Assessment of the Microbiota in Root Canals of Human Primary Teeth by Checkerboard DNA–DNA Hybridization

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ABSTRACT

Purpose: The purpose of this study was to evaluate *in vivo* the prevalence of microorganisms in root canals of human primary teeth, by checkerboard DNA-DNA hybridization.
Methods: Fifty-five root canals of primary teeth with irreversible pulpitis (group 1) and 51 root canals of teeth with pulp necrosis and apical periodontitis (group 2) were selected. Microbiological samples were collected and submitted to checkerboard DNA-DNA hybridization using 34 genomic DNA probes. The results were analyzed statistically by Mann-Whitney U-test at a 5% significance level.
Results: The most prevalent species in group 1 were: (1) *Campylobacter rectus* (87%); (2) *Gemella morbilorum* (78%); (3) *Streptococcus gordonii* (71%); (4) *Capnocytophaga ochracea* (69%); (5) *Treponema denticola* (58%); and (6) *Streptococcus intermedius* (49%). The most prevalent species in group 2 were: (1) *C. rectus* (90%); (2) *T. denticola* (88%); (3) *S. intermedius* (77%); (4) *G morbilorum* (73%); (5) *Streptococcus oralis* (67%); (6) *C. ochracea* (63%); (7) *S. gordonii* (55%); (8) *Streptococcus mitis* (51%); and (9) *Leptotrichia buccalis* (51%). Except for *Peptostreptococcus micros* and *Actinomyces israelii*, the most prevalent bacterial strains in the root canals with apical periodontitis were also those found in larger numbers ($P<.05$). Groups 1 and 2 differed significantly ($P<.05$) regarding the total number of bacterial cells detected in the root canal samples, with group 2 showing remarkably larger bacterial cell numbers.
Conclusions: Root canals of primary teeth have a great bacterial diversity, characterizing a polymicrobial endodontic infection with presence of: (1) anaerobic and facultative micro organisms; (2) black-pigmented rods; and (3) streptococci. A large number of anaerobic species were detected in teeth with necrotic pulp and apical periodontitis, and a significantly smaller number of bacterial cells were found in teeth with irreversible pulpitis.
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Keywords: Checkerboard dna-dna hybridization, primary teeth, microbiota, root canals

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Studies using microbial culture techniques in root canals of primary teeth with pulp necrosis and apical periodontitis, although scarce, have isolated bacterial species usually found in root canals of permanent teeth,[1-7] which indicates the predominance of anaerobic pathogens.[5-7] Nevertheless, in spite of its widespread use for assessment of endodontic infections, microbial culturing methods are not very sensible. On account of this, it is likely that pathogens might remain undetected until these days.[8,9]

Molecular biology methods are more specific, sensitive, and rapid than conventional microbial culture and can detect uncultivable and fastidious microorganisms. Checkerboard DNA-DNA hybridization was introduced as a method for hybridizing large numbers of DNA samples against large numbers of DNA probes on a single support membrane. It is faster than PCR technique because it uses several DNA probes at the same time and allows for simultaneous determination of the presence of a multitude of bacterial species in single or multiple clinical samples, which can be stored for long periods of time.[10,11] Checkerboard DNA-DNA hybridization has been used to examine the microorganisms present in root canals of permanent teeth.[10-14] As far as it could be ascertained, however, there are no studies reporting the use of biomolecular techniques to identify the endodontic microbiota in primary teeth

The purpose of this study was to asses *in vivo*, by checkerboard DNA-DNA hybridization, the prevalence and number of bacterial cells of 34 strains in root canals of human primary teeth under 2 clinical situations, teeth with irreversible pulpitis; and teeth with pulp necrosis and apical periodontitis.

METHODS

This research project was approved by the local Ethics in Research Committee (process no. 2003.1.1262.58.0) of the University of São Paulo, Ribeirão Preto, São Paulo, Brazil, and written informed consent was obtained from the parents or guardians.

The samples were collected from 3- to 7-year-old male and female patients who had been referred for dental treatment at the Pediatric Dentistry Clinic of the School of Dentistry of Ribeirão Preto, University of São Paulo. Excluded from the trial were children who, used antimicrobial mouthwashes, presented with any systemic disease, or had used antibiotics within the previous 3 months. The selected primary teeth:

1. had carious lesions of varied depths and extensions,
2. had intact roots or less than two thirds of root resorption,
3. required root canal therapy, and
4. presented no periodontal pockets or previous root canal intervention.

A total of 110 root canals were assigned to 2 groups: (1) 55 root canals of teeth with irreversible pulpitis; and (2) 55 root canals of teeth with pulp necrosis and apical periodontitis.

