Evaluation of the Susceptibility of Multispecies Biofilms in Dentinal Tubules to Disinfecting Solutions

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Abstract

Introduction: The present study evaluated the effect of the source of biofilm bacteria on their susceptibility in dentinal tubules to disinfecting solutions using an infected dentin model. **Methods:** Infected dentin blocks were prepared. *Enterococcus faecalis* strains VP3-181 and Gel 31 were introduced into dentinal tubules by centrifugation to form monospecies biofilms, whereas 3 specimens of pooled plaque bacteria collected from different donors were used to grow multispecies biofilms in dentin. After 1 and 3 weeks of incubation, the samples were subjected to sterile water, 2% chlorhexidine (CHX), and 2% sodium hypochlorite (NaOCl). After the 3-minute exposure, the proportions of killed bacteria in dentin canals were assessed by viability staining and confocal laser scanning microscopy. **Results:** The proportion of killed bacteria in mature (3 weeks) mono- and multispecies biofilms was lower than in young biofilms (1 week) after treatment ($P < .05$). *E. faecalis* Gel 31 biofilms and multispecies biofilms were more resistant than VP3-181 biofilms. No differences in the susceptibilities to the disinfecting agents of the 3 multispecies biofilms were detected; 2% NaOCl was more effective against multispecies biofilms in dentin than 2% CHX ($P < .05$), whereas no significant difference was detected between 2% CHX and 2% NaOCl against the *E. faecalis* strains. **Conclusions:** Mature mono- and multispecies biofilms in dentinal tubules are more resistant to disinfectants than corresponding young biofilms. The susceptibility of the monospecies *E. faecalis* dentin biofilm showed strain-related differences, whereas the multispecies biofilms from different donors showed similar susceptibility. *(J Endod 2016;42:1246–1250)*

Key Words

Biofilm, dentinal tubules, disinfection, maturation, monospecies, multispecies

Significance

Different disinfecting solutions have been reported to be able to kill monospecies bacteria in infected dentin. Clinically, bacteria in endodontic infections originate from multispecies plaque. The effectiveness of different antimicrobial strategies against multispecies plaque biofilm in infected dentin is about to be discovered.

Pulpal and periapical infections are caused by a mixed biofilm community acting as a multicellular organism embedded in an extracellular polymeric substance (EPS) (1). In primary apical periodontitis, the infectious microorganisms colonize and form sessile biofilms on the root canal wall of the tooth and invade into dentinal tubules, which may further contribute to the resistance of the biofilm microbes to instrumentation and disinfection (2). Contrary to primary infections, *Enterococcus faecalis* is often isolated in persistent endodontic infections, either as the only isolate or together with a few other bacteria. Several different bacteria have the ability to penetrate into dentin canals; with *E. faecalis*, this characteristic has been widely examined (3).

The eradication and killing of bacteria in infected dentin have been investigated in many studies. Most of them have used traditional approaches such as incubating liquid cultures of bacteria with dentin blocks or extracted teeth to create bacterial invasion into dentin (4, 5). The weakness of this method is that obtaining a strong and equal presence of bacteria in dentin in different specimens is difficult. Culturing the dentin sampled with bur shavings after periods of disinfection often gives results with high variation in bacterial counts, which makes it difficult to measure the effectiveness of the medicaments accurately (6). To overcome these shortcomings, centrifugation has been recently adopted to force bacteria into dentin canals in order to create a heavy, deep, and standardized presence of bacteria in dentin (7). In recent studies, the use of cell viability fluorescent dye in combination with confocal laser scanning microscopy (CLSM) has allowed *in situ* quantitative analysis of the effectiveness of dentin disinfection (8). This model has been used to test disinfecting efficacy of various antibacterial solutions, irrigation protocols, and endodontic materials against young and/or old *E. faecalis* biofilms in dentin canals (9–12). Results from different studies have given comparable results indicating the usefulness of the method. However, 1 major

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limitation of these studies is that only *E. faecalis* was used in the model, and often only a single strain was used (7, 9–12). No data about the effectiveness of antimicrobial strategies against multispecies dentin biofilm using this methodology are available so far.

Biofilm growth is a continuous, dynamic process. Studies by Shen et al (13) and Stojicic et al (14) using multispecies biofilms grown on collagen-coated hydroxyapatite disks from different donors showed that mature, 3-week-old biofilms were much more resistant to disinfection than younger biofilm. The same study showed that biofilms from 6 different sources had a similar, time-dependent susceptibility pattern (14). Studies using the infected dentin model with viability staining and CLSM as described earlier have reported that mature *E. faecalis* biofilms in dentin canals were also more resistant to antibacterial solutions than young *E. faecalis* dentin biofilms (9, 10). Corresponding information using viability staining and CLSM about the susceptibility of a multispecies biofilm in dentin canals to antimicrobial agents is not available.

The present study aimed to establish a multispecies dentin canal biofilm model using centrifugation of plaque bacteria into dentinal tubules and to evaluate the effect of the source of biofilm bacteria and the level of biofilm maturation on the effectiveness of disinfecting agents against biofilm bacteria in dentin canals using viability staining and CLSM. In addition, 2 *E. faecalis* strains with different sensitivity in planktonic killing tests in a previous study (15) were also included in the dentin biofilm experiments.

Materials and Methods

Dentin Sample Preparation

Thirty intact caries-free, single-rooted extracted teeth were collected and stored in 0.01% sodium hypochlorite (NaOCl) solution at 4°C until use. The study was approved by the University of British Columbia Clinical Research Ethics Committee Review Boards (certificate H12-02430). Written informed consent was obtained from the participants for collecting the plaque bacteria in this study. Specimen preparation was based on a previously described protocol (7). Sixty dentin block specimens were prepared.

