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Microbial culture and checkerboard DNA–DNA hybridization assessment of bacteria in root canals of primary teeth pre- and post-endodontic therapy with a calcium hydroxide/chlorhexidine paste

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Aim. To investigate the root canal microbiota of primary teeth with apical periodontitis and the *in vivo* antimicrobial effects of a calcium hydroxide/chlorhexidine paste used as root canal dressing.
Design. Baseline samples were collected from 30 root canals of primary teeth with apical periodontitis. Then, the root canals were filled with a calcium hydroxide paste containing 1% chlorhexidine for 14 days and the second bacteriologic samples were taken prior to root canal filling. Samples were submitted to microbiologic culture procedure to detect root canal bacteria and processed for checkerboard DNA–DNA hybridization.
Results. Baseline microbial culture revealed high prevalence and cfu number of anaerobic, black-pigmented bacteroides, *Streptococcus*, and aerobic microorganisms. Following root canal dressing, the overall number of cfu was dramatically diminished compared to initial contamination ($P < 0.05$), although prevalence did not change ($P > 0.05$). Of 35 probes used for checkerboard DNA–DNA hybridization, 31 (88.57%) were present at baseline, and following root canal dressing, the number of positive probes reduced to 13 (37.14%). Similarly, the number of bacterial cells diminished folowing application of calcium hydroxide/chlorhexidine root canal dressing ($P = 0.006$).
Conclusion. Apical periodontitis is caused by a polymicrobial infection, and a calcium hydroxide/chlorhexidine paste is effective in reducing the number of bacteria inside root canals when applied as a root canal dressing.

Introduction

The microorganisms inside the root canal play an important role in the development of apical periodontitis.[1,2] Although more than 468 species have been identified in contaminated root canals,[3] 15–30 bacterial species are involved in apical periodontitis.[4] These bacteria are mostly obligate anaerobic and gram negative and are disseminated throughout the root canal system.[5–7]

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Recently, molecular methods have been used to detect fastidious and difficult-to-cultivate bacterial species from the *Tannerella*, *Treponema*, *Prevotella*, and *Porphyromonas* genera,[8] which may not be identified by conventional culture methods.[4,7,9,10] Those findings lead to a new redefinition of the microbiota associated with apical periodontitis in permanent teeth. Conventional culture and molecular techniques have demonstrated that microorganisms present in root canal infections of primary teeth are similar to those found in permanent teeth.[6,9–13]

The goal of endodontic therapy is therefore to eliminate or at least reduce the number of microorganisms present in the root canal system.[14,15] It has been, however, demonstrated that bacteria may remain viable even after

mechanical preparation,[2,14] which may lead to an unfavourable root canal treatment outcome.[16] Therefore, the use of an intracanal medication with antimicrobial activity has been recommended to eliminate persistent microorganisms.[17,18]

Calcium hydroxide has been widely used as root canal dressing in endodontics owing to its overall biocompatibility, antibacterial effects, and ability to neutralize bacterial LPS.[19] Despite the favourable properties of calcium hydroxide, other compounds have been associated with calcium hydroxide to enhance its antimicrobial spectrum, including the antimicrobial agent chlorhexidine,[15,20] with the aim of targeting bacteria resistant to calcium hydroxide.[21–24] Chlorhexidine presents substantivity,[20,21] biocompatibility,[25] and a wide antimicrobial spectrum against bacteria, fungi, and viruses, including those present in contaminated root canals.[26] The calcium hydroxide–chlorhexidine association presents antimicrobial activity *in vivo*[27] and results in antimicrobial synergism[15,22,23] without loss of solubility or reduced activity of any of the compounds.[23,24] This association permits a favourable response in the subcutaneous connective tissue of mice,[28] without altering cell viability or anti-inflammatory properties of calcium hydroxide.[29]

The aim of this study was to investigate the root canal microbiota of primary teeth with apical periodontitis prior to and following root canal dressing using both microbial culture and checkerboard DNA–DNA hybridization technique.

Materials and methods

This research protocol was approved by the Ethics Review Committee for Human Subjects (process # 2004.1.571.58.5) at the University of São Paulo, Ribeirão Preto, São Paulo, Brazil. Prior to participating in the study, all parents were informed about its nature, and a signed consent form was obtained from each individual.

The samples were collected from 3- to 7-year-old male and female patients ($n = 19$) who had been referred for endodontic treatment at the Pediatric Dentistry Clinic because of the presence of a tooth with necrotic pulp and radiographic evidence of periradicular bone loss.

