Culture-based identification of pigmented Porphyromonas and Prevotella species in primary endodontic infections

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Abstract

Background. The most common species isolated from primary endodontic infections are black-pigmented bacteria. These species are implicated in apical abscess formation due to their proteolytic activity and are fastidious in nature. Therefore, the present study was carried out to evaluate the presence and identification of various pigmented Porphyromonas and Prevotella species in the infected root canal through culture-based techniques.

Methods. Thirty-one patients with primary endodontic infections were selected. Using sterile paper points, samples were collected from the root canals after access opening and prior to obturation, which were cultured using blood and kanamycin blood agar. Subsequently, biochemical test was used to identify the species and the results were analyzed using percentage comparison analysis, McNemar and chi-squared tests, Wilcoxon match pair test and paired t-test.

Results. Out of 31 samples 26 were positive for black-pigmented organisms; the predominantly isolated species were Prevotella followed by Porphyromonas. In Porphyromonas only P. gingivalis was isolated. One of the interesting features was isolation of P. gingivalis through culture, which is otherwise very difficult to isolate through culture.

Conclusion. The presence of Prevotella and Porphyromonas species suggests that a significant role is played by these organisms in the pathogenesis of endodontic infections.

Key words: Culture, infection, microorganism, Porphyromonas, Prevotella.
**Introduction**

All the pathoses of the pulp and periapical tissues is caused by microorganisms. To effectively treat endodontic infections, clinicians must recognize the cause and effect of microbial invasion of the pulp space and surrounding periapical tissues. The belief of the microorganisms associated with endodontic disease requires the development of a basic understanding of the disease process and effective management of patients with endodontic infection. The root canal infection is primarily a dynamic process. The microbiota involved in the primary endodontic infection is dominated by anaerobic bacteria which have been cultivated by culture-dependent approaches.

The most common species isolated from primary endodontic infections are black-pigmented bacteria. These bacteria are obligate anaerobes, non-motile and non-sporing, which are fastidious and oxygen-sensitive organisms. These organisms are implicated in apical abscess formation due to their proteolytic activity. *Prevotella* species such as *P. intermedia* and *P. nigrescens* have been cultured from 260% of infected root canals, suggesting an association with proteolytic activity.

Due to their fastidious nature these organisms are difficult to culture. Many studies have been carried out with controversial results regarding the isolation of pigmented *Porphyromonas* and *Prevotella* species through culture.

Culture, being considered the gold standard due to the identification of unexpected species, allows quantification of all the major viable microorganisms in the samples and broad range nature. Although molecular techniques appear to be pleasing and provide accurate results, it has certain limitation due to the chance of false positive results due to the detection of dead cells in the samples. Therefore, the present study was undertaken to review and detect black-pigmented organisms, mainly *Porphyromonas* and *Prevotella* species, in primary endodontic infections through a culture-based technique.

**Methods**

Ethical clearance was obtained from the Institute’s Ethical Review Board. (Reference code of certificate: 36/2014/MMNGHIDS).

A total of 31 patients who attended the Department of Endodontics with the need for endodontic treatment due to infected root canals were included in this study. The inclusion criteria were non-vital teeth presenting with a negative pulp test and deep carious lesion involving the pulp. All the permanent teeth having periapical radiolucency were included except for the third molars. The samples were obtained from single and multi-rooted teeth; in multi-rooted teeth, samples were collected from all the root canals which were non-vital. Exclusion criteria consisted of endodontically treated teeth, periodontal pocket >3 mm deep, periapical sinus/fistula, abnormal anatomy, calcified canals and any antibiotic therapy within the previous 3 months.

Patients who met both inclusion and exclusion criteria were enrolled for the study. Samples from the root canal were obtained after obtaining written informed consent forms from the patient. All the instruments and equipment were sterilized prior to the procedure. The procedure was carried by a single operator for all the 31 patients.

