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Porphyromonas gingivalis, Porphyromonas endodontalis, Prevotella intermedia and *Prevotella nigrescens* in endodontic lesions detected by culture and by PCR

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The aim of this study was to investigate the presence of four black-pigmented bacteria, *Porphyromonas gingivalis, Porphyromonas endodontalis, Prevotella intermedia* and *Prevotella nigrescens*, in endodontic infections by culture and polymerase chain reaction (PCR) analyses. Microbial samples were obtained from 50 teeth with untreated necrotic pulps (primary infection) and from 50 teeth with failing endodontic treatment (secondary infection). Microbiological strict anaerobic techniques were used for serial dilution, plating, incubation, and identification. For PCR detection, the samples were analyzed using species-specific primers of 16S rDNA and the downstream intergenic spacer region. Culture and PCR detected the test species in 13/100 and 50/100 of the study teeth, respectively. The organisms were cultured from 11/50 (22%) of primarily infected root canal samples and from 2/50 (4%) of secondary root canal samples. PCR detection identified the target species in 32/50 (64%) and 18/50 (36%) of primary and secondary infections, respectively. *P. gingivalis* was rarely isolated by culture methods (1%), but was the most frequently identified test species by PCR (38%). Similarly, *P. endodontalis* was not recovered by culture from any tooth studied, but was detected by PCR in 25% of the sampled teeth. PCR-based identification also showed higher detection rates of *P. intermedia* (33%) and *P. nigrescens* (22%) than culture (13%). In conclusion, *P. gingivalis, P. endodontalis*, *P. intermedia*, and *P. nigrescens* were identified more frequently in teeth with necrotic pulp than in teeth with failing endodontic treatment. Also, a higher frequency of black-pigmented species was detected by PCR than by culture.

Key words: black-pigmented anaerobes; endodontic pathosis; *Porphyromonas*; *Prevotella*

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Black-pigmented gram-negative anaerobic bacteria are relatively common in infected root canals and endodontic abscesses, and their presence is often accompanied by swelling and pain to percussion or palpation (7, 10–16, 28–36). Endodontopathogenic properties of black-pigmented anaerobic bacteria may include fimbriae, capsules, outer membrane proteins and, in particular, endotoxic lipopolysaccharides (5).

Culture and molecular methods are used to detect bacterial species in root canal infections. Bacterial culture identifies the predominant species and has played a key role in the association of specific bacteria

with signs and symptoms of endodontic infections (10–13, 15, 24, 35, 36). However, anaerobic cultivation is expensive, time-consuming, labor-intensive, and requires viable bacteria. On the other hand, molecular techniques can detect uncultivable or difficult-to-grow bacteria. However, a major limitation of molecular identification methods is the detection of only targeted microbial species.

Molecular techniques, particularly polymerase chain reaction (PCR), have been used to detect black-pigmented bacteria in primary endodontic infections and in teeth with failing endodontic therapy (3, 8, 27–30). PCR-based identification studies have reported a higher incidence of black-pigmented bacteria in endodontic infections when compared to cultural studies (12, 15, 25, 33, 35). However, few studies have compared the ability of culture and PCR to detect bacterial species in the same endodontic sample. Therefore, this study investigated the presence of four black-pigmented anaerobic bacterial species – *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia*, and *Prevotella nigrescens* – in teeth with primary and secondary root canal infections having periapical pathosis using culture and PCR analyses.

Material and methods

Study patients

A total of 100 patients in need of endodontic treatment were obtained from the Dental School of Piracicaba, SP, Brazil. No study patient had received antibiotic treatment during the preceding 3 months or had a systemic disease. The Human Volunteers Research and Ethics Committee of the Dental School of Piracicaba approved the study, and all patients signed an informed consent.

