INFLUENCE OF THE LENGTH OF REMAINING ROOT CANAL FILLING AND POST SPACE PREPARATION ON THE CORONAL LEAKAGE OF *ENTEROCOCCUS FAECALIS*

Alexandra Conca Alves Mozini[1]; Luis P. Vansan[1]; Manoel D. Sousa Neto[2]*; Rosimeire Pietro[1]

[1]Faculdade de Odontologia, Universidade de Ribeirão Preto, Ribeirão Preto, SP, Brasil; [2]Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

ABSTRACT

This study evaluated the sealing ability of different lengths of remaining root canal filling and post space preparation against coronal leakage of *Enterococcus faecalis*. Forty-one roots of maxillary incisors were biomechanically prepared, maintaining standardized canal diameter at the middle and coronal thirds. The roots were autoclaved and all subsequent steps were undertaken in a laminar flow chamber. The canals of 33 roots were obturated with AH Plus sealer and gutta-percha. The root canal fillings were reduced to 3 predetermined lengths (n=11): G1=6 mm, G2=4 mm and G3=2 mm. The remaining roots served as positive and negative controls. Bacterial leakage test apparatuses were fabricated with the roots attached to Eppendorf tubes keeping 2 mm of apex submerged in BHI in glass flasks. The specimens received an *E. faecalis* inoculum of 1 x 107 cfu/mL every 3 days and were observed for bacterial leakage daily during 60 days. Data were submitted to ANOVA, Tukey's test and Fisher's test. At 60 days, G1 (6 mm) and G2 (4 mm) presented statistically similar results (p>0.05) (54.4% of specimens with bacterial leakage) and both groups differed significantly (p<0.01) from G3 (2 mm), which presented 100% of specimens with *E. faecalis* leakage. It may be concluded that the shortest endodontic obturation remnant leaked considerably more than the other lengths, although none of the tested conditions avoids coronal leakage of *E. faecalis*.

Keywords: Bacterial infiltration, *E. faecalis*, root canal treatment.

INTRODUCTION

The advent of prefabricated intraradicular posts and adhesive materials allied to the trend of preservation of sound dental structure increased the restorative options and allowed better utilization of the tooth remnant. Teeth with extensive coronal destruction may be rehabilitated in a single appointment by the placement of an intraradicular post and core immediately after completion of root canal obturation, thus minimizing the risk of salivary and bacterial contamination of the remaining root canal filling (10).

The use of newer adhesive systems and prefabricated intraradicular posts has provided an adequate retention with cementation of smaller posts, thus maintaining a larger portion of obturating material to seal the middle and apical root thirds (5). Nevertheless, in teeth with short or curved roots, the length of the intraradicular post will be reduced, which may compromise its retention. In these cases, only few millimeters of root filling material will be left in the canal and will be responsible to maintain the health of the periradicular tissues.

Several studies have considered coronal leakage as an important cause of endodontic treatment failure. It has been demonstrated that root fillings exposed to the oral cavity are invariably contaminated by fluids, bacteria and their byproducts. Long-term contamination may lead to failure of endodontics and compromise the restorative/prosthetic treatment as a whole (1,8,21,22,25).

The maintenance of aseptic conditions during root canal therapy avoids microbial and fluid infiltration. However, the sequence of post space preparation procedures in endodontically

*Corresponding Author. Mailing address: Rua Célia de Oliveira Meirelles 350, 14024-070 Ribeirão Preto, SP, Brasil. Phone/Fax: +55 16 3603-6783. E-mail: sousanet@forp.usp.br

treated teeth, which includes impression, fitting of the intraradicular post to the prosthetic space and post cementation, may allow inadvertent contact of the remaining root canal filling with saliva and bacteria (10,12,14).

The length of the intraradicular post and the residual filling material are also very important. The intracanal retainer should have 2/3 of the bone-supported root length. When this length is not clinically feasible, the post should be at least as long as the clinical tooth crown (18). Some authors have advocated that a minimum of 4 mm of filling material should remain in the root canal to prevent bacterial leakage (15,24).

The purpose of this study was to evaluate *in vitro* the sealing ability of different lengths of remaining root canal filling (6 mm, 4 mm and 2 mm) after obturation and immediate post space preparation against coronal leakage of *Enterococcus faecalis* during a 60-day experimental period.

