
Detection of *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia*, and *Prevotella nigrescens* in chronic endodontic infection

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Black-pigmented anaerobic rods such as *Prevotella* spp. and *Porphyromonas* spp. are involved in the etiology and perpetuation of endodontic infections. The aim of this study was to evaluate the prevalence of these species in chronic endodontic infections by using culture and polymerase chain reaction (PCR) techniques. Samples of 100 patients with root canals displaying chronic endodontic infections were obtained by sterilized paper points. Bacterial identification was performed by using culture and PCR techniques. By culture, in 33% of the samples, *P. intermedia*–*P. nigrescens* (75.8%), *P. gingivalis* (27.3%), and *P. endodontalis* (9.1%) were identified, and by PCR 60% of the samples harbored *P. nigrescens* (43.3%), *P. gingivalis* (43.3%), *P. intermedia* (31.7%), and *P. endodontalis* (23.3%). The presence of these black-pigmented anaerobic rods alone or in association in chronic endodontic infections seems to be frequent. PCR is a very sensitive technique for detecting DNA from bacterial cells. Culturing is only able to reveal living bacteria and is less sensitive for the identification of low numbers of bacterial cells. (**Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;103:285-8**)

The development of anaerobic techniques has led to considerable progress in clarifying the etiopathogenesis of endodontic infections, and it has been shown that most are polymicrobial in nature with a high prevalence of anaerobic bacteria.^{1,2} The black-pigmented gram-negative anaerobic rods, such as *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia*, and *Prevotella nigrescens*, have been isolated from endodontic infections in symptomatic and asymptomatic teeth.³⁻⁴ Moreover, it is known that these organisms represent important pathogens in destructive periodontal disease and have been recovered from several other infectious processes, such as in the respiratory tract, in the female genital and intestinal tracts, and in acute endodontic infections.^{5,6}

The genus *Prevotella* is constituted by 13 saccharolytic or moderate saccharolytic species, and *P. intermedia* and *P. nigrescens* are the organisms most often isolated from oral sites.⁷ *P. intermedia* appears to be related to the periodontal disease, and *P. nigrescens* is

related to the healthy oral cavity.⁸ The genus *Porphyromonas* includes 3 asaccharolytic species of human origin, *P. gingivalis*, *P. endodontalis*, and *P. asaccharolytica*, and it has been suggested that *P. gingivalis* and *P. endodontalis* are key pathogens in adult periodontitis and in root canal infections, respectively.⁹

Traditionally, bacterial identification has included culturing on solid or liquid media and growth-based biochemical methods, but those procedures are laborious, expensive, and time-consuming. In the last decade, molecular methods have been used to identify bacteria in clinical samples which have been shown to be rapid, sensitive, and accurate, allowing the detection of fastidious organisms and organisms that are impossible to culture at this time. The polymerase chain reaction (PCR) method is used to identify a variety of microorganisms, including periodontal or endodontic pathogens.

The aim of this study was to evaluate, by culture and PCR, the prevalence of *P. gingivalis*, *P. endodontalis*, *P. intermedia*, and *P. nigrescens* in patients with chronic periapical lesions and compare the findings.

MATERIAL AND METHODS

Patients and bacterial isolation

Samples were collected from root canals of 100 patients seen in the School of Dentistry of the University of São Paulo (USP). None of the patients had received antibiotic therapy during the preceding 3 months. Patients with systemic diseases or severe acute apical periodontitis were excluded. All patients gave written informed consent to be recruited to this study,

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Initially, in each patient a single root canal was chosen. The tooth was isolated by rubber dam and the tooth and the surrounding field were cleansed with 30% of hydrogen peroxide and decontaminated with a solution of 5% of iodine. The iodine was inactivated with 5% of sodium thiosulfate. The sterility of the tooth surface after cleaning and disinfection was evaluated by samples taken from the surface and processed by PCR.

