canal (range, 1–4). At S3, only one case (7.7%) was positive for the presence of bacteria. The great majority of taxa found in posttreatment samples were gram-positive bacteria. A significantly high reduction in bacterial counts was observed between S1 and S2 and S1 and S3 ($p < 0.001$). Also, significant differences were observed for comparisons involving S2 and S3 samples with regard to both quantitative bacterial reduction ($p = 0.014$) and number of cases yielding negative cultures ($p = 0.01$). It was concluded that chemomechanical preparation with 0.12% CHX solution as an irrigant significantly reduced the number of intracanal bacteria but failed to render the canal free of cultivable bacteria in about one half of the cases. Application of a 7-day intracanal dressing with $Ca(OH)_2$/CHX paste further increased significantly the number of cases yielding negative cultures. (*J Endod* 2007;33:541–547)

A favorable outcome of the endodontic treatment of teeth with apical periodontitis depends on effective control of the root canal infection (1, 2). Ideally, an effective antimicrobial treatment protocol should be able to predictably render root canals free of bacteria so that the cause of apical periodontitis is eliminated. The most used protocols involve the use of sodium hypochlorite (NaOCl) as an irrigant and calcium hydroxide ($Ca(OH)_2$) in inert vehicles as an intracanal medication. Although protocols using these substances have been shown to be effective in eliminating bacteria from infected canals, they have their own limitations and the search for alternative or more effective antimicrobial protocols should be encouraged.

Chlorhexidine is a widely used antimicrobial agent that has emerged as a potential irrigant and interappointment medication to be used during the endodontic treatment of teeth with apical periodontitis. As an irrigant, CHX has shown antimicrobial effectiveness comparable to NaOCl in several clinical and laboratory studies (3–7), although controversy exists (8–10). Although CHX lacks tissue-dissolving ability, which is one of the obvious benefits of NaOCl (11), it has some advantages when compared with NaOCl because it has been reported to be less toxic to host tissues (8, 12) and present substantivity to dentin, which may result in residual antimicrobial effects for days to weeks (13–15). As an intracanal medication, CHX has been suggested to be used alone or combined with $Ca(OH)_2$ in a paste. Calcium hydroxide is the most commonly used intracanal medication, but it has a limited antibacterial spectrum that does not affect all members of the endodontic microbiota (16). In addition, physicochemical properties of this substance may limit its effectiveness in disinfecting the root canal system after a short-term use. Consequently, the addition of other antimicrobial mixing vehicles has been recommended to enhance the antimicrobial effectiveness of $Ca(OH)_2$ (16). Supplementing the antimicrobial activity of $Ca(OH)_2$ with CHX has been extensively studied in vitro. Several studies showed that the antimicrobial effects of $Ca(OH)_2$ are significantly increased when adding CHX in a paste (17–21). A clinical study (22) showed that canal dressing with a mixture of 2% CHX and $Ca(OH)_2$ was at least as effective as $Ca(OH)_2$ in an inert vehicle in the disinfection of root canal–treated teeth with apical periodontitis. The number of culture-negative canals after using this medication for 7 to 10 days was 13 of 20 (65%) (22). The antimicrobial effectiveness of dressings with $Ca(OH)_2$/CHX paste during the treatment of primarily infected canals has not been fully examined in vivo.

The present study is part of a series of investigations about the effectiveness of different antimicrobial treatment protocols to eliminate cultivable bacteria from infected root canals of teeth evincing apical periodontitis lesions (23, 24). The protocol tested in this study involves chemomechanical preparation using 0.12% chlorhexidine digluconate solution as an irrigant and a paste of $Ca(OH)_2$ in 0.12% chlorhexidine digluconate gel as an interappointment dressing for 7 days. Cultivable bacteria recov-

ered from infected root canals at three treatment stages were counted and identified by means of 16S ribosomal RNA (rRNA) gene sequencing analysis.

Materials and Methods
Clinical Material

Patients presenting to the endodontic clinic at the School of Dentistry, Estácio de Sá University, Rio de Janeiro, Brazil, for evaluation and treatment of apical periodontitis were considered for this study. Seventeen single-rooted teeth (5 maxillary central incisors, 2 maxillary lateral incisors, 4 mandibular incisors, 3 maxillary canines, and 3 mandibular premolars) from 14 patients (12 males and 2 females, aged 22–74 years old, mean 55.9 years old) were selected for this study based on stringent inclusion/exclusion criteria. Only teeth with intact pulp chamber walls, necrotic pulps as confirmed by negative response to sensitivity pulp tests, and clinical and radiographic evidence of chronic apical periodontitis lesions were included in this study. The size of the apical periodontitis lesions ranged from 2×3 mm to 12×12 mm. Teeth from patients who received antibiotic therapy within the previous 3 months, teeth with gross carious lesions; teeth with fractures of the root or crown, teeth that had received previous endodontic treatment, and cases showing periodontal pockets over 4 mm deep were excluded from the study. Approval for the study protocol was obtained from the Ethics Committee of the Estácio de Sá University.

