Comparative Evaluation of Endodontic Irrigants against *Enterococcus faecalis* Biofilms

Thomas R. Dunavant, DDS, John D. Regan, B.DentSc, MSc, MS,* Gerald N. Glickman, DDS, MS, MBA, Jd,* Eric S. Solomon, MA, DDS,† and Allen L. Honeyman, PhD‡*

Abstract

The aim of this study was to compare the efficacy of root canal irrigants against *E. faecalis* biofilms using a novel *in vitro* testing system. Biofilms grown in a flow cell system were submerged in test irrigants for either 1 or 5 minutes. Statistical analysis revealed a significant relationship between test agent and percentage kill of the biofilm bacteria ($P < 0.05$). No statistically significant relationship between time and percentage kill was found. The percentage kill of the biofilm bacteria was: 6% NaOCl (>99.99%), 1% NaOCl (99.78%), Smear Clear™ (78.06%), 2% chlorhexidine (60.49%), REDTA (26.99%), and BioPure™ MTAD™ (16.08%). Post-hoc analysis showed a significant difference between 1% and 6% NaOCl, and all other agents including Smear Clear™, 2% chlorhexidine, REDTA, and BioPure™ MTAD™ ($P < 0.05$). Within the parameters of this study, both 1% NaOCl and 6% NaOCl were more efficient in eliminating *E. faecalis* biofilm than the other solutions tested. *(J Endod 2006;32:527–531)*

Key Words

Biofilm, BioPure MTAD, chlorhexidine, EDTA, endodontic irrigants, SmearClear, sodium hypochlorite

From the *Department of Endodontics, †Department of Institutional Research, ‡Department of Biomedical Sciences, Baylor College of Dentistry, The Texas A&M University System Health Sciences Center, Dallas, Texas.

Address requests for reprint to Dr. Allen L. Honeyman, Department of Biomedical Science, Baylor College of Dentistry, The Texas A&M University System Health Sciences Center, 3302 Gaston Avenue, Dallas, TX 75246. E-mail address: ahoneyman@bcd.tamhsc.edu.

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Bacteria cause pulpal and periradicular disease (1). Hence, root canal treatment aims to eliminate bacteria from the infected canal system and to prevent reinfection. Clinical studies have demonstrated that chemo-mechanical preparation and use of antimicrobial medicaments are effective in reducing the bacterial load in root canal systems (2). However, some bacteria can persist despite these efforts (3). Clinical studies have also demonstrated that teeth from which a bacterial culture-negative specimen was obtained before obturation resulted in higher success rates than those with a culture-positive specimen (94% vs. 68%) after a 5 year follow-up (4).

Enterococcus faecalis is often isolated from previously treated teeth presenting with persistent disease (5). Consequently, recent laboratory studies have focused on evaluating the effectiveness of root canal irrigants and medicaments against *E. faecalis*. Many of these studies have grown the bacterial strains as planktonic cultures (bacteria in suspension) (6). However, planktonic bacteria do not generally represent the in vivo growth condition found in an infected tooth where bacteria grow as a biofilm on the dentinal wall (7). Consequently, recent laboratory studies have attempted to evaluate the efficacy of antimicrobial agents used in root canal treatment against *E. faecalis* grown as a biofilm (8).

A biofilm has recently been defined as a microbial community characterized by cells that are attached to a substratum, are in a matrix of extracellular polymeric substance (EPS), and exhibit altered growth phenotypes (9). Biofilms offer their member cells several benefits, the foremost of which is protection from killing by antimicrobial agents. Four mechanisms that confer antimicrobial tolerance to cells living in a biofilm have been elucidated. The first is the barrier properties of the EPS matrix. Extracellular enzymes such as β-lactamase may become trapped and concentrated in the matrix, thereby inactivating β-lactam antibiotics (10). The second mechanism involves the physiological state of biofilm microorganisms. Bacterial cells residing within a biofilm grow more slowly than planktonic cells; as a result, biofilm cells take up antimicrobial agents more slowly (11). Furthermore, the depletion of nutrients can force bacteria into a dormant or stationary growth phase in which they are protected from killing (12). The third suggested mechanism responsible for antimicrobial tolerance is that microorganisms within the biofilm experience metabolic heterogeneity. Studies have shown that oxygen can be completely depleted by cells at the biofilm surface leaving anaerobic niches deeper in the community (13). Some antibiotics like aminoglycosides are more effective against bacteria growing in aerobic conditions than the same microorganism growing in anaerobic conditions; therefore, not all cells within the biofilm will be affected in the same way (14). Finally, it has recently been speculated that a sub-population of microorganisms exists known as persisters (15). These microorganisms constitute a small percentage of the original population and are believed to constitute a highly resistant phenotypic state that is resistant to killing by antimicrobial agents.

