Antimicrobial Susceptibility of Monoculture Biofilms of a Clinical Isolate of *Enterococcus faecalis*

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Abstract

The purpose of this study was to create a monoculture biofilm of a clinical isolate of *Enterococcus faecalis* and to determine susceptibility against four antimicrobial irrigants. Biofilms were subjected to 1-, 3-, and 5-minute exposures to one of the following irrigants: 6% sodium hypochlorite (NaOCl), 2% chlorhexidine gluconate (CHX) or one of two new products, <6% NaOCl with surface modifiers (Chlor-XTRA) or 2% CHX with surface modifiers (CHX-Plus™) (Vista Dental Products, Racine, WI). It was hypothesized that NaOCl and CHX would be equally effective and that addition of surface modifiers would improve bactericidal activity of the respective irrigants compared to the original formulations. Results indicate that 6% NaOCl and Chlor-EXTRA™ were significantly superior against *E. faecalis* biolfilms compared to 2% CHX and CHX-Plus™ at all time points except five minutes. *(J Endod 2009;35: 95–97)*

Key Words

Biofilms, endodontic irrigants, *Enterococcus faecalis*

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The primary etiology of persistent periapical pathosis has been attributed to viable bacteria remaining in the undebrided areas of the root canal system (1). Numerous authors have identified *Enterococcus faecalis* as a predominant species found in previously treated root canals with persistent periapical disease (2–4). The ability of *E. faecalis* to adapt to environmental changes in the root canal after endodontic treatment and to remain as a pathogen in the root canal system makes the elimination of this species very difficult (5). *E. faecalis* has shown the ability to enter a viable but nonculturable state, a condition that protects the bacteria during extreme stress. Once the environmental stress has passed, the bacteria may return to normal function (6).

E. faecalis has been shown to have a high affinity for biofilm formation (7). Bacteria form biofilms as a means of defense and to facilitate physiologic processes. After a protein pellicle is deposited on the root surface, planktonic bacteria adhere and begin to multiply. Other planktonic bacteria adhere to the surface as the biofilm matures. This layering organization of the biofilm protects the bacteria from changes in pH and other antimicrobial insults (8). Furthermore, biofilm formation is enhanced with coaggregation, the adhesion of two or more bacterial species (9). An important virulence factor associated with *E. faecalis* is an aggregation substance that facilitates the ability of this species to form and remain viable in a biofilm (6).

Given that current root canal instrumentation techniques alone are unable to render root canals bacteria free, a chemical irrigant is necessary to assist in reducing bacterial numbers and their toxic byproducts. Ideal irrigants should remove organic and inorganic debris; have low tissue toxicity and low surface tension; and lubricate, disinfect, and remove the smear layer (10). Sodium hypochlorite possesses many properties of an ideal irrigant; however, it must be used cautiously to prevent extrusion into the periapical tissues where it may cause severe inflammatory reactions (11, 12).

In cases of concern (open apex, perforation, and so on), an alternate irrigant showing low tissue toxicity is desirable. Chlorhexidine (CHX) has been shown to have low tissue toxicity while retaining other favorable properties including its bactericidal activity and has been recommended as an alternative or potentially preferred irrigant for endodontic treatment (13). Based on previous studies, discrepancies exist regarding the efficacy of NaOCl and CHX with respect to bactericidal activity (14). Two new products, Chlor-XTRA and CHX-Plus (Vista Dental Products, Racine, WI), have recently been introduced to the market. According to the manufacturer, Chlor-XTRA contains a wetting agent and proprietary surface modifiers. Additionally, to increase the electrical capacity of the solution, alkylating agents have been incorporated into the solution. CHX-Plus contains proprietary surface modifiers to lower viscosity. It is unknown what effect the modification of NaOCl and CHX has on the bactericidal properties of these irrigants. Therefore, the purpose of this study was to compare the bactericidal effect of four antimicrobial formulations: group 1, 6% NaOCl; group 2, Chlor-XTRA; group 3, 2% CHX; and group 4, CHX-Plus, against a monoculture biofilm of a clinical isolate of *E. faecalis* at 1-, 3-, and 5-minute intervals.

