# Antimicrobial Substantivity over Time of Chlorhexidine and Cetrimide

*Pilar Baca, DDS, MD, PhD, Pilar Junco, DDS, MD, PhD, María Teresa Arias-Moliz, DDS, PhD, Francisca Castillo, BSN, Alberto Rodríguez-Archilla, DDS, PhD, and Carmen María Ferrer-Luque, DDS, MD, PhD*

## Abstract

**Introduction:** To reach an adequate control of dentin infection and to promote success in endodontic therapy, antimicrobial irrigating solutions with confirmed substantivity are recommended. The aim of the present study was to evaluate the antimicrobial substantivity against *Enterococcus faecalis* of a dentin-volumetric unit exposed for 1 minute to chlorhexidine (CHX) and cetrimide (CTR). **Methods:** Standardized coronal dentin blocks of human molars, with and without collagen, were treated for 1 minute with 0.2% and 2% CHX and 0.2% CTR. Afterwards, they were exposed to *E. faecalis* suspension to determine the antimicrobial substantivity over a period of 60 days. Results were analyzed by means of Kaplan-Meier survival analysis ($P < .05$). **Results:** A direct relationship was seen between CHX concentration and survival time, and the most statistically significant results were obtained in specimens with collagen. CTR showed intermediate survival values close to those of 2% CHX. **Conclusions:** The present study shows that 2% CHX used for 1 minute provides the longest substantivity followed by 0.2% CTR when applied to a dentin-volumetric model. *(J Endod 2012;38:927–930)*

## Key Words

Antimicrobial substantivity, biofilms, cetrimide, chlorhexidine, *Enterococcus faecalis*

From the Department of Preventive Dentistry, School of Dentistry, University of Granada, Granada, Spain.

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Address requests for reprints to Dr Pilar Baca, Department of Preventive Dentistry, School of Dentistry, Campus de Cartuja, Colegio Máximo s/n. E-18071, Granada, Spain. E-mail address: pbaca@ugr.es

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Root canal instrumentation produces the formation of the smear layer, which may contain bacteria and their byproducts; therefore, its removal is desirable (1). Because there is no single solution capable of dissolving organic tissue and demineralizing the smear layer, the sequential use of organic and inorganic solvents has been recommended (2, 3). Among the chelating agents used for removal, the most common is EDTA, which decalcifies dentin and exposes collagen but shows no antimicrobial activity (4, 5). Sodium hypochlorite (NaOCl), used during or after instrumentation, dissolves organic tissues including collagen (2), and it has a potent antimicrobial action (6), yet it does not exert any residual antimicrobial activity (7).

The use of a final irrigating solution that remains active not only at the time of application but over a fairly long period of time thereafter is one strategy for preventing bacterial recolonization or eliminating the bacteria that persist after root canal treatment. Antimicrobial irrigants such as chlorhexidine (CHX) and cetrimide (CTR) prove effective in eradicating *Enterococcus faecalis* biofilm both *in vitro* (8, 9) and *ex vivo* (5). These bacteria are one of the most commonly found species in root canal–treated teeth exhibiting emergent or persistent disease (10, 11). Moreover, CHX has the property of substantivity and is able to inhibit the adherence of *E. faecalis* to dentin (7, 12). In the root canal system, the antimicrobial substantivity of CHX has been reported to last from 48 hours to 28 days (13–16). When a concentration of 2% is applied for 10 minutes, it could even endure up to 12 weeks (17) although, from a clinical point of view, the recommended time is around 1 minute (18). Recently, Carrilho et al (19) showed that a high percentage of CHX is bound to dentin up to 56 days when exposed in concentrations of 0.2% or 2% for 30 seconds, but they did not study their antimicrobial effects. CTR is a cationic surfactant that, in our experience, eradicates *E. faecalis* biofilm and exerts effective residual antimicrobial activity, inhibiting 100% of the biofilm formation of these bacteria when evaluated at 24 hours (5).

