

Effect of Smear Layer and Chlorhexidine Treatment on the Adhesion of *Enterococcus faecalis* to Bovine Dentin

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Abstract

The aim of this *in vitro* study was to determine the effects of a smear layer and chlorhexidine (CHX) treatment on the adhesion of *Enterococcus faecalis* to bovine dentin. Forty dentin blocks from bovine incisors were prepared and randomly divided into four groups of 10 each. The blocks in group 1 were placed in sterile saline for 5 minutes, while those in group 2 were treated with 17% EDTA for 5 minutes. The blocks in group 3 were placed in 2% CHX for 7 days. The blocks in group 4 were treated with 17% EDTA for 5 minutes, and then placed in 2% CHX for 7 days. All the blocks were immersed in a suspension of *E. faecalis* for 3 hours. The bacteria adhering to the dentin surface were counted by examination using a scanning electron microscope. The most significant amount of bacteria was retained on the samples from group 1 ($p < 0.05$) and the smallest amount of bacteria adhered to the samples from group 4. These results suggest that a smear layer enhances the adherence of *E. faecalis* to the dentin, and CHX is effective in reducing the adherence of microorganisms. (*J Endod* 2006;32:663–667)

Key Words

Adhesion, chlorhexidine, *Enterococcus faecalis*, smear layer

Microorganisms play an important role in the development of pulpal and periapical diseases (1), and root canal instrumentation and fillings are generally performed to eliminate the organisms and prevent their re-colonization in the root canal. These treatments are normally toxic to most bacteria (2, 3), however, some bacteria can survive and grow despite the treatment. An example of such bacteria is *Enterococcus faecalis* (4). This microorganism is also known to be the species commonly recovered from the filled root canals (5–8). Therefore, many researchers have used *E. faecalis* to evaluate the antimicrobial effect of various intracanal medications or irrigants (9–14).

After a root canal instrumentation or filling, bacteria can re-invade the root canal via coronal leakage (15, 16). Once the coronal seal is lost, microorganisms and their products may reach the periradicular tissues and develop apical periodontitis. Adhesion of the cells to the canal wall is a prerequisite for the development of the infectious disease (17). Although there are few reports on the adhesion of bacteria to the root canal wall, several studies have examined whether or not the smear layer affects the adhesion of bacteria to the root canal wall (17–21). Calas et al. (18) reported that the adhesion of *Streptococcus sanguis* could be reduced by removing the smear layer using citric acid. Calas et al. (19) and Yang et al. (20) also reported that *Prevotella nigrescens* showed decreased adhesion after the removal of the smear layer. On the other hand, Love et al. (17) suggested that the presence of the smear layer has no effect on the degree of adhesion of *S. gordonii*, while Drake et al. (21) stated that the smear layer suppresses the colonization of *S. anginosus* in the root canal.

Because chlorhexidine (CHX) has a broad-spectrum antimicrobial effect and kills *E. faecalis* in the dentinal tubules more effectively than calcium hydroxide (11, 13), it

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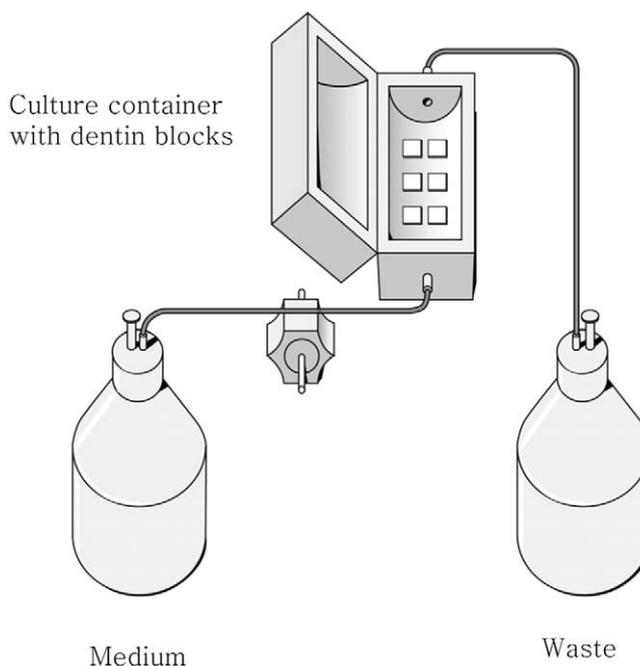


Figure 1. Schematic representation of the apparatus used for inoculation.

