

Endodontic Therapy Associated with Calcium Hydroxide As an Intracanal Dressing: Microbiologic Evaluation by the Checkerboard DNA-DNA Hybridization Technique

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Abstract

This study evaluated the predominant microbiota of infected necrotic pulps and the effects of calcium hydroxide therapy on these microorganisms by the checkerboard DNA-DNA hybridization technique. Conventional endodontic therapy associated with calcium hydroxide as intracanal dressing was performed in 12 single-rooted teeth with pulp necrosis and periradicular bone lesion. Samples were collected from the canal at baseline and 14 days after therapy, and the presence of 44 bacterial species was determined by the checkerboard method. Significant differences in the microbiota from baseline to post-therapy were sought by the paired-samples *t* test. The most prevalent species included *F. nucleatum* ss. *vincentii*, *C. sputigena*, *C. ochracea*, *S. constellatus*, *V. parvula*, *P. gingivalis*, *P. melaninogenica*, and *S. sanguis*. Most of the microorganisms were reduced after treatment, particularly *A. gerencseriae*, *A. israelii*, *A. naeslundii*, *C. gingivalis*, *C. ochracea*, *P. gingivalis*, *S. noxia*, *S. sanguis*, and *S. oralis* ($p < 0.05$). Conversely, *A. actinomycetemcomitans*, *C. sputigena*, and *E. corrodens* increased in numbers after therapy. These results indicate that conventional endodontic therapy with calcium hydroxide results in the reduction of pathogenic species associated with pulp necrosis. However, its use is limited, because it did not eliminate the whole spectrum of microorganisms.

Over the years, numerous investigations have demonstrated that microorganisms and their products play a fundamental role in the pathogenesis of pulp and periapical infections (1, 2, 3). Cultural studies of primary endodontic infections have shown that these infections are polymicrobial, with a predominance of obligate anaerobic bacteria, including species of the genera *Eubacterium*, *Fusobacterium*, *Peptostreptococcus*, *Porphyromonas*, and *Prevotella* (1, 2, 3).

Nevertheless, recent advances in molecular biology techniques have led to a new perspective and a redefinition of the microbiota associated with endodontic infections. Consequently, fastidious and difficult-to-cultivate species, such as *Tannerella forsythensis* (*Bacteroides forsythus*) and *Treponema* spp., have been detected more frequently in samples from canal with pulp necrosis (4, 5).

Based on this evidence, the main goal of endodontic therapy has been focused on the elimination, or at least a significant reduction of, microorganisms present in the root canal system, which can be achieved mostly by the chemomechanical preparation of the conduct. However, studies have demonstrated that bacteria may be viable in the root canal even after vigorous mechanical instrumentation (6), leading to persistent or secondary intraradicular infections and therefore to treatment failure. Therefore, the use of an intracanal medication with antimicrobial activity between therapy sessions has been recommended to eliminate possible persistent microorganisms (7), particularly in cases of pulp necrosis with periradicular bone loss (8). Among the available intracanal dressings, calcium hydroxide is the most indicated and frequently used in the clinical practice (9). Its antibacterial properties are related to its high alkalinity, which results in the inactivation of bacterial membrane enzymes (10). Despite good clinical outcome (11), the use of calcium hydroxide as an intracanal medication may be limited by the presence of resistant species such as *Enterococcus faecalis*, a species particularly associated with persistent infection and treatment failure (12). Therefore, the present investigation aimed to determine the predominant microbiota of teeth with pulp necrosis and periradicular lesion and to evaluate the effects of endodontic therapy associated with calcium hydroxide on these microorganisms by whole genomic DNA probes and the checkerboard DNA-DNA hybridization method.