Materials and Methods

A clinical isolate of *E. faecalis* recovered from an asymptomatic, failing root canal was used in all of the biofilm assays. Strain (ER3/2s) was provided by CM Sedgley. The strain was recovered from the root canal of a healthy 45-year-old female patient who presented for orthograde retreatment of a previously endodontically treated tooth (#7) with asymptomatic apical periodontitis. The procedure was approved by the University of Michigan Institutional Review Board, and patient written consent was obtained before

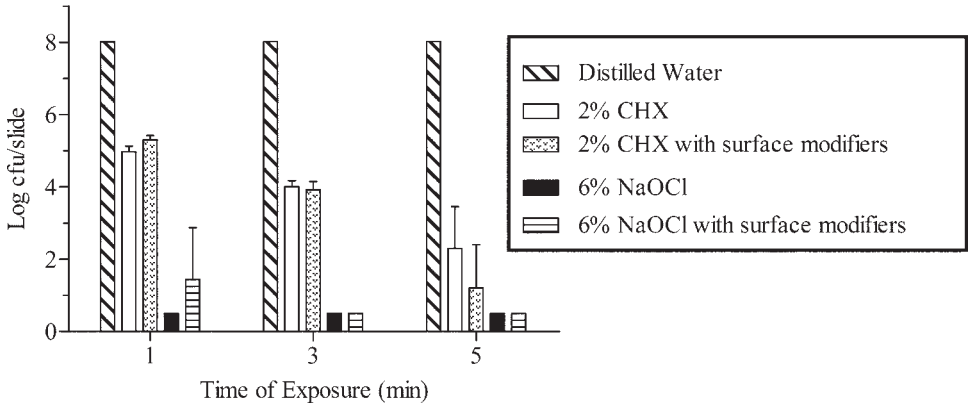

Figure 1. The bactericial activity of antimicrobial formulations on biofilms of *E. faecalis.*

treatment. Sample processing and species identification using culture and polymerase chain reaction–utilized methods have been described elsewhere (15). The organism was frozen down by quick freezing in liquid nitrogen and maintained at −80° C in cryovials.

For biofilm experiments, initial broth cultures were prepared in brain heart infusion broth (BHIB) and incubated in a 5% CO_2 incubator at 37°C overnight. Cells were then harvested by centrifugation and resuspended in fresh BHIB. Resulting suspensions were adjusted to a cell concentration of 10^8 colony forming units (cfu)/mL. Aliquots from these suspensions were spiral plated onto BHIB agar plates using an Autoplate 4000 (Spiral Biotech, Inc, Norwood, MA) to obtain the number of cells in the inocula described later.

The biofilm model system consisted of sterile glass microscope slides immersed in 30 mL of BHIB in sterile centrifuge tubes. These tubes were inoculated with 0.2 mL of the standardized suspensions prepared as described earlier. Tubes were then incubated in 5% CO_2 at 37°C for 48 hours. This allows for ample biofilm development on the slides. Pilot experiments showed stainable biofilms with crystal violet that could not be washed off but were recoverable upon scraping the slides carefully (data not shown). We routinely conduct monoculture and polymicrobial biofilm studies in our laboratory and are confident that we reproducibly obtained *E. faecalis* biofilms on the slides.

The exposure experiments were conducted as follows. Glass slides were removed from their media tubes and gently washed by repeated dipping in sterile distilled water. The slides were then immersed in centrifuge tubes containing 75 mL of each of the following treatments: group 1, 2% CHX; group 2, 2% CHX-Plus; group 3, 6% NaOCl; group 4, Chlor-XTRA; or group 5, sterile distilled water (control). The treatment solutions were in small beakers, with slow stirring achieved with small stir bars. Slides were exposed for different periods of time, 1, 3, and 5 minutes. After exposure, slides were removed and immediately immersed and dipped 10 times into tubes containing neutralizing broth (Benton, Dickinson, and Company, Sparks, MD) supplemented with 0.5% Tween 80. Pilot experiments by others in the laboratory (data not shown) have shown that this indeed does stop the killing reaction of the antimicrobials used in this study. After this treatment, slides with the biofilms were immersed in 5 mL of sterile BHIB in Petri dishes and the biofilms were scraped off the surfaces using a wire loop. The BHIB was collected and repeatedly vortexed to create homogeneous suspensions. Suspensions were then diluted into sterile BHIB, and then the original suspension plus dilutions were spiral plated onto BHIB agar. Numbers of viable bacteria in the biofilms were determined by sector plate counts according to standard spiral-plating methodology.

Each treatment group was done in triplicate per experiment, and three independent experiments were performed. Raw bacterial numbers were $\log_{10}$ transformed to normalize the data. Means of the transformed data were obtained (geometric means), and, therefore, there were three independent measures from the three independent experiments. Data were analyzed by two-way repeated-measures analysis of variance using Prism 5 software (GraphPad Software, La Jolla, CA). A test for equal variances was performed. If significant differences ($p < 0.05$) were found between treatment groups, Tukey-Kramer post-tests were performed.