Endodontic Treatment and Sampling Procedures

Rubber dam and an aseptic technique were used throughout the endodontic treatment. Before isolation with rubber dam, each tooth had supragingival plaque removed by scaling and cleansing with pumice. Caries and/or coronal restorations were removed with sterile high-speed and low-speed burs. After rubber dam application, dental floss was securely tied around the neck of the tooth. The operative field, including the tooth, clamp, and surroundings, were cleaned with 3% hydrogen peroxide until no further bubbling of the peroxide occurred. All surfaces were then disinfected by vigorous swabbing with a 2.5% NaOCl solution. After completing the access with another sterile bur under sterile saline irrigation, the operative field, including the pulp chamber, was then cleaned and disinfected once again the same way as described previously. NaOCl was neutralized with 5% sodium thiosulphate, and then sterility control samples were taken from the tooth surface with sterile paper points. For inclusion of the tooth in the study, these control samples had to be uniformly negative.

The first root canal sample (S1) was taken as follows. Three sterile paper points were consecutively placed in the canal to a level approximately 1 mm short of the root apex, based on diagnostic radiographs, and used to soak up the fluid in the canal. Each paper point was left in the canal for at least 1 minute. Paper points were then transferred aseptically to tubes containing 500 μL of reduced transport fluid.

Chemomechanical preparation was completed at the same appointment in all cases. The alternated rotation motion technique was used to prepare all canals (23, 24). Briefly, the coronal two thirds of the root canals were enlarged with Gates-Glidden burs. Working length was established 1 mm short of the root apex, and the patency length coincided with the radiographic root edge. Apical preparation was completed to the working length with hand nickel-titanium files (Nitiflex; Dentsply-Maillefer, Ballaigues, Switzerland), always using a back-and-forth alternated rotation motion. Master apical files ranged from #50 to #60, depending on both root anatomy and the initial diameter of the root canal. Apical patency was confirmed with a small file (#15 or #20 NitiFlex) throughout the procedures after each larger file size. Preparation was completed by using step back of 1-mm increments. A 0.12%

chlorhexidine digluconate solution was used as the irrigant during instrumentation. Two milliliters of this solution were used to rinse the canals after each instrument. Irrigant was delivered in the canals by means of a 5-mL disposable syringe with a 23-gauge needle.

Each canal was dried by using sterile paper points and then flushed with 5 mL of a mixture of 0.07% lecithin, 0.5% Tween 80, and 5% sodium thiosulfate to neutralize any residual CHX. This inactivating mixture was slightly modified from that proposed by Zamany and Spangberg (25). Subsequently, the root canal walls were gently filed and a postinstrumentation sample (S2) was taken from the canal as described earlier.

To remove the smear layer, 17% EDTA was left in the canal for 3 minutes followed by irrigation with 5 mL of 2.5% NaOCl. The canal was dried with paper points and dressed with a mix of $Ca(OH)_2$ in 0.12% chlorhexidine digluconate gel (2% natrosol), which was placed in the canals by means of Lentulo spiral fillers. The $Ca(OH)_2$/CHX paste was packed with a cotton pellet at the level of canal entrance. A radiograph was taken to ensure proper placement of the $Ca(OH)_2$ in the canal. The access cavities were filled with at least 4 mm thickness of a temporary cement (Coltosol; Coltène/Whaledent Inc., Cuyahoga Falls, OH).

The second appointment was scheduled for 1 week thereafter. At this time, the tooth was isolated with a rubber dam, the operative field disinfected, and the NaOCl neutralized, as outlined earlier. A sterility control sample of the operating field was obtained. The temporary filling was removed, and the $Ca(OH)_2$/CHX paste was rinsed out of the canal by using sterile saline solution and the master apical file. Five milliliters of the mixture of lecithin, Tween 80, and sodium thiosulfate were then used to inactivate CHX. The root canal walls were filed lightly and a postmedication sample (S3) was taken from the canals. Subsequently, the canals were filled with gutta-percha and Sealer 26 (Dentsply, Petrópolis, Brazil) by using cold lateral compaction. The tooth was temporized with glass ionomer cement and a permanent restoration planned. All clinical procedures were conducted by one experienced endodontist (SSMP).

Microbiological Analysis

Samples were transported to the laboratory within 15 minutes for microbiological processing. Samples in reduced transport fluid vials were dispersed with a vortex for 30 seconds and 10-fold serial dilutions to 10^{-3} (for S1 samples) or 10^{-2} (for S2 and S3 samples) were made in prereduced anaerobically sterilized buffered salt solution. Aliquots of 100 μL from the undiluted suspension and the highest dilution were each spread onto Brucella agar plates (BBL Microbiology Systems, Cockeysville, MD) supplemented with 5% defibrinated sheep blood, hemin (5 mg/L) and menadione (1 mg/L), and Mitis-salivarius agar plates (Difco, Detroit, MI). Plates were incubated anaerobically within anaerobic jars (GasPak system, BBL Microbiology Systems) at 37°C for 14 days. After incubation, the total colony-forming units (CFUs) were counted, and actual counts were calculated based on the known dilution factors. One or two colonies of each different colony type were isolated, and each one was individually placed in cryovials containing TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, and pH 8). Cryovials were then stored at −20°C until further bacterial identification by 16S rRNA gene sequencing.

