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Comparative analysis of endodontic pathogens using checkerboard hybridization in relation to culture

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Background/Aim: The purpose of this study was to detect bacterial species and to quantify the total number of bacteria from samples of infected root canals before (S1) and after chemo-mechanical preparation using 2% chlorhexidine (CHX) gel as auxiliary chemical substance (S2) and after 7 days of intracanal dressing (S3) to compare microbial changes.

Method: Twenty-four teeth were selected for this study. Chemo-mechanical preparation was performed using 2% CHX gel, then three different intracanal medicaments [M1: $Ca(OH)_2$ paste; M2: 2% CHX gel; and M3: $Ca(OH)_2$ paste plus 2% CHX gel] were used for 7 days. Checkerboard DNA–DNA hybridization was performed to detect 40 bacterial species. Aerobic and anaerobic culture techniques were used to determine the bacterial community by counting the colony-forming units (CFU).

Results: The species most frequently identified by checkerboard in S1 were: *Fusobacterium nucleatum* ssp. *polymorphum*, *Treponema socranskii* ssp. *socranskii*, *Parvimonas micra* and *Enterococcus faecalis.* In S2 and S3 a total of eight different species were identified; and only one of them was gram-positive (*E. faecalis*). Microorganisms were not identified after use of M2 for 7 days. The quantification obtained on agar plates ranged from 4×10^5 to 2.6×10^6 CFU/ml in S1, mean CFU was reduced by 99.96% in S2, and there was no statistical difference between the CFU in S2 and S3.

Conclusion: The antibacterial effect of the mechanical preparation supplemented by the use of an antibacterial auxiliary substance greatly reduced the microorganisms in the main root canal.

Key words: bacteria; calcium hydroxide; checkerboard DNA–DNA hybridization; chlorhexidine

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Microorganisms and their products are crucial factors in the development and maintenance of endodontic pathology (2, 20). However, cleaning, shaping, and thoroughly irrigating the canal greatly reduce the number of bacteria (3, 6, 14) to low levels; this allows periapical healing in most cases (27). Sodium hypochlorite (NaOCl) is the most commonly employed solution during root canal shaping. Recently, chlorhexidine (CHX) has been recommended as an alternative auxiliary chemical substance, especially in cases of open apex, because of its biocompatibility, or in cases of allergy related to bleaching solutions (44).

Even after chemo-mechanical preparation, it is impossible to obtain complete disinfection of the root canal system, especially because of its anatomical complexity (6, 12, 24). Sometimes an intracanal dressing with antibacterial properties is required to improve disinfection (3, 12). The need for intracanal dressings is recognized especially in those cases where therapy cannot be successfully completed because of the presence of pain or constant exudation (39).

Attempts have been made not only to identify the bacteria present in infected root canals but also to evaluate their susceptibility to endodontic procedures by means of culture-based techniques

(12, 26, 43) or molecular methods (41, 43). Diagnostic methods using DNA-based tools allow the identification of 'viable but noncultivable' cells that are metabolically active, but not dividing (23). Checkerboard DNA–DNA hybridization is an independent culture approach that screens for multiplex species identification in multiple clinical samples and so may lead to a more comprehensive picture of the microbiota associated with infected root canals. The technique was described by Socransky et al. (38) and has been used to study the oral microbiota, especially for investigating microbiological features of subjects with adult periodontitis (18, 37). The microbiota of apical periodontitis has also been studied by checkerboard analysis (40).

The purpose of this study was to identify 40 bacterial species and to quantify the total number of bacteria in samples taken from infected root canals before treatment (S1), after chemo-mechanical preparation using 2% CHX gel as auxiliary chemical substance (S2), and after 7 days of intracanal dressing (S3) using the checkerboard DNA–DNA hybridization technique and by counting the colony-forming units (CFU) on agar plates, to evaluate microbial changes after endodontic procedures.

Material and methods

Patient selection

Twenty-four patients, attending the Dental School of Piracicaba, SP, Brazil, for root canal treatment, were selected for this study. The age of the patients ranged from 18 to 59 years. The selected teeth (one tooth per patient) were uniradicular, containing a single root canal, their pulp chamber was without visual communication with the oral fluid, they presented with necrotic pulp tissue, and showed radiographic evidence of periapical periodontitis, but an absence of periodontal disease. None of the selected patients showed signs and symptoms of endodontic origin. One of the exclusion criteria for this study was the presence of signs and symptoms of endodontic cause on the day of the endodontic procedure or in the last 3 months. Patients who had received antibiotic treatment during the previous 3 months or who had a general disease were also excluded from the study. A detailed medical and dental history was obtained from each patient. The Human Volunteers Research and Ethics Committee of the Dental School of Piracicaba approved a protocol describing the specimen collection for this investigation, and all patients signed their informed consent to participate in the study.

