Recovery of Enterococcus faecalis after single- or multiple-visit root canal treatments carried out in infected teeth ex vivo

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Abstract


Aim To assess the presence of Enterococcus faecalis after root canal treatment in single or multiple visits in an ex vivo model.

Methodology Forty-five premolar teeth were infected ex vivo with E. faecalis for 60 days. The canals were then prepared using a crowndown technique with System GT and Gates–Glidden burs and irrigated with 2% chlorhexidine gel. The specimens were divided into five groups (G1, G2, G3, G4 and G5) according to the time elapsed between chemical–mechanical preparation and root canal filling, the irrigant solution used and the use or nonuse of a calcium hydroxide intracanal medicament. The teeth were then root-filled and incubated for 60 days at 37 °C. Dentine chips were removed from the canal walls with sequential sterile round burs at low speed. The samples obtained with each bur were immediately collected in separate test tubes containing Brain–Heart Infusion broth. These samples were placed onto agar plates and colony forming units were counted after 24 h at 37 ºC. Data were ranked and analysed using the Kruskal–Wallis statistical test.

Results Enterococcus faecalis was recovered from 20% (three of 15 specimens) of G1 (chlorhexidine irrigation and immediate root filling in a single visit), 25% (four of 15 specimens) of G2 (chlorhexidine irrigation and filling after 14 days use of a calcium hydroxide dressing in multiple visits), 40% (two of five specimens) of G3 (chlorhexidine irrigation and filling after 7 days), 60% (three of five specimens) of G4 (saline irrigation and filling after 7 days) and from 100% (five of five specimens) of G5 (saline irrigation and immediate filling without sealer).

Conclusions Neither single- nor multiple-visit root canal treatment ex vivo, eliminated E. faecalis completely from dentinal tubules. Up to 60 days after root filling, E. faecalis remained viable inside dentinal tubules. When no sealer was used, E. faecalis presented a higher growth rate.

Keywords: calcium hydroxide, chlorhexidine gel, Enterococcus faecalis, root canal treatment, single visit.

Introduction

The role of bacteria and their by-products in the initiation and perpetuation of pulpal and periapical disease has been well established (Gomes et al. 2002b). Most infecting bacteria, together with their principal substrate of necrotic pulp debris, may be removed by routine intra-canal procedures such as instrumentation and irrigation of the pulp space and the use of an intracanal medicament having antimicrobial activity. Nevertheless, this is not always fully achieved in clinical practice. The anatomical complexities of many root canals and consequent access limitations
of instruments, irritants and intra-canal medicaments are well-recognized factors (Biffi & Rodrigues 1989, Gomes et al. 2002b). Moreover, despite the antimicrobial effect of chemomechanical preparation and intra-canal medicaments, the elimination of microorganisms may not be uniform because of the vulnerability of the species involved (Gomes et al. 1996). Therefore, concern exists as to the fate and consequences of the remaining microorganisms in the canal. They may multiply rapidly, in some cases, to almost the initial number in 2–4 days, if the canal is left empty (Byström & Sundqvist 1981).

Many clinicians believe that the bacteria remaining after chemomechanical preparation can be eliminated or be prevented from repopulating the root canal space by placing an interappointment medicament. Another approach is to eliminate the influence of the remaining microorganisms or to render them harmless by entombing them in a filling, immediately after preparing and irrigating the canal space in the same visit. This way, the remaining microorganisms may be killed by the antimicrobial activity of the sealer or the Zn²⁺ ions of gutta-percha (Moorer & Genet 1982, Kaplan & Sundqvist 1981).

Root canal treatment in single or multiple visits should be viewed as part of a total endodontic treatment spectrum, with the choice of one over the other being determined by the circumstances peculiar to each particular case. The technique chosen would then best fit those circumstances (Ashkenaz 1984). The healing potential for teeth treated in one or two visits with an intra-canal disinfectant being applied appears to be similar (Katebzadeh et al. 1999, Fuss et al. 2000, Siqueira et al. 2000, Peters & Wesselink 2002).