MATERIAL AND METHODS

This research was approved by the Ethical Committee in Research of University of Ribeirão Preto, São Paulo, Brazil.

Selection and Preparation of Teeth

Extracted human maxillary central incisors were selected from a random collection and were checked for absence of root caries, cracks and structural defects. Soft debris were removed with hand curettes. Crowns were removed at the cementoenamel junction with a water spray-cooled carborundum disc, and the roots were trimmed coronally to a uniform length of 14 mm and stored in saline at 9ºC until use to prevent dehydration.

Endodontic Procedure

The working length (WL) was established 1 mm short of the anatomical apex. A size 40 K-file (Dentsply/Maillefer, Ballaigues, Switzerland) was selected as a master apical file (MAF) and 41 specimens whose canal diameter at WL coincided with the size of the MAF were included in the study. The apical plateau was prepared with the subsequent three file sizes larger than the MAF at the WL. The root canals were instrumented according to the step back technique and were irrigated with 2 mL of 1% sodium hypochlorite (Merck, Darmstadt, Germany) at every change of file. Biomechanical preparation was completed with enlargement of the coronal root third with #6 Largo burs (Dentsply/Maillefer, Ballaigues, Switzerland) to standardize the root portion that should further receive the bacterial inoculum. Final flushing was done with 5 ml of 17% EDTA (Merck, Darmstadt, Germany), which remained in root canals for 3 minutes, followed by irrigation with 10 mL of distilled water, aspiration and drying of the root canals with sterile absorbent paper points (Dentsply/Herpo, Petrópolis, RJ, Brazil).

The instrumented roots were autoclaved at 120ºC during 20 minutes (Autoclave model 103; Fabbe Primar, São Paulo, SP,

Brazil) and, thereafter, all procedures were performed in a laminar flow chamber (Veco, Campinas, SP, Brazil) for maintenance of the aseptic chain. All instruments and materials were sterilized before use.

The root canals were obturated with AH Plus sealer (Dentsply De Trey, GmbH, Konstanz, Germany) and lateral condensation of gutta-percha cones (Dentsply/Herpo, Petrópolis, RJ, Brazil), which were disinfected by immersion in 2.5% sodium hypochlorite for 3 minutes prior to use. After obturation, radiographs of all specimens were taken to evaluate the quality of the root canal filling. Specimens used this experiment had radiographically well-compacted fillings that extended to 1 mm short of the apical foramen.

Thirty-three roots were randomly assigned to three groups (n=11), according to the length of the remaining root canal filling: G1 - 6 mm, G2 - 4 mm and G3 - 2 mm. The other 8 roots served as positive (n=4) and negative (n=4) controls. The teeth used as positive controls were instrumented but not obturated to demonstrate the maximum bacterial leakage through the root canal system. The negative control teeth were instrumented, not obturated and thorough sealed externally with nail polish in order to check the method (confirm that the impermeabilization was able to avoid bacterial infiltration). Immediately after obturation, the root canal fillings were reduced to the predetermined lengths (2, 4 and 6 mm) using a heated plugger. Vertical force was applied with a cold instrument to compact the remaining mass. The obturated teeth were radiographed to check the homogeneity of the remaining filling material. A Hedstrom file (Dentsply/Maillefer, Ballaigues, Switzerland) with a rubber stopper was inserted into the canals to confirm the length of root filling remnants. The specimens were stored at 37ºC for 24 hours, which corresponds to 3 times the time required for complete set of the endodontic sealer, according to the manufacturer's instructions.

The roots in the experimental groups and positive control group were coated with two layers of cosmetic nail polish (Colorama, Maybelline, NY, USA), except for the apical 1 mm. The roots used as negative controls had the entire root surface sealed.

Leakage Apparatus Preparation

For the bacterial leakage test, one test apparatus was fabricated for each specimen. Briefly, the tapered end of sterile 1.5-mL-Eppendorf plastic tubes (Eppendorf-Elkay, Shrewsbury, MA, USA) was perforated with a scalpel blade (Med. S. N., Shanghai, China) and the obturated roots were individually inserted until their apical 2 mm protruded through the tube end. The junction between each tube and root was sealed with epoxy resin (Araldite, Brascola Ltda, São Paulo, SP, Brazil) to prevent leakage of the connection.