The structure of dentin, especially its collagen component, may be altered by the last irrigating solution used (20). Some recent studies underline that the presence of collagen is an important factor enhancing the substantivity of CHX (21) and playing a role in the adhesion of *E. faecalis* to dentin (22). The aim of the present study was to evaluate over time the antimicrobial substantivity against *E. faecalis* of a dentin-volumetric unit exposed for 1 minute to 0.2% and 2% CHX and 0.2% CTR.

## Materials and Methods

### Bacteria Strain and Irrigating Solutions

The bacteria used in this study were *E. faecalis* American Type Culture Collection 29212, taken from a 4°C stock culture and streaked out twice on brain-heart infusion (BHI; Scharlau Chemie SA, Barcelona, Spain) agar plates for 24 hours at 37°C. From the subculture of *E. faecalis*, a 1 McFarland standard suspension was prepared in BHI broth and then diluted 30-fold to obtain an initial bacterial suspension of $1 \times 10^7$ colony-forming units per milliliter. The solutions tested were 0.2% and 2% CHX (Guinama, Alboraya, Spain) and 0.2% CTR (Sigma-Aldrich Chemie, Steinheim, Germany).

### Preparation of Dentin Blocks

Twenty-three noncarious, unrestored, freshly extracted human molars were stored in 0.1% thymol solution at 4°C. In preparing the dentin blocks, we followed the methodology described in a previous article (5). The teeth were sectioned, and the 2 apical thirds of the roots were discarded as was the occlusal coronal enamel to obtain a flat coronal dentin surface. This slice was cut into serial blocks. Four dentin blocks without enamel/tooth were obtained and then adjusted using a calibrator and polished with 150-, 220-, and 600-grit silicon carbide papers to obtain 2 × 2 × 1.8 mm (width × length × height) specimens. After sterilization, they were kept in a sterile saline solution until use. The protocol was approved by the Ethics Committee of the University of Granada, Granada, Spain.

### Residual Antimicrobial Activity Test

Half of the dentin blocks of each tooth were randomly assigned to 1 of 2 groups, with and without collagen, which were each subdivided into the following 3 groups: 0.2% CHX, 2% CHX, and 0.2% CTR. All specimens were dried with sterile paper discs and submerged for 1 minute in 180 μL of 17% EDTA in a 96-well microtiter plate (Nunclon Delta Surface; Nunc, Roskilde, Denmark) for smear layer removal. In the group without collagen, the specimens were also dried and submerged for 1 minute in 180 μL of 2.5% NaOCl. Afterwards, all the dentin blocks were dried and immersed for 1 minute in 180 μL of the different antimicrobial solutions tested. Ten nontreated dentin blocks, 5 with collagen and 5 without, were inoculated with 180 μL of the initial bacterial suspension as positive controls. Another 10 nontreated dentin blocks (5 + 5) inoculated with 180 μL of BHI broth served as sterility controls.

The dentin blocks were dried and transferred to a microtiter plate with 180 μL/well of the initial bacterial suspension. To avoid evaporation, it was incubated at 37°C inside a plastic bag. Every 48 hours, a 2-μL sample/well was inoculated into a BHI broth tube. When a reading was positive, it was inoculated in a BHI agar plate to check the presence of *E. faecalis* and the lack of contamination. At this point, the specimens were considered positive. Then, 2 μL/well of a 3 McFarland standard bacterial suspension was added to achieve a number of colony-forming units per milliliter similar to the first day of the experiment, along with BHI broth to complete 180 μL/well. The follow-up time was 60 days.

The proportion of ungrown samples over 60 days was evaluated using the nonparametric Kaplan-Meier survival analysis. In our context, the term survival is used with the understanding that a sample survives when it does not grow at a given time. The overall evolution of growth of *E. faecalis* in the samples is analyzed, taking into account the entire time period not just one or more points in time. Differences among groups were tested using the log-rank test at a significance level of 0.05. All statistical analyses were performed by means of SPSS 15.0 software (SPSS Inc, Chicago, IL).