TABLE 1. Mean (\pm SD) numbers of *E. faecalis* adhering to dentin

Groups	n	No. of cells/ observation spot
Group 1: Smear	10	61.21 \pm 24.15 ^a
Group 2: Nonsmeared	10	7.65 \pm 6.9 ^b
Group 3: Smear & CHX treatment	10	4.7 \pm 5.9 ^{b,c}
Group 4: Nonsmeared & CHX treatment	10	0.95 \pm 1.6 ^c

Groups identified by the same superscript letters are not significantly different ($p > 0.05$). Groups identified by different superscript letters are significantly different ($p < 0.05$).

has received increasing recognition as an effective irrigant or an intracanal medicament in root canal therapy (22–25). In addition, its antimicrobial substantivity has been of interest to many researchers (26–28).

The aim of this study was to determine the effect of the smear layer and CHX treatment on the adhesion of *E. faecalis* to the bovine root dentin.

Materials and Methods

Preparation of Samples

The teeth were extracted from the jaws of adult bovines that had been recently slaughtered. The roots were cleaned with scalers and sodium hypochlorite (NaOCl), and the coronal and the apical third of the root were removed using a Microtome (Struers, Rodovre, Denmark). The remaining root (middle third) was split along the long axis, and the dentin including the pulpal surface was removed to facilitate its

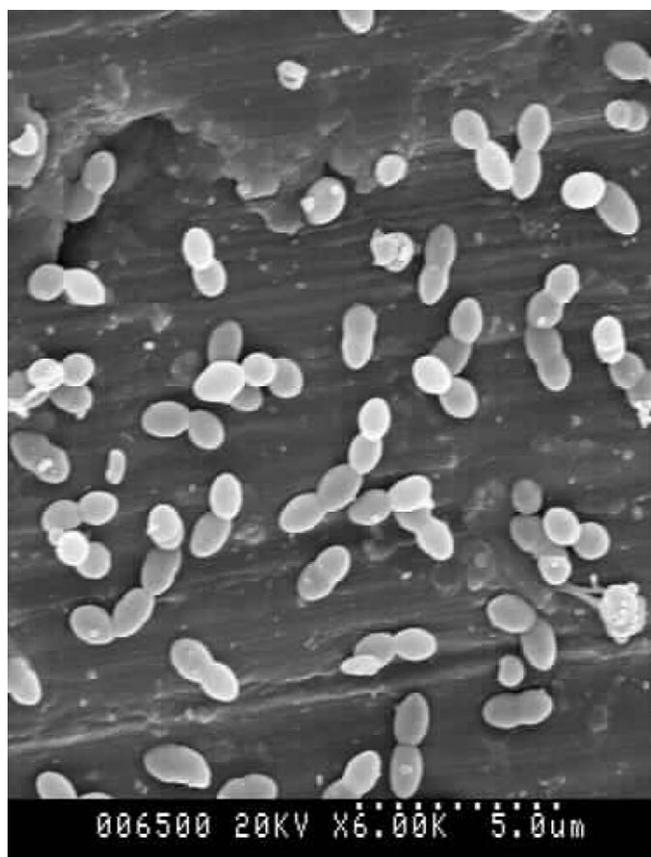


Figure 2. Many bacterial cells are seen on the dentin covered with a smear layer (group 1, original magnification $\times 6000$).