The Eppendorf tube/root assemblies were placed in sterile 13-mL glass flasks containing 9 mL of brain heart infusion (BHI)

broth (Oxoid Ltda, Basingstoke, Hampshire, England), in which the root apexes remained completely submerged. To avoid external contamination, the interface between the Eppendorf tubes and the glass flasks was sealed tightly with epoxy resin. The test assemblies were then incubated at 37ºC for 24 hours to confirm sterility. Fig. 1 presents a schematic illustration of the apparatus used for microbial infiltration.

Culture and Preparation of the *Enterococcus faecalis* suspension

Enterococcus faecalis ATCC 29212 was used for the leakage test. A total of 200 µL of the microbial strain were cultured overnight in 2 mL of BHI broth at 37ºC. The bacterial suspension was added to 2 mL of saline and adjusted to achieve a turbidity equivalent to 0.5 McFarland standard in order to ensure a bacterial concentration of approximately 1.5×10^8 colony forming units (cfu)/mL. For preparation of the inoculum, the culture was diluted by transferring 200 µL of the *E. faecalis* suspension to a hemolysis tube containing 1.8 mL of BHI, thus providing a concentration of 1×10^7 cfu/mL.

Bacterial Leakage Test

An inoculum of *E. faecalis* (10 µL) was injected into the post/canal space of the test specimens and positive controls using a micropipette (Finnpipette, Labsystems, Helsinki, Finland). The inoculum was changed with fresh inoculum every 3 days during a 60-day experimental period or up to the occurrence of bacterial growth. The negative controls did not receive bacterial inoculum. The test apparatuses were placed in plastic receptacles and incubated in a microbiological stove at 37ºC during the course of the experiment. Bacterial growth was checked every 24 hours.

Turbidity of the BHI broth in which the roots inoculated with *E. faecalis* were submerged was indicative of bacterial growth due to leakage of the test strain. The day of occurrence
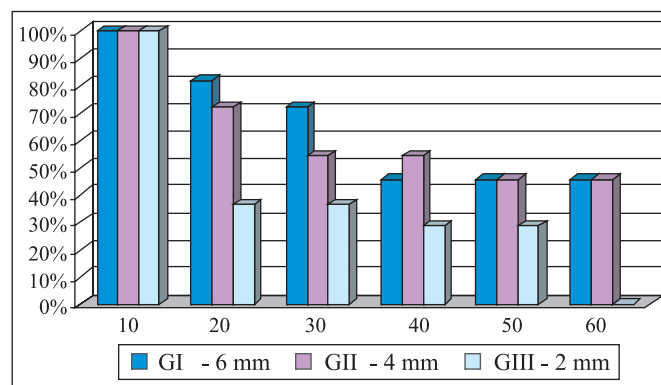
and code number of the specimen were recorded and the content was collected and submitted to microbiological assays. The specimens with bacterial leakage were removed from the test apparatuses and the root canals were dried with sterile absorbent paper points and a new inoculum was injected into the post/canal space. The soaked paper points were incubated overnight in BHI broth at 37ºC for analysis of the material collected from the root canals.

Bacterial growth was identified as *E. faecalis* by means of a series of microbiological tests, namely, colonial morphology, cell morphology, positive bile-esculin test and growth in 6.5% NaCl BHI broth. Samples were excluded if there were signs of contamination with other bacteria.

Analysis of colonial morphology in Müeller Hinton agar plates. Samples of BHI broth retrieved from the test apparatuses, cultured paper points and bacterial inoculum were collected, seeded onto agar Müeller Hinton plates (Merck, Darmstadt, Germany) and incubated at 37ºC for 24 hours to verify the occurrence of *E. faecalis* growth by analysis of colonial morphology.

Cell Morphology. After agar plate culture, smears were prepared on glass slides and stained with Gram's Method to identify microbial morphology.

Bile-esculin agar test. Samples of colonial growth on agar plates were collected, seeded on bile-esculin agar (Merck, Darmstadt, Germany) and incubated at 37ºC for 24 hours to verify the presence of *Streptococcus* sp. The bile-esculin agar test is based on the capacity of some bacteria to hydrolyze esculin in the presence of bile. Bacterial growth on this medium forms a black complex because the esculin reacts with ferric ions. A positive result is indicative of the presence of *Streptococcus* sp., among which there may be samples of *E. faecalis*.

