

An investigation of the presence of specific anaerobic species in necrotic primary teeth

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Abstract: Different microbial identification methods have shown that the microbial community profiles in endodontic infections are diverse and assorted. The aim of this study was to evaluate the frequency of selected endodontic pathogens in the pulp chambers (PCs) and root canals (RCs) of infected primary teeth using PCR methods. Paired PC and RC samples were collected from 15 subjects and analyzed by PCR for the presence of *Filifactor alocis*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Porphyromonas endodontalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Prevotella tanneriae*, *Tannerella forsythia*, *Treponema denticola*, and *Treponema socranskii*. The frequency of each species was determined in the PC and RC of each case. The species most frequently detected in PCs were *P. nigrescens* (86.7%), *P. gingivalis* (73.3%), and *F. alocis* (73.3%). Of the PC samples, 13.3% contained *P. micra* and *T. denticola*, and 6.7% contained *T. forsythia*. The species most frequently detected in RCs were *P. gingivalis* (100%) and *P. nigrescens* (93.3%). *P. tanneriae*, *P. micra*, and *T. denticola* were found in 40% of the RC samples; *T. forsythia* was found in 26.7% of the RC samples. The “red complex”, which comprises *P. gingivalis*, *T. denticola*, and *T. forsythia*, was not found in the PC of any tooth but was found in 30% of the RC samples. The detection of *P. nigrescens* in the PC was statistically associated with the presence of *P. nigrescens* in the RC ($p = 0.04$). The results suggest high heterogeneity among the samples, even among those from the same subject.

Descriptors: Tooth, Deciduous; Endodontics; Bacteria, Anaerobic; Polymerase Chain Reaction.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

Endodontic infections of primary teeth are related to bacterial incursion and multiplication in the pulp chamber (PC) and root canals (RCs). Depending on the virulence and number of microorganisms present in RCs, acute or chronic inflammation may be established in the periapical region.^{1,2}

In infected primary teeth, lesions normally develop in the furcation area instead of around the tooth apex, which could be related not only to the high incidence of accessory furcation canals³ but also to infections with certain bacteria at these sites. However, few studies have been performed using molecular methods to identify the presence of anaero-

Submitted: Sep 26, 2012
 Accepted for publication: Dec 21, 2012
 Last revision: Jan 18, 2013

bic bacteria in RCs of necrotic primary teeth.^{4,5} In addition, there are no reports in the literature that compare the microbial composition of PCs with that of RCs of infected primary teeth. Many anaerobic microorganisms are difficult to culture and identify precisely; thus, molecular methods have been used to identify microorganisms from endodontic infections.⁶

The aim of this study was to detect the presence of *Filifactor alocis*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Porphyromonas endodontalis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Prevotella tanneriae*, *Tanerella forsythia*, *Treponema denticola*, and *Treponema socranskii* in the PCs and RCs of primary teeth, as well as to compare the prevalence of these species in both environments.

Methodology

Study sample

The present research was approved by the Research Ethics Committee of the Pelotas Dental School (protocol no. 126/2009, Federal University of Pelotas, Brazil), and informed consent agreements were obtained from the parents of the children involved in this study. Fifteen subjects ranging from 3 to 8 years of age who presented with pulp necrosis in primary molars were selected at the Department of Pediatric Dentistry (UFPel). The following information was recorded for each patient, according to Jacinto *et al.*:⁷

- age,
- gender,
- previous episodes of pain,
- presence of tooth mobility,
- sinus tract,
- presence of swelling on periodontal tissues,
- presence or absence of periapical/interradicular bone resorption,
- RC status during sampling (such as dry or wet canals), and
- presence or absence of a foul odor.

The selected teeth had not received previous endodontic treatment. Children who received antibiotic treatment within the preceding 3 months or who

presented with systemic diseases were excluded from the study. Teeth with exposure of the PC to the oral cavity before pulp management and teeth with internal resorption were excluded from the study. All of the teeth had intact roots or resorption of less than one-third of the physiological root. In addition, all selected molars had clinical crowns that permitted effective rubber dam isolation and only occlusal caries, without involvement of proximal surfaces, and to an extent that did not expose the PC to the oral cavity. There was an absence of a history of trauma associated with the selected teeth and an absence of periodontal involvement.