CLINICAL PROCEDURES

After local anesthetic and rubber dam isolation, carious tissue was removed and antisepsis was performed with 1.0% chlorhexidine digluconate. Coronal access was gained, and the pulp chamber was irrigated with sterile saline and aspirated.

SAMPLE COLLECTION

For sample collection, a K-file was passively introduced up to 1 mm from the radiographic apex or the limit of root resorption and was gently moved in an apex-crown direction against the intracanal walls. The file containing the chips was placed in a 1.5-mL Eppendorf tube with sterile Milli-Q water, which was vigorously agitated for material desorption.

Thereafter, 2 sterile paper points were sequentially placed into the canals at the same level as the files for 1 minute and transferred to the same Eppendorf tube containing the material collected with the file. Next, the teeth were endodontically treated and restored.

MICROBIOLOGIC ASSESSMENT

The presence and numbers of 34 bacterial species (Table 1) were determined by a modification of the checkerboard DNA-DNA hybridization method described by Socransky et al (1994).[15] The cells of the collected samples were lysed and denatured. DNA was fixed in individual lanes on a nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Buckinghamshire, England) using a checkerboard slot blot device (Minislot 30, Immunetics, Cambridge, Mass). Thirty-four digoxigenin-labeled (Roche Applied Science, Indianapolis, Ind) 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), which were developed for chemiluminescence signal detection. Signals were evaluated visually by comparing to the standards of 10^5 and 10^6 bacterial cells of the test species present on the last 2 lanes of the same membrane. This provided the approximate number of bacterial cells *per* sample for each bacterial strain evaluated.

The sensitivity of this assay was settled to allow 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 all species (ie, the concentrations were adjusted in a way that all probes had similar signal intensity). Moreover, the probes were evaluated with a multitude of bacterial species from the collection of the Laboratory of Oral Microbiology of the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, for assessment of their specificity. To facilitate the semiquantitative examination of chemiluminescence signals, the intensity of infection by different species in each root canal was evaluated according to the following scores: (a) 0=not detected; (b) 1=<10^5 cells; (c) 2=~10^5 cells; (d) 3=10^5 to 10^6 cells; (e) 4=~10^6 cells; and (f) 5=>10^6 cells.

STATISTICAL ANALYSIS

The prevalence of the bacterial strains, the number of species, and the total number of bacterial cells in groups 1 and 2 were analyzed statistically by the Mann-Whitney nonparametric test at a 5% significance level using the GMC 8.1 statistical software package (School of Dentistry of Ribeirão Preto, University of São Paulo).

Table 1. Prevalence of Bacterial Species in 55 Samples From Root Canals of Primary Teeth With Irreversible Pulpitis (Group 1) and 51 Samples from Root Canals of Primary Teeth With Necrotic Pulp and Apical Periodontitis (Group 2)

	Group 1		Group 2	
Bacterial species	**No. of positive samples**	**Prevalence (%)**	**No. of positive samples**	**Prevalence (%)**
Veillnella parvula	0	0	0	0
Treponema denticola	32	58	45	88
Selenomonas noxia	2	4	15	29
Streptococcus mitis	14	26	26	51
Streptococcus sanguinis	12	22	22	43
Streptococcus oralis	15	27	34	68
Streptococcus gordonii	39	71	28	55
Streptococcus intermedius	27	49	39	77
Prevotella nigrescens	15	27	24	47
Prevotella melaninogenica	7	13	19	37
Porphyromonas gingivalis	1	2	13	26
Peptostreptococcus micros	3	6	13	26
Prevotella intermedia	5	9	11	22
Leptotrichia buccalis	14	26	26	51
Gemella morbilorum	43	78	37	73
Fusobacterium nucleatum spp nucleatum	4	7	11	22
Fusobacterium nucleatum spp polymorphum	20	36	20	39
Fusobacterium nucleatum spp vincentii	12	22	18	35
Fusobacterium periodonticum	6	11	9	18
Eikenella corrodens	0	0	0	0
Eubacterium nodatum	2	4	0	0
Campylobacter showae	0	0	0	0
Capnocytophaga sputigena	14	26	16	31
Capnocytophaga gingivalis	20	36	23	45
Capnocytophaga ochracea	38	69	32	63
Campylobacter rectus	48	87	46	90
Tannerela forsythia	2	4	0	0
Actinomyces viscosus	0	0	0	0
Actinomyces naeslundii genospecies 1	0	0	2	4
Actinomyces odontolyticus	3	6	10	20
Actinomyces israelii	0	0	10	20
Actinomyces gerencseriae	2	4	10	20
Aggregatibacter actinomycetemcomitans serotype b	8	15	16	31
Aggregatibacter actinomycetemcomitans serotype a	3	6	9	18

RESULTS

Of 110 samples, 4 were discarded for suspicion of salivary contamination during collection due to the children's uncooperative behavior. Therefore, the analysis of the bacterial microbiota was performed in 55 samples for group 1 and 51 samples for group 2.