16S rRNA Gene Identification

Genomic DNA was extracted from each colony by heating the suspension for 10 minutes at 97°C with a thermocycler. The vials were then stored for 5 minutes on ice and centrifuged, and 5-μL aliquots of the supernatant were further used as template in the polymerase chain reaction (PCR) assay.

PCR amplification of 16S rRNA genes was used for bacterial identification. The pair of universal 16S rRNA gene primers used was 5′-GAT TAG ATA CCC TGG TAG TCC AC-3′ and 5′-CCC GGG AAC GTA TTC ACC G-3′, corresponding to base positions 786-808 and 1,369-1,387, respectively, and spanning the variable regions V5-V8 of the *Escherichia coli* 16S rRNA gene. PCR amplification was performed in a reaction volume of 50 μL, consisting of 0.8 μmol/L concentration of each primer, 5 μL of 10$\times$ PCR buffer, 2 mmol/L MgCl$_2$, 1.25 U of *Tth* DNA polymerase, and 0.2 mmol/L concentration of each deoxyribonucleoside triphosphate (all reagents from Biotools, Madrid, Spain). Cycling parameters included an initial denaturation step at 95° C for 2 minutes, followed by 36 cycles of a denaturation step at 95°C for 30 seconds, a primer annealing step at 60°C for 1 minute, an extension step at 72°C for 1 minute, and a final step of 72°C for 2 minutes. The results of PCR amplification were examined by electrophoresis in 1.5% agarose gel. DNA was stained with ethidium bromide and visualized under short-wavelength ultraviolet light.

PCR products were purified by using a PCR purification system (Wizard PCR Preps; Promega, Madison, WI) and then sequenced directly on the ABI 377 automated DNA sequencer by using dye terminator chemistry (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Sequence data and electropherograms were inspected and corrected when obvious sequencing software errors were observed. Sequences generated were compared with the GenBank database to identify the closest relatives by using the BLAST algorithm. A $\geq$99% identity in 16S rRNA gene sequence was the criterion used to identify an isolate to the species level. A 97% to 99% identity in 16S rRNA gene sequence was the criterion used to identify an isolate at the genus level, whereas <97% identity in 16S rRNA gene sequence was the criterion used to define a potentially new bacterial species.

Statistical Analysis

Effectiveness of each treatment step in rendering root canals free of cultivable bacteria was recorded as percentage of cases yielding negative cultures. In this regard, the one-tailed Fisher exact test was used to compare S2 and S3 samples. The percent reduction in the number of CFUs after each treatment step was calculated based on quantitative data obtained from samples S1, S2, and S3. Quantitative data were statistically analyzed for differences by using the Mann-Whitney U test comparing pairs of groups. Significance level was always set at 5% (p < 0.05).

Results

Of the 17 teeth sampled, 13 followed in the study. Reasons for exclusion of 4 teeth were as follows: bacterial growth for the sterility control of the working field (one tooth), accidental loss of the third sample (one tooth), and flare-up requiring an emergency visit by an operator other than that involved in the project (two teeth). Bacteria were found in all initial samples (S1) from the 13 root canals. The median value of the number of CFUs in the initial samples was 1.1×10^6, ranging from 2.8×10^3 to 1×10^8.

After chemomechanical preparation using 0.12% CHX solution as an irrigant, 6 of the 13 canals (46.2%) showed negative culture results. The median number of CFUs in postinstrumentation samples was 4×10^2, ranging from 0 to 5.12×10^5. When compared with initial samples, chemomechanical preparation promoted reduction in the number of bacteria ranging from 53.45% to 100%.

After 7 days of dressing with Ca(OH)$_2$/CHX paste, 12 of the 13 canals (92.3%) yielded no cultivable bacteria. The only case positive for bacteria in S3 harbored 1.56×10^3 CFUs. The percent reduction in this case was 99.7%. All the other cases showed 100% reduction in the number of cultivable bacteria.

Analysis of the quantitative data revealed that the number of CFUs in S2 and S3 was significantly reduced in comparison to S1 (p < 0.001 for both comparisons, Mann-Whitney U test). Significant differences were also observed for comparisons involving S2 and S3 samples with regard to the number of cases yielding negative cultures (p = 0.01, Fisher exact test) or quantitative bacterial reduction (p = 0.014, Mann-Whitney U test). Quantitative data and percent reductions are depicted in Table 1 and shown in Figure 1.