Root canal treatment has been described as the disinfection of the root canal system, using endodontic instruments aided by an antimicrobial agent, before filling. Gomes et al. (2001) studied the disinfection time of several concentrations of chlorhexidine (either in gel or liquid vehicles) and sodium hypochlorite against E. faecalis. The 2.0% chlorhexidine in both presentation forms gave the shortest disinfection times, the same as with 5.25% sodium hypochlorite. Chlorhexidine has a broad antimicrobial activity against Gram-negative and Gram-positive bacteria, especially E. faecalis (Gomes et al. 2001, Vianna et al. 2004). Therefore, it has been used as irrigant or intra-canal medicament to improve disinfection.

Thus, the purpose of this study was to assess E. faecalis survival after root canal filling of infected extracted teeth in single and multiple visits, using chlorhexidine as irrigating solution in an ex vivo laboratory model.

### Material and methods

The method followed was a modification of one described previously by Gomes et al. (2003). Forty-five mandibular premolars freshly extracted for orthodontic reasons with complete apex formation and foramen diameter measuring up to a size 15 file were used. The teeth were cleaned with curettes to remove periodontal tissue and bone. The specimens were kept in 0.5% sodium hypochlorite solution for no longer than 7 days.

The crowns were removed, to enhance access, with carborundum disks (KG Sorensen, Barueri, Brazil) to the level of the amelo-dentinal junction resulting in roots 15 mm in length. The teeth were instrumented to
the apex using a size 25 file (Dentsply Maillefer, Ballaigues, Switzerland) to create a patency and facilitate the intra-canal contamination procedures. The external root cementum was completely removed using diamond burs (Haapasalo & Ørstavik 1987).

All teeth were submitted to an ultrasonic bath for 10 min in 17% EDTA, followed by 10 min in a 5.25% NaOCl bath, according to Perez et al. (1993) and a tampon phosphate bath (to eliminate EDTA and hypochlorite residues) followed by a distilled water bath (10 min each), in order to eliminate the smear layer produced during the initial preparation.

The teeth were individually placed in bijoux bottles containing 3.0 mL of Brain–Heart Infusion Medium (BHI; Oxoid, Basingstoke, UK) and autoclaved at 121 °C, 1 atm, for 15 min. They were then kept in an incubator at 37 °C for 24 h to check the efficacy of the sterilization treatment.

Isolated colonies (24 h) of pure cultures of E. faecalis (ATCC 29212) grown on 5% defibrinated sheep blood + BHI agar plates were suspended in 5.0 mL of BHI. The cell suspension was adjusted spectrophotometrically to match the turbidity of $1.5 \times 10^8$ colony forming unit (CFU) mL$^{-1}$ (equivalent to ± 0.5 McFarland standard).

The bijoux bottles containing each specimen were opened under laminar flow. Sterile pipettes were used to remove 2.0 mL of sterile BHI and to replace it with 2.0 mL of the bacterial inoculum. The bottles were closed and kept at 37 °C for 60 days, with the replacement of 1.0 mL of contaminated BHI for 1.0 mL of freshly prepared BHI every 2 days, to avoid medium saturation. The turbidity of the medium during the incubation period indicated bacterial growth. The purity of the cultures was confirmed by Gram staining, catalase production, colony morphology on BHI agar + blood and by the use of a biochemical identification kit (API 20 Strep; Bio-Mérieux SA, Marcy-l’Etoile, France). Bacterial penetration into the dentinal tubules using this technique was confirmed using SEM in a pilot study (Fig. 1).

Following the contamination period, each specimen was removed from its bijoux bottle under aseptic conditions and mounted in a sterile aluminium apparatus (Fig. 2). The canal was irrigated with 3.0 mL of 0.85% sterile saline and microbial samples (Sample 1) were collected with a sterile paper point. The paper point samples were placed individually in a 1.5 mL eppendorff tubes containing 1 mL BHI. The eppendorff tubes were then shaken for 30 s, followed by dilutions until 100 times. Fifty-microlitre aliquots of the dilutions were inoculated in BHIA + 5% of sheep defibrinated blood plates, which after 24 h at 37 °C incubation were inspected for CFU. The numbers counted were multiplied by 2000 to obtain CFU mL$^{-1}$. The purity of the cultures was confirmed by Gram staining, catalase production, colony morphology and by the use of a biochemical identification kit (API 20 Strep; Bio-Mérieux SA). These samples were used to confirm homogeneous infection in specimens.

