Polymerase Chain Reaction of Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia in Primary Endodontic Infections

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Abstract
The aim of this study was to investigate the correlation between endodontic clinical signs and symptoms and the presence of Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia or their association by nested polymerase chain reaction assay. Microbial samples were taken from 50 cases with necrotic pulp tissues in primary infections. DNA was extracted from the samples, which were analyzed for the presence of three endodontic pathogens by using species-specific primers. P. gingivalis, T. denticola, and T. forsythia were detected in 46%, 38%, and 22% of the symptomatic cases, respectively. The bacterial complex composed by T. forsythia, P. gingivalis, and T. denticola was found in 14% of the cases with spontaneous pain, tenderness to percussion, swelling, and pain on palpation. The high prevalence of P. gingivalis, T. denticola, and T. forsythia in the samples examined suggests that these bacteria are related to the etiology of symptomatic periapical diseases. (J Endod 2007;33:1049–1052)

Key Words
Endodontics, microorganisms, nested polymerase chain reaction, “red complex”

The polymicrobial nature of the endodontic microbiota suggests a bacterial interaction that might play an important role for their survival and virulence (1, 2). A restricted group of anaerobic and facultative microorganisms, especially Prevotella and Porphyromonas spp, Fusobacterium nucleatum, Streptococcus milleri group, and other gram-positive and gram-negative species are considered important in the pathogenesis of odontogenic infections in terms of sheer numbers and relevance toward clinical symptoms (3).

Studies have shown that molecular techniques can detect the presence of bacteria in endodontic infections when culture techniques yield a negative result. These techniques can also be used to identify a wider range of endodontic-infection-related bacteria including the presence of previously unidentified or unculturable species (4). The endodontic microbial diversity is greater than formerly described by culture methods and the structure of the microbiota varies significantly between asymptomatic and symptomatic infections (5). For instance, Tannerella forsythia (formerly Bacteroides forsythus), Treponema denticola, other Treponema species, Dialister pneumosintes, and Prevotella tannerae were detected in high prevalence in infected root canals by using polymerase chain reaction (PCR) analysis (6).

Gomes et al (7) detected a higher prevalence of black-pigmented species in symptomatic teeth by PCR analysis, and Porphyromonas gingivalis was present in nearly half of the samples. Siqueira et al (8) detected T. denticola in root canals of teeth that were tender to percussion and diagnosed as acute periapical abscesses, which suggests that this microorganism could also participate in the pathogenesis of periapical diseases.

Socransky et al (9) stratified the periodontal microbiota into groups or complexes, representing consortia that appear to occur together and that are associated with health and disease. These complexes have been related to the sequence of colonization on the tooth surface as well as with disease severity. The “red complex” is part of the climax community in biofilms and comprises species that are considered periodontal pathogens, namely, P. gingivalis, T. denticola, and T. forsythia (10). Roças et al (11) verified the occurrence of this complex in endodontic infections in 5% of the symptomatic cases, whereas at least one member of the complex was detected in 80% of the cases. The association of P. gingivalis and T. denticola tends to increase their growth potential (12). Kesavalu et al (13) showed that T. denticola/P. gingivalis complex exhibited enhanced virulence compared with monoinfection in a murine model.

Therefore, the purpose of this study was to investigate the correlation between clinical signs and symptoms and the presence of P. gingivalis, T. denticola, and T. forsythia isolated or forming the “red complex.” This work was accomplished at the molecular level by using nested PCR.

Material and Methods

Specimen Selection
The examined material was selected from 50 patients who had been referred for root canal therapy to the endodontic area of the Dental School of Piracicaba, State University of Campinas, Piracicaba, Sao Paulo, Brazil. None of the patients had received...
antibiotic treatment during the preceding 3 months. Patients with related systemic disease were not included in this study. The human Volunteers Research and Ethics Committee of the Dental School of Piracicaba approved the study, and all patients signed an informed consent form.

Clinical and Radiographic Examination

The 50 teeth selected had no prior endodontic treatment and exhibited either a necrotic pulp (primary infection), periradicular periodontitis, or periradicular abscess according to Torabinejad and Walton (14). 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 tract and its origin (endodontic or periodontal), presence of swelling of the periodontal tissues (ie, acute abscess), probing depth of any periodontal pockets, history of previous and present antibiotic therapy, and any other relevant medication. The internal status of the canal, such as a dry canal or the presence of clear, hemorrhagic or purulent exudates, was detected as a distinct dampening or stain on the sampling paper points. Each type of exudates was analyzed separately and also grouped with the other types under the denomination “wet canal.” Radiographically, 33 teeth showed radiolucent periapical areas, and 17 did not show radiolucent periapical areas. Forty-one teeth were single rooted and 9 were multirooted.