Few studies have evaluated the efficacy of endodontic irrigants against microorganisms grown as a biofilm. Therefore, the purpose of this study was (a) to quantify and compare the efficacy of contemporary irrigants currently used in root canal treatment; and (b) to introduce a new in vitro method for evaluating the efficacy of endodontic irrigants on biofilms.

Figure 1. Diagram of the continuous flow system.

Materials and Methods

Bacterial Strain

E. faecalis OG1X is a derivative of an oral isolate that has been shown to have cariogenic potential (16–18). *E. faecalis* was taken from frozen stock culture and inoculated into 10 ml Brain Heart Infusion (BHI) broth, grown overnight at 37°C, and streaked onto Todd Hewitt Broth (THB) agar plates. Single colonies were used to inoculate 30 ml BHI broth cultures that were grown statically overnight at 37°C. The cultures were then harvested and used to inoculate the bioreactor cell. The cultures were checked for purity by Gram stain and colony morphology.

Flow System

The continuous flow system consisted of a nutrient reservoir, a single channel flow cell (Custom Scientific, Dallas, TX), a peristaltic pump (Masterflex L/S, Cole Parmer, Niles, IL), and a waste vessel. All parts were connected with silicone tubing (Fig. 1). The flow cell (Fig. 2) was a Delrin poly-acetal resin (DuPont, Wilmington, DE) channel with a rectangular glass cover slip sealed with a rubber gasket and a Delrin poly-acetal resin flange. The flow channel (1-mm deep, 10-cm wide, and 12-cm long) contained eight circular recesses. Each recess (1-mm deep and 6.35 mm in diameter) allowed placement of coupons (1-mm thick and 6-mm in diameter). The coupons were made from low-fusing hydrothermal ceramic, dentin shade A3.5 (Duceram LFC, Degussa-Ney, Rosbach, Germany). The nutrient reservoir, silicone tubing, and waste vessel were sterilized by autoclaving at 121°C for 20 min. The flow cell, with coupons in place, was sterilized by ethylene oxide treatment overnight.

Inoculation and Biofilm Formation

The flow cell containing uninoculated coupons was filled with sterile THB media from the reservoir by reducing the atmospheric pressure on the waste side of the flow cell by activating the peristaltic pump. Once the flow cell was filled with media, the pump was shut off and the overnight inoculum was introduced into the bioreactor cell through a syringe injection port upstream of the coupons. The culture volume used covered all of the coupons and was maintained in the flow cell for 30 min to allow for bacterial attachment to the coupons. Laminar flow was resumed at an approximate flow rate of 20 ml/h to flush the inoculum from the flow cell and to allow biofilm formation on the coupons. Biofilms were grown at 37°C for 24 h. The coupons were then removed and used in the viability assay. It was determined that 24 h of biofilm growth was sufficient to yield an adequate density of bacterial growth on each coupon for the subsequent testing. Growth on each of the coupons was calculated to be approximately 10^8 colony-forming units (CFUs).

Assessment of Efficacy

After 24 h of bacterial growth, the coupons were removed aseptically from the flow cell and transferred into a well containing either 1 ml of the selected antimicrobial test agent or a buffer solution that served as the negative control. Coupons were submerged for either 1 min or 5 min. Following exposure to the test agent or the control solution, each coupon was transferred to three additional wells containing 1 ml of PBS for 20 s each to remove any residual test agent. The coupons were then transferred to test tubes containing 1 ml of PBS and vortexed for 1 min to disrupt the biofilm structure. Biofilm viability was determined using serial dilutions (10^{-1} to 10^{-7}) with PBS (pH 7.4) and these dilutions were plated onto THB agar plates. Duplicate plates were then incubated at 37°C in a 5% CO_2 atmosphere for 48 h and the number of CFUs per coupon determined. The coupons for each experimental run of the flow cell were allocated as follows: two for the negative control solution, two for the positive control solution, and four coupons for use with two test agents. All experiments were done in quadruplicate.