The results of groups 1 and 2 are described in Table 1.

GROUP 1—ROOT CANALS OF PRIMARY TEETH WITH IRREVERSIBLE PULPITIS

Of 34 DNA probes, checkerboard DNA-DNA hybridization identified 28 species in group 1. The number of bacterial species *per* root canal varied from 0 to 18 (mean=7.47). Of 55 root canal samples, 52 showed a positive signal for at least 1 probe (95%). Thirty-four (62%) presented less than 8 different species, while 21 samples (38%) presented 8 or more species per root canal. Six of the test species were not detected in the samples. In 3 root canals, there were no signals of any bacterial species.

Among the test species, 5 probes were detected in more than 50% of the samples. None of the DNA probes used in this study was detected in all samples of this group. *Campylobacter rectus*, however, was the most predominant

microorganism, being identified in 48 of the 55 root canal samples (87%).

Regarding the semiquantitative assessment of the bacterial cell number, infection intensity ranged from score 0 to 3. No cases were scored 4 and 5. Only 3 samples presented a large number of bacterial cells (approximately 550,000 cells).

The following different species were detected in group 1:

1. 14 out of 18 facultative species, with 6,910,000 total bacterial cells (mean=125,636).
2. 14 out of 16 anaerobic species, with 2,240,000 total bacterial cells (mean=40,727); and
3. 12 of 13 gram-negative anaerobic strains, with 2,010,000 total bacterial cells.

In group 1, there was a prevalence of: (1) black-pigmented rods (35%); (2) gram-negative anaerobes (75%); (3) streptococci (76%); (4) gram-negative facultative bacteria (89%); and (5) facultative micro-organisms (95%).

GROUP 2—ROOT CANALS OF PRIMARY TEETH WITH PULP NECROSIS AND CHRONIC PERIAPICAL LESION

Of 34 DNA probes, checkerboard DNA-DNA hybridization identified 28 species in group 2. The number of bacterial species *per* root canal sample varied from 0 to 27 (mean=11.45). Of 51 samples, 49 showed positive signal for at least 1 probe (96%). Twenty-nine samples presented 8 or more different bacterial species (57%), and 22 (43%) presented less than 8 species per root canal.

Nine DNA probes were present in more than 50% of group 2's root canals. On the other hand, 6 probes were not detected in the samples. Two root canals did not show a positive signal for any of the 34 test bacterial strains. In the same way as observed in group 1, *C. rectus* was the predominant bacterial strain, being identified in 90% of the root canals in group 2.

Regarding the semiquantitative assessment of the bacterial cell number, infection intensity ranged from a score of 0 to 3. No cases were scored 4 and 5. Thirteen samples presented a large number of bacterial cells (approximately 550,000 cells).

Fifteen of 18 facultative species were detected in group 2, with the total number of bacterial cells being 11,690,000 (mean=229,216). Of 16 anaerobic species, 13 were detected in group 2, with the total number of bacterial cells being 9,090,000 (mean=178,235). Of 13 gram-negative anaerobic species, 11 were detected, with 8,680,000 total bacterial cells.

In group 2, there was a prevalence of: (1) black-pigmented rods (51%); (2) gram-negative anaerobes (90%); (3) streptococci (82%); (4) gram-negative facultative bacteria (92%); and (5) facultative micro-organisms (96%).

STATISTICAL ANALYSIS

There was a statistically significant difference ($P<.05$) between groups 1 and 2 regarding the prevalence of the following species: (1) *Treponema denticola*; (2) *Selenomonas noxia*; (3) *Streptococcus mitis*; (4) *Streptococcus sanguinis*; (5) *Streptococcus oralis*; (6) *Streptococcus intermedius*; (7) *Prevotella nigrescens*; (8) *Prevotella melaninogenica*; (9) *Porphyromonas gingivalis*; (10) *Peptostreptococcus micros*; (11) *Leptotrichia buccalis*; and (12) *Actinomyces israelii*. The other 22 bacterial species had a similar prevalence in both groups ($P>.05$).

Except for P micros and A israelii, the most prevalent bacterial strains in the root canals with apical periodontitis (group 2) were also those found in larger numbers ($P<.05$).

Groups 1 and 2 differed statistically ($P<.05$) with respect to the total number of bacterial cells detected in the root canal samples. Group 2 showed remarkably larger bacterial cell numbers.