The samples were collected from root canals after rubber dam isolation from non-vital teeth using sterile paper points which were left in the canal for 60 seconds, after gaining access to the root canal using a sterile round bur and after gaining access to the root canal. Other steps such as cleaning, shaping and disinfection of the canal were carried out with 3% hydrogen peroxide and 2.5% sodium hypochlorite. Furthermore, another sample was collected prior to obturation, which was transferred to the laboratory in an appropriate transport medium (Reduced Transport Factor) immediately. The RTF samples were placed in a vortex (Biorad BR-2000 Vortexer) for 60 seconds and then centrifuged (500 rpm) for 1 minute. The deposits/sediments were cultured in blood agar enhanced with hemin and vitamin K and along with this in an anaerobic medium, i.e., Kanamycin blood agar. The culture plate was incubated in an anaerobic jar for 48–72 hours at 37°C. After completion of the incubation, the plates were removed and observed for suspected colony characteristics (pigmentation and hemolysis) and simultaneous counting of such suspected colonies. Gram staining was performed using the suspected colonies for the gram-negative bacilli.

Identification of species was carried out by performing a biochemical test, which included catalase, indole and nitrate reductase along with sugar fermentation test using key sugars like glucose, sucrose, lactose, maltose, xylose, arabinose and salicin cellobiose. The indicator used for biochemical test was bromocresol purple which imparted a purple color to the solution. Purple indicates negative and yellow indicates positive for the particular sugar. A standard table was used to compare the results of biochem-
Chemical test and identification of species was carried out. Statistical analysis was carried out by percentage comparison analysis. Further analyses for comparing access opening and pre-obturation samples were carried out using McNemar and chi-squared test and Wilcoxon match pair test. Colony forming units in all the samples were compared between access opening and pre-obturation samples using paired t-test.

Results
Of 31 samples 25 samples (80.6%) showed positivity for pigmented organisms in access opening samples and 5 samples showed positivity for pre-obturation cases (16.1%) (Figure 1). Of 31 patients, 16 were male and 15 were female. The patients’ ages were in the range of 18-58 years with an average of 29.6 years.

Twenty-five cases which were positive during access opening showed maximum positivity for Prevotella species, followed by Porphyromas species. There were various subspecies of Prevotella which was isolated (Table 1). In pre-obturation samples, Prevotella species were dominant. In the 25 samples, 5 were anterior teeth and 20 were posterior teeth.

In the anterior teeth the colony counts of Porphyromonas gingivalis were higher than Prevotella species (Table 2).

In 20 posterior teeth, 9 teeth were positive for Porphyromonas gingivalis with maximum number of colony counts (Table 3). Statistically the access opening and pre-obturation samples were analyzed using McNemar test (P = 0.00002) and Wilcoxon match pair test (P = 0.0008), which showed significant results (Table 4). The colony forming units in the samples were compared between access opening and pre-obturation samples using paired t-test (P < 0.00001), which also showed significant results.

Discussion
Pulp and periapical tissue pathoses are caused by microorganisms. Inflammatory reaction in the periapical tissue is due to the presence of bacteria within the root canal system. It is evident that an infected root canal system is a unique niche for the selective processes of microorganisms.

Endodontic microflora has been the focus of considerable research over the years. Apical periodontitis with bone resorption will occur only in infected necrotic pulps and in cases in which microorganisms have survived in the apical portion of the root canal system and/or outside the apical foramen. These factors are responsible for the treatment failures in the endodontic infection. Thus elimination of these microorganisms is of utmost importance to obtain root canal treatment success as endodontic infection is polymicrobial in nature.

Necrotic root canal environmental conditions are conducive to the establishment of microbiota conspicuously dominated by anaerobic bacteria. Black-pigmented bacteria are currently the focus of extensive basic science research and clinical studies as the main causative agent of endodontic infections, the severity of such infections and the resultant clinical symptoms associated with these infections. These anaerobic invaders have synergistic nature requiring specific nutrients for the growth and presence of other organisms to supply some of these products necessary for survival; therefore, they are not found clinically in pure cultures.