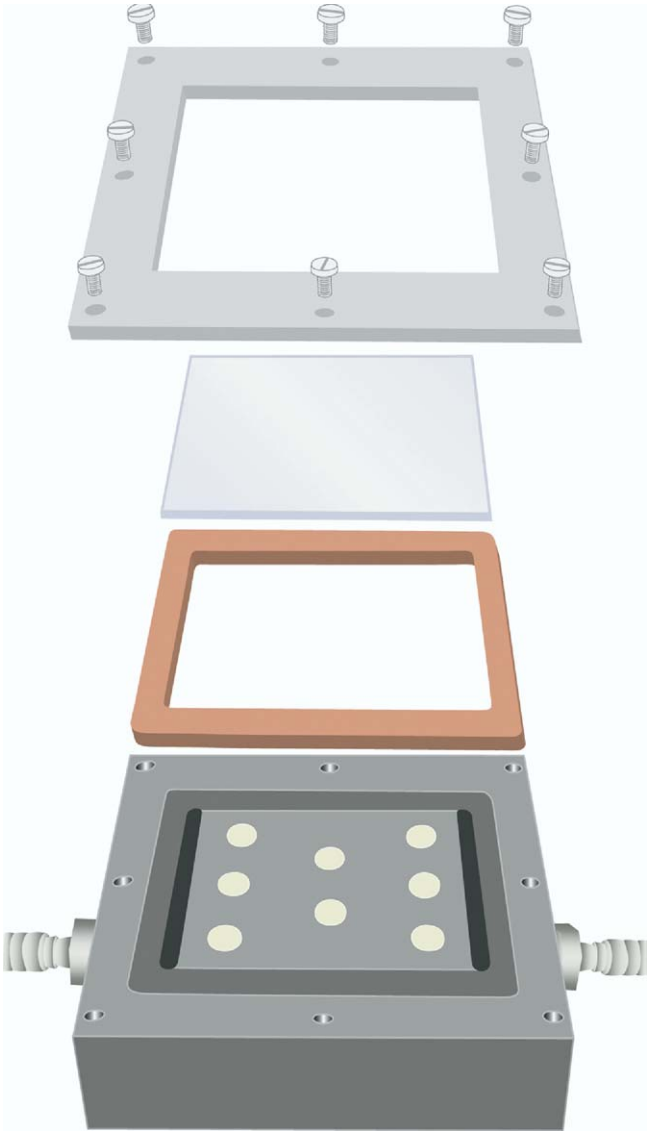

Figure 2. Diagram of the flow cell used to generate the biofilms.

TABLE 1. Test agents

Test Agent	Ingredients	pH	Manufacturer
NaOCl	hypochlorous acid, sodium hydroxide, water	12.3–12.6	Lasso Bleach, Labbco Inc., Houston, TX
SmearClear	17% EDTA, cetrimide, polyoxyethylene (10) iso-octylcyclohexyl ether, water	8.0 ± 0.2	SybronEndo, Orange, CA
2% CHX	2% chlorhexidine gluconate, water	5.7	Vista Dental Products, Racine, WI
REDTA	17% EDTA, water	7.5 ± 0.5	Roths International Ltd., Chicago, IL.
BioPure MTAD	Liquid–4.25% citric acid, 0.5% polysorbate 80, water; Powder–doxycycline hyclate	3	DENTSPLY Tulsa Dental, Tulsa, OK

Test Agents

The test agents used are shown in Table 1 and were 1% and 6% sodium hypochlorite (NaOCl) (Lasso Bleach, Labbco Inc., Houston, TX), BioPure MTAD (DENTSPLY Tulsa Dental, Tulsa, OK), 2% chlorhexidine gluconate (CHX) (Vista Dental Products, Racine, WI), REDTA (Roths International Ltd., Chicago, IL.), and SmearClear (SybronEndo, Orange, CA). All test agents were used as supplied by the manufacturer except for 1% NaOCl, which was diluted from 6% NaOCl using deionized water. Phosphate buffered saline (PBS, pH 7.4; Sigma Aldrich Co., St. Louis, MO) was used as the negative control while 6% NaOCl was used as the positive control.