Clinical features

Fifty teeth with no prior endodontic treatment had necrotic pulp therapy (primary infection), and 50 teeth endodontic therapy more than 4 years previously but showed radiographic evidence of apical periodontitis (secondary infection). Of the 100 study teeth, 61 presented coronal restorations, of which 49 were defective (i.e. restorations with open margins, fracture or recurrent decay). Thirty-nine teeth exhibited open root canals.

Age, gender, tooth type and pulp status were recorded for each patient. Clinical symptoms and signs included history of previous pain, tenderness to percussion, pain on palpation, mobility, presence of a sinus and its origin (endodontic or periodontal), presence of swelling of the periodontal tissues, probing depth of periodontal pockets, history of previous and present antibiotic therapy and any other relevant medication, radiographic findings, and the internal status of the canal such as dry canal or the presence of clear, hemorrhagic or purulent exudates, detected as a distinct dampening or stain on the sampling paper points. Each type of exudate was analyzed separately and also grouped with the other types under the denomination 'wet canal'.

Sampling procedure

The microbiological procedures used in this study have been previously described (11, 12). Aseptic techniques were used throughout the endodontic sample acquisition. Briefly, after a two-stage access cavity preparation, which was made under manual irrigation with sterile saline solution and employing sterile burs, the study teeth were individually isolated from the oral cavity with a rubber dam. Teeth that could not be fully isolated with a rubber dam were excluded from the study. Teeth and rubber dam were disinfected with 30% hydrogen peroxide followed by 2.5% sodium hypochlorite. The sterility of the operation field was checked after inactivation of the antiseptic solution with 5% sodium thiosulfate. Preexisting root-canal fillings were removed using Gates Glidden drills (Maillefer, Ballaigues, Switzerland) and endodontic files without the use of chemical solvents. Irrigation with sterile saline solution was performed in order to remove any remaining treatment material prior to sample collection. Sampling included a single root canal, even in the multirooted teeth, in order to confine the microbial evaluation to a single ecologic environment. The criterion used to choose the canal to be microbiologically investigated in the multirooted teeth was the presence of exudation or, in its absence, the largest canal or the canal with periapical radiolucency.

For microbial sampling, a sterile paper point was introduced into the full length of the root canal, as determined in a preoperative radiograph, and kept in place for 60 s. In the case of a dry root canal, a second paper point, moistened in sterile saline solution, was used to ensure adequate sample acquisition. In the case of a wet root canal, as many paper points as needed to absorb all fluid inside the canal were employed.

The paper points were immediately transferred to a test tube containing 1 ml of the VMGA III transport medium (6) and placed within 15 min inside an anaerobic workstation (Don Whitley Scientific, Bradford, UK). After thoroughly shaking the endodontic sample in a mixer for 60 s (Vortex, Marconi, São Paulo, SP, Brazil), 250 μl of the transport medium was used for cultivation, and 750 μl (including the paper points) was frozen for PCR analysis. Sample collection and processing took place within 4 h.

Microbial isolation

In the anaerobic chamber, the endodontic samples were serially diluted 10-fold in tubes containing Fastidious Anaerobe Broth (FAB, Laboratory M, Bury, UK). A 50 μl sample of each serial dilution as well as of the undiluted sample were plated, using sterile plastic spreaders, onto 5% defibrinated sheep blood Fastidious Anaerobe Agar (FAA, Laboratory M) containing 1 ml/l of hemin and 1 ml/l of Vitamin K1. Bacterial plates were incubated at 37°C under anaerobic conditions (in 10% CO_2, 10% H_2, and 80% N_2) for up to 14 days. Samples were also plated onto 5% sheep blood Brain Heart Infusion (BHI) agar (Oxoid, Basingstoke, UK) to allow growth of aerobic and facultatively anaerobic microorganisms. The BHI plates were aerobically incubated at 37°C for 2 days.

