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

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Abstract

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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 °C. 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 intra-canal 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

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of instruments, irrigants 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 Zn^{2+} ions of gutta-percha (Moorer & Genet 1982, Kaplan *et al.* 1999, Fuss *et al.* 2000, Siqueira *et al.* 2000, Peters & Wesselink 2002).

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, Weiger *et al.* 2000). Some studies report the presence of remaining microorganisms after cleaning and shaping, and then dressing with calcium hydroxide. But they do not relate this to healing (Byström *et al.* 1985, Yared & Bou Dagher 1994, Shuping *et al.* 2000).

Peters & Wesselink (2002) showed that positive cultures obtained immediately before root filling did not influence the outcome of treatment in either single or multiple visits. However, Sjögren *et al.* (1997) did report a strong relationship between bacterial contamination and treatment outcome. If teeth are symptom-free and the canals are dry, it should be possible to complete root canal treatment in a single visit. Weiger *et al.* (2000) observed that the success rate, after 5 years, in single-visit and two-visit root canal treatments with calcium hydroxide was 92 and 93% respectively. Complete radiographic healing was found,

up to 4.5 years after treatment, in 81% of the cases treated in one-visit and 71% of the cases treated in two visits with calcium hydroxide dressing (Peters & Wesselink 2002).

Enterococcus faecalis, a facultative gram-positive bacterium, has been considered one of the most resistant species in the oral cavity and one possible cause of post-treatment disease after root canal treatment. Several studies have reported their low susceptibility to irrigant solutions (Gomes *et al.* 2001, Vianna *et al.* 2004) and to intra-canal medicaments such as calcium hydroxide (Siqueira & Uzeda 1997, Valera *et al.* 2001, Gomes *et al.* 2002a,b, 2003, Menezes *et al.* 2004). Therefore, they may persist after instrumentation, medication and even after root filling, increasing the risk of post-treatment disease (Byström *et al.* 1987, Sjögren *et al.* 1991).

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 1.5×10^8 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
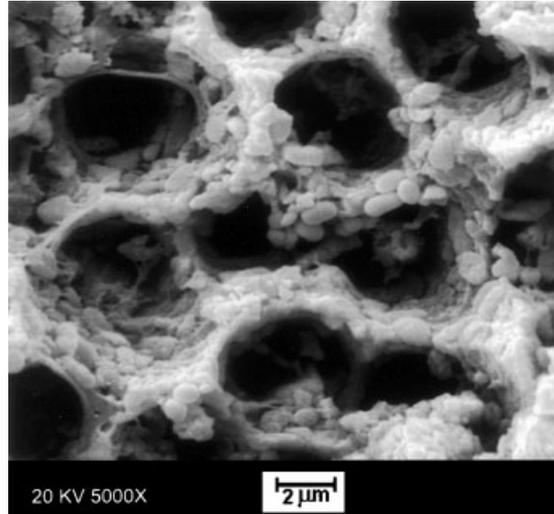

Figure 1 Dentin infection after 60 days.

Figure 2 Aluminium apparatus.

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^{TM}; 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^{TM}) 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^{TM}) 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^{TM}, Belo Horizonte, Brazil) and filled with Konne^{TM} 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^{TM} (3M-Espe, Seefeld, Germany) and incubated at 37 °C in eppendorff tubes with BHI (without entirely covering the root) for the established periods.

After being filled, all groups were sealed coronally with Cavit^{TM} again and incubated in eppendorff tubes with 50 μL BHI for 60 days at 37 °C. Every 72 h, the BHI was exchanged.

Coronal seals were removed and the specimens were transversally cut with a diamond disk (KG Sorensen) in the middle to the apical third, to obtain a 9–10 mm cervical fragment and a 5 mm apical fragment. The coronal and apical fragments were mounted and fixed in a sterile aluminium apparatus. The mean dentine width was 2 mm in the coronal fragment and 1.6 mm in apical one (Fig. 3).

Two sizes of round burs were used to sample the internal dentine and gutta-percha on both cervical and apical fragments. The first bur was adapted to the root canal diameter. The shallow collection (SC)

was obtained by removing all filling material and approximately 200–250 μm of the circumpulpal dentine over the complete extension of the fragment (round bur ISO 10 for the apical fragment and 16 for the cervical one). The second bur produced the deep collection (DC) removing up to approximately 650 μm of dentine from the apical and cervical fragments (round bur ISO 18 in the apical fragment and 25 in the cervical one) (Fig. 3). Debris was collected in a 5 mL glass flask containing 1 mL of BHI. These samples were shaken for 1 min and 100 μL was placed onto BHI agar plates that were incubated for 24 h at 37 °C to allow a count of CFU. All counts were multiplied by 10 to obtain CFU mL^{-1} (Almyroudi *et al.* 2002).