Materials and Methods

Study Participants

In an initial protocol, this investigation proposed the distribution of the study participants into two groups: a test group, treated with the calcium hydroxide intracanal medication, and a control group without this medication. However, for ethical reasons the inclusion of this control group was not approved by the Review Committee for Human Subjects. Therefore, this study focused only on the effects of the endodontic therapy associated with calcium hydroxide medication on the endodontic microbiota. Twelve systemically healthy adult patients (mean age 37 ± 0.8 years, range 21–82 years; 58% men) having a single-rooted tooth with necrotic pulp and radiographic evidence of periradicular bone loss were selected from the Clinic of Endodontics at the Odontoclinica Central da Marinha, Rio de Janeiro. None of the patients had experienced spontaneous pain or received antibiotics or root canal treatment of the affected tooth in the 3 months preceding entry into the study. In addition, teeth with deep periodontal

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pockets and/or that could not be isolated were excluded. In order to participate in the study, all patients were informed about its nature, and a signed consent form was obtained from each individual. The study protocol was approved by the Review Committee for Human Subjects of the Hospital Universitário Clementino Fraga Filho, Federal University of Rio de Janeiro.

Endodontic Treatment

After isolation of the tooth and access to the root canal, an initial bacteriologic sample was taken as described in Specimen Sampling, below. The root canals were cleaned and shaped by the step-down technique (13), using hand files and Gates-Glidden drills (Dentsply/Maillefer, Ballaigues, Switzerland) with 5.25% sodium hypochlorite irrigation. Then the canals were dried with sterile paper points and filled with a paste of calcium hydroxide (Dentsply Herpo, Petrópolis, RJ, Brazil) and saline solution in a creamy consistency by means of Lentulo spiral (Dentsply/Maillefer). The coronal cavities were sealed with a temporary filling, Coltosol (Vigodent, São Paulo, SP, Brazil). In all cases, the calcium hydroxide paste was left in the canals as a dressing for 14 days. After that, the dressing was removed by irrigation with saline solution, and the second bacteriologic samples were taken. The canals were then filled with gutta-percha points and cement (Endo Fill, Dentsply Herpo), using the lateral condensation technique.

Specimen Sampling

Samples were obtained from root canals using strict asepsis in a procedure previously described (14). Briefly, the tooth was isolated with the rubber dam, and a cotton applicator was used to clean the surface of the tooth and surrounding field with 3% hydrogen peroxide, followed by decontamination with 5.25% sodium hypochlorite. Complete access preparations were made with sterile burs without water spray. If the root canal was dry, a small amount of sterile saline solution was introduced into the canal. Samples were initially collected by means of a size 15 K-type file (Dentsply/Maillefer) introduced to a level approximately 1 mm short of the tooth radiographic apex, and a discrete

filing motion was applied. Then, two sequential sterile paper points were placed to the same level and used to soak up the fluid in the canal for 1 minute. Both paper points were transferred to Eppendorf tubes containing 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). The second sample was taken in the same way, right after removal of the calcium hydroxide dressing and before the root canal filling.

Microbiologic Assessment

The presence and levels of 44 bacterial species (Table 1) were determined by a modification of the checkerboard DNA-DNA hybridization method described by Socransky et al. (15). In brief, the samples collected were placed in separate Eppendorf tubes, the cells were lysed, and denatured DNA was fixed in individual lanes on a nylon membrane (Hybond N+; Amersham Biosciences do Brasil, São Paulo, Brazil), using the slot blot device Minislot 30 (Immunetics, Cambridge, MA, USA). Forty-four digoxigenin-labeled (Roche Applied Science, Indianapolis, IN, USA) whole genomic DNA probes were constructed and hybridized perpendicularly to the lanes of the clinical samples using the Miniblotter 45 (Immunetics). Bound probes were detected using phosphatase-conjugated antibody to digoxigenin (Roche Applied Science) and chemiluminescence (CDP-Star; Amersham Biosciences). Signals were evaluated visually by comparison with the standards at 10^5 and 10^6 bacterial cells for the test species on the same membrane. They were recorded as follows: 0, not detected; 1, $<10^5$ cells; 2, $\sim 10^5$ cells; 3, 10^5 to 10^6 cells; 4, $\sim 10^6$ cells; and 5, $>10^6$ cells. The sensitivity of this assay was adjusted to permit detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. This procedure was carried out to provide the same sensitivity of detection for each probe. Failure to detect a signal was recorded as zero, although counts in the 1 to 10,000 range could conceivably have been present.