Microbial identification

From each bacterial plate, representative colonies of each morphologic type were subcultured. Pure cultures were initially characterized according to their Gram stain characteristic, ability to produce catalase, and gaseous requirements. Black-pigmented colonies were then selected for further identification using the Rapid ID 32 A kit (Bio Merieux, Marcy-l'Etoile, France), colony fluorescence under long-wave (366 nM) UV light, hemagglutination of 3% sheep erythrocytes, lactose fermentation using the fluorogenic substrate 4-methylumbelliferyl-galactoside (Sigma Chemical Co., St. Louis, MO; M-1633) (1), and trypsin-like activity as detected by the synthetic fluorogenic peptide 7-(N-carbobenoxy-glycylglycylarginin-7-amido)-4-methyl coumarin hydrochloride (Sigma Chemical Co.; C-9396) (22).

For PCR detection of black-pigmented species, DNA was extracted according to Leys *et al*. (18). DNA isolated from the root canal specimens was first amplified

with prokaryotic universal ribosomal 16S and 23S primers (785 and 422, respectively), as described elsewhere (17, 21). Amplification was then performed of the 16S rDNA and the downstream intergenic spacer region (ISR). Inclusion of the ISR provided an additional check of the specificity of primers, since the length of the ISR region varies among bacterial species. *P. gingivalis, P. endodontalis, P. intermedia*, and *P. nigrescens s*pecies were then identified by a second, nested amplification with species-specific 16S primers paired with a universal primer located in the 23S gene (L189) (Fig. 1). Table 1 shows the primer sequences. All primers were synthesized by Biosynthesis (Lewisville, TX).

PCR reactions were performed in a total volume of 50 µl containing 1.25 U *Taq* DNA polymerase (Perkin-Elmer, Foster City, CA), 5 µl of 10X PCR buffer plus 3 mM $MgCl_2$, 0.25 mM of each primer and 0.2 mM (each) deoxynucleoside triphosphates. For the first amplification, samples were subjected to 22 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 2 min, and primer extension at 72°C for 3 min, and a final extension of 72°C for 10 min. For the second amplification, the PCR reaction conditions were 30 cycles of 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min. PCR amplification was carried out in an automated thermal cycler (Perkin-Elmer Cetus).

PCR products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, and viewed under UV translumination. A positive or negative identification was based on the presence of clear bands of the expected molecular size (Fig. 2) using a 21 kb lambda DNA ladder (Invitrogen Corporation, Carlsbad, CA). Each assay was repeated once, and in case of disagreeing results, the assay was repeated once more.

PCR primer specificity

Species-specific primers in the 16S rDNA coding region were selected based on sequences available in GenBank. The species specificity was confirmed by sequencing at least one PCR product from a clinical sample for each primer in an ABI Prism 310 automated sequencer (AME Bioscience Ltd, London, UK), and comparing the sequence generated with those available in GenBank.

Results

Table 2 shows the detection rates of *P. gingivalis*, *P. endodontalis*, *P. intermedia*, and *P. nigrescens* according to identification method and clinical endodontic status. Culture and PCR detection methods identified at least one of the test species in 13/100 and 50/100 root canals, respectively. Culture yielded the test organisms in 11/50 (22%) of root canal samples from primary infections and in 2/50 (4%) root canal samples from secondary infections. PCR yielded the test organisms in, respectively, 32/50 (64%) and 18/50 (36%) of the primary and secondary root canal infections studied. Additional black-pigmented species were cultured, including *Prevotella corporis, Prevotella loescheii, Prevotella denticola*, and *Prevotella melaninogenica.* Furthermore, black-pigmented species were always recovered in association with other species.

Six samples yielded the same test organisms by culture and PCR identification, 44 samples only revealed the test organisms by PCR, and seven samples showed *P. intermedia*/*P. nigrescens* by culture but not by PCR. In the 50 samples positive by PCR identification, 10 yielded two species, nine yielded three species, and 13 revealed all four target species.

By PCR identification, one or more black-pigmented species were detected in 65.8% of teeth with pain on palpation, in 76.7% of teeth with swelling, and in 71.4% of teeth with purulent exudates. In teeth with root-filled canals, black-pigmented bacteria were present in 2/2 cases with acute symptoms (pain to palpation and purulent exudates), and in 6/15 cases with tenderness on percussion; but the organisms also occurred in 10/35 asymptomatic teeth.

