Advanced Caries Microbiota in Teeth with Irreversible Pulpitis

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Abstract

Introduction: Bacterial taxa in the forefront of caries biofilms are candidate pathogens for irreversible pulpitis and are possibly the first ones to invade the pulp and initiate endodontic infection. This study examined the microbiota of the most advanced layers of dentinal caries in teeth with irreversible pulpitis. **Methods:** DNA extracted from samples taken from deep dentinal caries associated with pulp exposures was analyzed for the presence and relative levels of 33 oral bacterial taxa by using reverse-capture checkerboard hybridization assay. Quantification of total bacteria, streptococci, and lactobacilli was also performed by using real-time quantitative polymerase chain reaction. Associations between the target bacterial taxa and clinical signs/symptoms were also evaluated. **Results:** The most frequently detected taxa in the checkerboard assay were *Atopobium* genomospecies C1 (53%), *Pseudoramibacter alactolyticus* (37%), *Streptococcus* species (33%), *Streptococcus mutans* (33%), *Parvimonas micra* (13%), *Fusobacterium nucleatum* (13%), and *Veillonella* species (13%). *Streptococcus* species, *Dialister invisus*, and *P. micra* were significantly associated with throbbing pain, *S. mutans* with pain to percussion, and *Lactobacillus* with continuous pain ($P < .05$). Quantitative polymerase chain reaction revealed a mean total bacterial load of 1×10^8 (range, 2.05×10^5 to 4.5×10^8) cell equivalents per milligram (wet weight) of dentin. Streptococci and lactobacilli were very prevalent but comprised only 0.09% and 2% of the whole bacterial population, respectively. **Conclusions:** Several bacterial taxa were found in advanced caries lesions in teeth with exposed pulps, and some of them were significantly associated with symptoms. A role for these taxa in the etiology of irreversible pulpitis is suspected. *(J Endod 2015;41:1450–1455)*

Key Words

Dentinal caries, irreversible pulpitis, *Lactobacillus*, microbiota, molecular biology, permanent teeth, *Streptococcus*

Pulpitis is the inflammation of the dental pulp and is commonly a sequel to caries (1). Bacteria located in the advanced frontline of the caries biofilm are directly involved in inducing damage and consequent inflammation in the pulp tissue (2–4). The bacterial effects on the pulp are caused either by bacterial virulence factors and antigens that diffuse through the dentinal fluid or by the bacterial cells themselves, which may reach the pulp via dentinal tubules, especially in very profound caries cavities (5, 6).

Pulpitis can be clinically classified as reversible or irreversible (7). In the former condition, removal of the causative agent usually permits the pulp to return to normality, whereas in the latter condition, direct intervention in the pulp tissue may be required for improved treatment outcome (8). Irreversible pulpitis usually develops when the pulp is frankly exposed to the caries biofilm (6).

Many studies evaluating the microbiota associated with deep dentinal caries revealed that the bacterial composition is substantially different from enamel caries (4, 9–11). This is highly likely to be a result of different ecological conditions associated with these lesions. In addition to lactobacilli, which are very prevalent in dentinal caries (4, 12–14), asaccharolytic and/or proteolytic anaerobic bacteria have been frequently detected (9–11, 13–15). Most of the species in carious dentin have also been detected in infected root canals (11, 14, 16–22), suggesting that in addition to being involved with pulpal damage, these dentinal lesions might well be the primary source of bacteria that initiate endodontic infections. However, there are not many studies evaluating the microbiota of advanced caries lesions in association with pulp conditions. A study identified bacteria isolated from carious lesion biofilms and vital carious exposures of pulps of deciduous teeth and observed that the microbiota of the cariously exposed pulps were similar in composition to those of carious lesion biofilms except that fewer species/taxa were identified from the pulps (23). *Actinomyces* and *Selenomonas* species were associated with carious lesions, whereas *Veillonella* species were associated with pulps. Other studies have reported a close association between pain and the presence of *Prevotella*, *Porphyromonas*, and *Fusobacterium* species in deep dentinal caries (3, 4). Black-pigmented anaerobic bacteria and *Streptococcus mutans* have been positively related to pulpal pain triggered by heat, whereas *Fusobacterium nucleatum* and *Actinomyces viscosus* have been associated with cold sensitivity (24). Positive associations between *Parvimonas micra* and *Porphyromonas endodontalis* detection in carious dentin and irreversible pulpitis have been found (2).

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Bacterial taxa present in the forefront of deep dentinal caries biofilms are candidate pathogens for irreversible pulpitis and are possibly the first ones to invade the pulp and initiate endodontic infection. The present study used reverse-capture checkerboard assay to evaluate the prevalence of several caries and endodontic bacterial pathogens in the most advanced layers of dentinal caries in teeth with the clinical diagnosis of irreversible pulpitis. Quantification of total bacteria, streptococci, and lactobacilli was also performed by using real-time quantitative polymerase chain reaction (qPCR). Associations between the presence and levels of the target bacterial taxa and clinical symptoms of irreversible pulpitis were also evaluated.

Materials and Methods

Subject Population

This study included 30 patients (23 female and 7 male) with deep occlusal caries in permanent maxillary or mandibular molars diagnosed with irreversible pulpitis. Patients ranged in age from 12 to 33 years. Each patient contributed 1 tooth. Medical history revealed no significant systemic condition or disease. Ethical approval for the study was granted by the Ethics Committee of the Federal University of Rio Grande do Norte, and informed consent was obtained from all subjects or their parents/guardians.