Statistical Analysis

The microbiologic data were expressed as a percentage of positive samples (prevalence) and mean counts $\times 10^5$ (level) of each species. In the analysis of prevalence, only the absence or presence of the mi-

TABLE 1. Whole genomic DNA probes for 44 bacterial species tested against root canal samples of pulp necrosis by the checkerboard DNA-DNA hybridization technique.

Species	Strains	Species	Strains
<i>Actinobacillus actinomycetemcomitans</i> (sorotype a)	43718*	<i>Leptotrichia buccalis</i>	14201*
<i>Actinobacillus actinomycetemcomitans</i> (sorotype b)	29523*	<i>Neisseria mucosa</i>	19696*
<i>Actinomyces gerencseriae</i>	23860*	<i>Peptostreptococcus micros</i>	33270*
<i>Actinomyces israelii</i>	12102*	<i>Porphyromonas endodontalis</i>	35406*
<i>Actinomyces naeslundii</i> genospecies 1	12104*	<i>Porphyromonas gingivalis</i>	33277*
<i>Actinomyces odontolyticus</i>	17929*	<i>Prevotella intermedia</i>	25611*
<i>Actinomyces viscosus</i>	43146*	<i>Prevotella melaninogenica</i>	25845*
<i>Campylobacter gracilis</i>	33236*	<i>Prevotella nigrescens</i>	33563*
<i>Campylobacter rectus</i>	33238*	<i>Propionibacterium acnes I</i>	11827*
<i>Campylobacter showae</i>	51146*	<i>Propionibacterium acnes II</i>	11828*
<i>Capnocytophaga ochracea</i>	33596*	<i>Selenomonas noxia</i>	43541*
<i>Capnocytophaga gingivalis</i>	33624*	<i>Streptococcus anginosus</i>	33397*
<i>Capnocytophaga sputigena</i>	33612*	<i>Streptococcus constellatus</i>	27823*
<i>Eikenella corrodens</i>	23834*	<i>Streptococcus gordonii</i>	10558*
<i>Enterococcus faecalis</i> ‡	29212*	<i>Streptococcus intermedius</i>	27335*
<i>Eubacterium nodatum</i>	33099*	<i>Streptococcus mitis</i>	49456*
<i>Eubacterium saburreum</i>	33271*	<i>Streptococcus oralis</i>	33037*
<i>Fusobacterium nuc ss. nucleatum</i>	25586*	<i>Streptococcus sanguis</i>	10556*
<i>Fusobacterium nuc.ss. polymorphum</i>	10953*	<i>Tannerella forsythensis</i>	43037*
<i>Fusobacterium nucleatum ss. vincentii</i>	49256*	<i>Treponema denticola</i>	B1†
<i>Fusobacterium periodonticum</i>	33693*	<i>Treponema socranskii</i>	S1†
<i>Gemella morbillorum</i>	27824*	<i>Veillonella parvula</i>	10790*

* ATCC (American Type Culture Collection, Rockville, MD).

† FDC (Forsyth Institute, Boston, MA).

‡ Species not classified as a member of the oral microbiota.