Initial microbiological sampling

The microbiological procedures performed in this study have been previously described (13, 43). Briefly, the teeth were isolated with a rubber dam. The crown and the surrounding rubber dam were disinfected with 30% H_2O_2 (volume/volume) for 30 s followed by 2.5% NaOCl for an additional 30 s. Subsequently, 5% sodium thiosulphate was used to inactivate the disinfectant agents. An access cavity was prepared with sterile high-speed diamond burs under irrigation with sterile saline. Before entering the pulp chamber, the access cavity was disinfected following the same protocol described above. The disinfection was checked by taking a swab sample of the cavity surface and streaking this on blood agar plates with subsequent incubation at 37°C aerobically and anaerobically. All subsequent procedures were performed aseptically. The pulp chamber was accessed with sterile burs refrigerated by saline. The first samples (S1) were collected with five sterile paper points that were consecutively placed into the main root canal to a total depth calculated from the preoperative radiograph. Afterwards, the paper points were pooled in a sterile tube containing 1 ml Reduced Transport Fluid (Drogal Farmácia de Manipulação Ltda, Piracicaba, SP, Brazil). The samples were transferred to the laboratory within 15 min to perform the culture method (CFU counting).

Clinical procedures and post-treatment microbiological samplings

After the first microbiological sampling, the pulp chamber was thoroughly cleaned with 2% CHX gel (Endogel, Itapetininga, SP, Brazil). The CHX gel consisted of a gel base (1% natrosol) and CHX gluconate at pH 7.0. Natrosol gel (hydroxyethyl cellulose) is a non-ionic, highly inert, water-soluble agent (45). The K-file (DYNA-FFDM, Bourges, France) size 10 or 15 was inserted the total length of the root canal, as calculated from the preoperative radiograph. The coronal two-thirds of each canal were prepared initially using rotary files (GT® Rotary Files size 0.10/20 and 0.08/20; Malleifer-Dentsply, Bailagues, Switzerland) at 350 r.p.m. reaching to within 4 mm of the total length. Gates–Glidden drills sizes 5, 4, 3, and 2 (DYNA-FFDM) were used 2 mm before the length prepared with GT files. The working length (1 mm from the radiographic apex) was obtained by apical locator (Forum Technologies, Rishon Le-Zion, Israel), and the apical preparation was performed using K-files followed by Step-Back preparation (43).

The 24 root canals were treated with 2% CHX gel as a disinfectant. The working time for the chemo-mechanical procedure was 20 min for all cases. Before using each file, 1 ml CHX gel was introduced into the root canal with a syringe (27-gauge needle) and immediately after instrumentation 4 ml physiological saline solution was used to irrigate the canal. Before collecting the second (post-chemo-mechanical) sample (S2), the inactivation of CHX was accomplished by rinsing with 5 ml sterile solution containing 0.5% Tween-80 and 0.07% lecithin (weight/volume; chemical neutralizer of CHX) for 1 min. The second sample was then taken using five sterile paper points, which were consecutively placed into the main root canal to the working length. Afterwards, the paper points were pooled in a sterile tube containing 1 ml reduced transport fluid. The samples were transferred to the laboratory within 15 min to perform the culture method (CFU counting).

The canals were then thoroughly washed with 4 ml physiological saline solution and dried with sterile paper points, and the canals were randomly divided into three groups according to the intracanal medication used: $Ca(OH)_2$ paste (M1), 2% CHX gel (M2), and a combination of $Ca(OH)_2$ paste plus 2% CHX gel (M3) (16). The $Ca(OH)_2$ medicaments were plugged into the canal using Lentulo files (Malleifer-Dentsply) and with the blunt end of a sterile paper point. The access cavities were properly filled with two layers of Cavit (ESPE, Seefeld, Germany) and resin (Z-250, 3M Dental Products, St Paul, MN).

Seven days after the placement of the intracanal medication, the canal was aseptically accessed under rubber dam isolation using the protocol for disinfection described above. The medicaments were removed by rinsing the canals with 5 ml of their specific chemical neutralizers [Tween-80 plus 0.07% lecithin for CHX; 0.5% citric acid for $Ca(OH)_2$ and a combination of both for $Ca(OH)_2$ + CHX] and by carefully filing the canal with the master apical file. The third sample was then collected (S3) as described above. The root canals were re-instrumented; cleaned with 17% ethylenediaminetetraacetic acid (EDTA) and subsequently fully

washed with 4 ml physiological solution; dried with paper points; and obturated using the lateral condensation technique. The restorative procedures were performed with Cavit (ESPE) in the root canal entrance and resin to restore the crown (Z-250, 3M Dental Products).

Microbiological assessment – colony-forming unit counts

Inside the anaerobic chamber (Don Whitley Scientific, Bradford, UK), in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2, the transport medium was shaken thoroughly in a mixer for 60 s (Vortex, Marconi, São Paulo, SP, Brazil). Serial 10-fold dilutions (100 μl of the sample) were made up to $1 : 10^4$ in tubes containing Fastidious Anaerobe Broth (LabM, Bury, UK). First, 50 μl of each dilution was inoculated on Fastidious Anaerobe Agar (Lab M) supplemented with 5% defibrinated sheep blood, 5 mg/l hemin, and 1 mg/l menadione. Plates were incubated anaerobically (80% N_2, 10% H_2, 10% CO_2) at 37°C for 7 days. After incubation, the total CFU were counted using a stereomicroscope at 16 × magnification (Zeiss, Oberkoren, Germany). The remaining portions of the samples were frozen at – 70°C for molecular analysis.