Statistical Analysis

The effect of each test agent on the biofilm was determined by calculating the percentage kill of viable bacteria following treatment with a test agent. The percentage kill was calculated for each test agent by the following:

$$1 - \left\{ \frac{Average\ CFUs(TestAgent)}{Average\ CFUs(NegativeControl)} \times 100 \right\}.$$

The difference between the 1 and 5 min groups was analyzed by a two-by-two ANOVA including test agents and time period as fixed factors and percentage kills as the dependent variable. A one-way ANOVA was used to test the null hypothesis that there is no difference in percentage kill of *E. faecalis* biofilm following 1 and 5-min exposures to 1% NaOCl, 6% NaOCl, 2% CHX, REDTA, SmearClear, and BioPure MTAD. A post hoc analysis was employed using a multiple range test (Student-Newman-Keuls) to determine homogenuous subsets among the test agents. The level of significance was set at ($p \leq 0.05$).

Results

The two-by-two ANOVA results revealed a significant relationship between test agent and percentage kill ($p < 0.05$) but no statistically significant relationship between time and percentage kill or the interaction factor (Test Agent * Time). Consequently, both the 1 and 5 min groups were pooled for further analyses by a one-way ANOVA. The results demonstrated that there was a significant difference in percentage kill of *E. faecalis* among the solutions tested ($p < 0.05$). Further analyses using a multiple range test (Student-Newman-Keuls) provided five homogenous groups (Table 2). No statistical difference was found between 1% and 6% NaOCl. A significant difference was found between 1% and 6% NaOCl and all other test agents including SmearClear, 2% CHX, REDTA, and BioPure MTAD ($p < 0.05$).

Discussion

Although the testing of antimicrobial agents under in vivo conditions is the most definitive method for establishing efficacy, in vitro testing is also useful when evaluating potency and spectrum of activity. In vitro tests such as antimicrobial susceptibility allow experimental conditions to be controlled; however, this is often difficult to do when performing an experiment in vivo.

In this study, a novel biofilm model designed to evaluate the antibacterial efficacy of endodontic irrigants was introduced. Commonly used tests of antimicrobial efficacy rely on planktonic culture models (6, 19). Unfortunately, these tests that yield highly effective kills do not correlate well with clinical findings (4). In vitro susceptibility tests performed on biofilm models have demonstrated survival of bacterial biofilms after treatment with antibiotics at concentrations of 100 to 1000 times the minimum inhibitory concentration (MIC) of the same bacteria measured in suspension culture (20). A fundamental difference between planktonic and biofilm cultures is that all bacterial cells within the biofilm are not equivalent whereas they are in planktonic culture. In addition, Abdullah et al. demonstrated that *E. faecalis* grown as a biofilm was more resistant to CHX and povidone iodine than the same strain grown in planktonic suspension (8).

The flow cell used in this study to develop bacterial biofilms was modified from a previous study (21). Compared to other biofilm models that have evaluated the efficacy of endodontic irrigants, the model in this study is the first to incorporate fluid flow. Fluid flow is considered to be a principal determinant of biofilm structure (22). It provides nutrient exchange (23), influences density and strength (24, 25), and affects the dispersal of cells from the biofilm (26). When a tooth undergoes pulpal necrosis and subsequently develops periradicular periodontitis, an exudate may cycle in and out of the canal. This fluid exchange provides proteins, glycoproteins, and other nutrients to the bacteria growing as a biofilm in the root canal. This not only provides a sustainable nutrient source but also exerts shear force on the bacterial biofilm.