Results

The analysis of the data revealed an interaction between the treatment groups and time of exposure. All treatment groups exhibited a significant reduction in viable bacteria within the biofilms compared with the distilled water control (group 5) at all periods of exposure ($p < 0.001$) (Fig. 1). Groups 1 and 2 (6% NaOCl and Chlor-XTRA, respectively) reduced cfus in biofilms by 7 to 8 orders of magnitude. Conversely, groups 3 and 4 (2% CHX and CHX-Plus, respectively) reduced cfus in biofilms by 3 to 4 orders of magnitude. No statistically significant difference was observed between the biofilms in groups 1 and 2. Likewise, no statistically significant difference was observed between groups 3 and 4 ($p > 0.05$). Statistically significant differences were observed between the biofilms in either group 1 or group 2 compared with group 3 ($p < 0.001$). However, when comparing group 1 or group 2 against group 4, a statistically significant difference was noted at exposure intervals of 1 and 3 minutes ($p < 0.001$) but not at the 5-minute interval ($p > 0.05$).

Discussion

This study evaluated the bactericidal efficacy of four endodontic irrigants, 6% NaOCl, Chlor-XTRA, 2% CHX, and CHX-Plus, against a monoculture biofilm established from a clinical isolate of *E. faecalis* (ER3/2s). We found that both NaOCl groups reduced numbers of bacteria within the biofilms significantly more than either CHX group. These findings are in agreement with some studies in the literature but are different that what was shown in others. Dunavant et al. (16) compared the efficacy of 1% or 6% NaOCl and 2% CHX, among other irrigants, against *E. faecalis* biofilms in a novel in vitro model system. Their model consisted of biofilms grown in a flow cell system. Biofilms were immersed in test irrigants for 1 or 5 minutes. Results indicated that both concentrations of NaOCl provided statistically significantly better biofilm kill than any other of the tested agents. Although the results of the Dunavant study are consistent with our findings, direct comparisons cannot be made because of the differences in the biofilm model systems themselves. Another study evaluated the effectiveness of varying concen-

trations of NaOCl, 2% CHX, and 1% NaOCl followed by a final rinse of a mixture of tetracycline, acid and detergent (MTAD) on rendering bacteria nonviable and physically removing a polymicrobial biofilm established in an extracted tooth model (17). Results indicated that 6% NaOCl was the only irrigant tested that provided the intended outcome of bacterial kill and elimination of the biofilm. Although our results support their findings, our study used monoculture biofilms established on smooth glass surfaces. As such, we did not deal with the confines of tooth structure, which could potentially affect physical access of the irrigant solutions to the biofilm. We chose this approach because it would reduce variance among replicates and also provide a greater surface area of the biofilms to be exposed to the irrigant solutions.

Conversely, a study conducted by Jeansonne and White (14) used an in vitro root canal system comparing antimicrobial activity of 2% CHX and 5.25% NaOCl. They showed that although it is not statistically significant, the 2% CHX group rendered fewer positive cultures than the NaOCl group. Differences in results from their study could potentially be attributed to deficiencies in the methods if measures relating to the carryover effect of the irrigants were not performed.

There are limitations that have to be acknowledged in our study. The biofilm model system was chosen to be simple as to facilitate the ease of preparation and maximal surface area of biofilms for the exposure experiments. Moreover, we have found in other studies that one can obtain less variance in results in a more simple design as ours described here than in complex models involving extracted teeth. However, keeping it simple can be a limitation in that we do not include variables associated with root canals in terms of physical access to the biofilms and differences in the structure and development of biofilms in tooth models because of inherent physic-chemical differences. Nevertheless, we believe that a simple system as described here allows for the rapid assessment of the ability to reduce numbers of viable bacteria colonizing a smooth surface. The data obtained, of course, cannot be directly extrapolated to conditions of root canals in human patients, but the data can serve as a guide for future investigations on potential differences in antimicrobials used in endodontics.

The future direction for study involving specific irrigant effect on biofilms should include standardization of biofilm models. Additionally, of interest would be the comparison of the depth of penetration into the biofilm by specific irrigants and the resultant effect of penetration depth on biofilm kill.

Conclusions

Within the limitations of this study, the following conclusions may be made:

1. We developed a model system for monoculture biofilms of *E. faecalis* that was adopted and revised from previously-published models. Reproducible levels of bacteria colonized the slides from experiment to experiment and were shown to be strongly bound biofilms on the surfaces of the slides.
2. All of the antimicrobial formulations tested exhibited significant bactericidal activity against biofilms of *E. faecalis*. Sodium hypochlorite (6%) and Chlor-XTRA showed the highest levels of bactericidal activity across the spectrum of exposure times apart from biofilms exposed for 5 minutes.
3. Ongoing and future studies are focusing on polymicrobial biofilms and the efficacy of the various antimicrobial formulations on these complex communities of organisms.

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