Microbiological assessment – checkerboard DNA–DNA hybridization

Bacterial strains and growth conditions

The bacterial strains used for the preparation of DNA probes and standards are presented in Table 1. Thirty-eight of them were from the American Type Culture Collection (ATCC) and two were provided by the Forsyth Institute (Boston, MA).

The majority of the strains were grown on trypticase soy agar (Baltimore Biological Laboratories, Cocheysville, MD) supplemented with 5% defibrinated sheep blood, with the following exceptions: *Tannerella forsythia* was grown on trypticase soy agar supplemented with 5% defibrinated sheep blood (Baltimore Biological Laboratories) and 10 μg/ml *N*-acetylmuramic acid (Sigma Chemical Co., St Louis, MO); *Porphyromonas gingivalis* and *Porphyromonas endodontalis* were grown in trypticase soy agar (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated sheep blood, 0.3 μg/ml menadione (Sigma Chemical Co.) and 5 μg/ml hemin (Sigma Chemical Co.); *Eubacterium* and *Neisseria* species were grown on Fastidious Anaerobe Agar (Baltimore Biological Laboratories) supplemented with 5% defibrinated sheep blood. *Treponema denticola* and *Treponema socranskii* ssp. *socranskii* were grown in *Mycoplasma* broth (Difco, Laboratories, Detroit, MI) supplemented with 1 mg/ml glucose, 400 μg/ml niacinamide, 150 μg/ml spermine tetrahydrochloride, 20 μg/ml sodium isobutyrate, 1 μg/ml L-cysteine, 5 μg/ml thiamine pyrophosphate (Sigma Chemical Co.), and 0.5% bovine serum (Baltimore Biological Laboratories). All bacteria strains were incubated at 36°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2).

DNA isolation and preparation of DNA probes

After 3–7 days of growth, the cells were harvested and placed in 1.5-ml microcentrifuge tubes containing 1 ml TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.6). Cells were washed twice by centrifugation in TE buffer at 1300 ***g*** for 10 min. The cells were resuspended and the DNA extraction for gram-negative strains was performed with 10% sodium dodecyl sulfate (SDS) and proteinase K (20 mg/ml) (Sigma Chemical Co.). For gram-positive strains the DNA extraction was performed using 150 μl of a mixture containing 15 mg/ml lysozyme and 5 mg/ml achromopeptidase (both Sigma Chemical Co.) in TE buffer (pH 8.0). The pelleted cells were resuspended by 15 s of sonication in a micro-ultrasonic cell disrupter (Kontes, Vineland, NJ) at room temperature and incubated at 37°C for 1 h. DNA was isolated and purified as described by Smith et al. (36). The concentration of purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm.

The purity was assessed by calculating the ratio of the absorbance at 260 nm to that at 280 nm. Whole genomic DNA probes were prepared for each tested strain by labeling 1–3 μg DNA with dioxigenin (Boehringer Mannheim, Indianapolis, IN), using a random primer technique (10). The DNA was stored at – 20°C.

Checkerboard DNA–DNA hybridization

The samples stored in 150 μl of the transport media were transferred to sterile Eppendorff tubes and 0.1 ml 0.5 M NaOH was added to each tube. The checkerboard DNA–DNA hybridization method was performed based on the description by Socransky et al. (38). Briefly, the samples were boiled for 10 min and neutralized using 0.8 ml of 5 M ammonium acetate. The released DNA was then placed into the extended slots of a Minislot 30™ apparatus (Immunetics, Cambridge, MA), concentrated on a 15 × 15 positively charged nylon membrane (Boehringer-Mannheim) and fixed to the membrane by baking it at 120°C for 20 min. A Miniblotter 45™ (Immunetics) device was used to hybridize the 40 digoxigenin-labeled, whole-genomic DNA probes at right angles to the lanes of the clinical samples. Bound probes were detected by using phosphatase-conjugated antibodies to digoxigenin and chemiluminescence (CDP-Star Detection Reagent™, Amersham Biosciences, Chicago, IL). The signals of the probes were compared with the standards signals to obtain a semi-quantification of the number of cells present in the samples. The sensitivity of the assay permitted the detection of 10^4 cells of a given bacterial species by adjusting the concentration of each DNA probe.

Statistical analysis

The CFU counts were statistically analyzed using SPSS FOR WINDOWS, version 12.0 (SPSS Inc, Chicago, IL). The comparison among S1, S2, and S3 was performed using the Friedman test ($P < 0.05$) and the Wilcoxon test was used subsequently. Comparison among intracanal dressing groups was performed using Kruskal–Wallis test ($P < 0.05$).

Results

Overview of microbial composition by checkerboard DNA–DNA hybridization

Using the checkerboard DNA–DNA hybridization technique, 20 initial samples (S1, 83.3%) showed at least one bacterium (listed in Table 1). Bacteria were not detected in four cases (case nos 11, 17, 19, and 23).