6.5% NaCl tolerance test. This test is used to check microbial capacity to grow on the presence of 6.5% NaCl, distinguishing *Enterococcus* spp. (which grow on the presence NaCl) from *Streptococcus* spp. Samples of colonial growth on agar plates were collected, seeded on 6.5% NaCl BHI broth (Merck, Darmstadt, Germany) and incubated overnight at 37ºC. Turbidity of BHI broth within 24 hours indicated the presence of *E. faecalis*.

Data were submitted to ANOVA and Tukey's at 1% significance level to verify the differences among the groups and to determine which groups differed significantly to the others. Fisher's exact test was performed to identify the inter-group differences at the evaluated time points, with a value of $p < 0.05$ being considered statistically significant.

RESULTS

Mean values and standard deviations of specimens without bacterial leakage in the three experimental groups are given on Table 1.

Figure 1. Schematic illustration of the apparatus used for microbial infiltration.

There was statistically significant difference (p<0.01) among the lengths of remaining root canal filling. Group 1 (6 mm) and Group 2 (4 mm) did not differ significantly to each other (p>0.01) and presented a larger number of specimens without *E. faecalis* leakage during the course of the experiment. On the other hand, Group 3 (2 mm) presented a smaller number of specimens without bacterial leakage and was statistically significant different from the other groups (p<0.01).

Fisher's exact test was applied to the data obtained at 10-day intervals from the 20th to the 60th day because no turbidity of the medium (bacterial leakage) was observed up to the 10th day in either of the groups. Fig. 2 depicts graphically the number of specimens without *E. faecalis* leakage in the three groups at 10-day intervals. At 20 days, there was statistically significant difference (p<0.05) only between Group 1 (6 mm) and Group 3 (2 mm), the group with longer remaining root canal filling presenting a larger number of specimens without bacterial

Table 1. Means of the number of specimens without bacterial leakage in the three groups.

Lengths of remaining filling material	Means (±SD)
G1 - 6 mm	7.90 (±2.46) a
G2 - 4 mm	7.18 (±1.99) a
G3 - 2 mm	5.17 (±3.78) b

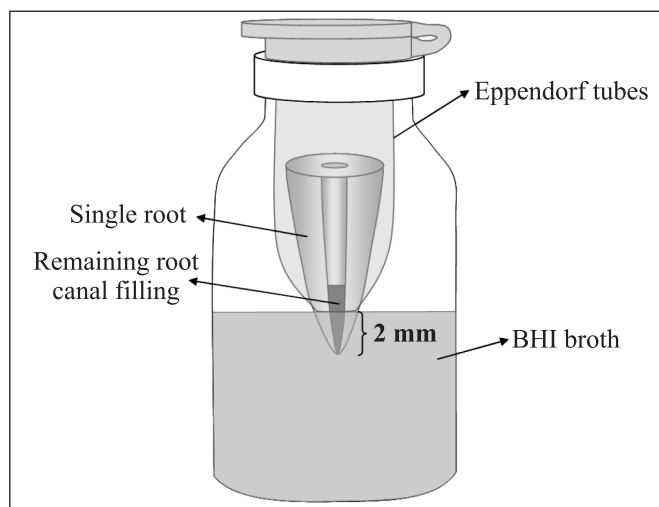Different letters indicate statistically significant difference at 1%; Tukey critical value = 1.46.

Figure 2. Graphic presentation of the number of specimens without *E. faecalis* leakage in the three groups at 10-day intervals.

infiltration. Nevertheless, at 30, 40 and 50 days, no statistically significant difference was observed among the groups (p>0.05). At 60 days, Group 1 (6 mm) was statistically similar to Group 2 (4 mm) (p>0.05) and both differed significantly from Group 3 (2 mm) (p<0.05), in which 100% of specimens presented *E. faecalis* leakage.

The positive controls demonstrated bacterial leakage after 1 day, whereas the negative controls did not show leakage for the entire observation period.

Samples of the broth retrieved from the test apparatuses, cultured paper points and bacterial inoculum were positive for *E. faecalis* as for counterproof, colonial morphology, 6.5% NaCl tolerance test, bile-esculin test and cell morphology.

DISCUSSION

Coronal leakage has been pointed out as one of the most important and challenging causes of endodontic failure because it compromises sealing of the root canal system (3,7,12,21,22).

