Evaluation of the Effect of Two Chlorhexidine Preparations on Biofilm Bacteria In Vitro: A Three-Dimensional Quantitative Analysis

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Abstract

Introduction: Microorganisms are essential in the development of periapical diseases and are the major causative factors associated with endodontic treatment failures. Microbial biofilms are communities of bacteria that attach to surfaces and form heterogeneous three-dimensional structures. The purpose of this study was to develop a biofilm model that closely mimicked in vivo biofilm to determine its susceptibility to endodontic antimicrobial irrigants by three-dimensional quantitative analysis. Methods: Collagen-coated hydroxyapatite (C-HA) and uncoated hydroxyapatite (HA) disks were inoculated with dispersed subgingival plaque for 3 weeks. Thick biofilms rich in spirochetes were formed on both substrates. Biofilms were subjected to 1-, 3-, and 10-minute exposures to CHX-Plus (Vista Dental Products, Racine, WI) and 2% chlorhexidine (CHX). After treatment, the volume ratio of dead bacteria to all bacteria in biofilms, indicated by the ratio of red and (red + green) fluorescence, was analyzed by confocal laser scanning microscopy for each medication. Results: The proportion of killed bacteria was dependent on the type of irrigant and the time of exposure in both C-HA and HA biofilm models (p = 0.00). CHX-Plus showed higher levels of bactericidal activity at all exposure times than 2% CHX (p < 0.001). The C-HA biofilm was thicker than the HA biofilm. Less bacteria were killed in C-HA biofilm than in the HA model. Conclusions: This multispecies biofilm model and quantitative analysis methodology may be useful for the evaluation of the antimicrobial effectiveness of endodontic disinfecting agents. (J Endod 2009;35: 981–985)  

Key Words  
Antimicrobial, biofilm, chlorhexidine, CHX-Plus, collagen, confocal laser scanning microscopy  

Successful treatment of apical periodontitis is dependent on the elimination of the infective microflora from the necrotic root canal system. Antimicrobial irrigating solutions and other locally used disinfecting agents and medicaments have a key role in the eradication of the microbes (1). One of the principle roles of root canal irrigation is to assist in the killing of bacteria and the removal of the bacterial biofilm from uninstrumented surfaces (30-50% of the root canal wall) (2, 3). Recent laboratory studies have focused on evaluating the effectiveness of root canal irrigants. Many of these studies have grown the bacterial strains as planktonic cultures (bacteria in suspension) (4, 5). Although it has long been acknowledged that pure cultures of bacteria are virtually absent in nature, it has been only in the past few years that the biofilm mode of growth has been recognized as the default state for most bacteria. It has become accepted that biofilm grown bacteria express different phenotypes and often exhibit quite different characteristics than do the same bacteria grown in planktonic cultures. Bacteria that are sessile (attached to a surface) express different genes and, therefore, behave differently from free-floating or planktonic bacteria (6). Notable among these differences is the increased resistance to antimicrobial agents that can be 100- to 1,000-fold greater for a species in a mature biofilm relative to that same species grown planktonically (7). Thus, to provide clinically more useful results, it is imperative to develop multispecies biofilm models to evaluate endodontic irrigants.

Currently available information on the relationship between endodontics and microbial biofilms consists chiefly of observations of bacterial condensations in the root canal system (8) and the ability of selected irrigants and medicaments to disrupt them (9–14). Knowledge of the structure of a biofilm is important in order to understand its special characteristics as well as to monitor the efficacy of procedures to eliminate or chemically remove it (15). Although various successful attempts to evaluate biofilm structure were made, producing a clear image of the biofilm or obtaining reliable data about its thickness, density, or biomass, biofilm still presents a challenge (16). Microscopy is a highly valuable approach to biofilm studies; a confocal laser scanning microscope (CLSM) in particular can create accurate three-dimensional (3D) reconstructions. To date, there have been no published data based on 3D analysis of the efficacy of endodontic irrigants against microorganisms grown as a biofilm. The aim of this study was therefore (1) to quantify and compare the efficacy of two endodontic irrigating solutions currently used in root canal treatment and (2) to introduce a new in vitro multispecies biofilm model that closely mimics the in vivo biofilm for evaluating the efficacy of endodontic irrigants.
Materials and Methods

Sterile hydroxyapatite (HA) discs (0.38-inch diameter by 0.06-inch thickness; Clarkson Chromatography Products, Williamsport, PA) were used as the biofilm substrate. The effect of collagen on biofilm formation and disinfectant performance was studied by using collagen-treated hydroxyapatite (C-HA) disc (16). The HA discs were coated with bovine dermal type I collagen (10 μg/mL collagen in 0.012 N HCl in water) (Cohesion, Palo Alto, CA). Collagen coating of the HA discs (C-HA) was done by overnight incubation at 4°C in the wells of a 24-well tissue culture plate containing 2 mL of the collagen solution.