After coronal access, the root canals were lightly irrigated with 0.9% of saline solution, avoiding flooding. The clinical specimens were aseptically obtained by inserting 4 sterile paper points (Dentsply, São Paulo, Brazil), remaining in the root canal for 60 s. Then 2 paper points were placed in a glass vial with a screw cap containing 3 mL of Möller's viability medium (VMGA III),¹⁰ prepared anaerobically and sterilized in oxygen-free gas (PRAS), and the 2 other paper points were placed into an Eppendorf tube containing 300 μ L sterile ultrapure water. Samples were processed within 4 h of sampling.

Bacterial detection by culturing.

The samples were vigorously vortexed. Undiluted and diluted (10^{-1} , 10^{-2} , 10^{-3}) samples (0.1 mL) of the VMGA III medium were streaked on trypticase soy agar (Difco Laboratories, Detroit, MI) supplemented with 5 μ g/mL hemin (Inlab, São Paulo, Brazil), 1 μ g/mL menadione (Inlab), 0.5% of yeast extract (Difco Laboratories), and 5% of defibrinated horse blood. Plates were incubated in anaerobic conditions (90% N_2 /10% CO_2), at 37°C for 14 days. One to five characteristic black colonies per plate were subcultured in horse blood agar to obtain pure cultures. Bacterial identification was performed by using a Rapid ID32A kit (bioMérieux, São Paulo, Brazil).

Bacterial detection by PCR

DNA extraction. The collected samples in 300 μ L sterile ultrapure water were vigorously mixed in vortex for 3 s and boiled for 10 min. After centrifugation at 14,000g for 10 min, the supernatant (DNA) was transferred to a new tube and used as template.

DNA amplification. DNA amplification was performed by using species-specific primer pairs as described by Bogen and Slots.¹¹ Amplifications were carried out by using final volumes of 25 μ L, containing 2.5 μ L of 10 \times PCR buffer, 1.25 μ L of 50 mmol/L $MgCl_2$, 1.0 μ L of 0.2 mmol/L dNTP, 1.0 μ L of 0.4 μ mol/L each primer, 0.25 μ L of 0.5 U Platinum Taq DNA polymerase (Invitrogen do Brasil, São Paulo, Brazil), 8 μ L sterilized ultrapure water, and 10 ng DNA. The primers used were as follows (5'... \rightarrow 3'): 1) to detect *P. gingivalis*, -AGG CAG CTT

GCC ATA CTG CG- and -ACT GTT AGC AAC TAC CGA TGT-; 2) to detect *P. endodontalis*, -GCT GCA GCT CAA CTG TAG TC- and -CCG CTT CAT GTC ACC ATC TC-; 3) to detect *P. intermedia*, -TTT GTT GGG GAG TAA AGC GGG- and -TCA ACA TCT CTG TAT CCT GCG T-; and 4) to detect *P. nigrescens*, -ATG AAA CAA AGG TTT TCC GGT AAG- and -CCC ACG TCT CTG TGG GCT GCG A-. Amplification reactions were carried out in a thermal cycler (GeneAmp PCR System 9700; Perkin Elmer, São Paulo, Brazil), programmed as follows: to detect *P. gingivalis* and *P. endodontalis* an initial denaturation step at 94°C for 5 min was included, followed by 36 cycles of a denaturation step at 94°C for 30 s, a primer annealing step at 60°C for 1 min, an extension step at 72°C for 1 min, and a final step at 72°C for 5 min. The same temperature profile used to detect *P. intermedia* and *P. nigrescens* was used to detect *P. gingivalis* and *P. endodontalis* but with an annealing temperature of 55°C. The expected amplicons were as follows: 404 bp for *P. gingivalis*, 672 bp for *P. endodontalis*, 575 bp for *P. intermedia*, and 804 bp for *P. nigrescens*. A negative control (without DNA) and a positive control (DNA of *P. gingivalis* ATCC 33277, *P. endodontalis* ATCC 35406, *P. intermedia* ATCC 25611, and *P. nigrescens* ATCC 33563) were included.

All PCR products were analyzed by 1% agarose gel electrophoresis performed at 60 V for 2.5 h, stained with 0.5 μ g/mL of ethidium bromide, and photographed under a UV transilluminator by using a Digital Kodak Science 120 system. A DNA ladder digest of 1 kb (Invitrogen, São Paulo, Brazil) was used as a molecular weight marker.