Clinical procedures

Clinical procedures and sample collection procedures were adapted from Jacinto *et al.*⁷ After application of local anesthesia, antiseptics of the child's oral cavity was performed with 0.12% chlorhexidine gluconate. The involved tooth received coronary polishing and was isolated with a rubber dam. The tooth surfaces, clamp, rubber dam, and arch were disinfected with sterile swabs soaked first in 30% hydrogen peroxide followed by 2.5% sodium hypochlorite for 30 seconds each, and then were neutralized with a sterile 5% sodium thiosulfate solution.⁸ After disinfection, a sample was taken with a sterile swab from the operational field and was analyzed by PCR. Carious tissues were removed using sterile burs, and preparation for complete access was carried out with an Endo-Z stainless steel bur (Dentsply Maillefer, Ballaigues, Switzerland) at high speed under manual irrigation with sterile 0.9% (w/v) sodium chloride until coronal access was gained.

Sample collection

Samples were taken from the PC of each tooth as follows: a sterile cotton ball moistened in sterile physiologic solution was inserted in the PC and kept in contact with the chamber floor for 60 seconds and then transferred to an empty sterile 1.5-mL tube (Axygen, Union City, USA). Then the PC was disinfected with cotton balls soaked in 2.5% sodium hypochlorite, which was inactivated with 5% sodium thiosulfate. Afterwards, three sterile absorbent paper points were sequentially placed into

the full length of the largest canal (palatal canal of maxillary molars and distal canal of mandibular molars)⁹ for 60 seconds and were then transferred to an empty sterile 1.5-mL tube. If the canal was dry, the paper point was moistened in sterile saline solution before transfer to the tube to ensure viable sample acquisition. The samples were stored at -80°C . All teeth involved in the study were endodontically treated after sampling. The technique for primary tooth endodontic treatment followed the American Association of Pediatric Dentistry guidelines,¹⁰ and all teeth were restored with modified glass ionomer resin (Vitremmer; 3M-ESPE, St. Paul, USA).

Detection of target species by PCR

PCR reactions were conducted according to Montagner *et al.*¹¹ Bacterial DNA was extracted with the PureLink Genomic DNA Mini kit (Invitrogen, Carlsbad, USA). The universal bacterial primers EuF and EuR, which are directed to the 16S rRNA

gene, were used to verify the presence of bacterial DNA in the samples, and specific primers were used to investigate specific species: *F. alocis*, *F. nucleatum*, *P. micra*, *P. endodontalis*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *P. tanneriae*, *T. forsythia*, *T. denticola*, and *T. socranskii* (Table 1). For each sample from the PC and RC, the PCR reaction was performed with 1.5 μL of extracted DNA added to the reaction mixture. The mixture consisted of 2.5 μL 10 \times reaction buffer, 0.75 μL 100 mM forward primer, 0.75 μL 100 mM reverse primer, 0.5 μL 25 mM dNTPs, 1.25 μL 25 mM MgCl_2 , 0.125 μL 5 U/mL Taq polymerase, and 17.625 μL milli-Q water.

The steps of the PCR cycle included an initial denaturation (95°C , 2 minutes); 36 cycles of denaturation (94°C , 30 seconds), annealing (temperature according to Table 1, 1 minute), and extension (72°C , 2 minutes); and a final extension (72°C , 10 minutes). PCR reactions were performed in a thermocycler (Mastercycler Family; Brazil Eppendorf;

Table 1 - Primer sequences, according to Montagner *et al.*,¹¹ and annealing temperature of each primer set used to detect 11 bacterial species.