Forty-five isolates belonging to 33 bacterial taxa were identified in initial samples. One additional isolate was not identified because of sequences with low-scoring homologies (<97% similarity) to sequences deposited in the GenBank. The mean number of bacterial taxa per canal was 3.5, ranging from 2 to 9. The most prevalent taxa were *Porphyromonas gingivalis* (3 cases), *Streptococcus mitis* biovar 2 (3 cases), *Propionibacterium acnes* (3 cases), *Fusobacterium nucleatum* (2 cases), *Prevotella* oral clone GU027 (2 cases), *Pseudoramibacter alactolyticus* (2 cases), *Propionibacterium propionicum* (2 cases), *Actinomyces odontolyticus* (2 cases), *Staphylococcus epidermidis* (2 cases), and *Streptococcus oralis* (2 cases) (Table 2).

Twelve isolates belonging to 11 distinct taxa were recovered from S2 samples (mean, 1.7 taxon per case; range, 1–4 taxa). Of the 7 cases showing culture-positive results, four cases harbored one taxon each (*S mitis* biovar 2, *P acnes*, *Staphylococcus aureus*, or *Actinomyces urogenitalis*), two cases showed two taxa each (*P alactolyticus* and an uncultured beta proteobacterium or *Streptococcus sanguinis* and *S oralis*) and another case harbored 4 taxa (*Prevotella* oral clone FM005, *S mitis* biovar 2, *Propionibacterium granulosum*, and *A odontolyticus*). All these bacteria were also found in S1, except for the

TABLE 1. Bacterial load and percent reduction determined for root canal samples of 13 teeth with apical periodontitis lesions

Case	Sample Code	Initial Samples (S1)	Postinstrumentation Samples (S2)	Postmedication Samples (S3)
1.	6TS	5.21×10^5	9×10^2 (99.83%)	1.56×10^3 (99.7%)
2.	4TS	1.1×10^6	5.12×10^5 (53.45%)	0 (100%)
3.	3TS	2.8×10^7	2.48×10^5 (99.11%)	0 (100%)
4.	8TS	1.63×10^7	2×10^5 (98.77%)	0 (100%)
5.	12TS	1.83×10^4	5×10^3 (72.68%)	0 (100%)
6.	11TS	6.08×10^4	4.4×10^3 (92.76%)	0 (100%)
7.	1TS	7.2×10^6	4×10^2 (99.99%)	0 (100%)
8.	15TS	1×10^8	0 (100%)	0 (100%)
9.	7TS	2.12×10^7	0 (100%)	0 (100%)
10.	5TS	2.2×10^6	0 (100%)	0 (100%)
11.	16TS	7×10^5	0 (100%)	0 (100%)
12.	13TS	5.3×10^3	0 (100%)	0 (100%)
13.	14TS	2.8×10^3	0 (100%)	0 (100%)
	Median	1.1×10^6	4×10^2	0

Samples were taken before treatment, after instrumentation with 0.12% CHX as an irrigant, and after a 7-day intracanal medication with a paste of Ca(OH)$_2$ in 0.12% CHX gel.
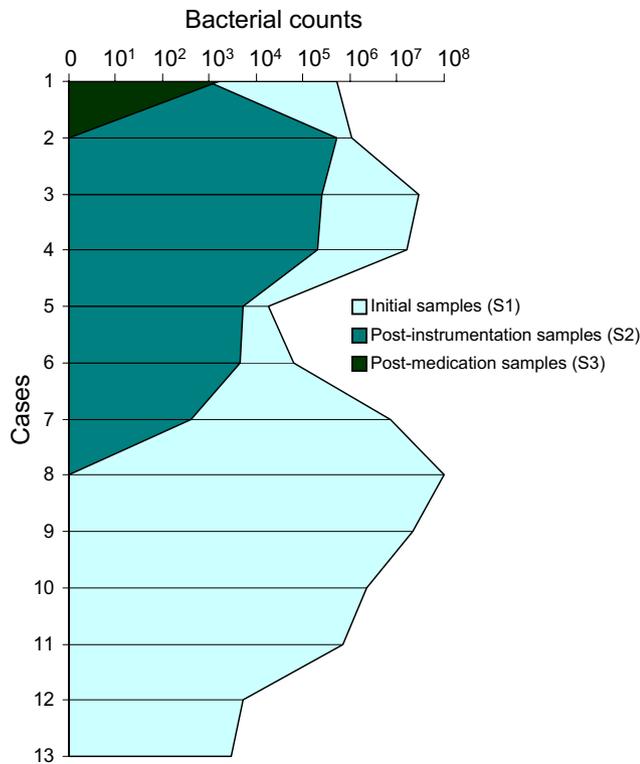
Bacterial counts

Figure 1. Bacterial reduction per case after chemomechanical preparation using 0.12% CHX solution as an irrigant and a 7-day intracanal dressing with a paste of Ca(OH)$_2$ in 0.12% CHX gel. Cases are ordered according to the bacterial counts after treatment steps.

uncultured beta proteobacterium, which was exclusively found in S2. This taxon may have been in S1 but in numbers below the detection levels of culture, or it may have been a contaminant introduced in the canal during intervention or during handling of samples. This sample was not eliminated from the study because the origin of this bacterium was unknown (it could have been in S1), and it was detected in S2 along with another species previously found in S1, confirming positiveness.