The diagnosis of irreversible pulpitis was based on clinical and radiographic findings and following the reports of the American Association of Endodontists Consensus Conference on diagnostic terminology (7). All cases had extensive caries lesions that led to pulp exposure. Intensity of pain was evaluated by using a visual analogue scale ranging from 0 (no pain) to 170 mm (severe pain). This scale permitted pain intensity to be ranked as mild, moderate, or severe. If present, pain was also recorded as provoked or spontaneous, intermittent or continuous, cold- or heat-evoked, localized or diffuse, and throbbing or after physical efforts. Pulp status was evaluated by thermal sensibility tests. Radiographic analysis involved extent of the caries lesion, presence of coronal restoration, stage of apical root formation, and conditions of the apical periodontal ligament space. Teeth with necrotic pulps or treated root canals and teeth with no evidence of pulp exposure after dentinal caries removal were excluded from the study.

Sample Taking and DNA Extraction

Selected teeth were cleaned with pumice, and the patient was anesthetized. Undermined enamel, superficial carious tissue, and debris were removed by using high-speed burs under water cooling. The target tooth was isolated with rubber dam, and the operative field, including the tooth, was cleaned with 6% hydrogen peroxide and disinfected with 2.5% sodium hypochlorite (NaOCl). The latter solution was inactivated with 10% sodium thiosulfate. The superficial layers of the caries lesion were removed by using a sterile spoon excavator and dismissed. Another sterile excavator was used to collect the deepest layer of dentinal caries in direct contact with the pulp, which was then transferred to cryotubes containing Tris-EDTA buffer. Transference of the material to the flasks in the clinical setting was always performed in the aseptic zone around a flame. Samples were immediately frozen at −20°C.

Caries dentin samples were weighed (wet weight), and DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), following the protocol recommended by the manufacturer for tissues. DNA from a panel of several oral bacterial species was also prepared to serve as controls (25).

Reverse-capture Checkerboard Assay

The reverse-capture checkerboard assay used in this study was as described previously (26–28). DNA extracted from clinical samples was used as template in a 2-step 16S rRNA gene-based PCR protocol. In the first step, a practically full-length 16S rRNA gene fragment was amplified by using universal primers 8f and 1492r (26, 29, 30). In the second step, the resulting PCR product from each sample was used as template to run 2 sets of partial 16S rRNA gene amplification; one set used primers digoxigenin-8f and 519r, and the other set used primers digoxigenin-515f and 1492r (27). PCR amplifications were performed in 50 μL reaction mixture containing 1 μmol/L of each primer, 5 μL of 10× PCR buffer (Fermentas, Burlington, ON, Canada), 3 mmol/L $MgCl_2$, 2 U *Taq* DNA polymerase (Fermentas), and 0.2 mmol/L of each deoxyribonucleoside triphosphate (Invitrogen Life Technologies, Carlsbad, CA). Negative controls consisted of sterile ultrapure water instead of sample and were included with each batch of samples analyzed. Temperature profile for the first PCR reaction was 95°C/1 min, 26 cycles at 94°C/45 s, 50°C/45 s, 72°C/1.5 min, and 72°C/15 min, and for the second step it was 95°C/5 min, 28 cycles at 94°C/30 s, 55°C/1 min, 72°C/1.5 min, and 72°C/20 min. PCR products were separated by electrophoresis in agarose gels, which were then stained with GelRed (Biotium, Hayward, CA) and visualized under ultraviolet transillumination.

Labeled PCR products were mixed by using equal proportions of each (40 μL) and used in the checkerboard assay to determine the presence and levels of 33 bacterial taxa by using probes described and validated previously (26, 27, 31). In addition to the taxon-specific probes, 2 universal probes were included in each checkerboard membrane to serve as controls. Two lanes in the membrane contained extracted DNA standards at the concentration of 10^5 and 10^6 cells, which were treated the same way as the clinical samples. The reverse-capture checkerboard assay was performed by using the Minislot-30 and Miniblotter-45 system (Immunetics, Cambridge, MA). First, 100 pmol of probe in TE buffer (10 mmol/L Tris HCl, 1 mmol/L EDTA, pH 8.0) was introduced into the horizontal wells of the Minislot apparatus and cross-linked to the Hybond- N+ nylon membrane (AmershamPharmacia Biotech, Little Chalfont, Buckinghamshire, UK) by ultraviolet irradiation by using a Stratalinker 1800 (Stratagene, La Jolla, CA) on autocross-link position. Each probe has a polythymidine tail that is preferentially cross-linked to the nylon and leaves the specific probe available for hybridization. The membrane was then prehybridized at 55°C for 1 hour. Subsequently, 80 μL of the labeled PCR products mixed with 60 μL of 55°C preheated hybridization solution was denatured at 95°C for 5 minutes and loaded on the membrane by using the Miniblotter apparatus. Hybridization was carried out at 54°C for 2 hours.

After blocking in a buffer with casein, the membrane was incubated in antidigoxigenin antibody conjugated with alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany) and then in ultrasensitive chemiluminescent substrate CDP Star (Roche Molecular Biochemicals). Finally, a square of x-ray film was exposed to the membrane in a cassette to detect the hybrids.

Quantitative Real-time PCR

To quantify the total bacterial load and levels of streptococci and lactobacilli in caries samples, 16S rRNA gene-targeted qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7500 Real-time PCR instrument (Applied Biosystems) in a total reaction volume of 20 μL. The primers used were as described and validated elsewhere (32–36) (Table 1).

Primers in a concentration of 0.5 μmol/L each and DNA extract volume of 2 μL were added to the PCR master mix in MicroAmp Optical