Species	Sequence	Size (bp)	A.T.
Non-specific (EuF/EuR)	5' TCC TAC GGG AGG CAG CAG T 3' 5' GGA CTA CCA GGG TAT CTA ATC CTG TT 3'	466	60°C
<i>F. alocis</i>	5' AAA CCC ATC TCT GAG TTC TTC TTC 3' 5' ATG CCA ACT TGA CGT TAA AT 3'	594	60°C
<i>F. nucleatum</i>	5' ATT GTG GCT AAA AAT TAT AGT T 3' 5' ACC CTC ACT TTG AGG ATT ATA G 3'	1000	55°C
<i>P. micra</i>	5' AGA GTT TGA ATC CTG GCT CAG 3' 5' ATA TCA TGC GAT TCT GTG GTC TC 3'	207	60°C
<i>P. endodontalis</i>	5' GCT GCA GCT CAA CTG TAG TC 3' 5' CCG CTT CAT GTC ACC ATG TC 3'	672	58°C
<i>P. gingivalis</i>	5' AGG CAG CTT GCC TAG AGT CGG 3' 5' ACT GTT AGC AAC TAC CGA TGT 3'	404	58°C
<i>P. intermedia</i>	5' TTT GTT GGG GAG TAA AGC GGG 3' 5' TCA ACA TCT CTG TAT CCT GCG T 3'	575	58°C
<i>P. nigrescens</i>	5' ATG AAA CAA AGG TTT TCC GGT AAG 3' 5' CCC ACG TCT CTG TGG GCT GCG A 3'	804	58°C
<i>P. tanneriae</i>	5' CTT AGC TTG CTA AGT ATG CCG 3' 5' CAG CTG ACT TAT ACT CCC G 3'	550	55°C
<i>T. forsythia</i>	5' TGC TTC AGT AGT TAT ACC T 3' 5' TGC TTC AGT GTC AGT TAT ACC T 3'	641	56°C
<i>T. denticola</i>	5' TAA TAC CGA ATG TGC TCA TTT ACA T 3' 5' TCA AAG AAG CAT TCC CTC TTC TTC TTA 3'	316	60°C
<i>T. socranskii</i>	5' GAT CAC TGT ATA CGG AAG GTA GAC A 3' 5' TAC ACT TAT TCC TCG GAC AG 3'	288	56°C

A.T.: annealing temperature; bp: base pairs.

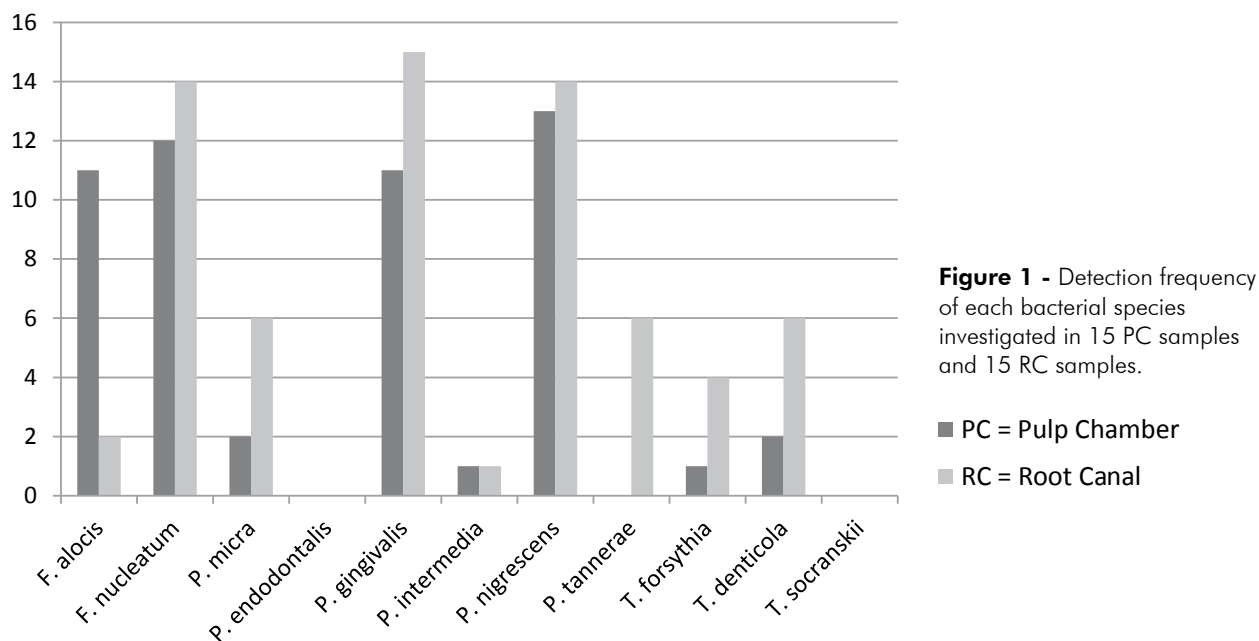


Figure 1 - Detection frequency of each bacterial species investigated in 15 PC samples and 15 RC samples.