An approximate flow of 20 ml/h was used in this study. However, the exact flow rate that occurs in vivo has not been determined. Potentially, a more appropriate in vitro test would be the growth of bacteria as biofilms in extracted teeth or on root dentin segments that are then subjected to antimicrobial agents. Although these methods more closely resemble the clinical situation with respect to anatomy, they do not take into account the hydrodynamics that profoundly influence biofilm growth. Furthermore, because of the complexities and variations in root canal anatomy, they may not provide an accurate representation of the antimicrobial efficacy of the agent itself.

E. faecalis, a Gram-positive facultative anaerobe, was selected because it is commonly found in the root canals of failing endodontically treated cases (5). It has been found to survive as a monoinfection in root canals (27). *E. faecalis* has displayed resistance to chemo-mechanical

TABLE 2. Percentage kill of *E. faecalis* by different test agents

Test Agent	N	Mean ± SD
1% NaOCl	32	99.78 ± 0.356[a]
6% NaOCl	96	99.99 ± 0.000[a]
SmearClear™	32	78.06 ± 33.257[b]
2% CHX	32	60.49 ± 44.596[c]
REDTA	32	26.99 ± 29.997[d]
BioPure™ MTAD™	32	16.08 ± 21.191[e]

Unit: %.

Different letters in the column indicate statistically significant homogeneous groups ($p < 0.05$).

preparation (28) and intracanal medication (29). The persistence of *E. faecalis* may stem, in part, from its capability to form biofilms in root canals (30).

In initial experiments, it was determined that 24 h of biofilm growth was sufficient to yield an equal and adequate density of growth (10^8 CFUs) on each coupon for the subsequent quantification of survivors. *E. faecalis* grew sufficiently under the conditions used in this study but an anaerobic setting would probably more closely replicate the in vivo environment of the root canal system.

To eliminate any variation in biofilm density in various flow cell runs, the percentage kill was calculated for each experimental run. Two coupons were treated for each test agent during the experimental runs to eliminate any variation between coupons. The experimental runs for each test agent were done in quadruplicate. Every coupon in the bioreactor cell was exposed to either a particular test agent or control solutions.

Porcelain coupons were selected for biofilm growth because they are inexpensive, durable, and easy to fabricate. Because Haapasalo et al. (31) have shown that dentin can inhibit the activity of some antimicrobial agents, a more relevant substrate for the evaluation of endodontic irrigants might be made from root dentin. However, variables such as the properties of the dentin and the degree of bacterial invasion into dentinal tubules are difficult to standardize.

In studies evaluating the efficacy of endodontic irrigants, a neutralizing broth is generally used to stop the antimicrobial action of test agents. An inactivating solution was not used in this study because one that would neutralize the action of all test solutions is not currently available. Instead, the coupons were submerged in three additional wells each containing 1 ml of PBS to dilute any residual test agent that remained. Furthermore, following washing, the coupons were then vortexed in test tubes containing 1 ml of PBS. Any remaining test agent would have potentially been diluted at least by a factor of 10,000.

The results of this study indicate that both 1% and 6% NaOCl were effective in killing *E. faecalis* grown in biofilms. These results compare favorably to those found in a recent study by Radcliffe et al. (32). They demonstrated that 0.5, 1.0, 2.5, and 5.25% NaOCl solutions eliminated *E. faecalis* to levels below detection after contact times of 30, 10, 5, and 2 min, respectively. Unlike the current study, these authors found a relationship between time and concentration (i.e. as the concentration of NaOCl increased, the time taken to reduce CFUs to zero decreased). The differences are likely to be explained in terms of methodological detail. In the present study, complete inhibition of growth was not evaluated. As such, percentage kill can be misleading in terms of remaining viable bacteria. It should be remembered that the percentage kill is based on the reduction from the initial bacterial count. Therefore, it is possible to have a high percentage kill and concurrently have a large number of viable bacteria. It may be more prudent to evaluate antimicrobial agents for the complete elimination of microbial growth, appreciating the importance of a culture-negative at the time of obturation (4).