Dentin Canal Infections

Two strains of *E. faecalis* (VP3-181 and Gel 31) originally isolated from persistent apical periodontitis cases (16) were grown on brain-heart infusion (BHI) agar (Becton-Dickinson, Sparks, MD) plates under anaerobic conditions (Bactron300 Shel Lab Anaerobic Chamber; Sheldon Manufacturing, Inc, Cornelius, OR) at 37°C overnight. The bacteria were harvested and suspended in BHI broth (Becton-Dickinson). The cell density was standardized to an optical density of 0.05 (150 μL, 405 nm; ELx808 Absorbance Reader, BioTek Instruments, Inc, Winooski, VT).

Pooled supragingival and subgingival plaque was collected from 3 adult volunteers aged 25–45 years. Plaque collected from each donor was suspended thoroughly in BHI broth and incubated under anaerobic conditions at 37°C overnight. The bacteria were collected and prepared with fresh BHI into the same optical density as described for *E. faecalis* earlier.

Following a previously described protocol (7), *E. faecalis* and plaque suspensions were centrifuged into dentinal tubules. The prepared dentin specimens were colonized either with one of the plaque cultures or with *E. faecalis* and incubated in BHI broth under anaerobic conditions at 37°C. Half of the specimens were randomly selected and incubated for 1 week, whereas the other specimens were incubated for 3 weeks to allow biofilm maturation. Fresh BHI broth was changed once a week for the 3-week samples (9).

Disinfection of Dentin

After 1 or 3 weeks of incubation, the infected dentin samples were removed from each tube. After a 1-minute rinse with sterile water and air drying, the cemental side of each dentin sample was sealed by nail varnish. A total of 60 dentin specimens were included in the study. These were first divided into 5 groups (3 plaque groups and 2 *E. faecalis* groups). Each of these groups was further divided into 1- and 3-week biofilm groups (6 dentin specimens in each). Finally, the dentin specimens were divided into 2 medicament groups, 2% chlorhexidine (CHX; Sigma-Aldrich Co, St Louis, MO) and 2% NaOCl (Clorox Company of Canada Ltd, Brampton, ON, Canada), and 1 control group of sterile water. Fifty microliters of each freshly prepared medication was added on the root canal side of the infected dentin samples for 3 minutes. After disinfectant exposure, each specimen was washed with sterile water for 1 minute and fractured vertically through the center of root canal into 2 halves to expose a fresh surface of longitudinally fractured dentin tubules (7). A total of 120 fractured dentin pieces were stained with viability staining before confocal laser scanning microscopic examination and analysis.

Confocal Laser Scanning Microscopic Examination

Fractured dentin specimens for confocal laser scanning microscopic imaging (Nikon Eclipse C1; Nikon Canada, Mississauga, ON, Canada) were stained using the LIVE/DEAD BacLight Bacterial Viability kit L-7012 (Molecular Probes, Eugene, OR) containing SYTO 9 (Molecular Probes, Eugene, OR) and propidium iodide according to the manufacturer's instruction followed by a 1-minute rinse with phosphate-buffered saline (7). Four areas (318.9 μm × 318.9 μm) edging the root canal and extending to the dentin were randomly selected and scanned on each fractured surface. Thus, a total of 480 scans were performed in the study, 16 in each treatment subgroup. A stack of 20 slices with a 0.5-μm step size was acquired for each confocal laser scanning microscopic scan using the EZ-C1 v.3.40 build 691 software (Nikon Canada) at a field resolution of 512 × 512 pixels. The volume of dead cells (red fluorescence) and live cells (green fluorescence) were calculated using Imaris 7.2 software (Bitplane Inc, St Paul, MN). The univariate analysis of variance test (SPSS 16.0; SPSS Inc, Chicago, IL) was performed to compare the proportions of dead cell volume in each group at different stages of growth. Furthermore, least significant difference post hoc multiple comparisons were used at a significance level of $P < .05$.

Results

A total of 60 infected dentin samples and 480 scanned areas were analyzed. Three-dimensional confocal laser scanning microscopic images confirmed the penetration of *E. faecalis* strains VP3-181 and Gel 31 and plaque bacteria (sampled from three different donors) deep into the dentinal tubules after centrifugation and incubation for 1 or 3 weeks (Figs. 1*A1–B3* and 2*A1–B3*). The amount of bacteria killed in each experimental group varied from 17.7% ± 3.9% to 32.8% ± 5.4% (Table 1). There were no significant differences in the control group in the proportion of dead bacteria between specimens of mono- and multispecies biofilms or biofilm of different ages ($P > .05$).

In the experimental groups, the percentage of killed bacteria was dependent on the level of biofilm maturation, the biofilm type (monospecies/multispecies), and the antimicrobial agent (Table 1). The killing ratio in 3-week-old biofilms was always significantly lower than in 1-week-old biofilms in each group ($P < .05$). Only 17.7%–27.5% of the bacteria were killed in the 3-week-old biofilms. *E. faecalis* VP3-181 biofilm in dentin was more sensitive to CHX and NaOCl than *E. faecalis* Gel 31 and plaque biofilms ($P < .05$), except for the 3-week-old *E. faecalis* biofilms exposed to 2% NaOCl ($P > .05$). No significant differences in killing by the same irrigant were found between the 3