Only two taxa (*P acnes* and *S mitis* biovar 2) were isolated from the single case that sampled bacteria after dressing with Ca(OH)$_2$/CHX paste. *P acnes* was present in both S1 and S2, whereas *S mitis* biovar 2 was isolated from S1 but not from S2. This case showed a slight increase in the number of CFUs when compared with S2, but because the species present also occurred in S1 and the temporary restoration was apparently intact, it was considered unlikely that it was because of contamination between appointments.

Discussion

A culture-dependent approach was used in the present investigation to assess the antimicrobial effectiveness of a CHX-based treatment protocol. As with any other method, culture has its own advantages and limitations. One of the greatest strengths of the culturing procedure is that it is one of the most reliable methods to detect viable bacteria after antimicrobial treatment. Furthermore, studies using culture-dependent techniques have shown a correlation between negative cultures and optimal treatment outcome (1, 2). However, an important limitation of the method refers to the fact that about one half of the endodontic bacteria have not been cultivated by standard culture techniques as the one used in this study (26). Also, because of the method's low sensitivity, some bacteria may pass unnoticed in the event that the number of bacterial cells sampled is below the detection limits of the culture

method. An additional shortcoming of the method used herein is that the samples obtained only reflect the bacteriological conditions of the main canal. As a consequence of these factors, the bacterial diversity in samples could have been actually broader, and a culture-negative canal cannot be regarded as a sterile root canal system. In fact, a negative culture is likely to mean that the bacterial reduction in the canal has reached levels below the detection limits of culture procedures, and these levels may be compatible with periradicular tissue healing.

Instead of the traditionally used phenotype-based approaches, bacteria isolated in this study were identified by 16S rRNA gene-sequencing analysis. One should be mindful that in some circumstances even the successful cultivation of a given bacterial species does not imply that this isolate can be successfully identified by phenotypic approaches. Phenotype-based identification methods can fail because the phenotype is inherently mutable and subject to biases of interpretation (27). When common bacteria with uncommon phenotypes are present, reliance on phenotypic characteristics can lead to misidentification. For slow-growing and fastidious bacteria, traditional phenotypic identification is difficult and time consuming. In addition, interpretation of phenotypic test results can involve a substantial amount of subjective judgment and personnel's expertise. The 16S rRNA gene sequencing approach has become the reference method for bacterial identification and taxonomy (28). By this method, an isolate can be identified after obtaining its 16S RNA gene sequence and comparing it with sequences deposited in public databases. This molecular technique can provide a more precise identification of bacteria that are difficult to identify by conventional techniques (28). The 16S rRNA gene sequencing approach also has the advantage of being able to accurately identify rare isolates, poorly described bacteria, as-yet-uncultivated and uncharacterized bacteria, and newly named species. By using this method, we succeeded in identifying several isolates from previously uncultivated species (e.g., *Prevotella* oral clones GU027 and FM005, *Fusobacterium* oral clones CZ006 and BS019, *Actinomyces* oral clone GU009, and two other uncharacterized clones), unnamed species of the genera *Acinetobacter* and *Flavobacterium*, and newly named species (*Dialister invisus* and *Prevotella salivae*).

All initial samples were positive for the presence of bacteria, and the number of CFUs per canal ranged from 10^3 to 10^8. Substantial bacterial reduction was observed after chemomechanical preparation with 0.12% CHX solution as an irrigant. Except for two cases (4TS and 12TS), the percent reduction of the bacterial counts was always more than 92%. These findings indicate that 0.12% CHX has a good potential to be used as an irrigant during treatment of infected root canals.

Chlorhexidine is highly effective against several gram-positive and gram-negative oral bacterial species as well as yeasts (6, 10). This cationic bisbiguanide can induce damage to the outer microbial cell layers but this effect is usually insufficient to induce lysis or cell death. Chlorhexidine crosses the microbial cell wall, presumably by passive diffusion, and subsequently attacks the cytoplasmic membrane. Damage to this delicate membrane is followed by leakage of intracellular constituents. At high concentrations, CHX causes precipitation of intracellular constituents, particularly phosphated entities, such as adenosine triphosphate and nucleic acids. As a consequence, the cytoplasm becomes congealed, with resultant reduction in leakage, so that there is a biphasic effect on membrane permeability (29). Chlorhexidine antimicrobial activity is pH dependent, with the optimal range of 5.5 to 7, and is greatly reduced or abolished in the presence of organic matter (29).

