Isolation of Alkaline-tolerant Bacteria from Primary Infected Root Canals

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Abstract

Introduction: Alkaline-tolerant bacteria in primary infected root canals could have enhanced survival capacity against antimicrobials commonly used in root canal treatment. The aims of this study were to isolate and characterize alkaline-tolerant bacteria before endodontic treatment (S1), after chemomechanical root canal preparation (S2), and after calcium hydroxide dressing (S3). **Methods:** Bacteriologic samples were obtained from 43 primary infected root canals. Samples were inoculated into culture media at a pH of 9 and incubated anaerobically. The identities of bacterial isolates were determined by 16S ribosomal RNA sequencing. **Results:** All S1 samples were culture positive, with 70% harboring bacteria tolerating a pH of 9. Gram-positive bacteria *Pseudoramibacter alactolyticus* and *Streptococcus* spp were the most frequently isolated strains with a prevalence of 54%. Of 13 culture-positive S2 samples, 8 isolates tolerated a pH of 9, namely *Streptococcus sanguinis*, *Enterococcus faecalis*, *Enterobacter cancerogenus*, *Streptococcus oralis*, and *Fusobacterium nucleatum*. Seven of these 8 isolates (88%) were correspondingly isolated at S1. All 3 culture-positive S3 samples tolerated a pH of 9, namely *S. sanguinis* and *E. faecalis,* which were also isolated in the corresponding S1 and S2 samples. **Conclusions:** We showed that the presence of alkaline-tolerant *Streptococcus* and *Enterococcus* spp in primary infected root canals could lead to their persistence during and after root canal treatment and could pose a challenge to current treatment efficacy. *(J Endod 2015;41:451–456)*

Key Words

Alkaline-tolerant bacteria, endodontic treatment, root canal infection

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Eradication of bacteria from the root canal system of teeth is an important goal of root canal therapy. Although radiographic healing was significantly better when no bacteria were recovered before filling (1, 2), studies have shown that mechanical instrumentation alone may reduce but not completely eliminate the microorganisms that reside in infected root canals (3, 4). Therefore, the use of irrigants and intracanal dressings with antimicrobial properties has been regarded as indispensable to endodontic therapy. Yet, even with sodium hypochlorite irrigation and calcium hydroxide ($Ca[OH]_2$) intracanal medication, bacteria-free root canals are not always attained (5–8). The broad, nonspecific antibacterial effect of $Ca(OH)_2$ comes from its high pH of 12.5, and its efficacy depends on maintaining this high pH to release hydroxyl ions during application. The availability of hydroxyl ions is influenced by the vehicle, additives, and dentin buffering effect. It has been reported in a recent study that pure $Ca(OH)_2$ aqueous paste and Calasept (Scania Dental Ab, Knivsta, Sweden) maintained alkalinity better than viscous gel products such as DS $Ca(OH)_2$ gel, Ultracal XS (Ultradent Products Inc, South Jordan, UT), Biokalkki (Dental Systems Oy, Helsinki, Finland), and Calxyl blue (OCO Präparate GMBH, Dirmstein, Germany) when challenged with acid. However, these products were neutralized by dentin powder within 24 hours, reducing their antimicrobial effects (9).

Along with the observation that the clinical outcome of single-visit endodontic treatment is comparable with that of multiple-visit treatments (10, 11), the single-visit treatment approach has gained popularity in recent years. A drawback of single-visit endodontic treatment is the potential for root canals to be left with residual bacteria and run the risk of treatment failure. Recently, a histobacteriologic investigation comparing the effectiveness of $Ca(OH)_2$ in single- and 2-visit endodontic treatment showed that interappointment medication with $Ca(OH)_2$ significantly reduced the bacterial load in ramifications, isthmuses, and dentinal tubules. However, bacteria-free root canals were not consistently achieved, and long-term follow up data were not reported (12).