It has been demonstrated that oral fluids, bacteria and their byproducts may infiltrate through the marginal gaps of defective restorations and penetrate the interface between the root filling material and the root canal walls, reaching the periapical region within a relatively short time (3,4,7,8,21,22). This scenario is worsened in case of teeth with extensive coronal tooth loss that implicates in the placement of intraradicular posts because of following factors associated with post space preparation and post cementation: break of the aseptic chain during procedures undertaken without rubber dam isolation, possibility of dislodging the obturation during removal of the coronal portion of the filling material, placement of temporary restorations between the prosthetic working sessions and length of the remaining root canal filling.

Among the currently available methods to evaluate the quality of root filling seal, dye marginal leakage is the most frequently used model. However, this methodology is not the most effective and has some limitations, as the size of dye molecules may overestimate the infiltration or the interaction between dye and sealer may lead to false results (23).

It has been demonstrated that tests using bacteria as tracer agents of leakage may simulate in a more reliable manner what actually occurs under clinical conditions, as they allow a dynamic interaction between the tested bacterial strain and the root canal sealer (11,14,20,21,24,25). *E. faecalis* is the most often retrieved pathogen in asymptomatic endodontic infections because of its capacity to invade dentinal tubules, compete with other microorganisms and resist nutritional privation. It is a facultative anaerobic Gram-positive coccus that may present either isolated, in pairs or in chains (4,16,17,20).

Regarding the methodology used in the present study (coronal leakage of *E. faecalis*), root length, MAF and diameter of the coronal root third were standardized to allow that the

same amount of bacterial inoculum was placed in contact with the root filling material within the post space. The roots were autoclaved after instrumentation and thereafter all procedures were performed in a laminar flow chamber. Sterility of the test assemblies was confirmed by absence of turbidity of the medium. Purity of the bacterial inoculum used for contamination of the specimens and purity of the material retrieved from the specimens at each renewing of the inoculum were confirmed by a set of microbiological assays including colonial morphology in agar Müeller Hinton plates, cell morphology, bile-esculin test and 6.5% NaCl tolerance test. The results of all tests were positive to the presence of *E. faecalis* as the sole microbial strain.

Two aspects should be discussed in the light of the results of the present study: bacterial leakage through the residual root canal obturation and the time elapsed until its occurrence. *E. faecalis* penetration through the obturating material was observed in all experimental groups. Bacterial leakage was statistically similar in the groups with 6 and 4 mm of remaining filling material and, comparatively, significantly smaller in the group with 2 mm of root filling remnant. At the end of an experimental period of 60 days, Group 3 (2 mm of remaining filling material) exhibited 100% of specimens with *E. faecalis* leakage. The greatest coronal bacterial leakage observed in Group 3 may be explained by the high incidence of lateral and accessory canals in the apical region (6). It is likely that the specimens in which the length of the root filling remnant was 6 or 4 mm (Groups 1 and 3) had better apical sealing. However, even in these groups the residual obturation was not capable of preventing bacterial leakage, probably due to the presence of gaps at the interface between the filling material and the root canal walls. These interfacial defects may be attributed to variables such as internal anatomy of the root canal system, biomechanical preparation, proprieties of endodontic irrigants, physicochemical proprieties of endodontic materials and root filling techniques (16).

The findings of the present study, in which bacterial leakage occurred regardless of the length of the remaining root canal filling, are consistent with those of previous investigations (1,3,7,8,10,21,22,25). Regarding the time elapsed until the occurrence of *E. faecalis* leakage, broth turbidity was not observed in any of the groups up to the 10th day of the experiment. According to Pizzo *et al.*, (13), AH Plus sealer, used in this study, presents bacterial inhibition within the first 24 hours, which may have delayed bacterial penetration but was not able to completely prevent coronal leakage of *E. faecalis*. Therefore, an important role of the endodontic obturation is the formation of a physical barrier to prevent bacteria from reaching the apical region and periapical tissues (16). In Group 1 (root filling remnant of 6 mm), this physical barrier was 3 times larger than that in Group 3 (root filling remnant of 2 mm), which may possibility explain the difference observed between these

groups at 20 days concerning the number of specimens with bacterial leakage.