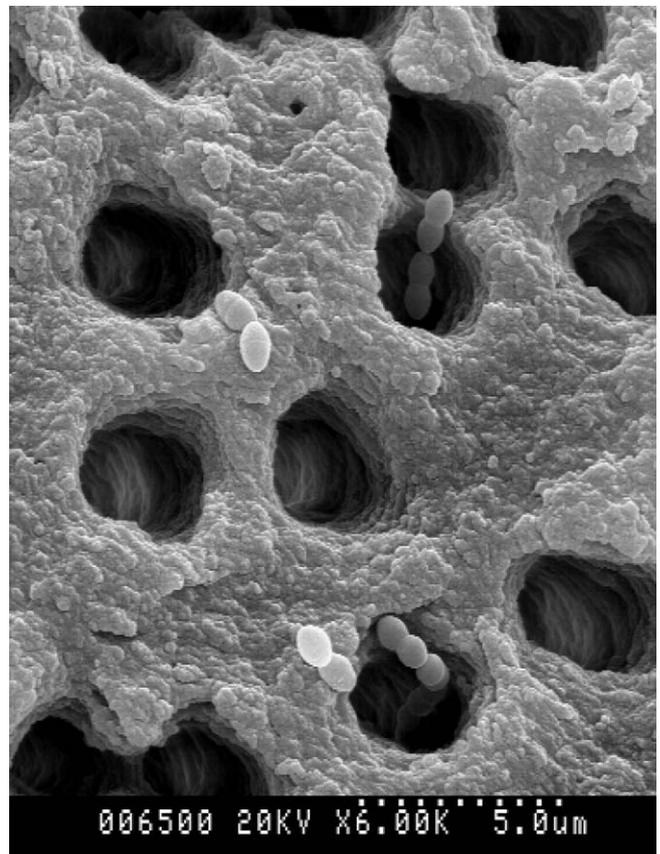
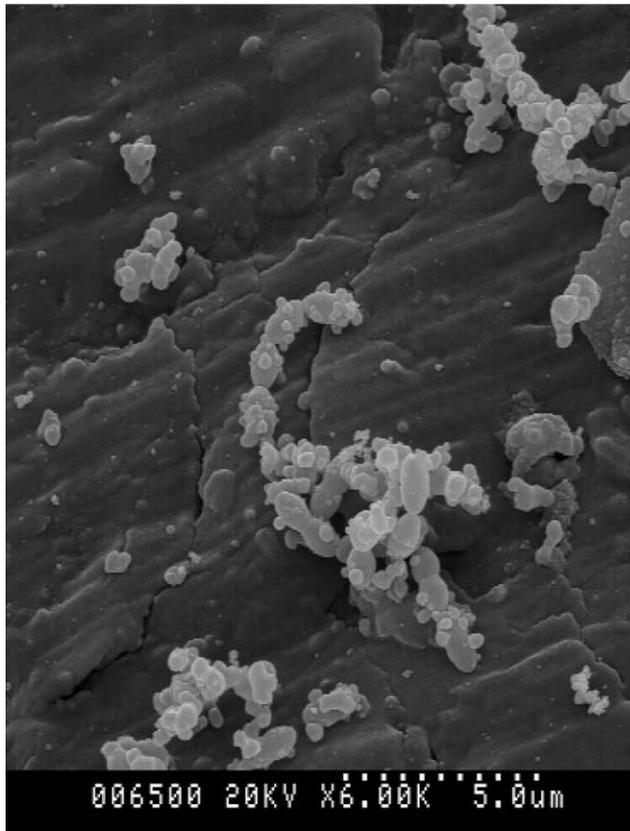


Figure 3. Smear layer was removed in the samples of group 2. Some cells are penetrating into dentinal tubules (original magnification $\times 6000$).