One way forward is to characterize the types of root canal bacteria that are able to withstand endodontic treatment procedures. We hypothesize that bacterium with the ability to tolerate an alkaline environment, at which common antimicrobial irrigants and dressing operate, have a survival edge that may allow them to persist in the various stages of root canal treatment. It is reasonable to assume that the colonization of root canals with alkaline-tolerant microorganisms can lead to a higher probability of such microorganisms surviving chemomechanical procedures. Some conditions can enhance the ability of bacteria to survive exposure to alkaline treatments. Brändle et al (13) studied the impact of growth conditions on the susceptibility of 5 taxa of microbes to alkaline stress. They found that planktonic *Enterococcus faecalis* and *Candida albicans* could withstand $Ca(OH)_2$ exposure up to 100 minutes. Dentin adhesion greatly enhanced the resistance of *E. faecalis* and *Actinomyces naeslundii* to $Ca(OH)_2$, whereas multispecies biofilm coaggregates enhanced the resistance of *Streptococcus sobrinus*.

Nakajo et al (14) isolated 29 alkaline-resistant bacterial species from 37 primarily infected teeth. The authors reported that the predominant alkaline-resistant bacteria belonged to the *Enterococcus* genus. Their report obtained bacteria from teeth that were extracted because of severe root canal infection. Therefore, their results only describe bacteria present at this end stage. Furthermore, their methods did not allow the study of bacteria from different stages of endodontic treatment.

In this study, we sought to isolate alkaline-tolerant bacteria before endodontic treatment (S1), after chemomechanical root canal preparation (S2), and after the placement of an interappointment $Ca(OH)_2$ dressing (S3).

Materials and Methods

Selection Criteria

A total of 50 patients were recruited in this study. Ethics approval was obtained from the Domain Specific Review Board, National Healthcare Group. Informed consents were obtained from all patients. Included were teeth with pulp necrosis, clinical and radiographic evidence of periapical periodontitis, sufficient tooth structure for adequate isolation, a periodontal pocket depth of less than 4 mm, and no history of previous endodontic treatment. Patients who had received antibiotic therapy 3 months before treatment or suffered from immunocompromised disease and cracked teeth were excluded. Of the 50 cases, 43 passed the sterility control check. These teeth consisted of 15 anterior teeth, 8 premolars, and 20 molars. A total of 23 symptomatic (tenderness to percussion only) and 20 asymptomatic cases were examined.

Sampling Procedures

One endodontist and 9 full-time residents enrolled in the Postgraduate Endodontics Residency Program were trained and performed the same sampling procedures. A written checklist and a data collection form were used to ensure consistency among operators. Briefly, teeth were cleaned, and care was taken not to encroach into the pulp chamber during caries or restoration removal. Significant losses of tooth structure were rebuilt to secure leakage-free sampling conditions. After rubber dam isolation, dental floss was tightened around the cervical region. The operation field and tooth were sterilized using 30% hydrogen peroxide and 10% iodine tincture (15). Iodine tincture was inactivated with 5% sodium thiosulfate before a sterility check performed by scrubbing a cotton pellet against the tooth surface where the access opening was to be performed. The cotton pellet was aseptically transferred to a sterile tube containing thioglycollate broth (Sigma-Aldrich, St Louis, MO) and incubated anaerobically for 5 days. Samples that failed the sterility check were excluded from the study.

An access opening to the pulp chamber was prepared with a sterile bur. A small amount of reduced transport fluid (RTF) (16) was added to the canal. The canal was instrumented with a sterile stainless steel #15 K-file 1 mm short of the radiographic apex (estimated from the diagnostic radiograph) to release dentinal shavings. The smeared fluid in the canal was absorbed with 5 successively applied paper points, with each paper point left in the canal for at least 30 seconds. This bacteriologic sample, termed S1, was placed in a sterile Eppendorf tube containing 250 μL RTF. The tube was immediately transported to the laboratory for microbiological analysis. After coronal preflaring and working length determination, the canal was instrumented sequentially with hand/rotary files to the desired master apical file size. Canal irrigation with 1.25% sodium hypochlorite was delivered with a 5-mL disposable syringe and a 27-G needle after each instrumentation.