Only a few in vivo studies have investigated the antimicrobial efficacy of CHX as an irrigant. Leonardo et al. (14) reported that 9 of 22 canals (40.9%) showed negative cultures after chemomechanical preparation using 2% CHX. Ercan et al. (3) concluded that both 2% CHX and

TABLE 2. Cultivable bacterial taxa detected during treatment of 13 infected root canals of teeth associated with apical periodontitis

Bacteria Only in Initial Samples (S1)*	Persisting Bacteria	
	Postinstrumentation (S2)†	Postmedication (S3)‡
Porphyromonas gingivalis (3)	*Streptococcus mitis* biovar 2 (2)	*Streptococcus mitis* biovar 2 (1)‖
Fusobacterium nucleatum (2)	*Propionibacterium acnes* (1)	*Propionibacterium acnes* (1)
Prevotella oral clone GU027 (2)	*Prevotella* oral clone FM005 (1)	
Propionibacterium acnes (2)	*Pseudoramibacter alactolyticus* (1)	
Propionibacterium propionicum (2)	*Actinomyces odontolyticus* (1)	
Staphylococcus epidermidis (2)	*Actinomyces urogenitalis* (1)	
Dialister invisus (1)	*Streptococcus oralis* (1)	
Pseudoramibacter alactolyticus (1)	*Streptococcus sanguinis* (1)	
Mogibacterium neglectum (1)	*Propionibacterium granulosum* (1)	
Prevotella salivae (1)	*Staphylococcus aureus* (1)	
Prevotella oralis (1)	Uncultured beta proteobacterium clone FAC20 (1)§	
Fusobacterium oral clone CZ006 (1)		
Fusobacterium oral clone BS019/		
Fusobacterium periodonticum (1)		
Campylobacter gracilis (1)		
Campylobacter curvus (1)		
Micromonas micros (1)		
Uncultured bacterium clone rRNA007 (1)		
Anaerococcus prevotii (1)		
Actinomyces naeslundii (1)		
Actinomyces oral clone GU009 (1)		
Actinomyces odontolyticus (1)		
Streptococcus oralis (1)		
Acinetobacter sp. (1)		
Flavobacterium sp. (1)		
Staphylococcus pasteuri (1)		
Staphylococcus saccharolyticus (1)		
Unidentified (1)		

Data are based on 16S rRNA gene sequencing identification of isolates.

*Bacteria eliminated after treatment and not found in S2 or S3.

†Bacteria found in both S1 and S2, except where indicated.

‡Bacteria found in S1, S2, and S3, except where indicated.

§Isolate not found in S1.

‖Isolate found in S1 but not in S2.

5.25% NaOCl were significantly effective in reducing the bacterial population in infected root canals. In the CHX group, 12 of 15 (80%) canals were free of cultivable bacteria, whereas in the NaOCl group 11 of 15 (73.3%) canals were rendered bacteria free (3). Vianna et al. (9) compared the degree of bacterial reduction after chemomechanical preparation of infected root canals by using 2.5% NaOCl or 2% CHX gel as irrigants. The bacterial load was reduced substantially in both groups (over 96%). Data from quantitative real-time PCR revealed that the bacterial reduction in the NaOCl group was significantly greater than in the CHX group. Culture analysis showed that 12 of 16 (75%) canals were free of cultivable bacteria after chemomechanical preparation in the NaOCl group, whereas 8 of 16 (50%) canals were free of bacteria in the CHX group. In the present study, 6 of 13 (46.2%) canals yielded no cultivable bacteria after instrumentation and irrigation with 0.12% CHX solution. Our results are comparable to the culturing findings reported by Vianna et al. (9), who also performed inactivation of CHX before sample collection. The present findings also suggest that CHX in lower concentrations may produce similar effects to more concentrated solutions under in vivo conditions.

Although a significant reduction in bacterial counts occurred after chemomechanical procedures, about one half of the root canals still yielded positive cultures in S2. These findings are also comparable with previous studies using NaOCl as an irrigant (23, 24, 30–32). Several gram-negative species were isolated from S1 samples, but most taxa found in S2 samples were gram-positive bacteria. Streptococci (4 isolates), *Actinomyces* (2 isolates), and *Propionibacterium* (2 isolates) were the most frequent groups recovered from S2 samples. These findings confirm that gram-positive species are the most common taxa persisting after chemomechanical procedures (33).

Virtually all of the taxa found in S2 samples were eliminated after a 7-day intracanal medication with the paste of Ca(OH)$_2$ in 0.12% CHX gel. The only exceptions were *P acnes* and *S mitis* biovar 2, which coinfected the canal of case 6TS. Although *P acnes* was found in all three samples from this case, *S mitis* biovar 2 was isolated from S1 but not S2. Its presence in S3 strongly suggests that this species was also present in S2 but in undetectable numbers. The increase in the number of CFUs in S3 as compared with S2 also indicates that conditions in this particular case in some way favored the growth of bacteria already present in the canal. However, the possibility also exists that this increase in bacterial numbers was in fact an artifact generated by a possible flaw during S2 sample collection. It was noteworthy that these 2 species were present in 3 initial samples from different cases. In two cases, *P acnes* was eliminated after chemomechanical procedures, and in two other cases *S mitis* biovar 2 was eliminated after intracanal medication. The reason why these species were eliminated in some cases and not in case 6TS are not apparent but may be related to more resistant clonal types, unnoticeable errors during some steps of the treatment, and/or bacterial presence in regions inaccessible to intracanal disinfection procedures.