The number of bacterial species detected in the positive samples ranged from 1 to 35 species. Three of the bacteria, *Actinomyces gerencseriae*, *Actinomyces israelii*, and *Actinomyces naeslundii* 1, were absent in the initial samples (S1), as well as after chemo-mechanical preparation (S2) and after 7 days of intracanal dressing (S3).

After chemo-mechanical preparation (S2), most of the samples were negative for the 40 evaluated bacteria, with only 16.6% (four cases) of the samples being positive for at least one bacterium (Fig. 1, Table 2). After 7 days of intracanal dressing (S3), again four cases were positive. However, these cases were negative in S2 (Fig. 1, Table 2).

Table 1. Bacterial strains used for DNA probes preparation

Species	Strain	Species	Strain
Aggregatibacter actinomycetemcomitans A and B	ATCC 43718* ATCC 29523*	*Gemella morbillorum*	ATCC 27824*^{T}
Actinomyces gerencseriae	ATCC 23860*^{T}	*Leptotrichia buccalis*	ATCC 14201*^{T}
Actinomyces israelii	ATCC 12102*	*Neisseria mucosa*	ATCC 19696*^{T}
Actinomyces naeslundii 1	ATCC 12104*^{T}	*Parvimonas micra*	ATCC 33270*^{T}
Actinomyces odontolyticus	ATCC 17929*	*Porphyromonas endodontalis*	ATCC 35406*^{T}
Actinomyces naeslundii 2	ATCC 43146*	*Porphyromonas gingivalis*	ATCC 33277*^{T}
Campylobacter gracilis	ATCC 33236*^{T}	*Prevotella intermedia*	ATCC 25611*^{T}
Campylobacter rectus	ATCC 33238*^{T}	*Prevotella melaninogenica*	ATCC 25845*^{T}
Campylobacter showae	ATCC 51146*^{T}	*Prevotella nigrescens*	ATCC 33563*^{T}
Capnocytophaga ochracea	ATCC 33596*	*Propionibacterium acnes* I and II	ATCC 11827* ATCC 11828*
Capnocytophaga gingivalis	ATCC 33624*^{T}	*Selenomonas noxia*	ATCC 43541*^{T}
Capnocytophaga sputigena	ATCC 33612*^{T}	*Streptococcus constellatus*	ATCC 27823*^{T}
Eikenella corrodens	ATCC 23834*^{T}	*Streptococcus gordonii*	ATCC 10558*^{T}
Enterococcus faecalis	ATCC 29212*	*Streptococcus intermedius*	ATCC 27335*^{T}
Eubacterium saburreum	ATCC 33271*	*Streptococcus mitis*	ATCC 49456*^{T}
Fusobacterium periodontium	ATCC 33693*^{T}	*Streptococcus oralis*	ATCC 35037*^{T}
Eubacterium nodatum	ATCC 33099*^{T}	*Treponema socranskii* ssp. *socranskii*	S1[a]
Fusobacterium nucleatum ssp. *nucleatum*	ATCC 25586*^{T}	*Treponema denticola*	B1[a]
Fusobacterium nucleatum ssp. *polymorphum*	ATCC 10953*^{T}	*Tannerella forsythia*	ATCC 43037*^{T}
Fusobacterium nucleatum ssp. *vincentii*	ATCC 49256*^{T}	*Veillonella parvula*	ATCC 10790*^{T}

*ATCC (American Type Culture Collection, Rockville, MD).
[a]Strains from Forsyth Institute.
^{T}Type strains.

Species diversity

The bacteria recovered by checkerboard DNA–DNA hybridization before treatment (S1), after chemo-mechanical preparation (S2), and after 7 days of intracanal dressing (S3) are shown in Fig. 1. Before treatment (S1), the most prevalent taxa were *Fusobacterium nucleatum* ssp. *polymorphum*, corresponding to 12 of 20 positive cases (60% of the positive cases), followed by *T. socranskii* ssp. *socranskii*, *Parvimonas micra*, and *Enterococcus faecalis*, each of them present in 11 of 20 cases (55%). Other species also detected frequently are listed in decreasing order as follows: *Eubacterium nodatum* (nine cases; 45%), *Streptococcus oralis* (eight cases; 45%), *Prevotella melaninogenica* (eight cases, 45%), *Prevotella intermedia* (eight cases; 45%), *P. gingivalis* (eight cases; 45%), *Capnocytophaga sputigena* (eight cases; 45%), *T. forsythia* (eight cases; 45%), *Streptococcus gordonii* (seven cases; 35%), *Prevotella nigrescens* (seven cases; 35%), *Leptotrichia buccalis* (seven cases; 35%), *Gemella morbillorum* (seven cases; 35%), *Capnocytophaga gingivalis* (seven cases; 35%), *Capnocytophaga ochracea* (seven cases; 35%), *T. denticola* (seven cases; 35%), *Streptococcus intermedius* (six cases; 30%), *Selenomonas noxia* (six cases; 30%), *F. nucleatum* ssp. *nucleatum* (six cases; 30%), *Campylobacter showae* (six cases; 30%), and *Campylobacter gracilis* (six cases; 30%) (Fig. 1).