A total of 30 root canals were selected from primary incisor and molar teeth that presented carious lesions of varied depths and extensions, had intact roots or less than two-thirds of root resorption, required root canal therapy, and presented no deep periodontal pockets or previous root canal intervention. Children who had used antimicrobial mouthwashes, presented with any systemic disease, or had received antibiotics within 3 months preceding entry into the study were excluded from the trial.

Specimen sampling and root canal treatment

After local anaesthetic and rubber dam isolation, carious tissue was removed and antisepsis was performed with 1.0% chlorhexidine digluconate. Complete access preparations were made with sterile burs, and a small amount of sterile saline solution was introduced into the canal. Initial bacteriologic samples (baseline) were first collected by means of a size 15 K-type file (Dentsply/Maillefer, Ballaigues, Switzerland) introduced to a level approximately 1 mm short of the tooth radiographic apex or the limit of root resorption. A discrete filing motion in an up-and-down movement was applied. Then, two sequential sterile paper points were placed at the same level and used to soak up the fluid in the canal for 1 min. Both paper points were transferred to tubes containing 2.5 mL of reduced transport fluid. The root canals were cleaned and shaped by the step-back technique, using hand files (Dentsply/Maillefer) with 2.5% sodium hypochlorite irrigation (pH 12.0).[12,13,30] Then, the canals were dried with sterile paper points and filled with a paste of calcium hydroxide (Calen®; S.S. White Artigos Dentários, Rio de Janeiro, RJ, Brazil) containing a final volume of 1% chlorhexidine (Chlorhexidine Stock Solution: 20%). The coronal cavities were sealed with a composite resin (Z-100, 3M ESPE Sumaré, 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,

the root canals were left empty for 7 days, and the second bacteriologic samples were taken. The root canals were left empty for 7 days after chemomechanical preparation to permit the proliferation of the remaining microorganisms in the root canal system, which can reach numbers equivalent to baseline.[31,32] After that, root canals were filled with a resorbable calcium hydroxide paste thickened with zinc oxide. Calcium hydroxide-based paste was prepared using 1 g of Calen® paste and 0.65 g of zinc oxide as described previously.[33,34]

Microbiologic culture procedure

Four-to-six glass beads and a sterile metal wing were added to the test tubes containing the samples. The tubes were agitated for 2 min in a mixer (Mixtron, Toptronix, SP, Brazil) at maximum speed. Subsequently, serial decimal dilutions up to 10^{-4} were made in phosphate-buffered saline under laminar airflow. A volume of 50 μL of the pure samples and of each dilution was seeded, with a sterile calibrated pipette, onto plates containing modified SB-20 culture medium (SB-20M), blood agar (Difco, Detroit, MI, USA) and blood agar supplemented with 5.0 μg/mL hemin and 1.0 μg/mL menadione (Sigma Chemical Co., St Louis, MO, USA). For the remaining undiluted sample, 5.0 mL of sodium thioglycolate was added (without glucose or pH indicator; Difco) in order to detect microorganisms present at levels lower than or equal to 20 cfu/mL. Blood agar plates were incubated aerobically for 24–48 h at 37°C, and blood agar plates enriched with hemin and menadione were incubated anaerobically using the GasPak system for 7–10 days. SB-20M plates were incubated microaerobically by the candle jar system for 2–3 days. Following incubation, colonies were counted with a stereomicroscope (Nikon, Tokyo, Japan) under reflected light and the cfu/mL value was calculated.

Checkerboard DNA–DNA hybridization

The presence and relative quantification of 35 bacterial species (Table 1) were determined by a modification of the checkerboard DNA–DNA hybridization method.[13] Briefly, the cells of the collected samples were lysed, and denatured DNA was fixed in individual lanes on a nylon membrane (Hybond N+;

Table 1. Whole-genomic DNA probes for 35 bacterial species tested against root canal samples of pulp necrosis by the checkerboard DNA–DNA hybridization technique.