Sampling Procedure

Aseptic techniques were used throughout the endodontic sample acquisition. Briefly, after caries removal without the exposure of the canals, the teeth were individually isolated from the oral cavity with a rubber dam. Teeth and rubber dam were disinfected with 30% hydrogen peroxide followed by 2.5% sodium hypochlorite. Then, the canals were exposed under manual irrigation with sterile saline solution using sterile burs. Sampling included a single root canal. In the multirooted teeth, only the largest canal in the root with the periapical radioluscency was sampled to confine the microbial evaluation to a single ecological environment.

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 seconds. 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 were used to absorb all fluid inside the canal. The paper points were transferred to a test tube containing VMGA III transport medium (15) and stored at −70°C for further PCR analyses.

The Detection of P. gingivalis, T. forsythia, and T. denticola by Using PCR

DNA was extracted according to Leys et al (16). Briefly, 250 μL of the sample were centrifuged at 10,000g for 30 seconds, after which the supernatant was removed and discarded. The pellet was suspended in 300 μL of 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, and 1% sodium dodecyl sulfate. Proteinase K was added to a concentration of 1 mg/mL, and the sample was incubated at 37°C for 1 to 2 hours. The DNA was purified by the standard GeneClean (Bio 101, Inc, LaJolla, CA) protocol, with the addition of one additional washing step. This simplified purification method provided DNA of suitable integrity for PCR amplification.

DNA isolated from the root canals specimens was first amplified with prokaryotic universal ribosomal 16S and 23S primers (785 and 422, respectively), as described elsewhere (17). The annealing of these universal primers to conserved regions amplified a fragment that included the 16S rDNA and the downstream intergenic spacer region. Because the length of the intergenic spacer region varies among species, the inclusion of this region provided an additional check of the specificity of the primers. 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 5 mmol/L MgCl₂, 0.25 mmol/L of each primer, and 0.2 mmol/L (each) deoxynucleoside triphosphates. For each sample, 0.5 μL of extracted DNA was added to the reaction mixture. PCR was also performed by using a positive control (0.5 μL of DNA extracted from the P. gingivalis ATCC 33277, T. forsythia ATCC 43037, and T. denticola ATCC 35405) and several negatives control (only the reaction mixture without DNA). Samples were subjected to 22 cycles of denaturation at 94°C for 1 minute annealing at 42°C for 2 minutes, and primer extension at 72°C for 3 minutes, and a final extension of 72°C for 10 minutes in an automated thermal cycler (Perkin-Elmer Cetus).

P. gingivalis, T. forsythia, and T. denticola were then identified by a second, nested amplification with species-specific 16S primers paired with a universal primer located in the 23S gene (L189). Primer sequences are shown in Table 1. All primers were synthesized by Biosynthesis, Lewisville, TX. The PCR reaction conditions were as follows: 27 cycles of 94°C for 1 minute, 52°C for 2 minutes, and 72°C for 3 minutes. PCR products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, and viewed under ultraviolet transillumination.

A positive or negative identification was based on the presence of clear bands of the expected molecular size by using a 21-kb lambda DNA ladder (Invitrogen Corporation, Carlsbad, CA). All assays were repeated, and, if the results were not in agreement, they were repeated again.

Primer Specificity

The species-specific primer in the 16S rDNA coding region was selected based on previous investigation of endodontic bacteria by cloning and sequencing of the bacterial 16S gene (17, 18) and on sequences available in GenBank. The species specificity was further confirmed by sequencing at least one PCR product from a clinical sample for the specific primer in an ABI Prism 310 automated sequencer (AME Bioscience Ltd, London, UK) as described by Rumpf et al (19). Direct sequencing of PCR products eliminated the problem of misincorporation that is associated with cloning PCR products, and comparison of the

<table>
<thead>
<tr>
<th>Primers and Probes</th>
<th>Specificity/Location/Orientation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pg13M</td>
<td>P. gingivalis/Pg/16S/F</td>
<td>CATCGGTAGGCTAACACGTTCG</td>
</tr>
<tr>
<td>Tdent</td>
<td>T. denticola/Td/16S/F</td>
<td>CAAGGACAAATGACATAGATGGG</td>
</tr>
<tr>
<td>Bf4R</td>
<td>T. forsythia (B. forsythus) Bl/16S/F</td>
<td>TTGGAATATAGTGAAGCTTACAG</td>
</tr>
<tr>
<td>Sm785</td>
<td>Universal primer/16S/785 bp from 5’end/F</td>
<td>GGATTAATAGCCCTGTAGTC</td>
</tr>
<tr>
<td>422</td>
<td>Universal primer/23S/422 bp from 5’end/F</td>
<td>GGATATTAGCTT</td>
</tr>
<tr>
<td>L189</td>
<td>Universal primer/23S/F</td>
<td>GGTACTTAGATGTTTCAGTTC</td>
</tr>
</tbody>
</table>
sequence was generated with those available in GenBank by using the BLAST software available on line at http://www.ncbi.nlm.nih.gov/blast.