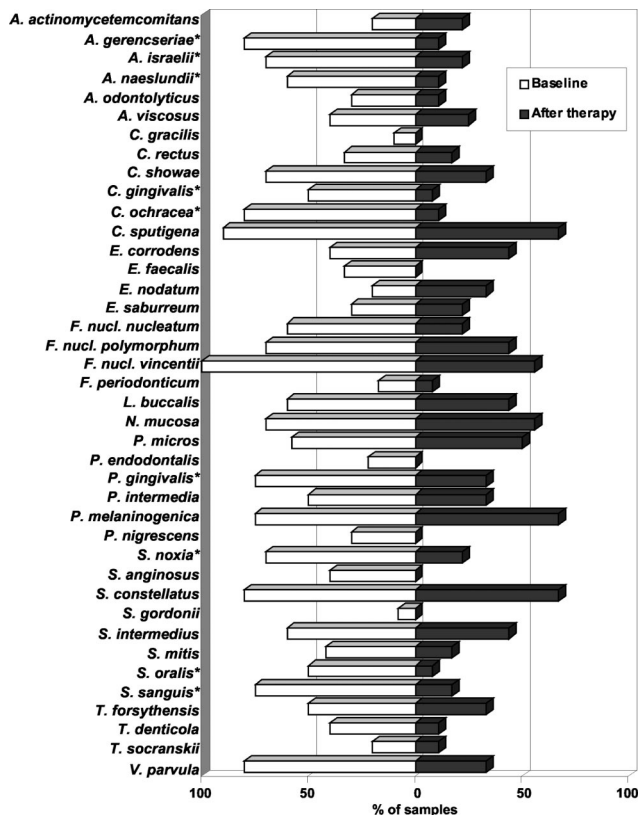


Fig 1. Bar chart of the frequency of detection (prevalence) of 44 oral species examined in 12 samples from pulp necrosis at baseline and after endodontic therapy with calcium hydroxide, using the checkerboard method. The significance of differences in prevalence of bacterial species from baseline to post-therapy was determined by the paired-samples *t* test ($p < 0.05$).

croorganism before and after therapy was considered. The levels of different species were determined by transforming the scores 0 to 5 obtained from de control specimens 10^5 and 10^6 in bacterial counts, as previously described. Significant changes in the microbiota composition from baseline to post-therapy sampling were sought by the paired-samples *t* test. The level of significance determined in all analyses was 5%.

Results

The results of the checkerboard DNA-DNA hybridization method showed that 42 of the 44 DNA probes tested were reactive with one or more samples. All samples were positive for at least one species. The mean number of bacterial species per sample was 17.3, ranging from 5 to 33 species (data not shown).

The prevalence and levels of the species analyzed by DNA probes in the root canal samples before and after therapy with calcium hydroxide are shown in Figures 1 and 2, respectively. The majority of the species were detected in a high frequency and mean levels at baseline (pre-therapy). In particular, the most predominant microorganisms included species of the genera *Actinomyces*, *Capnocytophaga*, *Fusobacterium*, and *Streptococcus*, as well as black-pigmented bacteria. Among those, *F. nucleatum* ss. *vincentii* was detected in 100% of the samples, *C. sputigena* in 90%, *A. gerencseriae*, *C. ochracea*, *S. constellatus*, and *V. parvula* in 80%, *P. gingivalis*, *P. melaninogenica*, and *S. sanguis* in 75%. Other species that were quite prevalent (70%) included *A. israelii*, *C. showae*, *F. nucleatum* ss. *polymorphum*, *N. mucosa*, and *S. noxia*. The periodontopathogenic species *T. forsythensis* was detected in 50% of the samples. *A. actinomycetemcomitans*,

