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.

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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 (Bactron 300; 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. 1A1–B3 and 2A1–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
plaque biofilms in dentin when biofilms of the same age were compared ($P > .05$). In 1-week-old and 3-week-old biofilms, the proportions of killed bacteria after exposure to 2% NaOCl in *E. faecalis* Gel 31 and each plaque group showed no significant differences ($P > .05$). Two percent NaOCl was more effective than 2% CHX against the 3 multispecies plaque biofilms in dentin ($P < .05$).

**Discussion**

Supragingival and subgingival plaque consists mainly of gram-positive and gram-negative facultative and anaerobic bacteria (17, 18). Tooth surface plaque is easy to collect and in a much greater amount than bacteria/biofilm from an infected root canal. Therefore, biofilms from the tooth surface rather than the root canal have been used to develop *in vitro* oral biofilms for the testing of different disinfection strategies (14, 19). Bacteria in periodontal and endodontic infections and in dental caries all originate from tooth surface plaque (20). In previous studies, the open biofilm model, grown on collagen-coated hydroxyapatite discs or dentin, has been used for the multispecies biofilm (13, 14), whereas *E. faecalis* has been studied using both the open biofilm model (15) and the dentin infection model in which dentin canals have been filled by forcing the bacteria in using centrifugation (7).

*E. faecalis* seems to tolerate well the potentially damaging forces of centrifugation as indicated by the observations in several previous studies (7, 9–12). The round cell shape, the relatively small cell size, and the ability to survive in a harsh environment may contribute to the usability of *E. faecalis* in this model (7). The present study is the first time that the same centrifugation protocol was adopted for the multispecies biofilm. The differences between plaque and *E. faecalis* suspensions are many and include the presence of species with a cell length longer than the dentin canal diameter and a possible presence of other materials such as food rests or even human cells in the pooled plaque. Plaque samples were notably difficult to disperse homogeneously in BHI broth. Therefore, based on extensive pilot experiments, after gentle but thorough pipetting of the initial sample, the plaque suspension was transferred into an incubator and cultured in BHI broth under anaerobic conditions at 37°C overnight. This allowed the growth of bacteria in suspension and sedimentation of debris in the medium. Pilot experiments had shown this to be necessary to prevent blockage of the dentinal tubule openings during centrifugation. Gram staining of the BHI-grown plaque before centrifugation confirmed the presence of multiple cell morphotypes, including cocci and rod-shaped bacteria of different diameters and cell lengths (data not shown). Despite the concerns about the effect of centrifugation on plaque bacteria, the proportion of dead cell volume in negative controls was only 6.8%–7.9%, which is similar to that in *E. faecalis* biofilms (Table 1). Another limitation of the methodology is that although mixed bacterial flora was used in the centrifugation into the dentin, no analysis was done to examine which species ultimately penetrated and established themselves in the dentin canal biofilms. However, the differences between CHX and NaOCl against gram-positive *E. faecalis* biofilms and mixed species biofilms indicate the presence of more varied flora in the latter biofilms.

To measure the portion of killed bacteria after exposure to the antibacterial solutions, viability staining and CLSM of fractured, intact dentin blocks were used instead of culturing infected dentin from bur shavings for colony-forming unit counting. CLSM collects the fluorescent signal from bacteria as well as the background fluorescence of dentin. Possibly because of the heavy presence of bacteria in the dentin canals because of centrifugation, the fluorescence signal from the bacteria and biofilm is strong, which allows using low gain values in the

![Figure 1.](image-url)
confocal scanning. Therefore, the background fluorescence, which could be a confounding factor, was very low and not an issue in any of the specimens. This has also been shown in earlier studies using the same model (7, 9–12).

We investigated the susceptibility to 2% CHX and 2% NaOCl of bacteria in young (1 week) and old (3 weeks) biofilms in dentinal tubules using 2 monospecies (*E. faecalis*) biofilms and 3 multispecies plaque biofilms. CHX and NaOCl were chosen because they are the most commonly used endodontic irrigants with antibacterial potential during root canal treatment in the clinic. In the present study, equal amounts of irrigants were placed on the dentin surface to measure their antibacterial efficacy, whereas in a clinical situation irrigants are usually applied as a continuous flow. Therefore, it is possible that the killing ratio by irrigants presented here is different and lower than in the clinical situation during the same exposure time. Generally, our results showed that bacteria cannot be completely killed in dentin canals during a 3-minute exposure to the tested medicaments (Table 1). Interestingly, a recent study that applied 2%–6% NaOCl or 2% CHX to infected dentin specimens showed that after 3 minutes of exposure, killing of *E. faecalis* in dentin canal biofilm slowed down considerably when compared with the first 3 minutes. Furthermore, after 10 minutes of repeated disinfectant exposures, killing of the bacteria almost ceased (10).