The canals were instrumented using Greater Taper instruments (Tulsa Dentsply, Tulsa, OK, USA) from tapers 12 through 6, tip size 20 (Buchanan 1994). Gates–Glidden burs were then used from sizes 6 to 2 in the crowndown technique, followed by manual apical preparation up to a size 35 file (master apical file;...
Maillefer Dentsply). Two test and three control groups were distributed as follows:

**G1.** Single visit – 0.5 mL of 2% Chlorhexidine gel (Endogel™, Essencial Pharma, Itapetininga, SP, Brazil) irrigation between each file and immediate filling (15 specimens).

**G2.** Multiple visit – 0.5 mL of 2% Chlorhexidine gel (Endogel™) irrigation between each file and filling after 14 days of calcium hydroxide intra-canal dressing (15 specimens).

**G3.** 0.5 mL of 2% Chlorhexidine gel (Endogel™) irrigation between each file and filling after 7 days of BHI culture medium intra-canal dressing (five specimens; Chlorhexidine substantivity control).

**G4.** 1 mL saline solution irrigation between each file and filling after 7 days of BHI culture medium intra-canal dressing (five specimens; positive control).

**G5.** 1 mL saline solution irrigation each file and immediate filling only with gutta-percha cones and without sealer (five specimens; positive control).

All groups were irrigated 5 mm short apical foramen with 5 mL saline solution before filling (20 × 5.524 3/4 needle), including the calcium hydroxide group. After that, the specimens were dried with sterile medium paper points (Konne™, Belo Horizonte, Brazil) and filled with Konne™ medium gutta-percha points (taper .06) and a few accessory points (when necessary). Grossman sealer was also applied using warm vertical condensation (De Deus 1992), except for G5 where only gutta-percha points were used.

The teeth where intra-canal dressings were used (G2, G3 and G5) were sealed coronally with Cavit™ (3M-ESPE, Seefeld, Germany) and filled with Konne™ medium gutta-percha points (taper .06) and a few accessory points (when necessary). Grossman sealer was also applied using warm vertical condensation (De Deus 1992), except for G5 where only gutta-percha points were used.

The Kruskal–Wallis statistical test was applied with 5% significance. All initial samples presented high CFU counts (mean of 10⁶ each specimen); there were no statistically significant differences between the five experimental groups (P > 0.05).

Up to 60 days following filling, *E. faecalis* was viable inside dentinal tubules. *E. faecalis* cells were recovered from 20% (three of 15 specimens) of G1 root blocks (chlorhexidine irrigation and immediate root filling in a single visit), 25% (four of 15 specimens) of G2 root blocks (chlorhexidine irrigation and filling after 14 days of calcium hydroxide dressing in multiple visits), 40% (two of five specimens) of G3 root blocks (chlorhexidine irrigation and filling after 7 days), 60% (three of five specimens) G4 root blocks (saline irrigation and filling after 7 days) and 100% (five of five specimens) G5 root blocks (saline irrigation and immediate filling without sealer).

No statistical differences were found between G1, G2 and G3 (P > 0.05). G4 had significantly higher CFU counts than G1 and G2 (P < 0.05), but without a difference to G3 group (P > 0.05). G5 was statistically different to all other groups (P < 0.05), except for the apical G3 shallow collection (P > 0.05), cervical G4 deep collection and all apical G4 collections (P > 0.05) (Figs 4 and 5).
Discussion

The dentine block model, suggested by Gomes et al. (2003), was used with some modifications. *E. faecalis* has been detected within dentinal tubules up to 500–700 \( \mu \text{m} \) after 60 days of incubation. Saleh et al. (2004) used 3 weeks of incubation and found microorganisms up to 300–400 \( \mu \text{m} \) within the tubules. The results of the present study are not compatible with those of Haapasalo & Ørstavik (1987) who reported that the average depth of penetration of the tubules by bacteria increased only slowly with time.