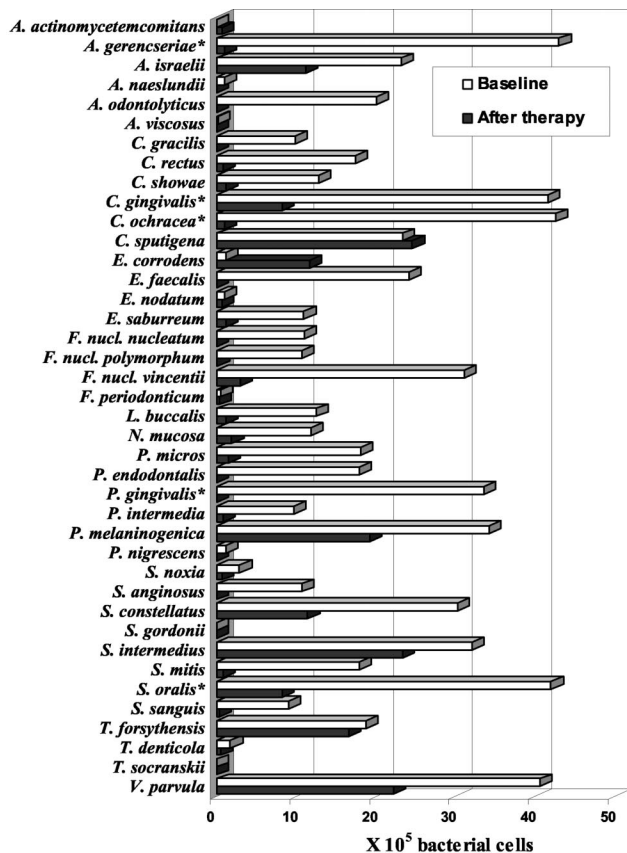


Fig 2. Bar chart of the mean levels ($\times 10^5$ cells) of 44 species examined in 12 samples from pulp necrosis at baseline and after endodontic therapy with calcium hydroxide, using the checkerboard method. The significance of differences in mean counts of bacterial species from baseline to post-therapy was determined by the paired-samples *t* test ($*p < 0.05$).

C. gracilis, *E. nodatum*, *F. periodonticum*, *S. gordonii*, and *T. socranskii* were the least prevalent species, whereas *G. morbillorum* and *P. acnes* were not detected in any sample (Fig. 1). The post-therapy results showed a significant reduction in the prevalence of most of the species examined, with the exception of *A. actinomycetemcomitans*, *E. corrodens*, and *E. nodatum*, which increased in frequency after treatment. Statistically significant reductions were observed for *A. gerencseriae* ($p = 0.031$), *A. israelii* ($p = 0.046$), *A. naeslundii* ($p = 0.046$), *C. gingivalis* ($p = 0.006$), *C. ochracea* ($p = 0.046$), *P. gingivalis* ($p = 0.025$), *S. noxia* ($p = 0.046$), *S. sanguis* ($p = 0.016$), and *S. oralis* ($p = 0.006$). By contrast, *C. sputigena*, *E. saburreum*, *N. mucosa*, *P. melaninogenica*, *P. micros*, *S. constellatus*, and *S. intermedius* decreased slightly ($<30\%$) in prevalence. The species not detected after treatment included *C. gracilis*, *E. faecalis*, *P. endodontalis*, *P. nigrescens*, *S. anginosus*, and *S. gordonii* (Fig. 1). An average of 8.3 species per sample was observed after therapy, resulting in a mean decrease of approximately 52% from baseline. Surprisingly, 32 species were still detected in a single sample after treatment (data not shown).

In the analysis of the mean counts (levels) of bacterial species in the pre-therapy samples, *A. gerencseriae* (43.1×10^5 cells), *C. ochracea* (42.7×10^5 cells), *S. oralis* (42.1×10^5 cells), *C. gingivalis* (41.8×10^5 cells), *V. parvula* (40.8×10^5 cells), *P. melaninogenica* (34.4×10^5 cells), *P. gingivalis* (33.6×10^5 cells), *F. nucleatum* *vincentii* (31.2×10^5 cells), *S. intermedius* (32.1×10^5 cells), and *S. constellatus* (30.3×10^5 cells) were observed in quite high levels (Fig.