Statistical Analysis

The data collected for each case (clinical features) were typed onto a spreadsheet and statistically analyzed by using SPSS for Windows (SPSS Inc, Chicago, IL). The Pearson chi-square test or the one sided Fisher exact test, as appropriate, was chosen to test the null hypothesis that there was no relationship between endodontic clinical symptoms and signs and the presence of P. gingivalis, T. denticola, and T. forsythia.

Results

All samples were positive for bacterial DNA as determined by the use of ubiquitous bacterial primers. Some signs and symptoms were present in 50 canals as follows: 42 with spontaneous pain, 41 with tenderness to percussion, 39 with pain to palpation, 30 with swelling, 20 with purulent exudate, 27 with abscesses, and 15 with acute periapical periodontitis. Dental abscesses were the cases with purulent exudates or presence of sinus tract.

By using the nested PCR, it was possible to detect P. gingivalis, T. forsythia, and T. denticola from 22, 12, and 19 canals, respectively. Statistically significant relationships were found between P. gingivalis and swelling, mobility, and periapical radiolucency (all p < 0.05). T. denticola was statistically significant associated with both wet and dry canals (p < 0.05).

Significant associations were found between the presence of P. gingivalis/T. denticola and T. forsythia/T. denticola (both p < 0.05). The pair P. gingivalis/T. denticola had statistically significant relationships with spontaneous pain, tenderness to percussion, pain on palpation, swelling, and periapical lesions (all p < 0.05). The simultaneous presence of T. forsythia and T. denticola was related to tenderness to percussion, pain on palpation, spontaneous pain, swelling, and wet canals (all p < 0.05).

The “red complex” composed by T. forsythia, P. gingivalis, and T. denticola was found in 16% (7/42) of the cases with abscess. However, no statistically significant difference was found between such complex and the presence of abscess (p > 0.05). Nevertheless, the presence of the “red complex” was related particularly to mobility (p < 0.05) and also to periapical lesions (p < 0.05). Table 2 summarizes the clinical features and the presence of the target species either isolated, forming pair or in the “red complex.”

Discussion

Even though nested PCR assay has an extremely high sensitivity, a great care must be taken during the procedures to avoid cross-contamination. The major drawback of nested-PCR protocol is the high probability of contamination during transfer of the first-round amplification products to a second reaction tube (20). Many blank reactions are recommended throughout the first and second rounds of amplification to monitor for the increased likelihood of false-positive results (20). In the present report, several negative controls were used in every PCR test, and all showed negative results.

The development of acute endodontic signs and symptoms might depend on synergy between black-pigmented bacteria and other bacterial species (1). P. gingivalis is a small, gram-negative anaerobe that produces a substantial array of putative virulence factors (21). Gomes et al (7) showed that P. gingivalis, which was rarely isolated by culture methods, was the most frequently “black-pigmented bacteria” identified by PCR in almost 38% of the cases. Seol et al (22) detected P. gingivalis in 22.5% of endodontic abscesses by multiplex PCR and in 5% of the cases by culture. In the present study, we detected this species in 44% (22/50) of the cases, which is in agreement with Siqueira et al (8), who found that almost 40% of the symptomatic cases were positive to P. gingivalis. Moreover, Roças et al (23) detected this species in 70% of endodontic abscesses of a Brazilian population.

T. denticola and T. forsythia are very fastidious anaerobes and barely showed up in cultivation methods, being overlooked, bypassed, and hardly mentioned in previous studies (24). Molecular techniques allowed their association with severe periodontal diseases (10), periodontal lesions (25, 26) and endodontic abscesses (23). Our results showed that T. denticola was present in 19 of 50 cases, in which abscess, acute periapical periodontitis, tenderness to percussion, pain on palpation, wet canals, and swelling were the most common clinical features. Foschi et al (27) also showed that this species was highly associated with symptomatic endodontic infections and was found in higher incidence in primary infections than in secondary infections. The high prevalence of T. forsythia in acute periapical abscesses was also reported by Gonçalves & Mouton (25) and Siqueira and Roças (28). In our study, a significant association was found between T. forsythia and tenderness to percussion, mobility, wet canals, and purulent exudate. These results suggest that T. forsythia does play an important role in the development of symptomatic cases. For instance, treponemes and T. forsythia have the capacity of activating a wide range of immu-

### Table 2. Clinical Features and the Presence of P. gingivalis, T. denticola, and T. forsythia Isolated or in Association in Primary Endodontic Infections