In the current study, all the experimental groups had viable residual *E. faecalis*, either in gutta-percha and sealer or in the inner dentine layers. However, the number of cells recovered in G1 and G2 was smaller than in other groups. In the single-visit group (G1), *E. faecalis* was equally recovered from both root

![Figure 3](image1.png)

**Figure 3** Debris collection using round burs. A: cervical and medium thirds; B: apical third.

![Figure 4](image2.png)

**Figure 4** Ranked colony forming unit (CFU) averages, per group. Values followed by different letters presented significant statistical differences using the Kruskal–Wallis statistical test \( (P < 0.05) \).
fragments, whilst in the multiple-visit group (G2) they were recovered more frequently in the apical fragment. This may be due to the inherent difficulties of inserting calcium hydroxide into the apical third of the root canal and also because of the anatomical characteristics of this region, where the dentine tubules are smaller and less frequent making the diffusion of hydroxyl ions more difficult. For these reasons bacterial growth in that region between treatment visits may have occurred. Haapasalo et al. (2000) stated that the poor diffusion of hydroxyl ions into infected-dentine and the buffering capacity of dentine can limit the raise of pH. These are some of the reasons why hydroxide pastes are ineffective against E. faecalis even after an extended incubation time.

Chlorhexidine gel was chosen to irrigate the canals of the test groups because of its broad-antimicrobial spectrum (Jeansonne & White 1994, White et al. 1997, Gomes et al. 2001, 2003, Vianna et al. 2004), good lubrication (Ferraz et al. 2001) and especially its substantivity (i.e. residual antimicrobial activity) (Kuruvilla & Kamath 1998, Tanomaru Filho et al. 2002). Such residual antimicrobial activity may explain the reduced number of bacteria recovered from group G3 (40%) after irrigation with chlorhexidine and 1 week with BHI medium intra-canal dressing. On the other hand, for G4, where the canals were irrigated with saline solution followed by 7 days BHI medium intra-canal dressing, bacteria were recovered from 60% of the canals.

The root canal sealer is also of great importance in root canal filling, removing all space and nutrients necessary to maintain bacterial viability. In G5, where the canals were irrigated with saline and filled with gutta-percha only, E. faecalis was recovered from 100% of the canals. This result confirms the findings of Saleh et al. (2004) that the use of Grossman sealer in vitro killed all bacteria in the dentinal tubules within a 300 μm zone around the root canal.

Several studies (Byström et al. 1985, Haapasalo & Ørstavik 1987, Safavi et al. 1990, Saleh et al. 2004) have shown that calcium hydroxide has limited capability to completely kill bacterial cells inside dentinal tubules. The present study showed that after single and multiple visits, the number of E. faecalis cells inside the root canals can be reduced, but cannot be eliminated completely. However, the E. faecalis cells were maintained viable because the cementum was intentionally removed and the dentinal tubules were in contact in the middle third of the canals containing the BHI medium. The root canal cementum is of greater importance in isolating patent dentinal tubules on the periodontal side, preventing residual bacteria from reaching exit portals to the periodontal space, except in larger lateral canals.

Thus, when the cementum is present, residual bacteria inside dentinal tubules will not survive without nutrients and space for their growth (Haapasalo & Ørstavik 1987, Safavi et al. 1990). The cementum was confirmed by Berutti et al. (1997) to be a barrier against the penetration of bacteria.

Clinically, the nutrition required for microbial cell recovery may come from coronal leakage, from fluid within dentinal tubules or from periapical tissues. This demonstrates the importance of complete filling of the root canal system after chemomechanical disinfection, followed by a high quality coronal seal, in order to prevent post-treatment failure.

**Conclusion**

Within the limits of this study:

1. No statistical differences in E. faecalis counts were found ex vivo between single-visit and multiple-visit root canal treatments.
2. Even after 60 days of root filling E. faecalis remained viable inside dentinal tubules ex vivo.
When no sealer was used E. faecalis had a higher growth rate ex vivo.

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