In two previous studies using similar methodology (23, 24), we found that 45.5% and 54.5% of the canals sampled bacteria after chemomechanical preparation with 2.5% NaOCl. The number of cases with positive culture decreased to 18.2% and 9.1% after a 7-day medication with Ca(OH)$_2$/glycerin or Ca(OH)$_2$/camphorated paramonochlorophenol paste, respectively. A significant difference between the

number of culture-negative cases after chemomechanical preparation and intracanal medication was only observed when $Ca(OH)_2$/camphorated paramonochlorophenol paste was used. In the present study, 53.8% of the cases cultured bacteria after chemomechanical preparation using 0.12% CHX solution as an irrigant. A 7-day medication with $Ca(OH)_2$/CHX paste further reduced the number of culture-positive cases to 7.7%. This difference was statistically significant. Although based on a small sample size, these findings seem to suggest the need for adding other disinfectants to $Ca(OH)_2$ to enhance the antimicrobial activity in the canal. However, further comparative clinical studies using a larger sample size are necessary to validate this assumption.

So far, there is no consensus among in vitro studies investigating the antimicrobial effectiveness of $Ca(OH)_2$ mixed with CHX. Although some studies showed that the antimicrobial effects of $Ca(OH)_2$ are significantly increased when adding CHX in a paste (17–20), others have shown no significant increase in activity (34, 35). However, it is well established that the efficacy of CHX is significantly reduced when mixed with $Ca(OH)_2$ (19, 34, 35). Chlorhexidine remains stable at pH 5-8 and, as the pH is increased, ionization decreases. Association of $Ca(OH)_2$ with CHX maintains a high pH value, which is similar to $Ca(OH)_2$ paste using water as a vehicle (20, 22). At high pH values, the CHX precipitates and may be unavailable as an antimicrobial agent (22). Despite the remarkable high loss of CHX when mixed with $Ca(OH)_2$, the combined resulting effect may be of clinical significance (22). The association of $Ca(OH)_2$ and CHX presented significant antibacterial effects in the present study, and this may have been because of a small residue of active CHX still present in the paste, although the effects of the high pH of the paste should not be discarded.

Given the anatomic complexity of the root canal system and taking into account the extant treatment techniques and medications, it is fair to assume that it is virtually impossible to render the whole system sterile. Therefore, the purpose of the antimicrobial endodontic treatment should be to reduce bacterial populations to levels that are undetectable by contemporary culturing techniques (culture-negative canals), which is compatible with periradicular tissue healing. In the present study, comparisons of the absolute bacterial counts and the number of culture-negative cases between S2 and S3 samples revealed statistically significant differences. These findings confirm the importance of using an interappointment dressing to supplement the antibacterial effects of the chemomechanical preparation (24, 30, 31). Because the intracanal medication remains in the canal longer than the irrigation solution, it has more time to diffuse, reach, and eliminate bacteria in areas not affected by chemomechanical procedures, even if the substance used as medication is less potent than the irrigant.

Findings from the present study support the assertion that negative cultures are consistently obtained from the main canal after adequate instrumentation and irrigation with an antimicrobial agent, smear layer removal, and application of an intracanal antimicrobial dressing. The CHX-based protocol used was highly effective in reducing the bacterial populations within infected root canals and rendering most canals free of cultivable bacteria. Only 7.7% of the cases showed cultivable bacteria after the whole protocol. The impact of a 7-day intracanal dressing with $Ca(OH)_2$/CHX on the observed antibacterial effects was indisputable. These findings indicate that this CHX-based antimicrobial clinical protocol can be successfully used for the treatment of primary intraradicular infections in teeth with apical periodontitis.