Subgingival plaque on the first or second upper molars of each of three healthy volunteers was collected and mixed in brain heart infusion broth (BHI) (Difco, Detroit, MI). The C-HA and HA discs were placed in the wells of a 24-well tissue culture plate containing 1.80 mL of BHI. Each well was inoculated with 0.2 mL of dispersed dental plaque, containing a minimum bacterial cell concentration of 3.2 × 10^7 CFU/mL. The discs were incubated under anaerobic conditions (AnaeroGen; Oxoid, UK) at 37°C for 21 days; fresh medium was changed once a week.

After 21 days of anaerobic incubation in BHI broth, specimens of four C-HA and HA biofilms for scanning electron microscopy (SEM) were washed with phosphate-buffered saline and immersed in a fixative solution containing 4% glutaraldehyde in sodium cacodylate buffer at 4°C for 3 hours. The specimens were then subjected to increasing concentrations of ethanol for serial dehydration. The dehydrated specimens were dried using a critical point drier (Samdri-795; Tousimis Research Corporation, Rockville, MD), sputter coated with gold-palladium (Hummer VI; Technics West Inc, CA), and examined by SEM at 8 kV (Stereoscan 260; Cambridge Instruments, Cambridge, UK).

The specimens for the CLSM were rinsed in 0.85% physiological saline to remove the culture broth. The discs were then immersed in 2 mL of either 2% chlorhexidine digluconate (CHX) freshly prepared from 20% stock solution (Sigma Chemical Co, St Louis, MO) or CHX-Plus (Vista Dental Products, Racine, WI) for 1, 3, or 10 minutes. Samples treated with saline for corresponding time periods were used as a negative control. Following the exposure to the disinfecting agents or saline, the specimens were gently rinsed in saline. Four biofilm discs were used for each disinfecting agent for the indicated times. The LIVE/DEAD BacLight Bacterial Viability kit L-7012 (Molecular Probes, Eugene, OR) for microscopy and quantitative assays contains separate vials of the two component dyes (SYTO 9 and propidium iodide. The dyes were used in 1:1 mixture for staining the biofilm bacteria following manufacturer’s instructions. The excitation/emission maxima for these dyes are 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide. Fluorescence from stained cell was viewed using a CLSM (Nikon Eclipse C1; Nikon Canada, Mississauga, ON). Simultaneous dual-channel imaging was used to display green and red fluorescence.

CLSM images of the biofilms were acquired by the software EZ-C1 v. 3.40 build 691 (Nikon) at a resolution of 512 × 512 pixels. Individual biofilm images covered an area of 1.64 mm² per field of view. The mounted specimens were observed using 10× lens. The scanned images for each disinfecting agent were obtained by using five randomly chosen areas of each of the four samples (20 samples per agent per time). Confocal LIVE/DEAD images were analyzed by using a color segmentation algorithm in the MeVisLab package (available from www.mevislab.de/), which separated the red (dead cells) and green (viable cells) fluorescence signals by color threshold and measured the total volume (pixels³) covered by each segmented color. The volume ratio of red fluorescence (dead cells) to green-and-red fluorescence (live and dead cells) indicated the portion of killed cells for each medicament. The results were analyzed by using univariate and post hoc analysis, when necessary, at a significance level of p < 0.05.

Results

After 3 weeks of incubation, the CHA biofilm (around 155 μm) was thicker than the HA biofilm (around 100 μm) (Fig. 1A and B). SEM images of the C-HA and HA discs showed the presence of multispecies, heterogeneous biofilm consisting of cocci, rods, and filaments as well as small clusters of cocci dispersed across the HA surface (Fig. 1C). Spirochetes were found in high numbers throughout the biofilm surface. The biofilm was organized in network structures typical of natural biofilms.
Typically, 120 to 160 slices with a voxel size of 2.5 x 2.5 x 0.5 mm were scanned in each sample per stack. After segmentation and reconstruction of the data, the distribution of microbial populations in a 3D biofilm model was analyzed by the MeVisLab package. The proportion of killed bacteria was dependent on the type of disinfecting agent and the time of exposure in both C-HA and HA biofilm models (univariate analysis, F = 122.35, p = 0.00) (Table 1). All treatment groups showed a significant reduction of viable bacteria within the biofilms compared with the saline control (post hoc test, p < 0.001) (Fig. 2). The proportion of killed cells increased significantly with the increasing time of medicament exposure for 1, 3, and 10 minutes (post hoc test, p < 0.001). The volume of killed cells in the CHX-Plus group was significantly higher than that in the 2% CHX group at all time periods and in both C-HA and HA groups (p < 0.001). The proportion of killed bacteria was higher in the CHX-Plus group at 3 minutes than in the 2% CHX group at 10 minutes. More bacteria survived in the C-HA biofilm than in the HA model in both medicament groups (Table 1).