Species	Strains	Gram staining	Species	Strains	Gram staining
Aggregatibacter actinomycetemcomitans wild-type A	ATCC 43718	Gram-negative	*Peptostreptococcus micros*	ATCC 33270	Gram-positive
Aggregatibacter actinomycetemcomitans wild-type B	ATCC 29523	Gram-negative	*Porphyromonas gingivalis*	ATCC 33277	Gram-negative
Actinomyces gerencseriae	ATCC 23860	Gram-positive	*Prevotella intermedia*	ATCC 25611	Gram-negative
Actinomyces israelii	ATCC 12102	Gram-positive	*Prevotella melaninogenica*	ATCC 25845	Gram-negative
Actinomyces naeslundii I	ATCC 12104	Gram-positive	*Prevotella nigrescens*	ATCC 33563	Gram-negative
Actinomyces odontolyticus	ATCC 17929	Gram-positive	*Propionibacterium acnes*	ATCC 11827	Gram-positive
Actinomyces viscosus	ATCC 43146	Gram-positive	*Selenomonas noxia*	ATCC 43541	Gram-negative
Campylobacter rectus	ATCC 33238	Gram-negative	*Streptococcus anginosus*	ATCC 33397	Gram-positive
Capnocytophaga gingivalis	ATCC 33624	Gram-negative	*Streptococcus constellatus*	ATCC 27823	Gram-positive
Capnocytophaga ochracea	ATCC 33596	Gram-negative	*Streptococcus gordonii*	ATCC 10558	Gram-positive
Capnocytophaga sputigena	ATCC 33612	Gram-negative	*Streptococcus intermedius*	ATCC 27335	Gram-positive
Eikenella corrodens	ATCC 23834	Gram-negative	*Streptococcus mitis*	ATCC 49456	Gram-positive
Eubacterium nodatum	ATCC 33099	Gram-positive	*Streptococcus oralis*	ATCC 35037	Gram-positive
Eubacterium saburreum	ATCC 33271	Gram-positive	*Streptococcus sanguinis*	ATCC 10556	Gram-positive
Fusobacterium nucleatum sit *nucleatum*	ATCC 25586	Gram-negative	*Tannerella forsythia*	ATCC 43037	Gram-negative
Fusobacterium nucleatum sit *polymorphum*	ATCC 10953	Gram-negative	*Treponema denticola*	*B1	Gram-negative
Fusobacterium periodonticum	ATCC 33693	Gram-negative	*Veillonella parvula*	ATCC 10790	Gram-negative
Neisseria mucosa	ATCC 19696	Gram-negative			

ATCC (American Type Culture Collection, Rockville, MD, USA).
*Forsyth Institute, Boston, MA, USA.

Amersham Pharmacia Biotech, Buckinghamshire, England) using a checkerboard slot blot device (Minislot 30; Immunetics, Cambridge, MA, USA). In total, 35 digoxigenin-labelled (Roche Applied Science, Indianapolis, IN, USA) whole genomic DNA probes were constructed and hybridized perpendicularly to the lanes of the clinical samples using a Miniblotter 45 apparatus (Immunetics). Bound probes were detected using phosphatase-conjugated antibody to digoxigenin (Roche Applied Science). After incubation in a solution containing the CDP-Star substratum (Amersham Pharmacia Biotech), the membranes were placed in an autoradiography cassette under a radiographic film (Kodak X-Omat, Rochester, NY, USA), which was developed for chemiluminescence signal detection. 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, $\sim10^5$ cells; 3, 10^5–10^6 cells; 4, $\sim10^6$ cells; and 5, $>10^6$ cells. The sensitivity of this assay was adjusted to permit the 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 1–10,000 range could conceivably have been present.

Statistical analysis

Prevalence data expressed as a percentage of positive samples for both culture and checkerboard techniques were analysed by Fisher's exact test. For microbial culture, the baseline or post-therapy concentration of microorganisms in cfu/mL was compared using one-way ANOVA followed by Tukey test. Significant changes in the microbiota composition from baseline to post-therapy sampling were sought by the paired-samples *t*-test. For checkerboard relative quantification of the concentration of different species prior to and following therapy, scores 0–5 transformed from 10^5 and 10^6 bacterial counts were analysed by Fisher's exact test. The level of significance determined in all analyses was set at 5%.

Results

Microbial culture (N = 19; 38 root canals)

Initial prevalence of anaerobic microorganisms was 94.7% of the samples, with variation from 60 to 446,000 cfu/mL. In ten specimens (52.6%), black-pigmented bacteroides (BPB; 160–17,000 cfu/mL) and *Streptococcus* (20–4800 cfu/mL) were detected. Aerobic microorganisms were present in 18 samples (94.7%), with variation from 60 to 202,000 cfu/mL. At the second appointment, following calcium hydroxide/chlorehexidine root canal dressing, the prevalence of samples that were positive for the presence of microorganisms did not change, although the overall number of cfu was dramatically diminished compared with initial contamination for anaerobic (20–72,000 cfu/mL), BPB (20–1200 cfu/mL), aerobic (20–47,000 cfu/mL), and streptococcal bacteria (20–100 cfu/mL) ($P < 0.05$) (Table 2).