■ PC = Pulp Chamber
 ■ RC = Root Canal

São Paulo, Brazil). Aliquots of extracted DNA from ATCC strains and aliquots of ultrapure water were used as positive and negative controls, respectively. The reference strains were as follows:

- *F. alocis* ATCC 35896,
- *F. nucleatum* ATCC 25586,
- *P. micra* ATCC 33270,
- *P. endodontalis* ATCC 35406,
- *P. gingivalis* ATCC W83,
- *P. intermedia* ATCC 25611,
- *P. nigrescens* ATCC 33563,
- *P. tanneriae* ATCC 51259, and
- *T. forsythia* ATCC 43037.

PCR products were analyzed by 1% agarose gel electrophoresis stained with GelRed (Biotium, Hayward, USA) and viewed under ultraviolet light transillumination (Major Science, Saratoga, USA). Detection was based on the presence of clear bands of the expected molecular size using a 50-bp DNA ladder (Invitrogen Corporation).

Statistical analysis

The data collected for each subject were entered into a spreadsheet and statistically analyzed using SPSS for Windows (SPSS Inc., Chicago, USA). Fisher's exact test was used to test the null hypothesis

that there was no statistically significant association between the presence of specific species in the PCs and RCs. The level of significance was 5% ($p < 0.05$).

Results

All samples were positive for the presence of bacterial DNA when the primers directed to the 16S rRNA gene were used. Figure 1 shows the frequency of the 11 species found by species-specific 16S rRNA-directed PCR among the 15 samples from both the PCs and RCs. In general, more species were detected in the RC (mean, 3.6) than in the PC (mean, 2.7). The highest number of species found in the same tooth was seven, and all teeth presented at least one of the target species, either in the PC or in the RC. The species most frequently detected in the PC were *P. nigrescens* (86.7%), *P. gingivalis* (73.3%), and *F. alocis* (73.3%). Of the PC samples, 13.3% contained *P. micra* and *T. denticola*, and 6.7% contained *T. forsythia*. The species most frequently detected in RC samples were *P. gingivalis* (100%) and *P. nigrescens* (93.3%). *P. tanneriae*, *P. micra*, and *T. denticola* were found in 40% of RC samples; *T. forsythia* was found in 26.7%, *F. alocis* in 13.3%, and *P. intermedia* in 6.7% of RC samples. The species *T. socranskii* and *P. endodontalis* were

not detected in RC or PC samples.

The concomitant presence of microbial species in PC and RC paired samples occurred in some cases. The detection of *P. nigrescens* in the PC was statistically associated with the presence of *P. nigrescens* in the RC ($p = 0.04$). Interradicular bone resorption was detected in nine cases; the most frequent species that were associated with these cases were *F. alocis* and *P. nigrescens* in the PC and *P. gingivalis* and *P. nigrescens* in the RC.

There was no statistically significant association between the presence of bacteria and clinical signs and symptoms.

The simultaneous presence of *P. gingivalis*, *T. forsythia*, and *T. denticola*, which together form the “red complex”,¹² was not found in the PC of any teeth but was found in the RC in three cases.

Discussion

The RCs of primary teeth with necrotic pulp and chronic interradicular/apical lesions present a high number of bacterial species and often exhibit polymicrobial infection with a high prevalence of anaerobic bacteria.^{2,13,14} In the present study, bacterial DNA was detected in all PC and RC samples investigated. Samples collected from the operational field were DNA-free, confirming that the DNA detected in the PC and RC samples did not result from trace external contamination.

The most commonly isolated genera in endodontic infections include *Fusobacterium*, *Prevotella*, and *Porphyromonas* spp.¹⁵ In the present study, *P. gingivalis* was detected in all RCs investigated, and species such as *P. nigrescens* were also highly detected. Tavares *et al.*⁴ detected *P. intermedia* in 96.9% of the samples from the RC system of primary teeth exhibiting pulp necrosis with or without radiographically detectable interradicular bone resorption, followed by other obligate anaerobes, such as *P. nigrescens*, *T. forsythia*, *P. denticola*, and *F. nucleatum ss vincenti*. In a study by Ruvieri *et al.*,¹⁶ *Campylobacter rectus*, *T. denticola*, and *Gemella morbillorum* were the most prevalent taxa, whereas Cogulu *et al.*⁵ found that *T. denticola* and *P. gingivalis* were the most prevalent species.