**TABLE 1.** The Proportion of the Dead Cell Volume of the Entire Biofilm Volume in the C-HA and HA Biofilms

<table>
<thead>
<tr>
<th></th>
<th>CHX-Plus†</th>
<th>2% CHX†</th>
<th>Saline</th>
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<tr>
<td></td>
<td>C-HA</td>
<td>HA</td>
<td>C-HA</td>
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<td></td>
<td>1 min*</td>
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<tr>
<td></td>
<td>0.24 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.19 ± 0.02</td>
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<tr>
<td></td>
<td>3 min*</td>
<td></td>
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<tr>
<td></td>
<td>0.69 ± 0.03</td>
<td>0.72 ± 0.03</td>
<td>0.31 ± 0.03</td>
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<tr>
<td></td>
<td>10 min*</td>
<td></td>
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<tr>
<td></td>
<td>0.75 ± 0.02</td>
<td>0.79 ± 0.01</td>
<td>0.56 ± 0.03</td>
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</table>

C-HA, collagen-coated hydroxyapatite; HA, hydroxyapatite; CHX, chlorhexidine.

*Significant difference between different exposure times (p < 0.001).
†Significant difference between CHX-plus and 2% CHX at all time periods and in both C-HA and HA groups (p < 0.001).

Figure 2. Three-dimensional constructions of CLSM scans of 3-week-old H-CA biofilms after treatment with disinfecting agents and viability staining. Biofilm treated with physiological saline (control) ([A] top view and [B] bottom view), ([C]) 2% CHX treatment for 3 minutes, ([D]) CHX-Plus treatment for 3 minutes, ([E]) 2% CHX treatment for 10 minutes, and ([F]) CHX-Plus treatment for 10 minutes. Green (viable cells) and red (dead cells).
Discussion

Dentin is a composite material made up of an organic fraction (around 20 wt%), which is mainly collagen, and an interpenetrating inorganic fraction (around 70 wt%). The latter is composed primarily of a poorly crystalline carbonated HA with needle and/or plate-like morphology, which exists both within the collagen fibrils (intrafibrillarly mineralized) and between fibers (interfibrillarly mineralized) on a nanometric scale (1). It has been established that the forming capacity and structural organization of a biofilm are influenced by the chemical nature of the substrate (18). Type I collagen is the major organic component (90%) of dentin, although several other noncollagenous proteins are also present in small amounts. It is known that certain bacteria can attach to type I collagen in dentin (19) through the expression of surface adhesins and form biofilms (17, 20). Biofilm experiments conducted on polycarbonate or glass substrate may not provide a true indication of the bacteria-substrate interaction. In the present study, HA, with and without coating of type I collagen, provided an excellent substrate for multispecies biofilm growth. Chemical similarity with the teeth/dentin and the excellent growth of the multispecies biofilm indicate that this model has the potential to serve as a standard biofilm model for studies of in vitro endodontic biofilms. The corresponding growth of oral spirochetes in multispecies in vitro biofilm has not been described before. Kuramitsu et al (21) described a two-species in vitro biofilm by Porphyromonas gingivalis and Treponema denticola grown on special media. However, P. gingivalis cells died after 3 days of culture and the spirochetes after 5 days. In the present study, 90% of the bacteria were viable after 3 weeks of culture (Table 1), indicating that the interbacterial ecologic relationships together with the weekly addition of fresh external nutrition succeeded in creating a viable multispecies system in which the microbial species have adapted to long-term survival in the biofilm.

Conditions under which biofilms exist in infected root canals in vivo are not fully understood (22). In vitro studies have focused on the efficacy of selected irrigants and medicaments to remove biofilms grown in wells (9), on membrane filters (23), and on dentin samples (8, 24–26) by using one or a few strains of selected species found in root canal infections. In the present study, HA with collagen coating and nutrient-rich media were used. Biofilms were not grown under agitation in order to simulate the situation in the root canal in which the fluid flow is supposed to be minimal. Chemically, the new model resembles dentin (HA and collagen); on the other hand, it does not simulate the fine details of dentin microanatomy. However, the standard shape of the discs makes it possible to grow biofilms with consistent characteristics, which has proven difficult when using dentin as the biofilm substrate. Additional local factors in the root canal environment may affect the function of the various irrigating solutions. Therefore, conclusions from the present study must be drawn with caution.