Checkerboard DNA–DNA hybridization (N = 14; 28 root canals)

Of 35 probes used, 31 (88.57%) were detected prior to performing root canal treatment, with 1–31 (mean = 5.71) bacterial species per sample. Following root canal dressing, 13 (37.14%) probes were positive, with 1–6 (mean = 2.64) bacterial species per sample (Fig. 1). *Aggregatibacter actinomycetemcomitans wild-type A* was not detect either at baseline or following treatment.

Of 31 probes identified prior to treatment (baseline), 16 were from facultative bacteria, six from facultative gram-negative species, and ten from facultative gram-positive species. We found 14 anaerobic species; among those, 11 were from anaerobic gram negatives and three were from anaerobic gram positives. Aerobic microorganisms were found in all samples.

Following root canal dressing, 13 probes were found. Of these, four represented facultative microorganisms; one, gram negative; and three, gram positive. Also, eight anaerobic probes were detected; six were gram negative and two were gram positive. One aerobic probe was present in all samples.

The species predominant prior to and following root canal dressing were *Neisseria*

Table 2. Prevalence of microorganisms from root canals of primary teeth with necrotic pulp and apical periodontitis pre- and post-root canal dressing with $Ca(OH)_2$ 1% CHX.

		Baseline				After root canal dressing			
		Anaerobic	BPB	Aerobic	*Streptococcus*	Anaerobic	BPB	Aerobic	*Streptococcus*
Prevalence (%)		18 (94.7%)	10 (52.6%)	18 (94.7%)	10 (52.6%)	16 (84.2%)	8 (42.1%)	16 (84.2%)	5 (26.3%)
cfu/mL	Median (quartile range)	1600 (470–33,667)	160 (0–780)	1530 (280–18,700)	20 (0–280)	180 (20–1190)	0 (0–60)	140 (40–1186)	0 (0–20)
	Median (quartile range)	4640 (910–67,447)				420 (60–3000)			

BPB, black-pigmented bacteroides.

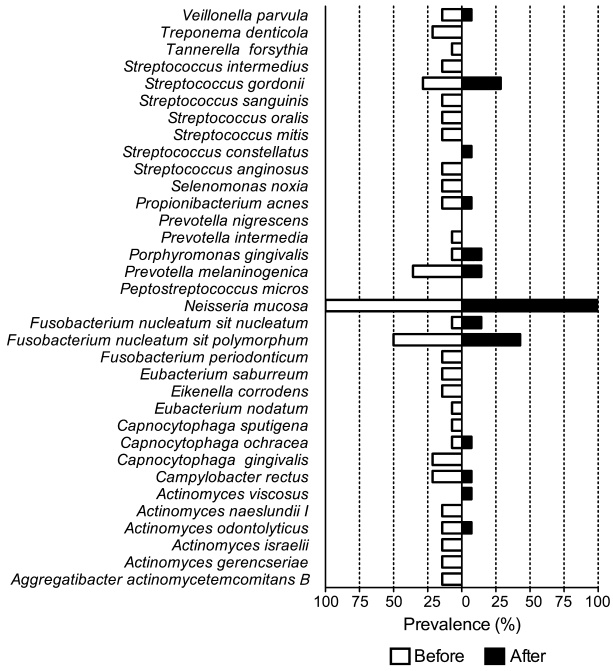

Fig. 1. Prevalence of bacterial species in the root canals of primary teeth with pulp necrosis and apical periodontitis prior to (baseline) and following root canal dressing with a calcium hydroxide/1% chlorhexidine paste.

mucosa (100%), *Fusobacterium nucleatum* sit *polymorphum* (50% *vs* 42.8%), *Prevotella melaninogenica* (35.7% *vs* 14.2%), and *Streptococcus gordonii* (28.5%).

The frequency of the score representing more than 10^6 bacteria decreased following root canal dressing ($P = 0.006$), whereas the frequency of undetected bacteria increased following treatment ($P = 0.0009$) (Fig. 2).