In the present study, 2% CHX was determined to be less effective than 1% and 6% NaOCl. This finding is consistent with that reported by Abdullah et al. (8) who grew *E. faecalis* biofilms on cellulose nitrate membrane filters. They found 3% NaOCl to be more effective than 0.2% CHX. In addition, they found that 17% EDTA exhibited minimal activity against *E. faecalis* grown as a biofilm, which is also in agreement with the results of this study. However, these results are in contrast to those reported by Oncag et al. (33). In their study, extracted teeth infected with *E. faecalis* were irrigated with 2 ml of 5.25% NaOCl, 2% CHX, or 0.2% CHX plus 0.2% cetrimide (Cetrexidin) for 5 min. Based on the presence of broth turbidity, they found 2% CHX and Cetrexidin to be more effective than 5.25% NaOCl. The differences could be attributed to the inability of NaOCl to penetrate into confined areas of the root canal. In addition, the smear layer was not removed in their study. This could further prevent the penetration of the antibacterial solutions into infected dentinal tubules or accessory canals. Furthermore, NaOCl reacts with organic component of the smear layer thus facilitating its removal; however, this reaction also inactivates NaOCl and reduces its antibacterial capacity. In the present study, *E. faecalis* biofilms were grown under fluid shear that may also explain the increased resistance to 2% CHX solution.

Torabinejad et al. (6) reported that BioPure MTAD possessed superior bactericidal activity compared with NaOCl or REDTA when tested against *E. faecalis*. The results of the present study do not corroborate those findings. In this study, the microorganisms were grown as a biofilm whereas planktonic bacteria were used in their study. An inoculum effect could have happened in this study whereby a less than optimal effect of the antimicrobial agent resulted because of the dense microbial population. In addition, the efficacy of BioPure MTAD against *E. faecalis* is reported to result from doxycycline that is present in the solution (34). However, doxycycline is a bacteriostatic antibiotic that does not kill bacteria; it prevents the multiplication of susceptible bacteria. With the experimental procedure described in this report and most endodontic procedures, one would not expect a bacteriostatic agent to enhance the killing effect. In addition, enterococci are known to naturally possess resistance to a wide range of antimicrobials. They can also acquire resistance to other antimicrobials such as tetracyclines through the acquisition of resistance genes on plasmids or transposons from other organisms. Although the strain used in this study was genetically different from the one used in the Torabinejad et al. (6) study, it was tested for tetracycline resistance and found to be sensitive (data not reported).

An interesting finding in this study was the fact that SmearClear demonstrated significant antibacterial activity. To the authors' knowledge, the antibacterial effects of SmearClear have not been previously evaluated. Although it contains approximately the same ingredients (17% EDTA) as REDTA, SmearClear more effectively eliminated *E. faecalis* biofilm than 2% CHX, REDTA, or BioPure MTAD. One possibility might be because of the addition of the surfactant cetrimide. Cationic surfactants have been reported to have bactericidal and fungicidal properties (35). Potentially, the surfactants could have been effective at disrupting the biofilm so that when the coupons were washed in the three wells of PBS a portion of the biofilms was removed. However, one would not expect a 78% decrease in bacterial numbers during the rinse procedure.

Recognizing the anaerobic environment of the root canal system and the fact that oxygen limitation may enhance biofilm resistance to antimicrobial agents, additional studies are needed to assess antimicrobial susceptibility of biofilms grown under anaerobic conditions. The age or maturity of a biofilm is known to influence tolerance to killing by antimicrobial agents, thus a biofilm that is grown for a longer period should be evaluated. Considering that root canal infections can be polymicrobial, further in vitro tests should evaluate multi-species biofilms. Because various endodontic irrigants have different modes of activity, combinations of irrigants should be tested in a biofilm model.

Based on the results of this study, NaOCl was highly effective in eliminating *E. faecalis* grown in biofilm. In addition, SmearClear eliminated *E. faecalis* biofilm more effectively than 2% CHX, REDTA, and BioPure MTAD and may prove to be a useful adjunct to the root canal irrigation regimen. BioPure MTAD was the least effective solution tested against *E. faecalis* biofilm in this study.

The biofilm model used in this study was effective in determining the in vitro antimicrobial efficacy of root canal irrigants. Because biofilms form on root surfaces in vivo (7), therefore, it would appear that

a biofilm model is more clinically relevant for the evaluation of antimicrobial efficacy.

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