2). Of interest, *C. gingivalis* and *S. oralis* were found in approximately 50% of the samples but in high levels, whereas *S. noxia* and *S. sanguis* were detected in low levels and high prevalence (Figs. 1 and 2). Similarly to the prevalence data, a decrease in the mean counts was observed for most of the species evaluated after treatment. *A. gerencseriae* ($p = 0.026$), *C. gingivalis* ($p = 0.034$), *C. ochracea* ($p = 0.016$), *P. gingivalis* ($p = 0.026$), and *S. oralis* ($p = 0.035$) showed statistically a significant decrease in levels after therapy. By contrast, *A. actinomycetemcomitans*, *C. sputigena*, and *E. corrodens* showed an increase in the mean number of bacterial cells, whereas *E. nodatum*, *F. periodonticum*, *S. intermedius*, *T. forsythensis*, and *T. denticola* showed a modest decrease in levels after treatment (Fig. 2).

Discussion

It has been demonstrated that the elimination of microorganisms from the root canal system determines the full success of endodontic therapy, particularly in cases of pulp necrosis and periradicular lesion (16). To increase the effectiveness of the endodontic therapy, various intracanal dressings have been used as adjuncts, calcium hydroxide being one of the most used (9). Despite the high success rate, the effects of this antimicrobial canal medication on different species constituting the endodontic microbiota are not completely understood. Thus, the present investigation evaluated the predominant endodontic microbiota in cases of pulp necrosis with periradicular lesion and the effects of conventional therapy associated with calcium hydroxide in its composition.

Regarding the endodontic microbiota, our results reinforce the concept that endodontic infections are mixed infections of polymicrobial etiology, with a predominance of obligate anaerobic bacteria (2). The high prevalence and levels of *F. nucleatum ss. vincentii*, detected in 100% of the samples before therapy with mean counts of 31.2×10^5 bacterial cells, is in agreement with other studies that have described this species as the most isolated from these infections (17). This and other subspecies have been described as key microorganisms in the process of co-aggregation between different genera of facultative bacteria, precursors of the dental biofilm formation, and strictly anaerobic bacteria, the following colonizers (18). Therefore, the presence of these species may have a critical role in endodontic infections. Likewise, the predominance of black-pigmented rods observed in the present investigation also confirms the data reported by other studies (19). The highest prevalence was found for *P. melaninogenica* (75%), whereas other authors have demonstrated that *P. nigrescens* was the most frequently detected species of this group (20, 21). Our data showed that only 30% of the samples were positive for *P. nigrescens*, whereas *P. intermedia* was detected in 50% of those. Given that these species are closely related, and that whole genomic DNA probes were employed in the checkerboard method, it is conceivable that some cross-reaction between them may have occurred. Nevertheless, other studies like that of Jung et al. (5) have found similar results regarding *P. intermedia*. Of the *Porphyromonas* species, *P. gingivalis* was detected in 75% of the cases; however, *P. endodontalis* was present in 22% of the samples at baseline. Our data are in agreement with those of Siqueira et al. (22), who also found high levels of *P. gingivalis* by using two molecular methods, polymerase chain reaction and checkerboard. The relatively high prevalence of *T. forsythensis* (50%), a species related to alveolar bone loss in patients with destructive periodontal disease (15), is in contrast to previous studies where the isolation and identification of this microorganism from canals with pulp necrosis was carried out by conventional cultural techniques (3). In these studies, this bacterium was barely cited as a possible endodontic pathogen. Despite that, recent investigations based on molecular methods have observed a greater frequency of *T. forsythensis* in infections of endodontic origin (14, 23).

These differences may be due to the fastidious nature of this microorganism and difficulties in its cultivation. Other fastidious species not usually isolated from endodontic infections by cultural methods are the spirochetes, particularly *T. denticola*. We detected this species in 40% of the samples, but in low levels. Similarly to our data, Siqueira et al. (24) and Baumgartner et al. (25) also reported a high prevalence of spirochetes in samples from endodontic infections using a different molecular method. Thus, it seems that these difficult-to-grow microorganisms have been underestimated in the past, as well as their role in the etiology of endodontic infections. In fact, we performed cultural analysis under anaerobic conditions in 50% of the baseline samples studied. As expected, fastidious species, such as *T. forsythensis* and *T. denticola*, were detected in much lower frequency (data not shown).