After chemo-mechanical preparation (S2), bacteria were detected in four cases. The most prevalent taxon was *C. ochracea*, which was found in 50% of the positive cases (two of the four), followed by *T. socranskii* ssp. *socranskii* (one of the four, 25%), *F. nucleatum* ssp. *polymorphum* (one of the four, 25%), *E. faecalis* (one of the four, 25%), and *C. showae* (one of the four, 25%) (Fig. 1, Table 2).

After 7 days of intracanal dressing (S3), bacteria could still be detected in three cases. The bacterial species found were: *G. morbillorum* (two of the four, 50%), *L. buccalis* (one of the four, 25%), *F. nucleatum* ssp. *polymorphum* (one of the four, 25%), *E. faecalis* (one of the four, 25%), and *C. sputigena* (one of the four, 25%) (Fig. 1, Table 2).

Overview of microbial detection by culture analysis

An overview of the quantitative data and illustrations of the CFU/ml, in other words, the amount of cultivable bacteria at the different steps S1, S2, and S3, is provided in Fig. 2. Bacteria were present in all initial samples (S1, $n = 24$). The amount of cultivable bacteria ranged from 1.9×10^5 CFU/ml to 3.0×10^6 CFU/ml, with a mean of 2.0×10^5 CFU/ml. After chemo-mechanical preparation (S2), cultivable bacteria were detected in eight cases and their amount ranged from 4.0×10^2 to 6.0×10^2 CFU/ml, with a mean of 1.0×10^2 CFU/ml. There was a 99.96% reduction of bacteria in S2 compared to S1. After 7 days of intracanal dressing (S3), bacteria were detected by culture analysis in 13 cases. The amount of cultivable bacteria in the positive cases ranged from 2.0×10^1 to 8.0×10^3 CFU/ml, with a mean of 6.0×10^2 CFU/ml. This represented a reduction of 99.79% compared to the baseline S1. Differences in the amount of cultivable bacteria between S1 and S2 were statistically significant ($P < 0.05$) but there were no significant differences between S2 and S3.

The highest bacterial amount was found in the initial samples (S1). After chemo-mechanical preparation using 2% CHX gel as auxiliary substance (S2), a major reduction in bacteria was achieved. On the other hand, the use of an intracanal dressing for 7 days did not improve the bacterial reduction achieved by chemo-mechanical preparation. Moreover, it contributed to a slight increase in bacterial numbers and in the number of positive cases.

Microbial susceptibility to chemo-mechanical preparation

Bacteria were recovered in four cases by checkerboard DNA–DNA hybridization after chemo-mechanical preparation using 2% CHX gel as auxiliary substance. After chemo-mechanical preparation (S2) *T. socranskii* ssp. *socranskii* was present in one case (case no. 13, Table 2) and also in the initial sample (S1). By comparing the sample concentration to the standard