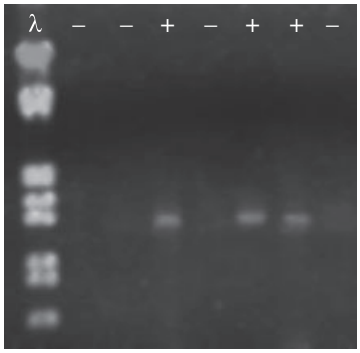

Fig. 2. Amplified *P. endodontalis* DNA fragments. The markers in lane 1 are *Eco*RI and *Hind*III digestion products of bacteriophage lambda DNA. The other lanes contain DNA amplified with *P. endodontalis* specific primer, which appears at 1.3 kb. Scoring for the presence of *P. endodontalis* (+ or –) are indicated in the diagram above the gel.

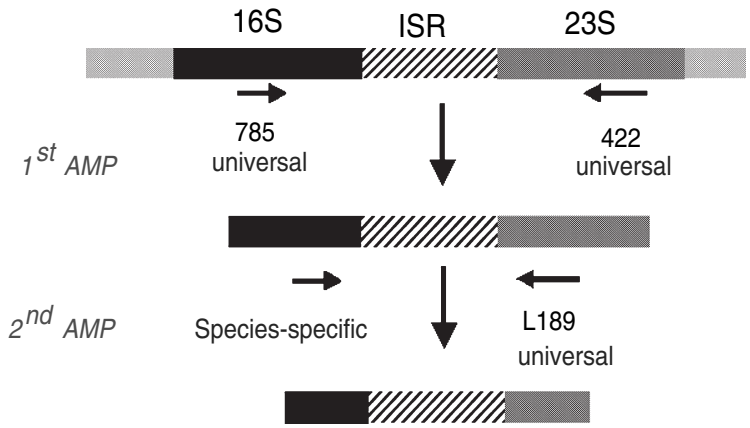

Fig. 1. Schematic representation of the prokaryotic ribosomal operon and the locations and orientations of the primers and resulting DNA fragments. Universal primers 785 and 422 were used to generate a DNA fragment in a first nonspecific amplification step, and species-specific primer and the universal primer L189 were used in a second amplification.

Table 1. Primers used in this study

Primers	Specificity/location/orientation	Sequence
PRIN	*Prevotella intermedia*/Pi/16S/Forward	TGTTAGCGCCTTGCGCTA
PRNI	*Prevotella nigrescens*/Pn/16S/Forward	CGTTGGCCCTGCCTGCGG
Pg13	*Porphyromonas gingivalis*/Pg/16S/Forward	CATCGGTAGTTGCTAACAGTTTTC
Pe1	*Porphyromonas endodontalis*/Pe/16S/Forward	TTTAGATGATGGCAGATGAGAG
Sm785	Universal primer/16S/785 bp from 5′end/Forward	GGATTAGATACCCTGGTAGTC
422	Universal primer/23S/422 bp from 5′end/Forward	GGAGTATTTAGCTT
L189	Universal primer/23S/Forward	GGTACTTABATGTTTCAGTTC

Table 2. Prevalence of four black-pigmented bacteria in root canals with untreated necrotic pulp and with failed endodontic treatment using culture and PCR analyses

	Culture			PCR		
Bacteria	Necrosis*	Failed treatment*	Total*	Necrosis*	Failed treatment*	Total*
P. intermedia/P. nigrescens	11/50(22%)	2/50(4%)	13/100(13%)	–	–	–
P. intermedia	–	–	–	28/50(56%)	5/50(10%)	33/100(33%)
P. nigrescens	–	–	–	16/50(32%)	6/50(12%)	22/100(22%)
P. gingivalis	1/50(2%)	0	1/100(1%)	22/50(44%)	16/50(32%)	38/100(38%)
P. endodontalis	0	0	0	14/50(28%)	11/50(22%)	25/100(25%)
Total black-pigmented species**	11/50(22%)	2/50(4%)	13/100(13%)	32/50(64%)	18/50(36%)	50/100(50%)

***** Number of positive cases/number of teeth examined.
****** One or more species of black-pigmented bacteria isolated per root canal.