Table 1. Cases positive for species in access opening samples

| Serial | Species                        | No. of cases positive |
|--------|--------------------------------|-----------------------|
| 1.     | Porphyromonas gingivalis       | 11                    |
| 2.     | Prevotella loeschnii           | 5                     |
| 3.     | Prevotella corporis            | 2                     |
| 4.     | Prevotella tannerae            | 2                     |
| 5.     | Prevotella nigrescens          | 2                     |
| 6.     | Prevotella denticola           | 1                     |
| 7.     | Prevotella melaninogenica      | 1                     |
| 8.     | Prevotella intermedia          | 1                     |
| 9.     | Prevotella corporis/nigrescens | 1                     |
| 10.    | Prevotella nigrescens/intermedia| 1                     |

Total cases positive for access opening = 25
Pathogenicity of bacteria is mainly related to the presence of lipopolysaccharides and peptidoglycans. They induce hormones like cytokines which play an important role in inflammation, stimulate B-lymphocytes, activate complement cascade, release various enzymes like collagenase, and enhance production of various pain mediators like bradykinin, histamine and prostaglandins; lipopolysaccharides (LPS) once released (as endotoxin) cause biological effects, including inflammation and bone resorption.13

The virulence potential of dark-pigmented bacteria has the ability to confront phagocytosis, degrade immunoglobulins and increase pathogenesis when in combination with other specific strains of bacteriodes.13 The majority of these organisms are fastidious in nature and difficult to isolate through culture. Thus the study was undertaken to isolate these microorganism through culture-based techniques.

In the current study the number of samples positive for black-pigmented organisms through culture in access opening samples was 80.6%, with 16.6% during pre-obturation. Various studies6,14,15 have shown a higher prevalence of black-pigmented species by PCR but these species can be efficiently identified through simple and cost-effective methods like culture.

In a study Gomes et al16 found that isolation of *Porphyromonas gingivalis* was rare through culture methods (1%) but it was the most commonly identified species by PCR because *Porphyromonas* species are oxygen-sensitive and fastidious microorganisms.

| Table 2. Number of colony counts with species in the anterior teeth |
| --- | --- | --- | --- |
| Serial No. | Sample No. | Tooth | Species | Colony count (CFU/mL) |
| --- | --- | --- | --- | --- |
| 1. | A-1 | 11 | PG | 1000 |
| 2. | A-3 | 22 | *Prevotella loeschii* | 1000 |
| 3. | A-6 | 12 | PG | 6000 |
| 4. | A-13 | 31 | *Prevotella corporis* | 4000 |
| 5. | A-53 | 12 | *Prevotella loeschii* | 1000 |
| Total No. of anterior teeth | -5 |

| Table 3. Number of colony counts with species in the posterior teeth |
| --- | --- | --- | --- |
| Serial No. | Sample No. | Tooth (FDI) | Species | Colony count (CFU/mL) |
| --- | --- | --- | --- | --- |
| 1. | A-2 | 36 | PG | 1000 |
| 2. | A-5 | 26 | PG | 6000 |
| 3. | A-9 | 36 | *Prevotella corporis* | 4000 |
| 4. | A-12 | 16 | PG | 2000 |
| 5. | A-15 | 15 | *Prevotella tannerae* | 8000 |
| 6. | A-16 | 16 | *P. nigrescens-2* | 25000 |
| 7. | A-18 | 16 | *P. tannerae-1* | 15000 |
| 8. | A-19 | 15 | PG | 15000 |
| 9. | A-20 | 26 | PG | 1000 |
| 10. | A-21 | 25 | *P. intermedia* | 25000 |
| 11. | A-22 | 36 | *P. corporis/Nigrescens* | 6000 |
| 12. | A-23 | 24 | *P. nigrescens/intermedia* | 34000 |
| 13. | A-26 | 16 | PG | 3000 |
| 14. | A-28 | 26 | PG | 4000 |
| 15. | A-30 | 14 | PG | 200000 |
| 16. | A-41 | 46 | *P. melaninogenica* | 20000 |
| 17. | A-42 | 45 | *P. denticola* | 12000 |
| 18. | A-59 | 14 | *P. loeschii* | 3000 |
| 19. | A-66 | 16 | *P. loeschii* | 30000 |
| 20. | A-69 | 26 | PG | 15000 |
| Total No. of posterior teeth | -20 |

| Table 4. Comparison of access opening and pre-obturation samples |
| --- | --- | --- | --- |
| Access opening | Negative | Positive | Total | % |
| --- | --- | --- | --- | --- |
| Negative | 6 | 0 | 6 | 19.35 |
| Positive | 20 | 5 | 25 | 80.65 |
| Total | 26 | 5 | 31 | 100.00 |
| % | 83.87 | 16.13 | 100.00 |