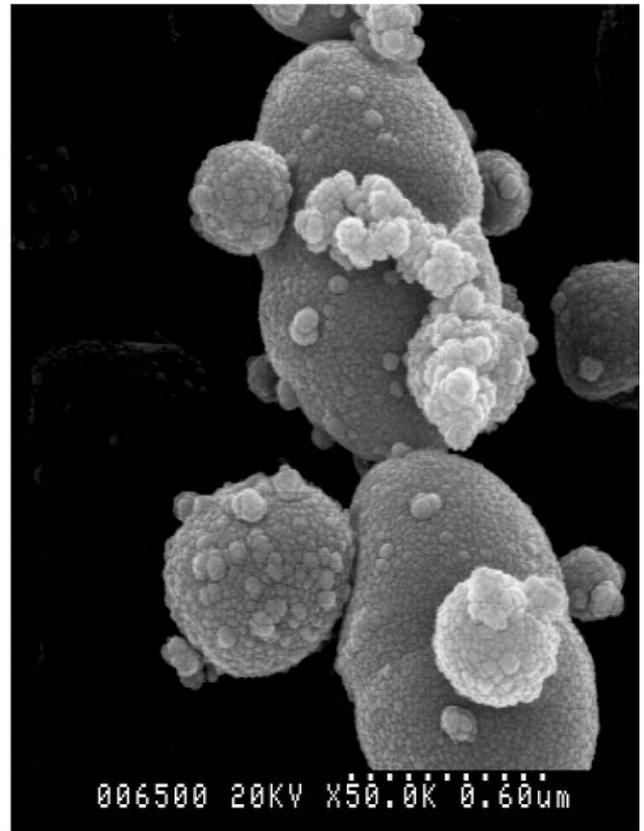
preparation for a scanning electron microscope. Each dentin block measured 2.5 \times 2.5 mm. The smear layer was produced on the pulpal surface using a round bur (SS White, Lakewood, NJ). The resulting 40 dentin blocks were placed in a 2.5% NaOCl solution for 5 minutes and sterilized using ethylene oxide gas. The blocks were then randomly divided into four groups of 10. The blocks in group 1 were placed in sterile saline for 5 minutes, while those in group 2 were treated with 17% EDTA for 5 minutes. The blocks in group 3 were placed in 2% CHX for 7 days. The blocks in group 4 were treated with 17% EDTA for 5 minutes before being placed in 2% CHX for 7 days. These samples were used immediately in the adhesion assay.

Adhesion Assay

E. faecalis (KCCM 11814, Korean Culture Center of Microorganisms) was used in this study. There was 100 μ l of a 24-hour culture of *E. faecalis* inoculated into 10 ml of Brain Heart Infusion (BHI, Difco Laboratories, Detroit, MI) broth, which was further incubated for 8 hours before being tested for the adhesion assay. The number of bacterial cells in the 8-hour cultures ranged from 3×10^8 to 8×10^8 cells/ml. All incubations were performed aerobically at 37°C. The adhesion assays were carried out using the simple apparatus reported by Luppens et al. (Fig. 1) (29). The apparatus consisted of a vessel containing 10-fold-diluted BHI, a pump (Micro Tube Pump, Tokyo Rikakai Co., Ltd., Tokyo, Japan), a culture container (perfusion culture container 1301; Minucells and Minutissues, Bad Abbach, Germany), and a vessel with waste, all connected by silicone tubing. Before inoculation, the dentin blocks were placed in a culture container, and BHI, diluted 10-fold, was pumped through the system for 30 minutes. The



A.



B.

Figure 4. (A) CHX-treated dentin showing small particles adhering to bacteria (original magnification $\times 6000$). (B) Higher magnification of (A) (original magnification $\times 50,000$).

blocks were then inoculated by removing 4.5 ml of the medium from the culture container, pipetting 4.5 ml of the 8-hour culture of *E. faecalis* into the container, and waiting for 30 minutes. Next the pump was restarted and adhesion was allowed to develop for 3 hours at 37°C with a constant nutrient flow (0.8 ml/minute), after which the pump was stopped, and the blocks were removed from the container and washed three times in sterile saline.

SEM Analysis and Statistical Analysis

The washed dentin blocks with the adherent bacteria were fixed in 2% glutaraldehyde at 4°C for 24 hours and prepared for a scanning electron microscope (JEOL, JSM-6320F, Tokyo, Japan). After the central beam of the SEM was directed to the surface area of each dentin block under $\times 45$ magnification, 10 areas of each specimen on the screen were randomly selected. For each selected area, the magnification was increased to $\times 1500$ and a transparent grid was placed on the SEM screen. The number of bacteria adhering to dentin surface was evaluated for 10 preselected squares of the grid. Areas of special interest were photographed at $\times 6000$ magnification. The final result for the mean number of *E. faecalis* binding to the dentin was obtained by calculating the mean scores of the 10 selected areas of each specimen. The data was analyzed using a one-way ANOVA and the comparison of means was conducted using a post hoc multiple comparison Tukey test.