Statistic analyses

The statistic analyses were performed by using a binomial test to compare the efficiency of detection by culture and PCR techniques.

RESULTS

By using a standard culture technique, 33 root canal samples harbored at least 1 of the studied black-pigmented rods, and a total of 81 isolates were recovered. Furthermore, 75.6% of the samples harbored *P. intermedia*-*P. nigrescens*, 15.2% *P. gingivalis*, and 9.1% *P. endodontalis*. Moreover, bacterial associations in 21.2% of the samples (*P. intermedia*-*P. nigrescens*/*P. gingivalis*) and in 3% of them (*P. intermedia*-*P. nigrescens*/*P. endodontalis*) were observed.

Control samples of the tooth surface after cleaning and disinfection did not show bacteria or DNA detectable by PCR.

By using PCR, of the 60 analyzed samples the DNA of the following organisms were detected: *P. gingivalis* (43.3%), *P. nigrescens* (43.3%), *P. intermedia* (31.7%),

Table I. Bacterial identification of the 33 and 60 analyzed root canal samples by culture and polymerase chain reaction (PCR), respectively

Culture (n)	PCR (n)
<i>P. intermedia</i> - <i>P. nigrescens</i> (21)	<i>P. gingivalis</i> (14)
<i>P. gingivalis</i> (6)	<i>P. nigrescens</i> (13)
<i>P. endodontalis</i> (2)	<i>P. endodontalis</i> (8)
<i>P. intermedia</i> - <i>P. nigrescens</i> / <i>P. gingivalis</i> (3)	<i>P. intermedia</i> (5)
<i>P. intermedia</i> - <i>P. nigrescens</i> / <i>P. endodontalis</i> (1)	<i>P. intermedia</i> / <i>P. gingivalis</i> (5)
	<i>P. intermedia</i> / <i>P. nigrescens</i> (4)
	<i>P. nigrescens</i> / <i>P. gingivalis</i> (2)
	<i>P. gingivalis</i> / <i>P. endodontalis</i> (2)
	<i>P. nigrescens</i> / <i>P. endodontalis</i> (2)
	<i>P. intermedia</i> / <i>P. nigrescens</i> / <i>P. gingivalis</i> (3)
	<i>P. intermedia</i> / <i>P. nigrescens</i> / <i>P. endodontalis</i> (2)

and *P. endodontalis* (23.3%) (Table I). Additionally, double bacterial associations were observed as follows: *P. intermedia* with *P. gingivalis* (8.3%) or *P. nigrescens* (6.7%), *P. nigrescens* with *P. gingivalis* (3.3%), *P. nigrescens* with *P. endodontalis* (3.3%), and *P. gingivalis* with *P. endodontalis* (3.3%). Moreover, triple bacterial associations were also observed in 5 samples as follows: *P. intermedia* with *P. nigrescens* and *P. gingivalis* (5.0%) and *P. intermedia* with *P. nigrescens* and *P. endodontalis* (3.3%). The statistic analyses showed significant differences between PCR and culture techniques ($P = .0002$).

DISCUSSION

Culture and molecular methods are used to detect bacterial species in root canal infections. However, culture identifies predominant or specific species in endodontic infections,^{12,13} but the sensitivity to bacterial culturing can be rather low, especially for nonselective media, with detection limits averaging 10³-10⁴ bacterial cells. Molecular techniques are more sensitive and can detect uncultivable or difficult-to-growth bacteria. The major limitation, however, is that the technique used here is unable to differentiate between living bacteria and DNA remnants of dead bacteria. In addition, the technique only detects targeted microbial species.¹⁴

The presence of black-pigmented organisms in root canal infections by using culture and PCR techniques has been described previously.^{8,15} Siqueira et al.¹⁶ and Gomes et al.¹⁴ have analyzed root canal samples from teeth with necrotic pulp by using conventional culture technique and PCR, and they identified, by the respective techniques, black-pigmented anaerobic rods in 15% and 37.5% of the clinical samples. Data show that there are geographical differences in detecting these anaerobic organisms in endodontic infections, and dif-

ferences in clinical diagnosis, sampling, DNA extraction, sensitivity of primers, or PCR techniques must also be considered.¹⁵

The biochemical differentiation between *P. intermedia* and *P. nigrescens* is difficult because of their great phenotypical similarities. Furthermore, biochemical identifications are limited by the quality of the biochemical databases used. Polymerase chain reaction, however, is a suitable tool to distinguish both organisms.