The most commonly isolated Gram-positive coc-

ci from RCs of primary teeth include *Peptostreptococcus* spp.¹⁷ The present study found the presence of *P. micra*, which formerly belonged to the genus *Peptostreptococcus*, in 40% of the RCs. *P. micra* is associated with the pathogenesis of *P. gingivalis* in endodontic abscesses.¹⁸ Ruvieri *et al.*¹⁶ detected *P. micra* in 26% of the primary teeth examined, whereas Cogulu *et al.*⁵ detected this species only in 3% of primary teeth exhibiting periapical/interradicular radiolucency. The differences in these results could be attributed to the different sets of primers used or the different features of the samples.

Socransky *et al.*¹² stratified the periodontal microbiota into groups or complexes. The “red complex” is part of the climax community in biofilms and comprises species that are considered oral pathogens, namely *P. gingivalis*, *T. denticola*, and *T. forsythia*. These species were found in association in three RC samples in this study.

The occurrence of endodontic symptomatology may be the result of an increased virulence of microorganisms. Cogulu *et al.*⁵ found that *T. denticola* and *Enterococcus faecalis* are highly associated with previous pain and that *P. gingivalis* is associated with tenderness to percussion in primary teeth. However, the different incidences of the microorganisms detected in the present report, as compared with those of other studies that used different methods for microbial identification, suggest that, similar to infections in RCs of permanent teeth, the microbial composition of endodontic infections of primary teeth is heterogeneous. This finding confirms that it is not possible to attribute the etiology of these pathologies to specific microorganisms.

Even though DNA sequence analysis is the gold standard in microbial identification, PCR also represents a sensitive, fast, and accessible method for the study of endodontic bacteria.¹⁹ Therefore, PCR was used in the present study. A disadvantage of this method is that by targeting specific bacteria, it does not allow a broad examination of the microbial profiles related to these particular PCs and RCs. However, based on our results, it is possible to speculate that PCs and RCs exhibit different microbial profiles. Furthermore, the anaerobes investigated generally had a higher prevalence in the RCs than in the PCs, possi-

bly because of factors such as the gradual decrease in oxygen tension in RCs, together with the nutritional needs of microorganisms and the food chain that influence the process of bacterial succession from the saccharolytic cariogenic flora in the PCs to a more anaerobic and proteolytic flora in the RCs.

The ample medullar bone spaces of children favor infection dissemination. In rare situations, bacteria and their toxic products that enter the trabecular bone in the interradicular area of primary teeth²⁰ and induce acute inflammation and pus formation²¹ might lead to a spread of these dental abscesses, threatening the life of the child. Another and more likely consequence of pulp necrosis in primary teeth is that these infections may potentially affect the permanent tooth germ.²² In the present study, no statistical association was found between specific species and the presence of radiolucent interradicular areas. When analyzing 79 patients with infected primary teeth, Cogulu *et al.*⁵ found that *T. denticola* and *E. faecalis* are highly associated with periapical radiolucency. These differences in results could be attributed to sample size. Furthermore, in cases with interradicular radiolucency in the present

study, *P. gingivalis* was found in five PC and nine RC samples, and *P. nigrescens* was found in six PC and eight RC samples. The pathogenesis of these species, especially when associated with other anaerobes, may influence bone loss in the interradicular area.

Conclusion

The role of bacteria in lesion pathogenesis is undeniable, although modern diagnostic techniques have not identified a single causative pathogen. The results suggest a high bacterial heterogeneity among PCs and RCs. Strict anaerobes were frequently detected in PC and RC samples, but specific anaerobic species were not associated with interradicular radiolucent lesions or other signs and symptoms of infection. Therefore, studies comprising a larger number of cases and a wider range of species are necessary to investigate these associations.

Acknowledgements

We would like to thank the CCDB - Pelotas Dental School, Federal University of Pelotas and CNPq (483809/2007-1).

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