Results

The data is summarized in Table 1. Samples from group 1 had significantly more bacteria than those of the other groups ($p < 0.05$).

The cells adhered homogeneously to the smear layers (Fig. 2). In group 2, some cells were penetrating into the dentinal tubules (Fig. 3). In the samples from groups 3 and 4, which were treated with CHX, small particles that adhered to the bacteria were observed (Fig. 4A, B). Interestingly, cells with damaged membranes were frequently found as well (Fig. 5). The lowest number of cells was observed in the samples from group 4 (Fig. 6). The difference between groups 2 and 4 was statistically significant ($p < 0.05$), while there was no significant difference between groups 3 and 4 ($p > 0.05$).

Discussion

The inoculation system used in this study was the method utilized by Luppens (29), which was different from systems used in previous investigations. The first difference is that the apparatus allows investigators to observe the adhesion of bacteria under a constant flow, and the other is the use of 10-fold-diluted broth. Because the bacteria grown in static broth and/or rich media may not be representative of bacteria in the patient's root canals (14), the system seems to be more closely related to clinical cases.

The mechanism by which bacteria adhere to the root canal wall has not been fully determined. Love et al. (4) reported that the dentin invasion by *E. faecalis* is related to their binding to exposed unmineralized collagen. Hubble et al. (30) also suggested that the collagen binding protein contributed to the adhesion of bacteria to the dentin. However, it does not appear that the collagen played an important role in the adhesion observed in this study because the number of bacteria

adhering to the dentin was decreased when the smear layer was removed. Therefore, it seems that the adhesion observed in this study might be a result of a nonspecific physical interaction based on surface properties rather than specific binding to collagen.

The present finding that *E. faecalis* can adhere to smeared dentin more than nonsmeared dentin is similar to the previous results reported by Calas et al. (18, 19) and Yang et al. (20). However, Love et al. (17) and Drake et al. (21) reported contradictory results to those of this study. These disparities may be the result of different strains of bacteria used or the study methodology.

Many researchers have investigated the effect of the smear layer on the outcome of root canal treatment (31–35). With respect to bacteria, some researchers have insisted on removing the smear layer, because the smear layer itself may be infected and can decrease the antimicrobial effect of an irrigant or an intracanal medication (31, 32). However, as seen in this study, as well as those previously conducted (20, 21), bacteria could easily invade the dentinal tubule after the smear layer was removed. In such cases, the elimination of bacteria becomes even more difficult. Love et al. (36) reported that the dentinal smear layer is an effective barrier against dentinal tubule invasion of *S. gordonii*. The results of this study show that one of the concerns associated with the smear layer, which is the increased binding of *E. faecalis*, can be solved by treating the canal with CHX. However, the smear layer needs to be evaluated in terms of other aspects like prevention of leakage and durability. Therefore, whether smear layer should be removed still remains controversial.

CHX has been noted for its antimicrobial substantivity, which is because of the release of positively charged molecules from CHX-treated