References

1. Sjögren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J 1997;30:297–306.
2. Waltimo T, Trope M, Haapasalo M, Orstavik D. Clinical efficacy of treatment procedures in endodontic infection control and one year follow-up of periapical healing. J Endod 2005;31:863–6.
3. Ercan E, Ozekinci T, Atakul F, Gul K. Antibacterial activity of 2% chlorhexidine gluconate and 5.25% sodium hypochlorite in infected root canal: *in vivo* study. J Endod 2004;30:84–7.
4. Jeansonne MJ, White RR. A comparison of 2.0% chlorhexidine gluconate and 5.25% sodium hypochlorite as antimicrobial endodontic irrigants. J Endod 1994;20:276–8.
5. Vahdaty A, Pitt Ford TR, Wilson RF. Efficacy of chlorhexidine in disinfecting dentinal tubules *in vitro*. Endod Dent Traumatol 1993;9:243–8.
6. Vianna ME, Gomes BP, Berber VB, Zaia AA, Ferraz CC, de Souza-Filho FJ. In vitro evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;97:79–84.
7. Ruff ML, McClanahan SB, Babel BS. In vitro antifungal efficacy of four irrigants as a final rinse. J Endod 2006;32:331–3.
8. Oncag O, Hosgor M, Hilmioglu S, Zekioglu O, Eronat C, Burhanoglu D. Comparison of antibacterial and toxic effects of various root canal irrigants. Int Endod J 2003;36:423–32.
9. Vianna ME, Horz HP, Gomes BP, Conrads G. In vivo evaluation of microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue. Int Endod J 2006;39:484–92.
10. Ohara P, Torabinejad M, Kettering JD. Antibacterial effects of various endodontic irrigants on selected anaerobic bacteria. Endod Dent Traumatol 1993;9:95–100.
11. Naenni N, Thoma K, Zehnder M. Soft tissue dissolution capacity of currently used and potential endodontic irrigants. J Endod 2004;30:785–7.
12. Tanomaru Filho M, Leonardo MR, Silva LAB, Anibal FF, Faccioli LH. Inflammatory response to different endodontic irrigating solutions. Int Endod J 2002;35:735–9.
13. White RR, Hays GL, Janer LR. Residual antimicrobial activity after canal irrigation with chlorhexidine. J Endod 1997;23:229–31.
14. Leonardo MR, Tanomaru Filho M, Silva LA, Nelson Filho P, Bonifacio KC, Ito IY. In vivo antimicrobial activity of 2% chlorhexidine used as a root canal irrigating solution. J Endod 1999;25:167–71.
15. Rosenthal S, Spangberg L, Safavi K. Chlorhexidine substantivity in root canal dentin. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;98:488–92.
16. Siqueira JF Jr, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J 1999;32:361–9.
17. Evans MD, Baumgartner JC, Khemaleelakul SU, Xia T. Efficacy of calcium hydroxide: chlorhexidine paste as an intracanal medication in bovine dentin. J Endod 2003;29:338–9.
18. Podbielski A, Spahr A, Haller B. Additive antimicrobial activity of calcium hydroxide and chlorhexidine on common endodontic bacterial pathogens. J Endod 2003;29:340–5.
19. Gomes BP, Vianna ME, Sena NT, Zaia AA, Ferraz CC, de Souza Filho FJ. In vitro evaluation of the antimicrobial activity of calcium hydroxide combined with chlorhexidine gel used as intracanal medicament. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102:544–50.
20. Siren EK, Haapasalo MP, Waltimo TM, Orstavik D. In vitro antibacterial effect of calcium hydroxide combined with chlorhexidine or iodine potassium iodide on *Enterococcus faecalis*. Eur J Oral Sci 2004;112:326–31.
21. Gomes BP, Souza SF, Ferraz CC, et al. Effectiveness of 2% chlorhexidine gel and calcium hydroxide against *Enterococcus faecalis* in bovine root dentine *in vitro*. Int Endod J 2003;36:267–75.
22. Zerella JA, Fouad AF, Spangberg LS. Effectiveness of a calcium hydroxide and chlorhexidine digluconate mixture as disinfectant during retreatment of failed endodontic cases. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005;100:756–61.
23. Siqueira JF Jr, Guimarães-Pinto T, Rôças IN. Effects of chemomechanical preparation using 2.5% NaOCl and intracanal medication with calcium hydroxide on cultivable bacteria in infected root canals. J Endod (in press).
24. Siqueira JF Jr, Magalhães KM, Rôças IN. Bacterial reduction in infected root canals treated with 2.5% NaOCl as an irrigant and calcium hydroxide/camphorated paramonochlorophenol paste as an intracanal dressing. J Endod (in press).
25. Zamany A, Spangberg LS. An effective method of inactivating chlorhexidine. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;93:617–20.
26. Sakamoto M, Rôças IN, Siqueira JF Jr, Benno Y. Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections. Oral Microbiol Immunol 2006;21:112–22.
27. Petti CA, Polage CR, Schreckenberger P. The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. J Clin Microbiol 2005;43:6123–5.

28. Siqueira JF Jr, Rôças IN. Exploiting molecular methods to explore endodontic infections: part 1—current molecular technologies for microbiological diagnosis. J Endod 2005;31:411–23.

29. McDonnel G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 1999;12:147–79.

30. McGurkin-Smith R, Trope M, Caplan D, Sigurdsson A. Reduction of intracanal bacteria using GT rotary instrumentation, 5.25% NaOCl, EDTA, and Ca(OH)$_2$. J Endod 2005;31:359–63.

31. Sjögren U, Figdor D, Spangberg L, Sundqvist G. The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. Int Endod J 1991;24:119–25.

32. Byström A, Sundqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. Int Endod J 1985;18:35–40.

33. Chávez de Paz LE. On bacteria persisting root canal treatment. Identification and potential mechanisms of resistance to antimicrobial measures [PhD thesis]. Göteborg, Sweden: Göteborg University, 2005.

34. Schafer E, Bossmann K. Antimicrobial efficacy of chlorhexidine and two calcium hydroxide formulations against *Enterococcus faecalis*. J Endod 2005;31:53–6.

35. Siqueira JF Jr, Rôças IN, Lopes HP, Magalhaes FA, de Uzeda M. Elimination of *Candida albicans* infection of the radicular dentin by intracanal medications. J Endod 2003;29:501–4.