Endodontic diseases are polymicrobial infections in which microbial interactions play a significant role in the ecologic regulation and establishment of an endodontic habitat-adapted multispecies microbiota. Duggan and Sedgley (27) found that the biofilm-forming capacity of root canal and oral strains may not be an important contributory factor for their presence in these environments. However, the fact remains that bacteria and other microbes in the root canal infections are organized in biofilms. The model described here provides a method for the study in vitro of multispecies biofilm that closely mimics the in vivo biofilm. The versatility of this model, combined with its simplicity and high reproducibility, may make it an effective model to study bacterial biofilm colonization and development. The model should also be useful for investigating the mechanisms of enhanced antimicrobial resistance that has been attributed to biofilm-grown bacteria. Further research is required to detect species composition in multispecies biofilm.

Biofilms are communities of microorganisms attached to surfaces that may develop a complex heterogeneous 3D structure. Analysis of microbial biofilms by CLSM yields stacks of digital images that can be combined to give a 3D view of the biofilm. CLSM technique can be useful as a complement to the established microbiological, histological, electron microscopic, and polymerase chain reaction-based techniques for the identification of viable bacteria. MeVisLab as an advanced software package has been used to solve 3D problems in contemporary endodontic research (28). This sophisticated tool is also used to quantitatively analyze and compare biofilm changes under different conditions. However, the precision of the calibration procedure may be biased by imperfections during the conversion of a physical quantity into a numeric value (e.g., signal-to-noise) and sample preparation techniques. The size of contour can be used to define the level of resolution and hence the extent of analysis. Finer details are represented by small-size contours, whereas coarser details are represented by larger contours or borders. Because the number of clusters is decreasing, the level of resolution is also lower (29). In the current study, a high resolution of 512 × 512 pixels was chosen. Contours of all objects in the image were automatically generated and used to segment and label each object. This procedure had the capability of filtering out specific objects of small contour sizes, normally considered as noise, which is an interesting feature that proved suitable for biofilm analysis.

In the present study, as observed by CLSM examination, the basal layer of the biofilm appeared to have a slightly higher proportion of red fluorescence than the superficial layer in both C-HA and HA models. This is likely to be a consequence of nutrient depletion in the basal layer because it was a universal finding in both treatment and control groups. This finding is consistent with a previous report by Kishen et al (25) with Enterococcus faecalis biofilm (25). The fluid flow in biofilms is reduced, and long diffusional distances are likely to create areas of different solute concentrations. As a consequence, transport of may become the rate-limiting process of the various biotransformations (30). Solute gradients account for the formation of microridges within the biofilm matrix. The various physical and chemical characteristics of the biofilm influence the rates of liquid transport and bioconversions. The current results showed the C-HA biofilm was about 50% thicker than the HA biofilm. Hence, it is not surprising that the proportion of dead bacteria in C-HA biofilm was lower than that in HA model. Adhesion to and colonization of the host by microorganisms are the first steps in the establishment of endodontic infections. Kayaoglu et al (31) found that collagen association at a planktonic state increased the bacterial resistance to chlorhexidine. The authors hypothesized that bacterial cell encountering extracellular matrix proteins (eg, collagen, laminin, and fibrinogen) as the elements to be exploited for the colonization of the host may modify its gene expression for the protection and adaptation to the stressful condition (31, 32). Their finding lends support to our result here; adhesins produced on the bacterial cell surface as response to collagen can partly explain the greater thickness of the C-HA biofilm and thereby also increased resistance to the medicaments.

CHX-Plus is a relatively new product for endodontic disinfection. In addition to chlorhexidine gluconate, it contains proprietary surface modifiers to reduce the surface tension. The results of the present study showed that CHX-Plus killed bacteria much faster in anaerobic multispecies biofilm than 2% CHX. Recently, Williamson et al (14) similarly showed a better effect by CHX-Plus than by normal 2% CHX against E. faecalis in a simple, monospecies biofilm model.
Within the limitations of this in vitro study, the multispecies biofilm model that closely mimics in vivo biofilm served as a promising platform for the evaluation of the antimicrobial efficacy of two different chlorhexidine products used in endodontic disinfection. Three-dimensional quantitative measurements of the biofilm viability with CLSM provided a powerful tool for the analysis of the dynamics of killings by the antibacterial agents. The described methodology has excellent potential for biofilm research.

**References**