DISCUSSION

To date, few authors have investigated the microorganisms present in root canals of primary teeth and the available data came from studies using microbial culturing methods. As far as it could be ascertained, there are no published studies referring to the endodontic microbiota of primary teeth that have used molecular techniques, which hinders the comparison of this study's results to those of previous works.

In this study, of 55 root canals with irreversible pulpitis (group 1), only 3 were free of the test species. Of the 34 DNA probes, 28 showed a positive signal in at least 1 sample, although with bacterial cell numbers significantly smaller than that of group 2.

Regarding the possibility of bacterial contamination in inflamed pulps, Kronfeld[16] affirmed in the early 1930s that when the pulp tissue is exposed—due to a deep carious lesion—its surface becomes infected. In fact, according to Kronfeld, the presence of bacteria in the pulp tissue can already be detected when the healthy dentin layer separating the cavity floor from the pulp tissue is less than 0.2-mm thick.

The group 1 teeth were cases of pulp exposure secondary to caries progression with irreversible pulpitis, which certainly resulted in much greater bacterial contamination than cases of mechanical, accidental, or traumatic exposure. Therefore, it may be inferred that, although several bacterial strains were detected in group 1, the microorganisms did not have enough time and number to cause necrosis of the entire radicular pulp tissue.

Of 51 root canals with apical periodontitis in group 2, 96% showed positive evidence for the test species, with the total number of bacterial cells being significantly larger than in group 1.

The total number of bacterial cells of the test anaerobic species was larger for group 2 (9,090,000) than group 1 (2,240,000). Particularly, the gram-negative anaerobes were present in a larger number in group 2 (8,680,000) than group 1 (2,010,000). Furthermore, of 51 group 2 samples, 46 presented at least one of the test gram-negative anaerobic bacteria.

Gram-negative bacteria play an extremely important role in the etiology of periapical lesions because, in addition to being highly virulent, bacterial endotoxin (LPS) is found in the outer membrane of these microorganisms. LPS is released during microbial multiplication or death, causing undesirable biological effects that lead to the occurrence of an inflammatory reaction and bone resorption in the periapical region, which fa-

cilitates the onset and progression of periapical lesions.[17,18]

T. denticola was the most prevalent gram-negative anaerobe in the primary root canals of group 2 (88%). This result is particularly interesting, because this species has never been isolated in endodontic microbial culture studies.

Among the gram-negative anaerobes, black-pigmented rods were also detected in the root canal samples of group 2, which are frequently observed in root canals of permanent teeth with necrotic pulps. Using microbial culture techniques, black-pigmented rods were detected in primary teeth with pulp necrosis in 36%, 36%, and 30% of the cases examined by Tomic-Karovic and Jelinek (1971),[3] Pazelli et al (2003),[5] and Silva et al (2006),[7] respectively. Of 51 samples in the present study, 26 (51%) showed at least 1 black-pigmented bacterial species, which is a considerably higher prevalence. Nevertheless, microbial culture techniques have limitations that probably explain the lower prevalence of these micro-organisms in studies using conventional culture methods.

The test facultative bacteria and streptococci in group 2 were detected in 96% and 82% of the root canals, respectively. This result indicates that endodontic infection in primary teeth has a polymicrobial nature regarding development of interactions among the micro-organisms, as previously described.[5,7]

At least one of the gram-negative facultative bacteria examined in this study was detected in 92% of the group 2 samples. This result also disagrees with the findings of previous studies that assessed the endodontic microbiota of primary teeth using microbial culture techniques. Cohen et al (1960)[1] found these species in only 17% of the root canals. This difference in prevalence might be attributed to the greater sensitivity of checkerboard DNA-DNA hybridization compared to conventional culturing methods.

Of all bacterial strains evaluated in this study, *C. rectus* was the most prevalent (90%). This outcome contrasts with the findings of previous studies in permanent teeth, which reported that the prevalence of this species ranged from 7% to 17%.[10,12]

Based on *their* prevalence in the microbial population detected in group 2 samples, it may be inferred the following pathogens are components of the microbiota of root canals of primary teeth with apical periodontitis: (1) black-pigmented rods (51%); (2) streptococci (82%); (3) gram-negative anaerobes (90%); (4) gram-negative facultative bacteria (92%); and (5) facultative bacteria (96%).

CONCLUSIONS

The root canals of human primary teeth examined in this *in vivo* study exhibited a great bacterial diversity—characterizing a polymicrobial endodontic infection similar to that found in permanent teeth—with presence of anaerobic and facultative bacteria, black-pigmented rods, and streptococci. A large number of anaerobic species were detected in teeth with necrotic pulp and apical periodontitis, and a significantly smaller number of bacterial cells were found in teeth with irreversible pulpitis.

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