## Results

All positive controls exhibited bacterial growth within 48 hours, whereas the negative controls remained uncontaminated throughout the study. The number of grown samples and the mean day of bacterial growth are given in Table 1. At 60 days, *E. faecalis* growth was detected in all samples except the 2% CHX with collagen group, in which only 6 samples exhibited growth.

The results of the Kaplan-Meier survival analysis are shown in Figure 1. Pair comparisons determined by the log-rank test showed longer survival overall of the samples with collagen than without collagen, with statistically significant differences observed for 2% and 0.2% CHX. A direct relationship was observed between CHX concentration and survival time. The highest survival value was for 2% CHX with collagen, which showed statistically significant differences with respect to the other groups. It was followed by 2% CHX without collagen, 0.2% CTR with collagen, 0.2% CTR without collagen (no significant differences among these 3), 0.2% CHX with collagen, and 0.2% CHX without collagen (both significantly different than the other groups).

## Discussion

Antimicrobial agents with proven substantivity have shown promise in promoting successful endodontic therapy and appear to reduce the recolonization of the dentin by *E. faecalis* (23). Our objective was to determine such antimicrobial substantivity over time after exposing dentin (with and without collagen) to 2 concentrations of CHX and CTR for 1 minute, a time recommended for clinical practice (18). We used as a carrier a dentin-volumetric unit that is effective as a biological unit of biofilm formation (5). The smear layer was treated with 17% EDTA in half of the specimens, leaving the collagen exposed, whereas in the rest the collagen was eliminated.

The results of this study show that CHX and CTR, when used as final irrigating agents, can fully inhibit *E. faecalis* growth for a considerable time (ie, a substantial amount of CHX or CTR remained on the dentin carrier in an active form). However, there were differences between the effects of 0.2% and 2% CHX regardless of the presence of collagen. With the 0.2% concentration, the mean survival was 28.42 and 24.58 days, respectively, for specimens with and without collagen. In contrast, for the 2% CHX group, the mean survival was 58.75 and 49.16 days, respectively. This relationship between concentration and substantivity time is consistent with previous results (15), and it is clinically interesting that it involves only 1 minute of exposure. Kadhemi et al (16) reported that after 5 minutes of exposure the antimicrobial substantivity of 2% CHX remained for 28 days. It has also been shown that the substantivity of CHX determined by spectrophotometry after 30 seconds of exposure can last up to 8 weeks for concentrations of 0.2% and 2% (19).

Our results pointed to significant differences in survival between specimens with and without collagen in the 2 groups

**TABLE 1.** The Number of Grown Samples and the Mean ± Standard Deviation of the Day of Growth

| Protocol | Number of grown samples | Lower bound | Higher bound | Mean ± standard deviation |
|---|---|---|---|---|
| 0.2% CHX with collagen[a] | 12 | 2 | 41 | 28.42 ± 10.01 |
| 2% CHX with collagen[b] | 6 | 55 | >60 | 58.75 ± 1.76 |
| 0.2% CTR with collagen[c] | 12 | 20 | 57 | 44.08 ± 11.67 |
| 0.2% CHX without collagen[d] | 12 | 18 | 34 | 24.58 ± 5.01 |
| 2% CHX without collagen[c] | 12 | 27 | 60 | 49.16 ± 8.1 |
| 0.2% CTR without collagen[c] | 12 | 22 | 54 | 42.16 ± 9.68 |

The same superscript letter shows differences that were not statistically significant determined by the log-rank test ($P < .05$).