All samples were identified by Gram staining, catalase production, colony morphology on BHI agar + blood and by the use of a biochemical identification kit (API 20; Strep – bioMérieux SA). Miniapi software (BioMérieux SA) was used to visually read the API range tests from BioMérieux.

Results

The Kruskal–Wallis statistical test was applied with 5% significance. All initial samples presented high CFU counts (mean of 10^6 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) of 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).
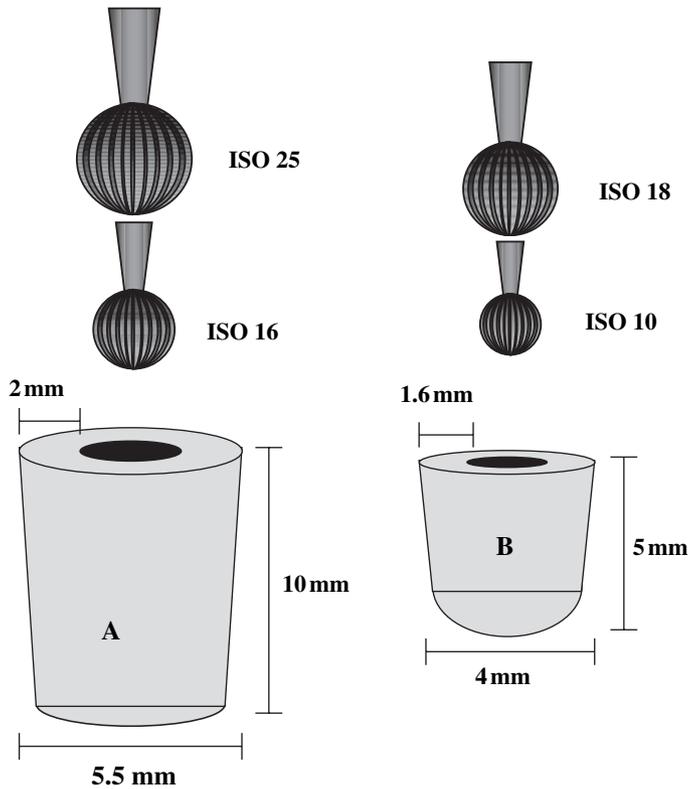
Figure 3 Debris collection using round burs. A: cervical and medium thirds; B: apical third.
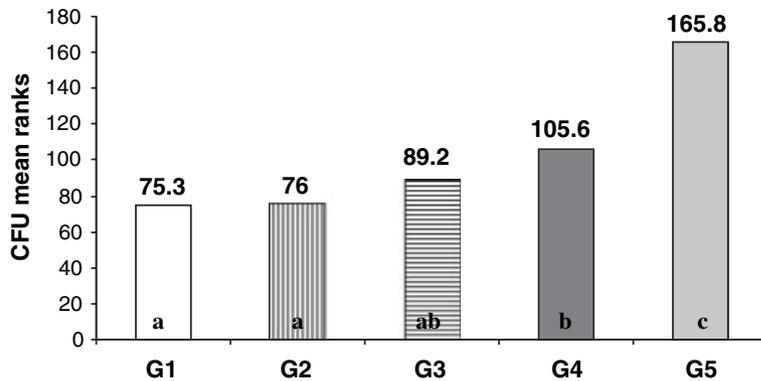

Figure 4 Ranked colony forming unit (CFU) averages, per group. Values followed by different letters presented significant statistical differences using the Kruskal–Wallis statistical test ($P < 0.05$).

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 μm after 60 days of incubation. Saleh *et al.* (2004) used 3 weeks of incubation and found microorganisms up to 300–400 μm 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
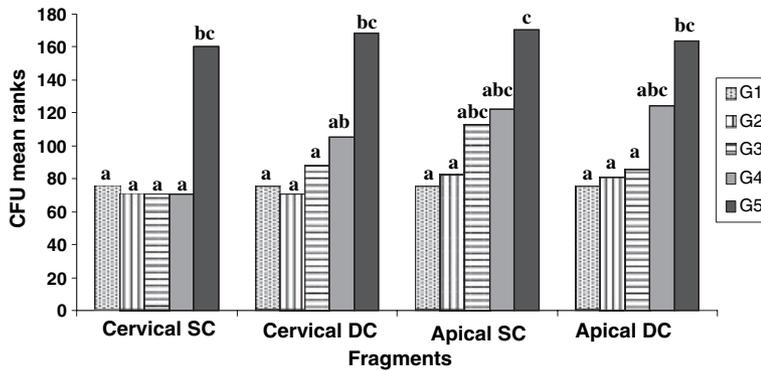
Figure 5 Colony forming units (CFU) per group, per fragment. SC, shallow collection; DP, deep collection. Values followed by different letters presented significant statistical differences using the Kruskal–Wallis statistical test ($P < 0.05$).

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*.

3 When no sealer was used *E. faecalis* had a higher growth rate *ex vivo*.

Acknowledgements

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