Discussion

The goal of endodontic therapy requires the effective elimination of pathogenic bacteria inside the root canal. As the microbiota of primary root canals in teeth with apical periodontitis have been poorly investigated,[13,30] we first evaluated the microorganisms present in the root canal by culture techniques and by checkerboard DNA–DNA hybridization. We then evaluated the prevalence of microorganisms in the root canal system following application of a calcium hydroxide/1% chlorhexidine paste.

Baseline microbial culture results corroborate previous findings indicating that the microbiota of primary teeth with apical

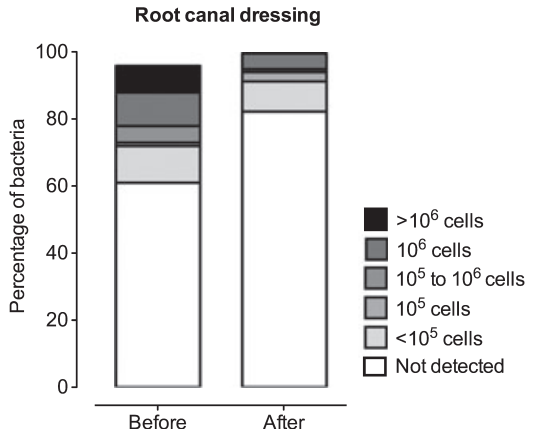

Fig. 2. Frequency of the scores relative to the amount of bacterial species in the root canals of primary teeth with pulp necrosis and apical periodontitis prior to and following root canal dressing with a calcium hydroxide/1% chlorhexidine paste.

periodontitis comprise anaerobic, aerobic, black-pigmented bacteria, and mutans streptococci,[11,13,30] similar to permanent teeth.[35]

Molecular techniques represent an advance in the identification of bacteria in endodontic infection.[3,4] At baseline, our checkerboard hybridization data indicated the presence of some difficult-to-cultivate bacteria such as *Tannerella forsythia* and *Treponema denticola*, corroborating previous findings.[3,4] *Peptostreptococcus micros* and *Prevotella nigrescens* were, however, not detected in this study.

Adequate root canal treatment led to an 84–93% rate of successful outcomes.[35] Technical issues related to the access of endodontic files to the bacteria present in the root canal, however, might prevent effective cleaning of the root canal, and therefore bacteria can remain in the system, especially in the external areas of resorption of cementum.[36,37] Therefore, root canal dressing has been used as a clinical step to enhance the disinfection of the root canal, dentin tubules, and apical cementum resorption areas.[2,35,38] It has been demonstrated that 14 days of root canal dressing is enough for hydroxyl ions from calcium hydroxide to penetrate the root canal dentin and reach out the cementum surface, increasing the pH of this area.[39]

Although calcium hydroxide has been widely used for this purpose, its antimicrobial spectrum of action has been questioned,[14,24] and therefore, an association of calcium hydroxide with potent antimicrobial medications is desirable.[24] Chlorhexidine is a potent antimicrobial agent effective against bacteria resistant to calcium hydroxide.[21] In this study, we used a calcium hydroxide paste associated with 1% chlorhexidine to obtain a synergism of action in order to enhance antimicrobial activity, as previously described for *in vitro* studies.[23]

Following root canal dressing, the prevalence of microorganisms inside the root canal did not change; however, there was a decrease in the number of bacteria revealed by either culture or checkerboard technique, demonstrating that a calcium hydroxide/1% chlorhexidine paste reduced the number of microorganisms inside the root canal. A mean of 2.6 bacterial species (range 1–6) was detected, unlike the mean 8.3 bacterial species detected in another study using a pure calcium hydroxide paste.[10]

Our findings indicate that a calcium hydroxide/1% chlorhexidine paste is effective in reducing the number of bacteria inside root canals when applied as a root canal dressing evaluated by either culture or molecular techniques. Nevertheless, research should be conducted to evaluate tissue response induced by calcium hydroxide/chlorehexidine mixture in different concentrations.

What this paper adds?

- This paper provides novel information regarding the microbiota present in the root canals of primary teeth with apical periodontitis.
- Clinically, this information might be useful because we demonstrated that a calcium hydroxide/chlorhexidine paste is effective in reducing the number of pathogenic microorganisms inside the root canal, thereby leading to a more favourable root canal treatment outcome.

Why this paper is important for paediatric dentists?

- Paediatric dentists are encouraged to perform root canal treatment in deciduous teeth using effective antimicrobial non-hazardous medication.

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