Discussion

In the present study, the black-pigmented species studied were recovered from 13% and 50% of the root canals examined by culture and PCR analyses, respectively. This finding confirmed those reported in previous studies (2, 19, 20), showing a higher sensitivity of the PCR technique than of culture for detecting black-pigmented anaerobes. The higher prevalence of black-pigmented species by PCR analysis may be due to the possible loss of difficult-to-grow bacteria during culture procedures (32).

P. gingivalis, which was rarely isolated by culture methods (1%), was the most frequently identified test species by PCR (38%). *P. endodontalis*, which was not recovered by culture from any root canal, was identified in 25% of the teeth by PCR. Previous studies using culture methods have also revealed a low recovery of *P. gingivalis* and *P. endodontalis* from endodontic infections (4, 10, 14, 15). *Porphyromonas* species are fastidious and oxygen-sensitive microorganisms, which may explain the failure to culture these species (35). It may also be that some test bacteria were killed during the disinfection procedures of the teeth. PCR-based identification, not being dependent on bacterial viability, may not be as technique-sensitive as culture (30).

PCR identification has the ability to differentiate strains of the species *P. intermedia* and *P. nigrescens*, which is not possible using culture and current commercial identification kits (2, 9, 26). In this study, 13 strains were identified by culture as *P. intermedia/P. nigrescens*. PCR identification detected one species in three samples and both species in three samples, but seven culture-positive samples did not reveal *P. intermedia* or *P. nigrescens*. The disagreement between PCR and culture results may be due to a possible misidentification by the commercial identification kit. The black-pigmented anaerobic organisms detected by culture may be in fact have belonged to another, closely related species, such as the newly described *Prevotella tannerae,* which may be a common pathogen in endodontic infections (37).

In this study, *P. gingivalis* was the most frequent test species found (38%), followed by *P. intermedia* (33%), *P. endodontalis* (25%), and *P. nigrescens* (22%). Siqueira *et al*. (29) reported a higher percentage of *P. endodontalis* (42.6%) and smaller occurrences of *P. intermedia* (5.6%) and *P. nigrescens* (7.4%). Conversely, Fouad *et al*. (8) found *P. nigrescens* to be the most frequent species (32%), followed by *P. endodontalis* (18%), *P. gingivalis* (9%), and *P. intermedia* (5%). These discrepancies may be due to differences in patient selection, sampling methodology, DNA extraction or PCR techniques. Also, differences in bacterial prevalence in endodontic infections may be related to geographical location (3).

Our study detected the target species in about 65% of teeth with pain and in about 75% of teeth with swelling or purulent exudates. Siqueira *et al*. (29) reported a similar occurrence of black-pigmented bacteria in purulent endodontic exudates. The black-pigmented species studied were detected at a higher frequency in teeth with necrotic pulp than in teeth with failing endodontic treatment, despite most study teeth with primary endodontic infection being symptomatic. In secondary endodontic infections, black-pigmented bacteria were identified in both symptomatic and asymptomatic teeth. Since the development of acute endodontic signs and symptoms depends on synergy between black-pigmented bacteria and other bacterial species, and also on the number of bacterial cells present in the root canal (34), the data of this study cannot shed light on the precise role of black-pigmented species in the pathogenesis of acute endodontic pathosis.

In conclusion, *P. gingivalis, P. endodontalis, P. intermedia*, and *P. nigrescens* were detected more frequently in untreated teeth with necrotic pulp than in teeth with failing endodontic treatment. Furthermore, PCR provided more frequently detected endodontic black-pigmented bacteria than did culture.

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