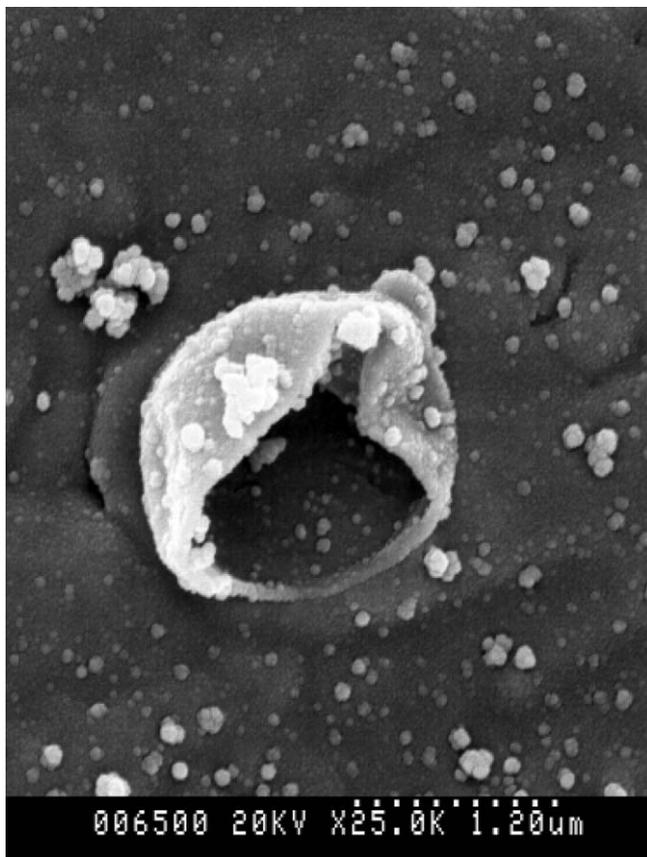


Figure 5. The cells with damaged membranes were observed only in groups 3 and 4 (original magnification $\times 25,000$).

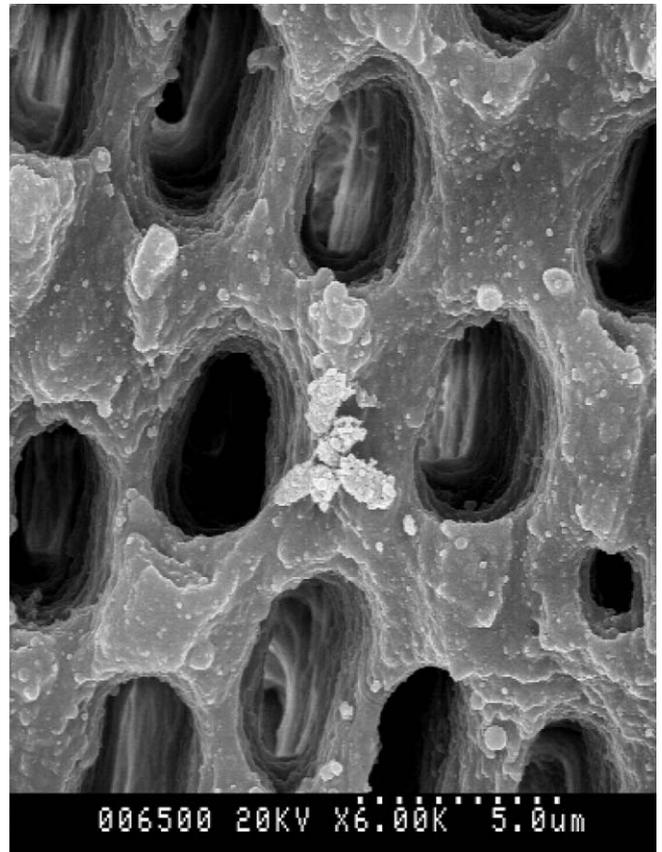


Figure 6. Cells are hardly seen in the samples of group 4 (original magnification $\times 6000$).

dentin (26–28). The released molecules bind to the bacteria, thereby interfering with their adhesion to the dentin and causing them membrane damage. There was difficulty in counting the number of the bacteria in some samples treated with CHX because in several instances there were particles covering the bacteria. It appears that these particles were from CHX. Some bacteria with damaged membranes were also observed in the samples of CHX-treated groups. These findings are related to the cationic molecule of CHX binding to the negatively charged bacterial cell walls, thereby altering the bacterial osmotic equilibrium.

Because it is unlikely that a sufficient amount of irrigant can be supplied to the canal, as was done in this study, it is unclear whether such antimicrobial substantivity occurs in actual clinical cases. However, some investigators (11) have reported that it is possible to solve this problem using a CHX-containing-release device. Another concern is how long the antimicrobial effect is maintained. However, such antimicrobial effect appears to remain for the duration of the normal interappointment period (11). Therefore, it may be effectively used as an intracanal medication.

Conclusions

This study showed that the initial colonization of *E. faecalis*, which is one of the bacteria most frequently found in root canals after canal instrumentation or obturation, can be prevented and controlled by either removal of the smear layer or by treatment with CHX.

Acknowledgments

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