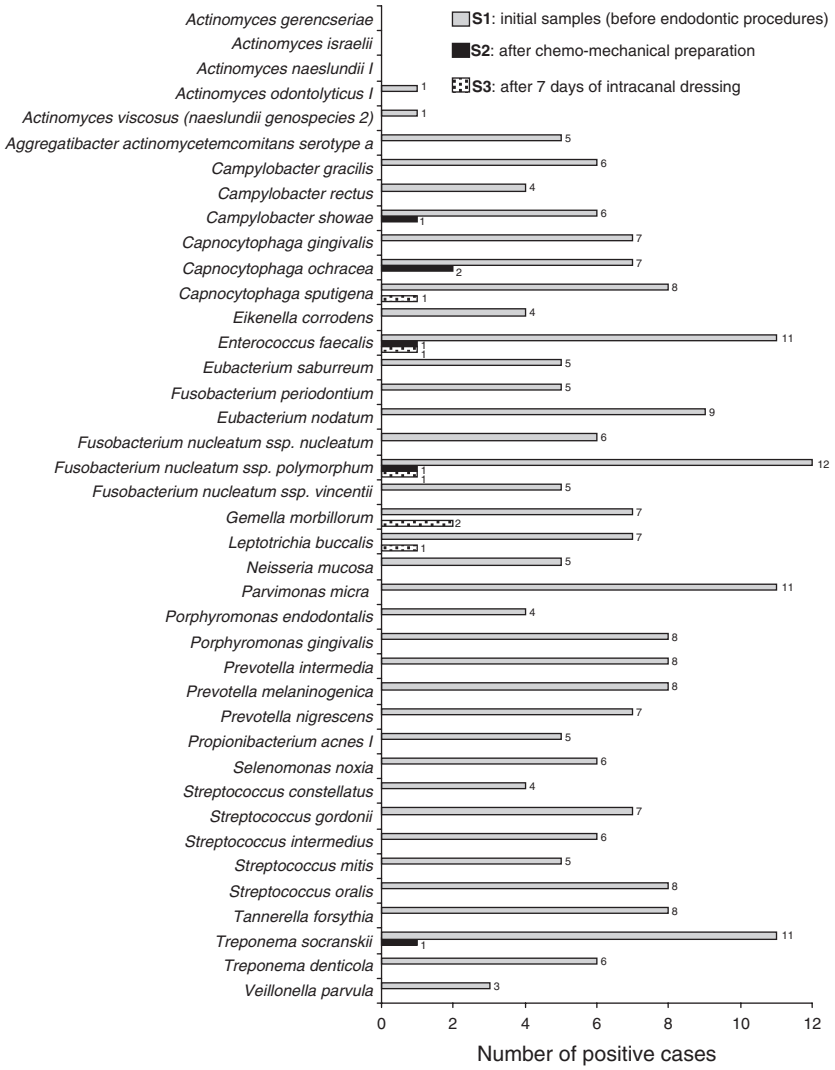


Fig. 1. Frequency of bacterial species found in hybridization before treatment (S1), after chemo-mechanical preparation (S2), and after 7 days of intracanal dressing (S3) by checkerboard DNA–DNA hybridization.

Table 2. Bacterial taxa detected by checkerboard DNA–DNA hybridization after chemo-mechanical preparation and after 7 days of intracanal medicaments

After chemo-mechanical preparation (S2)			After 7 days of intracanal medicaments (S3)		
Species	Number of identifications	Case number	Species	Number of identifications	Case number
Campylobacter showae	1	5	*Capnocytophaga sputigena*	1	9*
Treponema socranskii ssp. *socranskii*	1	13	*Enterococcus faecalis*	1	9*
Capnocytophaga ochracea	2	18,19	*Gemella morbillorum*	2	9*, 17*
Fusobacterium nucleatum ssp. *polymorphum*	1	18	*Fusobacterium nucleatum* ssp. *polymorphum*	1	9*
Enterococcus faecalis	1	19	*Leptotrichia buccalis*	1	23**

*M3: $Ca(OH)_2$ + 2% CHX gel.
**M1: $Ca(OH)_2$ paste.

concentrations, it was possible to estimate that the amount of *T. socranskii* ssp. *socranskii* was approximately 10^6 bacterial cells in the initial samples, and after chemo-mechanical preparation this number had declined to 10^4 bacterial cells.

C. showae was present in one case after chemo-mechanical preparation (S2; case no. 5, Table 2) and also in the initial sample (S1). The number of *C. showae* was around 10^5 before and after chemo-mechanical preparation.

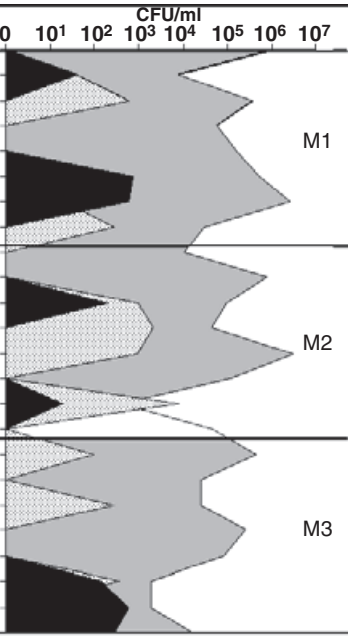
C. ochracea, *F. nucleatum* ssp. *polymorphum*, and *E. faecalis*, detected in S2 (Table 2), were absent in S1.

It should be emphasized that more cases were positives (eight cases, Fig. 2) in S2 by culture than by checkerboard DNA–DNA hybridization (four cases).

Comparison of the different intracanal dressings

Bacteria were recovered in three cases by checkerboard DNA–DNA hybridization in different treatment groups in S3

Case number	S1	S2	S3
3	1.0×10^6	0	0
8	7.6×10^3	4.0×10^1	4.0×10^1
10	3.7×10^5	0	6.0×10^2
11	6.0×10^4	0	0
14	1.5×10^5	0	0
18	5.5×10^5	7.4×10^2	7.2×10^2
20	2.7×10^6	6.0×10^2	2.0×10^1
23	3.0×10^4	0	2.8×10^2
1	1.0×10^4	0	0
5	7.6×10^5	0	0
6	9.6×10^4	2.0×10^2	1.0×10^3
7	4.4×10^4	0	2.1×10^3
13	3.0×10^5	0	9.0×10^2
16	1.2×10^5	0	0
19	4.0×10^2	2.0×10^1	8.0×10^3
22	4.6×10^4	0	0
2	4.4×10^5	0	1.0×10^2
4	2.5×10^4	0	0
9	2.5×10^4	0	2.8×10^2
12	2.6×10^5	0	0
15	8.1×10^4	0	0
17	1.9×10^3	1.4×10^2	3.6×10^2
21	2.0×10^3	6.0×10^2	0
24	1.6×10^4	3.0×10^2	4.0×10^1
Mean values*	2.5×10^5	1.0×10^2	6.0×10^2
Bacterial reduction**	100%	99.97%	99.79%



S1 : initial samples, S2: after chemo-mechanical preparation, S3: after 7 days of intracanal dressing;
M1 : $Ca(OH)_2$ paste, M2: 2% CHX gel, M3: $Ca(OH)_2$ + 2% CHX gel
*** only positive samples were used to calculated mean values**
**** bacterial reducation calculated using S1 (100%) as baseline for S2 and S3.**

Fig. 2. Colony forming units per ml (CFU/ml) per case before and after chemo-mechanical preparation using 2% CHX gel as auxiliary substance, and after 7 days of intracanal dressing. Cases are ordered according to different intracanal dressings (M1: $Ca(OH)_2$ paste, M2: 2% CHX gel, M3: $Ca(OH)_2$ + 2% CHX gel). The area graph gives an overview of microbial susceptibility.