It is worthy mentioning that, owing to its high flow capacity and long setting time, AH Plus epoxy resin-based sealer is able to penetrate deeply into micro-gaps and micro-defects. These properties have been claimed to enhance the dentin/sealer interweaving (19), increase the resistance to removal and/or dislodgment from dentin surface and reduce marginal infiltration (9,16,21). Some factors that may contribute to attainment of different results with respect to bacterial leakage time include study protocol, type of microorganism and its concentration, dental anatomy, amount of remaining tooth structure and sample size (3). Therefore, comparison of the findings of the present study to those published elsewhere should be done with care because of methodological differences.

Torabinejad *et al.* (22) have demonstrated that *P. vulgaris* was able to penetrate through obturated root canals in a mean time of 49 days and that *S. epidermidis* reached the root apex within 24 days. Gish *et al.* (7) reported that penetration of *S. anginosus* occurred within 71 days, while Khayat *et al.* (8) observed bacterial leakage between 4 and 48 days. Barrieshi *et al.* (3) assessed bacterial leakage of a mixed anaerobic community of organisms in obturated canals after post space preparation and observed that the time of bacterial penetration ranged from 48 to 84 days.

E. faecalis may remain viable in the root canals for up to 13 months, being the main microorganism involved in the onset of secondary endodontic infections (17,20). Histological analysis using H.E. and Brown & Brenn staining techniques reveals the presence of bacteria in the root canal lumen and lodged into the dentinal tubules, which confirms the capacity of penetration of this microorganism.

Despite the currently available wide array of root canal filling materials and techniques, the findings of different studies (12,14,25) have systematically demonstrated that, to date, none of them is able to completely prevent the occurrence of coronal or apical leakage, which makes the concept of a hermetic sealing hardly achievable in Endodontics.

The findings of the present study and the research-based evidences discussed herein emphasize the need to establish treatment protocols for endodontically treated teeth that require the placement of intraradicular posts in order to prevent bacterial invasion and failure of the obturation. Recommendable measures include the fabrication of plugs to protect the remaining filling material (2) and use of prefabricated posts and adhesive systems to reduce the number of clinical sessions and avoid exposure of the root filling to the oral cavity.

Within the limitations of an *in vitro* study, it may be concluded that, it may be concluded that the shortest endodontic obturation remnant leaked considerably more than the other lengths, although none of the tested conditions avoid coronal leakage of *E. faecalis*.

RESUMO

Influência do comprimento do material obturador remanescente no canal radicular e preparo do espaço protético na infiltração coronária do *Enterococcus Faecalis*

Este estudo avaliou a capacidade de diferentes de remanescentes de material obturador do canal radicular e preparo do espaço protético na infiltração do *Enterococcus faecalis*. Quarenta e uma raízes de incisivos superiores foram preparadas biomecanicamente, mantendo-se diâmetro padronizado nos terços médio e cervical. As raízes foram autoclavadas e todos os passos subseqüentes foram realizados em capela de fluxo laminar. Os canais de 33 raízes foram obturadores com AH Plus e guta-percha. As obturações foram reduzidas a 3 comprimentos (n=11): G1=6 mm, G2=4 mm e G3=2 mm. As raízes remanescentes serviram de controles positivo e negativo. O dispositivo para testar a microinfiltração bacteriana foi confeccionado com as raízes fixas a *Eppendorfs*, mantendo-se 2 mm do ápice submergido em vidro contendo BHI. Os dentes receberam o inóculo de 1×10^7 UFC/ml de *E. faecalis* a cada 3 dias, com observação diária por 60 dias. Os dados obtidos foram submetidos à análise de variância, teste de Tukey e Fisher. Foi possível observar que aos 60 dias, o G1 (6 mm) e G2 (4 mm) apresentou resultados estatisticamente semelhantes (p>0,05) (54% dos espécimes com infiltração bacteriana) e ambos os grupos foram diferentes estatisticamente (p<0,01) do G3 (2 mm), o qual apresentou 100% de espécimes com microinfiltração. Concluiu-se que apesar da infiltração do *E. faecalis* ter ocorrido em todas as condições testadas, aparentemente houve uma correlação positiva entre o comprimento do remanescente radicular e a eficácia do selamento, uma vez que, a menor a obturação remanescente infiltrou consideravelmente mais que os outros comprimentos.

Palavras-chave: Infiltração bacteriana, *E. faecalis*, tratamento dos canais radiculares

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