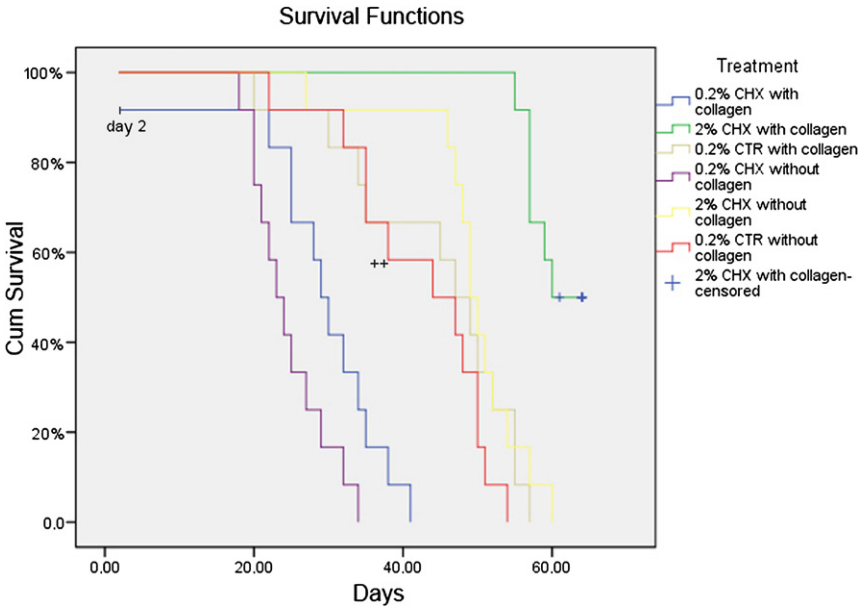


**Figure 1.** Kaplan-Meier survival probabilities at 60 days (probability of no growth) for all groups. Censored represents the proportion of the samples showing no growth at the end of the time period. Cum survival is the percentage of samples that did not show *E. faecalis* growth at a given time. For clarity, ++, 0.2% CTR without collagen. At day 38, there was growth of *E. faecalis* in 5 of 12 samples, meaning that 58.33% survived without growing.

treated with CHX. The best results, which were obtained in specimens pretreated with EDTA, can be explained by the nature of the CHX-dentin interaction. CHX, a cationic molecule, reacts with negatively charged dentin molecules, including the mineralized matrix (24, 25) and the collagen matrix in particular (19). EDTA creates an ideal substrate. It allows CHX to bind to the collagen matrix and underlying mineralized matrix and also enhances the porosity of the dentin (26) so that CHX is trapped within the collagen network (27).

The intermediate values obtained for CTR, a cationic surfactant, were closer to 2% CHX (44.08 and 42.16 mean survival days, respectively, with and without collagen). Although less studied, CTR has shown substantivity when combined with CHX (23, 28), and alone (0.2%) it may exert residual antibacterial activity for 24 hours, which is comparable with CHX (5). This could stem from its cationic nature, which lends it the capacity to interact with dentin, resulting in an action close to that of 2% CHX. However, the fact that there are no differences between specimens with and without collagen suggests that the mechanism(s) of CTR uptake in mineralized dentin would not be very different from those of demineralized dentin.

Although we had anticipated that CHX and CTR would both exert antimicrobial residual activity, the results obtained over time were better than expected. A previous article (29) evidenced the need to expose dentin to CHX for more than 1 minute. In addition, our methodology included *E. faecalis* reinoculated every 2 days in order to replace killed bacteria. It is noteworthy that the 2% CHX with collagen group had only 6 specimens with *E. faecalis* growth after 60 days. We must emphasize that the CHX concentration was high, and the presence of collagen enhances the substantivity (21), but it also enhances the adherence of *E. faecalis* to dentin (22, 30). In 60 days, one might expect that the CHX molecules were neutralized. Further studies will be needed to verify the results clinically.

From a clinical point of view, leaving the collagen exposed by the use of a chelating agent before root canal obturation results in other benefits. One of them is avoiding the use of NaOCL as the final irrigating solution because it affects the polymerization of methacrylate resin sealers to radicular dentin (31). On the other hand, the use of EDTA improves the bond strength of an adhesive sealer to the human dentin surface (32), which is not affected (33) or may even improve (34) with the posterior use of 2% CHX.

The present study shows that 2% CHX, especially with collagen, used for 1 minute ensures the longest substantivity when applied to a dentin-volumetric model. CTR at 0.2% also obtained high substantivity values and was not affected by the presence of collagen.

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