McNemar/chi-squared=18.0501, P=0.00002* Wilcoxon matched pairs test, Z=3.9199, P=0.00008*
which explains failure to culture these species, but in the present study we isolated *Porphyromonas gingivalis* in 44% of the access opening samples through culture. This suggests that if adequate precaution is taken and good anaerobic condition is maintained, *Porphyromonas* species can be cultivated through simple method as culture and biochemical assay. *Prevotella intermedia* and *Prevotella nigrescens*, which are not possible to be differentiated using culture, can be identified through PCR. In the present study *P. intermedia* (4%) and *P. nigrescens* (8%) were identified through culture in access opening samples.

The isolated black-pigmented organisms were compared to clinical signs and symptoms such as pain, tenderness to percussion and abscess, which were similar to the findings reported by Gomes et al.,16,17 Griffee et al,18 Sundquist et al19 and Yoshida et al.20 *Prevotella melaninogenica* isolated from access opening samples was significantly associated with clinical features such as foul odor, pain, sinus, swelling and pain on percussion. In our study, we isolated *P. melaninogenica* from 4% of access opening samples. *Prevotella intermedia* is associated with acute apical periodontitis. In our study, we could isolate *P. intermedia* (4%) in access opening samples.

*Porphyromonas gingivalis* showed the maximum colony counts in the anterior teeth (7000 CFU/mL) and the posterior teeth during access opening. *Porphyromonas gingivalis* produces the collagenase gene, enhancing its particular virulence.

*Prevotella loescheii* is associated with previous episodes of pain; in our study we could isolate 2 cases in anterior teeth and 2 cases in posterior teeth and made a distinction between anterior teeth and posterior teeth in relation to pigmented organisms during access opening but could not correlate them with respective teeth because of small sample size.

The colony count of *Porphyromonas gingivalis* (1000–200000 CFU/mL) outnumbered *Prevotella* species. *Porphyromonas* species which are considered more virulent than *Prevotella* species and major pathogen in periodontitis are less frequent in acute endodontic infections but our findings showed *Porphyromonas gingivalis* was more virulent in endodontic infections with higher colony counts when compared to *Prevotella* species.

The five pre-obturation samples which were positive for *Prevotella nigrescens*, *Porphyromonas gingivalis*, *P. melaninogenica* and *P. corporis* could be due to the resistance of these organisms to biomechanical preparation. In these cases obturation should be referred to a later appointment because it may further lead to periapical infection such as periapical abscess formation.

**Conclusion**

The presence of *Prevotella* and *Porphyromonas* species in the majority of samples shows that they play a significant role in the pathogenesis of endodontic infections. No other unusual pigmented species of *Prevotella* and *Porphyromonas* were identified through culture in this study. But we could isolate *Porphyromonas gingivalis* in greater numbers only through culture. This suggests that culture-based techniques can be effectively used for the isolation of these fastidious organisms in endodontic infection and can also be used as a cost-effective method in small laboratories.

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**Authors’ contributions**

AR was responsible for the study design, data collection and compiling the manuscript. VSK was responsible for designing the research protocol and manuscript writing and reviewing. PDS was responsible for collecting review for the study. PI was responsible for designing the methodology and performing the analysis and data compilation. KB was responsible for review and editing the manuscript. All the authors have read and approved the final manuscript.

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**Competing interests**

The authors declare no competing interests with regards to the authorship and/or publication of this article.

**Ethics approval**

This study was approved by Central Research and Ethics committee of Maratha Mandal’s NG Halgekar Institute of Dental Sciences and Research Centre (Code of ethical certificate No.36/2014/MMNGHIDS).