After completion of this treatment sequence, the remaining sodium hypochlorite was neutralized with 5% sodium thiosulfate. The canal was dried with paper points, and RTF was added. A sterile K-file 1 size larger than the master apical file was inserted to the working length and rotated to release dentinal shavings. The K-file was removed, cut off at the apical 5 mm, and transferred to the second RTF vial according to the technique designed by Ørstavik et al (4). The remaining RTF was absorbed with 5 paper points and was transferred to the same RTF vial as the cut K-file. This sample served as the postinstrumentation sample (S2). In all cases, the root canal instrumentation was completed during the first appointment. After these procedures, a slurry paste of $Ca(OH)_2$ mixed with sterile water was placed into the canal with a Lentulo spiral, and the access cavity was dressed with Intermediate Restorative Material (IRM; Dentsply Caulk, Milford, DE).

$Ca(OH)_2$ dressing was left in the canal for at least 1 week. During the second appointment, a sterility control sample was obtained. The IRM dressing was removed aseptically under rubber dam isolation. The intracanal $Ca(OH)_2$ was removed with ultrasonically agitated irrigation of the physiological saline. Inspection with microscopic magnification confirmed the removal of $Ca(OH)_2$. A third bacteriologic sample (S3) was then taken according to the procedure described for the S2 sample, except that a K-file 2 sizes larger than the master apical file was used. After sampling, the canal was dried and filled with gutta-percha and sealer.

Monitoring of pH under Culture Conditions

The pH of the brain-heart infusion (BHI) broth (14) (Acumedia, Lansing, MI) supplemented with yeast extract (Acumedia), hemin (Sigma-Aldrich), and vitamin K (Sigma-Aldrich) was adjusted to a pH of 9 with 10 mol/L potassium hydroxide (Sigma-Aldrich) using a pH meter and incubated at 37°C anaerobically. Changes in pH were monitored daily up to a period of 5 days. Experiments were performed 3 times and each time in triplicate.

Bacterial Culture

Samples were vortexed to dislodge bacteria from the paper points. The amount of bacteria present in the root canal sample was determined by serial dilution in BHI broth and plating on Tryptic soy agar (TSA) supplemented with 5% sheep blood agar (Oxoid, Hampshire, UK). A 50-μL bacterial suspension was inoculated into 3 mL buffered BHI broth supplemented with yeast extract, hemin, and vitamin K at a pH of 9 and incubated at 37°C for 3 days. Cultures were incubated at 37°C in a DG250 Anaerobic Workstation (Don Whitley Scientific, West Yorkshire, UK) supplied with 80% N_2, 10% H_2, and 10% CO_2. Uninoculated (pH = 9) adjusted media served as an aseptic control. If turbidity was observed after 3 days of culture, the bacterial culture was subcultured at a ratio of 1:20 into a tube containing 2 mL BHI broth supplemented with yeast extract, hemin, and vitamin K with the pH adjusted to 9 to further confirm that the bacteria were capable of growing under alkaline conditions. When turbidity was observed, the bacterial suspension was streaked on a TSA sheep blood agar plate.

Identification of Bacterial Isolates

Isolated colonies, which were monocultures at this point, were inoculated into 2 mL buffered BHI broth supplemented with yeast extract, hemin, and vitamin K at a pH of 9 and incubated anaerobically at 37°C. Genomic DNA was extracted using the QiaAmp Minikit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The 16S ribosomal RNA gene was amplified by polymerase chain reaction (PCR) using a universal primer set (forward, 5′-GAGAGTTTGATYMTGGCTCAG; reverse, 5′-GAAGGAGGTGWTCCARCCGCA) (17). A PCR mixture consisted of a 50-ng template of DNA, 20 pmol each of forward and reverse primers, and 2× GoTaq PCR mastermix (Promega, Madison, WI) in a final reaction volume of 100 μL. The following thermal cycling conditions were used: initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute 45 seconds. A final extension at 72°C for 10 minutes was performed. PCR was performed for 25 cycles in an iCycler (BioRad, Hercules, CA). An aliquot of the PCR product was analyzed by electrophoresis on a 1% agarose gel. The PCR product was purified using Wizard SV Gel and the PCR Clean Up System (Promega) according