<table>
<thead>
<tr>
<th>Primary Endodontic Infection (n=50)</th>
<th>P gingivalis (n=22)</th>
<th>T denticola (n=19)</th>
<th>T forsythia (n=12)</th>
<th>P gingivalis/ T denticola (n=13)</th>
<th>P gingivalis/ T forsythia (n=8)</th>
<th>T denticola/ T forsythia (n=7)</th>
<th>Red Complex (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous pain (n=42)</td>
<td>18</td>
<td>15</td>
<td>9</td>
<td>10*</td>
<td>6</td>
<td>6*</td>
<td>5</td>
</tr>
<tr>
<td>Periapical radiolucency (n=33)</td>
<td>10*</td>
<td>10*</td>
<td>6</td>
<td>6*</td>
<td>2</td>
<td>3*</td>
<td>2*</td>
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<tr>
<td>Previous pain (n=22)</td>
<td>8</td>
<td>6</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>TTP (n=41)</td>
<td>19</td>
<td>16</td>
<td>11</td>
<td>11*</td>
<td>10*</td>
<td>2*</td>
<td>7</td>
</tr>
<tr>
<td>Pain on palpation (n=39)</td>
<td>19</td>
<td>15</td>
<td>8</td>
<td>11*</td>
<td>6</td>
<td>7*</td>
<td>6</td>
</tr>
<tr>
<td>Mobility (n=11)</td>
<td>8*</td>
<td>7</td>
<td>5</td>
<td>6*</td>
<td>4</td>
<td>5*</td>
<td>4*</td>
</tr>
<tr>
<td>Dry canals (n=20)</td>
<td>6</td>
<td>4*</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Wet canals (n=30)</td>
<td>16</td>
<td>15*</td>
<td>8</td>
<td>11*</td>
<td>6</td>
<td>7*</td>
<td>6</td>
</tr>
<tr>
<td>Purulent exudate (n=20)</td>
<td>11</td>
<td>10</td>
<td>6</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Swelling (n=30)</td>
<td>17*</td>
<td>12</td>
<td>6</td>
<td>10*</td>
<td>5</td>
<td>5*</td>
<td>5</td>
</tr>
<tr>
<td>Foul odor (n=30)</td>
<td>16</td>
<td>14</td>
<td>8</td>
<td>10*</td>
<td>7</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Abscess (n=27)</td>
<td>16*</td>
<td>12</td>
<td>6</td>
<td>10*</td>
<td>5</td>
<td>5*</td>
<td>5</td>
</tr>
<tr>
<td>Acute periapical periodontitis (n=15)</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>3*</td>
<td>0</td>
</tr>
</tbody>
</table>

TTP, tenderness to percussion.
*Statistical significant association (p < 0.05).
Clinical Research

opathologic mechanisms while resisting, downregulating, or degrading mediators of innate immunity pathways (10, 24).

The presence of some relationships among bacterial species and between bacterial species and the host should be outlined to understand the disease process (29). For instance, bacterial synergism may be an important factor in the development of signs and symptoms of endodontic origin (1), and the presence of certain potentially pathogenic species composing the root canal microbiota may predispose patients to acute periradicular inflammation (30).

The presence of “red complex” formed by P. gingivalis, T. forsythia, and T. denticola has been related to periodontal disease, local clinical characteristics, and the systemic background of the host (31). In endodontic infections, its occurrence was initially shown by Roças et al (30) at a relatively low prevalence and without association with signs and symptoms. In the present study, the “red complex” was only found in 7 cases. However, at least one member of the complex was found in the 50 cases investigated. Our results found an association of the red complex with tenderness to percussion, swelling, and pain on palpation.

Bacteria have to live and survive under continuously changing environmental conditions and therefore are forced to adapt to them (32), which may be accomplished by their association in complexes. In the periodontal tissues, this intrinsic dependence is a determinant to the microorganism survival to avoid host defenses. On the other hand, in the apical third of the root canal, which is considered a critical territory because of its anatomical complexity, the host tissues can only prevent periradicular damage (33) because the defense system is not able to act inside the root canal system during pulp necrosis. Therefore, the bacterial associations in stratified complexes forming biofilms might not be a critical factor for their survival inside the root canal. However, their interactions are the key for the development of pulpal and periradicular pathosis (1).

Conclusions

Because of the high prevalence of P. gingivalis, T. denticola, and T. forsythia in the samples examined in the present study and considering their virulence factors and pathogenicity, the results suggest that these bacteria are related to the etiology of periapical abscesses. The “red complex” was detected in few cases and was associated with specific signs and symptoms of endodontic origin. However, further studies must be done to stratify the microbiota of root canals and determine its relationship with the development of periradicular diseases.

Acknowledgments

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References

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