[M1 = $Ca(OH)_2$ paste and M3 = $Ca(OH)_2$ with 2% CHX gel]. In M1, one case was positive for *F. nucleatum* ssp. *polymorphum* after 7 days of intracanal medication (case no. 23, S3, Table 2). The relative quantification, obtained by checkerboard DNA–DNA hybridization, showed the presence of 1×10^5 bacterial cells of this species at S3. In the same case (no. 23), this species was not detected in S1 and S2.

Bacteria were detected in two cases in the M3 treatment group. In one of them (case no. 9) four bacterial species were detected: *C. sputigena*, *E. faecalis*, *G. morbillorum*, and *L. buccalis* (Table 2). For all these species, the relative quantification, obtained by checkerboard DNA–DNA hybridization, showed that fewer than 1×10^5 bacterial cells were present in each sample at M3. *E. faecalis* was also detected at similar levels in the initial sample (S1). In the other case (no. 17), fewer than 1×10^5 bacterial cells of *G. morbillorum* were detected, and this species could not be detected in S1 and S2.

It is noticeable that in S3 the number of positive cases was higher (13 cases) by culture, when compared to checkerboard DNA–DNA hybridization (three cases). Moreover in M3, by CFU counting, a trend was observed towards a lower bacterial count when compared to M1 and M2 (2% CHX gel) (Fig. 2).

Discussion

The purpose of this study was to detect 40 bacterial species by checkerboard DNA–DNA hybridization and to quantify the total number of bacteria by a culture-based technique, to evaluate microbial changes after endodontic procedures.

The molecular technique used in the present study does not require viable bacterial strains (38). However, the bacterial DNA integrity and its quantity are important factors for microbial detection. The latter fact is highly important because of the detection limit of checkerboard DNA–DNA hybridization (i.e. numbers below 10^4 are not detectable). This fact seems to be highly important in the evaluation of the postendodontic procedure samples (S2 and S3), in which bacterial cell numbers were substantially reduced. Negative samples did not necessarily mean, therefore, that the screened bacteria were absent but that they could be below the levels of detection. The introduction of multiple displacement amplification to provide abundant target DNA and DNA probes for checkerboard DNA–DNA hybridization may be a valuable tool for characterizing the microbiota in clinical samples (42), especially for post-treatment samples. On the other hand, it should be considered that not only the presence of the bacteria is an important factor for development or maintenance of disease, but also the population size. Therefore the checkerboard DNA–DNA hybridization technique might be useful for predicting the outcome of the endodontic treatment.

CFU counting is a reliable method for evaluating the potential of the antimicrobial agents. However, it is worth pointing out that the bacterial diversity and bacterial counts are usually underrepresented by culture analysis (43). Moreover, negative culture does not imply sterility. This fact might be a result of limitations of the sampling procedure, the culture techniques, and the presence of as yet uncultivated bacteria. Negative results might again mean that cultivable bacterial populations were reduced to levels below the detection ability of culture-dependent

methods. However, from a clinical point of view it seems that these low levels of bacteria are compatible with periradicular tissue healing, because negative cultures are reported to be correlated to favorable treatment outcome (34, 46).

Microbial reduction

Most of the root canals included in the present study (S1) showed at least one of the 40 bacteria screened. Four samples were negative for the species evaluated by checkerboard DNA–DNA hybridization (cases nos 11, 17, 19, and 23).

By culture analysis, all the root canals included in this study contained bacteria on initial sampling (S1), confirming the correlation between bacteria and periapical periodontitis. The CFU found in S1 were in accordance with the results of other studies that used culture-based techniques (4, 5, 24, 26, 35, 43).

There was a substantial reduction in bacterial levels after chemo-mechanical preparation, agreeing with studies that had used NaOCl as the chemical auxiliary substance (5, 6, 12, 26, 34, 43) and also CHX (43).

Even though cultivable bacteria still remained in some root canals after chemo-mechanical preparation, the pronounced bacterial reduction observed (over 99%) confirmed the important role of cleaning and shaping root canals using an auxiliary substance with an antimicrobial property. These results are in accordance with other studies (4, 5, 12, 26, 43).

Intracanal dressings in teeth with closed apices are mainly used to control persistent exudation or persistent pain (9). In the present study, the intracanal dressing was used to test if the number of microorganisms that might have survived after chemo-mechanical preparation would decrease. A wide variety of intracanal medicaments have been employed, with $Ca(OH)_2$ being the most frequently used (19). $Ca(OH)_2$ has been recommended owing to its biological and antimicrobial properties (9, 14, 15, 32), as well as to its mechanical property – it acts as a barrier against microbial ingress and cuts off nutrient supply (15). $Ca(OH)_2$ has been reported to be effective against several bacteria. However, some studies have demonstrated that it does not effectively eliminate alkali-tolerant species, such as enterococci (16, 17, 33, 45). Combinations of $Ca(OH)_2$ with different substances are used to enhance the antimicrobial properties (15, 43), especially in combination with CHX (16).