More recently, studies have demonstrated the presence of pathogens associated with various community and/or hospital diseases in endodontic infections (26). Among those, *E. faecalis* has been observed quite frequently in cases of persistent or secondary endodontic infections (26). In the present study, this species was detected in only 33% of the samples before treatment, in agreement with previous studies of primary endodontic infections (14, 27).

To increase the rate of bacterial elimination in the system of radicular canals and improve therapeutic efficacy, mechanical instrumentation associated with the use of an antimicrobial intracanal dressing has been proposed (7, 11). The use of calcium hydroxide as an intracanal medication resulted in the reduction of most of the species initially detected. *A. gerencseriae*, *A. israelii*, *A. naeslundii*, *C. gingivalis*, *C. ochracea*, *P. gingivalis*, *S. noxia*, *S. sanguis*, and *S. oralis* showed statistically significant reductions in prevalence and/or mean counts after treatment, but they were still detectable. Moreover, *C. gracilis*, *E. faecalis*, *P. endodontalis*, *P. nigrescens*, *S. anginosus*, and *S. gordonii* were not detected after therapy. These results demonstrated that the use of calcium hydroxide as a dressing was not able to completely eliminate the microorganisms from the root canal system. This may imply a problem, given that studies have reported that the persistence of bacterial species, especially the *Actinomyces* species is related to endodontic infections refractory to therapy (28). In fact, there has even been a moderate increase in the prevalence of *A. actinomycetemcomitans*, *E. corrodens*, and *E. nodatum*. It is possible that the reduction of other competitive species might have favored the increase of these species. These results are in agreement with the data reported by Barbosa et al. (11), who observed bacterial growth using cultural techniques in 26.9% of root canal samples after therapy with calcium hydroxide.

In the in vitro evaluation of the antimicrobial activity of calcium hydroxide, different results have been obtained, depending on the vehicle used and the microorganisms tested (29, 30). However, the in vivo characteristics of the radicular canal system are very complex and may favor the persistence of some microorganisms. Calcium hydroxide activity is related to close contact with the lethal hydroxyl ions. Some bacteria can be lodged in the dentinal tubules (31) and thus evade the ions. In addition, these microorganisms may be organized in microcolonies located in the interior of a biofilm, being protected from the effects of the medication that will act only on microorganisms located in the periphery (32). One should also consider that the detection of a bacterial species after treatment by molecular methods may result from the detection of DNA molecules still present in the specimen, which does not mean cell viability.

Because calcium hydroxide is so widely used, it has been speculated that it could eliminate sensitive bacteria, favoring the proliferation of more resistant species, such as *E. faecalis* (33). Of interest, the combination of mechanical instrumentation with intracanal medication of calcium hydroxide was effective in eradicating *E. faecalis*. Similar results were demonstrated by Sjögren et al. (27). Other authors, how-

ever, have reported difficulties in eliminating the enterococci (34). It should be pointed out that this species is resistant to an alkaline environment and to several antimicrobials, being described as an important member of the microbiota in cases of persistent endodontic infections (12). Therefore, this treatment seems to be sufficient for the elimination of *E. faecalis* present in low mean counts, as in primary endodontic infections, but not in teeth with failed endodontic therapy, when it is found in high numbers (35).

In conclusion, the data obtained in the present study indicated a great microbial diversity in cases of pulp necrosis with periradicular lesions, confirming the polymicrobial cause of these infections. Fastidious species not previously related to these infections, such as *P. gingivalis*, *T. forsythensis*, and *T. denticola* were observed in high prevalence and levels. In addition, calcium hydroxide proved to be an effective adjunctive in mechanical endodontic therapy. Nevertheless, its use is limited, given that it decreases but does not completely eliminate the microorganisms from the root canal system. Future investigations should be conducted to define the role of specific species in the development of infections of endodontic origin, as well as to search for new adjunctive medications in endodontic treatment.

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