In the 60 PCR samples, it was observed that *P. gingivalis* (43.3%) and *P. nigrescens* (43.3%) were the most prevalent, followed by *P. intermedia* (31.7%) and *P. endodontalis* (23.3%); however, when using the culture technique, the detection of *P. gingivalis* and *P. endodontalis* detection was lower (15.2% and 9.1%, respectively). This result can be explained because of the fastidious nature of those organisms, and it can overestimate their presence in some infections. Moreover, this finding confirmed previous studies^{17,18} showing a higher sensitivity of the PCR technique than culture for detecting black-pigmented anaerobic bacteria. On the other hand, differences may also occur when different methodologies of collection and identification are used.

Although the PCR technique returns more positive samples than culturing, this information must be carefully considered in each case, because PCR is unable to differentiate between living microorganisms and DNA traces of dead microbes. The PCR technique returns as positive the presence of viable culturable bacteria, nonculturable bacteria, dead bacteria, and DNA traces from bacteria.^{19,20}

Molecular techniques such as PCR are sensitive, rapid, and more convenient than conventional bacterial cultures, because the samples do not require a special transport.²¹ The high sensitivity of PCR makes it possible to detect a low number of bacterial cells, which often is an advantage. It may, however, record so few cells that it may be irrelevant in terms of pathogenicity.²² The use of specific primers represents an appropriate tool to identify or to detect endodontic organisms from mixed bacterial populations.

The presence of these anaerobic rods in chronic endodontic infections can suggest some role in the etiology and in the perpetuation of periradicular diseases. The studied organisms are constituents of mixed infections and do not seem to occur as mono-infection.^{8,9} Moreover, because bacterial associations such as *P. intermedia* with *P. gingivalis* or *P. nigrescens*, *P. nigrescens* with *P. gingivalis* or *P. endodontalis*, *P. endodontalis* with *P. gingivalis*, *P. intermedia* with *P. nigrescens* and *P. gingivalis*, and *P. intermedia* with *P. nigrescens* and *P. endodontalis* were observed, it is suggested that these associations are commonly found in endodontic processes. On the other hand, studies have shown that the bacterial participation in a mixed infection with consequent synergism appears as a

requirement to the pathogenicity of *Porphyromonas* spp. and *Prevotella* spp.²³

Several studies have shown the isolation of *P. gingivalis* and *P. endodontalis* from acute periapical lesions associated with untreated root canals; however, their high virulence could explain the involvement of the organisms in purulent periapical inflammation and in acute periapical exacerbations. *Porphyromonas endodontalis* has been almost exclusively associated with endodontic infections, and its pathogenicity seems to be dependent on the presence of the other species in a consortium.²⁴

In our study, a difference between culture and PCR methods was observed in the detection (33% and 60%, respectively) of *P. gingivalis*, *P. endodontalis*, *P. intermedia*, and *P. nigrescens* from periapical lesions, which could be explained by the sensitivity to oxygen and the unfavorable environmental conditions for these organisms when they are transferred to a new growth medium. Because these organisms are strictly fastidious anaerobes, suitable techniques of anaerobiosis are required. Our results show that *P. intermedia*, *P. nigrescens*, *P. gingivalis*, and *P. endodontalis* are frequent organisms isolated along or in association with chronic periapical infections, but the precise role of these organisms in the pathogenesis of those infectious processes could not be determined. Furthermore, PCR technique was more sensitive than culture for detecting the cells or the DNA of these anaerobic organisms from root canals.

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