Regardless of the type of intracanal dressing used for 7 days, the number of bacteria found by culture analysis was slightly higher in S3 than in S2. Peters et al. (26), who used 2% NaOCl as an irrigating solution and $Ca(OH)_2$ with sterile saline for 4 weeks as intracanal dressing, also found culture-positive cases, with the bacterial load not significantly reduced after this step.

Other studies have evaluated the use of intracanal medications for different periods of time, which ranged from 1 to 4 weeks (4, 8, 24, 30, 35). However, complete bacterial elimination was accomplished in only a few works (4, 8) and seems to be not necessarily dependent on the period that the intracanal dressing remained in the root canals. We chose a period of 7 days for evaluation because this is the usual time between visits (11, 31, 45). However, the use of intracanal medication to predictably render root canals bacteria-free before filling seems to be questionable, at least in cases where signs and symptoms of endodontic origin are absent.

Microbial susceptibility

Culture-based studies have shown the variety of microorganisms that may colonize the root canals of necrotic pulp tissues, and revealed that they are mainly anaerobes (2, 13). On the other hand, treated teeth have revealed a much higher proportion of facultative anaerobes (28). The reduction of gram-negative microorganisms following endodontic treatment, and the subsequent proportional increase of gram-positive facultative anaerobes (4, 7, 12, 26) supports the thought that antimicrobial treatment procedures in endodontics are more efficient against obligate anaerobes and less efficient towards a whole set of facultative species.

Lactobacilli, streptococci, and enterococci are frequently found between visits, whereas gram-negative rods are less frequent, when using culture analysis (7, 12, 26). Furthermore, it is believed that in cases where total eradication has failed, endodontic treatment may select the most robust segment of the root canal microbiota, which is the facultative anaerobic microorganisms. Such microorganisms may be responsible for post-treatment apical periodontitis (7, 12, 13).

The present study showed that gram-negative species were the most frequent bacteria recovered by checkerboard DNA–DNA hybridization after chemo-mechanical preparation and after 7 days of intracanal dressing. This fact raises a question. Are these microorganisms usually in a dormant state?

A great number of pathogens and non-pathogens are now known to enter a state of dormancy and its significance in medicine is becoming increasingly evident [reviewed in Kell et al. (21) and in Oliver (23). Cells in the viable but non-cultivable (VBNC) stage typically demonstrate very low levels of metabolic activity, but on resuscitation are again cultivable (23). A large number of human pathogens, such as *Campylobacter* spp. and *E. faecalis*, are able to achieve this VBNC stage (23). Whether or not cells in the VBNC state are capable of causing human infection is an important aspect that is not well understood. Because of the near dormancy exhibited by such cells, it would not be expected that they would be capable of initiating infection while in this state (1, 31). However, this fact is not clear, at least for several pathogens (23). The ability of *E. faecalis* cells in the VBNC state to adhere to cultured heart and urinary tract epithelial cells has been shown by Pruzzo et al. (29).

E. faecalis has been considered one of the most resistant species in the oral cavity and a possible cause of root canal treatment failure (13, 25, 28). Traditional methods have demonstrated that enterococci constitute a small percentage of the microbial species isolated from root canals of teeth with necrotic dental pulps. However, in the present study using checkerboard DNA–DNA hybridization *E. faecalis* was found in 55% of the initial positive samples (11 of 20), supporting the findings of Gomes et al. (16), who also found this species in high prevalence and proportions in root canals with necrotic pulp tissues.

E. faecalis has demonstrated the capacity to survive in an environment in which there are scant available nutrients and in which commensality with other bacteria is minimal. It has been postulated that a virulence factor of these species in failed endodontically treated teeth may be related to the ability of *E. faecalis* cells to invade dentinal tubules and adhere to collagen in the presence of human serum (22). The present study confirms that this species is resistant to endodontic procedures. In one case, *E. faecalis* could be detected after the chemo-mechanical preparation using 2% CHX, and in another case, it could still be detected after using the combination of $Ca(OH)_2$ with 2% CHX gel for 7 days as intracanal medicament.

Indeed, stress conditions such as those provoked by chemo-mechanical prepara-

tion and the use of intracanal medicaments may lead gram-positive and gram-negative bacteria to enter a dormant stage. There is much yet to learn of the physiology, biochemistry, and genetics of cells to understand how they enter and resuscitate from this physiological state. While this point is not yet fully clear, it appears that cells in the dormancy state retain virulence, which might be associated with persistent endodontic infections.

Conclusion

In conclusion, the checkerboard DNA–DNA hybridization technique confirmed that primary endodontic infections are characterized by a large variety of bacterial species. Hence, the primary aim of endodontic treatment is to remove as many bacteria as possible from the root canal system, creating an environment in which any remaining organisms cannot survive. The antibacterial effect of the mechanical preparation supplemented by the use of an antibacterial auxiliary substance largely reduced the microorganisms in the main root canal.

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