Susceptibilities of different strains of *E. faecalis* dentin biofilms or different multispecies dentin biofilms to antibacterial agents have not been previously compared. *E. faecalis* strains VP3-181 and Gel 31 have different susceptibility in planktonic killing experiments (15). Strain VP3-181, which was clearly more sensitive in planktonic killing, was also more sensitive in the present study when tested after forming a biofilm (Table 1). Interestingly, the differences between the 2 *E. faecalis* strains were smaller in the 3-week-old biofilms, and the difference in sensitivity to NaOCl at 3 weeks was no longer statistically significant. Although not enough strains were compared, the result might indicate that *E. faecalis* biofilm maturation (eg, continuing production of EPS) increasingly affects the sensitivity/resistance of the biofilm to external threats.

**TABLE 1.** Proportion of Dead Cell Volume (mean ± standard deviation, %) in Dentinal Tubules of Biofilms of Different Ages (1 and 3 weeks) Exposed to Different Disinfecting Solutions for 3 Minutes

<table>
<thead>
<tr>
<th>Source of biofilm bacteria</th>
<th>Sterile water</th>
<th>2% CHX</th>
<th>2% NaOCl</th>
<th>Sterile water</th>
<th>2% CHX</th>
<th>2% NaOCl</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em> VP3-181</td>
<td>6.8 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.9 ± 5.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.8 ± 5.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.5 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.2 ± 3.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27.5 ± 3.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. faecalis</em> Gel 31</td>
<td>6.8 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.8 ± 4.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.3 ± 4.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.5 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.1 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.4 ± 5.2&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plaque 1</td>
<td>6.9 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.4 ± 3.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>28.4 ± 2.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.9 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.8 ± 4.3&lt;sup&gt;f&lt;/sup&gt;</td>
<td>23.2 ± 4.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plaque 2</td>
<td>6.9 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.6 ± 4.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>28.1 ± 3.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.8 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.6 ± 3.7&lt;sup&gt;f&lt;/sup&gt;</td>
<td>22.5 ± 3.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plaque 3</td>
<td>6.8 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.8 ± 2.9&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>26.8 ± 5.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.5 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.7 ± 3.9&lt;sup&gt;f&lt;/sup&gt;</td>
<td>21.0 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

CHX, chlorhexidine; NaOCl, sodium hypochlorite.

Different superscript letters indicate statistically significant differences between groups (*P* < .05).
The multispecies biofilms in dentin grown from pooled plaque from 3 different donors showed no difference in their susceptibility to either 2% CHX or 2% NaOCl. The result may indicate that the source and possible differences in the species composition of the multispecies biofilm have less impact on its susceptibility than biofilm maturation, which is in accordance with a previous study (14). CHX was less effective than NaOCl against young multispecies biofilm, but after 3 weeks of biofilm growth, the difference was much smaller (Table 1). Overall, the effect of the 3-minute exposure of both agents against the multispecies biofilms was relatively poor. CHX and NaOCl showed a similar antibacterial effect against gram-positive *E. faecalis* biofilms in dentin, whereas in multispecies biofilms less bacteria were killed by CHX than NaOCl. CHX has been shown to be more effective on gram-positive bacteria than gram-negative bacteria (21). The differences in killing between the present study and the early study by Emilon (21) may be attributed to different microbial ecology (biofilms vs planktonic bacteria) and a different technique for analysis.

Old biofilms in dentin were more resistant than young biofilms to the disinfectants in all groups, even though no significant differences were found in 1-week-old and 3-week-old *E. faecalis* Gel 31 biofilms after exposure to 2% NaOCl. This is consistent with an earlier study using the dentin biofilm model with *E. faecalis* (9) and another study with an open biofilm model on plaque-infected hydroxyapatite disks (14). Several protective mechanisms underlying biofilm antimicrobial resistance have been reported. During biofilm development, increasing the biofilm matrix such as EPS acts as a physical or chemical diffusion barrier against antibiotics penetrating into the biofilm (22). In addition, environmental stresses in biofilms such as pH variations trigger certain bacterial stress responses that can affect gene expression (23). Slow-growing bacterial cells because of nutrient limitation can change into the viable but nonculturable state (24). With biofilm maturation in dentin, it is perhaps more difficult for bacterial cells located deep in dentinal tubules to get enough nutrients. The formation of subpopulations known as persisters also contributes to the adaptation of bacteria in biofilms and leads to enhanced resistance (25). Besides the previously described mechanisms, in the present scenario, limited penetration depth of irrigants into the dentinal tubules and inactivation of solutions by dentin could also contribute to the overall weak antimicrobial performance (5, 26).

In conclusion, within the limitation of the present study, a multispecies dentin canal biofilm model was established by centrifugation of plaque bacteria into dentin. Exposure of 1- and 3-week-old biofilms to 2% CHX and NaOCl revealed that only a small proportion (less than a third) of bacteria was killed after 3 minutes. The source of the multispecies plaque biofilms had no effect on the proportion of killed bacteria after exposure to the antibacterial solutions.

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The authors deny any conflicts of interest related to this study.

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