**References**

1. Ingle JI, Bakland LK, Baumgartner JC. *Ingle Text book of Endodontics.* 6th ed. New Delhi: CBS Publishers; 2008. p. 221.
2. Shailaja S, Suresh BS. Endodontic microflora: a review. *J Oral Health Comm Dent* 2014;8(3):160-5.
3. Siqueira JF Jr, Rocsas IN. Exploiting molecular methods to explore endodontic infections: Part 2. Redefining the Endodontic Microbiota. *J Endod* 2005; 31:488-98. doi: 10.1097/01.don.0000157990.86638.49
4. Stanley, Martin D, Falkow. The prokaryotes: a handbook on the biology of bacteria. 2006; 7:428-54.
5. Gomes BPFA, Jacinto RC, Pinheiro ET, Sousa ELR, Zaia AA, Ferraz CCR, Souza-Filho FJ. Porphyromonas gingivalis, Porphyromonas endodontalis, Prevotella Intermedia and Prevotella nigrescens in endodontic lesions detected by culture and PCR. Oral Microbiol Immunol 2005; 20:211-15. doi:/10.1111/j.1399-302x.2005.00214.x
6. Loesche WJ, Lopatin DE, Stoll J, Van popen N, Hujoel PP. Comparison of various detection methods for periodontopathic bacteria: can culture be considered the primary reference standard? J Clin Microbiol 1992;30:418-26.
7. Siqueira JF Jr, Rocas IN. Exploiting molecular methods to explore endodontic infections: Part 1. Current Molecular Technologies for Microbiological Diagnosis. J Endod 2006; 31:411-23. doi: 10.1097/01.don.0000157989.44949.26
8. Murray PR, Baon JE, Jorgensen JH, Landry ML, Pfaller MA. Manual of clinical microbiology. 9th ed; Vol. 1. Washington, D.C.: ASM Press; 2007. p. 911-28.
9. Tronstad L, Sunde PT. The evolving new understanding of endodontic infections. Endodontic Topics 2003; 6:57-77. doi:10.1111/j.1601-1546.2003.00039.x
10. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. Crit Rev Oral Biol Med 2004;15:348-81. doi:10.1177/154411304015006004
11. Rocas In, Siqueira JF Jr. Root canal microbiota of teeth with chronic apical Periodontitis. J Clin Microbiol 2008; 46:3599-606. doi:10.1128/jcm.00431-08
12. Weine FS. Endodontic therapy. 6th ed. Chicago: Mosby. p.500.
13. Garg N, Garg A. Textbook of endodontics. 3rd ed. New Delhi: Jaypee Publishers; 2014. p. 51-60.
14. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced Periodontitis lesions. Oral Microbiol Immunol 1996;11:266-73. doi: 10.1111/j.1399-302x.1996.tb00180.x
15. Mättö J, Saarela M, Alaluusua S, Oja V, Joukimies-Somer H, Asikainen S. Detection of Porphyromonas gingivalis from saliva by PCR by using a simple sample processing method. J Clin Microbiol 1998;36:157-60.
16. Gomes BPFA, Drucker DB, Lilley JD. Association of specific bacteria with some endodontic signs and symptoms. Int Endod J 1994; 27:291-98. doi: 10.1111/j.1365-2591.1994.tb00271.x
17. Gomes BPFA, Lilley JD, Drucker DB. Association of endodontic symptoms and signs with particular combination of specific bacteria. Int Endod J 1996; 29:69-75. doi: 10.1111/j.1365-2591.1996.tb01164.x
18. Griffie MB, Patterson SS, Miller CH, Kafrawy AH, Newton CW. The relationship of Bacteroides melaninogenicus to symptoms associated with pulpal necrosis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1980;50:457-61. doi: 10.1016/s0030-4220(80)80015-6
19. Sundqvist G, Johansson E, Sjogren U. Prevalence of Black-pigmented bacteroides species in root canal infections. J Endod 1989;15:13-19. doi: 10.1016/s0099-2399(89)80092-5
20. Yoshida M, Fukushima H, Yamamoto K, Ogawa K, Toda T, Sagawa H. Correlation between clinical symptoms and microorganisms isolated from root canal of teeth with Periapical pathosis. J Endod 1987;13:24-28. doi: 10.1016/s0099-2399(87)80088