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ORIGINAL ARTICLE

Bacterial colonization in the apical part of extracted human teeth following root-end resection and filling: a confocal laser scanning microscopy study

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Abstract
Objectives The purpose of this study was to evaluate *Enterococcus faecalis* colonization at the apical part of root canals following root-end resection and filling using confocal laser scanning microscopy (CLSM).
Materials and methods The apical 3-mm root-ends of 55 extracted single rooted human teeth were resected, and 3-mm retrograde cavities were prepared and filled using either mineral trioxide aggregate (MTA), intermediate restorative material (IRM), or Biodentine (n = 10 each); 25 teeth served as controls. The roots were placed in an experimental model, sterilized, and coronally filled with *E. faecalis* bacterial suspension for 21 days. Then, the apical 3-mm segments were cut to get two slabs (coronal and apical). The slabs were stained using LIVE/DEAD BacLight Bacterial Viability Kit and evaluated using CLSM.
Results The fluorescence-stained areas were larger in the bucco-lingual directions compared with the mesio-distal directions ($p < 0.05$). The mean and maximal depths of bacterial colonization into the dentinal tubules were 755 and 1643 μm, respectively, with no differences between the root-end filling materials ($p > 0.05$). However, more live bacteria were found in the MTA group in comparison to IRM and Biodentine groups ($p < 0.05$).
Conclusions CLSM can be used to histologically demonstrate bacterial root-end colonization following root-end filling. This colonization at the filling-dentine interfaces and deeper into the dentinal tubules may be inhomogeneous, favoring the bucco-lingual aspects of the root.
Clinical relevance Following root-end resection and filling bacterial colonization may lead to inflammatory reactions at the periapical tissues; the viability of the colonized bacteria may be affected by the type of root-end filling material.

Keywords Endodontic surgery · Root-end filling · Bacterial colonization · *Enterococcus faecalis* · Confocal laser scanning microscopy

Introduction

The association between bacteria in the root canal system and periapical pathosis has been well established [1]. Root canal colonization of microbial biofilms as a result of either continuing bacterial contamination of the root canal-treated teeth or residual infection in the root canal system can prevent periapical healing of endodontically treated teeth [2–6].

For teeth with apical periodontitis, surgical endodontic treatment may be indicated when non-surgical retreatment is impractical [4, 5, 7, 8]. The main goal of the surgical endodontic treatment is to prevent the invasion of bacteria and their by-products from the root canal system into the periradicular tissues by adequate root-end management and filling [4, 5, 8, 9]. Several root-end filling materials have been used in modern endodontic surgery, such as mineral trioxide aggregate (MTA) [10–13], intermediate restorative material (IRM) [14–17], and recently also Biodentine [18–20]. A very high success rate was reported for modern surgical endodontic treatments [4, 5]. However, in some cases, failure may occur as a result of insufficient retrograde seal, followed by bacterial penetration and colonization [4, 5]. The exact mode of

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bacterial colonization in the apically resected and filled root-ends is not fully elucidated.

The most common experimental model that was traditionally used to evaluate bacterial penetration through root canals that have been apically resected, prepared, and filled has been the two-chamber leakage model [21–23]. This model presumably evaluates the penetration of bacteria from the upper chamber through the retrograde filling into the suspension located in the lower chamber, evident mainly by the appearance of turbidity in the suspension. However, inherent problems with this in-direct model include the basic assumption that the leakage occurs only through the root canal space and not through additional potential routes, as well as the lack of appropriate negative controls [22, 23]. In addition, in most of these leakage studies, the routes of microbial leakage were not traced histologically, and thus it was difficult to quantify the bacterial colonization or to evaluate its colonization routes within the root canal space [22–24]. Therefore, both the reliability of these leakage models and their capability to provide a comprehensive evaluation of the root canal bacterial colonization is questionable [22, 23]. Thus, alternative microscopic techniques that are able to directly evaluate the bacterial colonization in the apical part of the resected and filled root canal are warranted.

Confocal laser scanning microscopy (CLSM) has the advantage of providing a direct and quantitative information about the presence and distribution of bacteria inside dentinal tubules and in the total circumference of the root canal walls [25–27]. A direct evaluation of the bacterial colonization in the apically treated and filled part of the root canal using CLSM could potentially overcome the inherent limitations of the traditional leakage models, thus providing more reliable and clinically relevant data.

The aim of this study was to evaluate *Enterococcus faecalis* colonization at the apical part of root canals in extracted human teeth following root-end resection and filling by different root-end filling materials, using confocal laser scanning microscopy.

Materials and methods

Teeth selection, preparation, and allocation to groups

Fifty-five freshly extracted single rooted human teeth were stored in 0.05% sodium hypochlorite solution. Only fully developed teeth with one root canal with curvature not exceeding 5° were included [28]. Teeth with long oval canals (when the ratio of long to short canal diameter was >2, [6]), teeth with no apical patency, teeth with an apical diameter of more than k-file #25, teeth with more than one root canal, teeth that were previously endodontically treated, teeth with incomplete root development, or teeth with root resorption were excluded.

The crowns of the selected teeth were removed in order to obtain root specimens of 13 mm length, and the working length was confirmed using a standard #10 k-file protruding from the apical foramen. The root canals were prepared to apical size #30 with stainless steel hand files (Dentsply Maillefer, Tulsa, OK, USA) using a "balanced force technique" [29]. During instrumentation, copious irrigations were performed using 5% sodium hypochlorite solution. At the completion of the instrumentation, a final flush of 17% EDTA followed by 5% sodium hypochlorite solution was used to remove the smear layer [30].

The apical 3-mm root ends were resected without bevel using Zakaria high-speed bur (Maillefer, Ballaigues, Switzerland). Retrograde cavities were prepared to a depth of 3 mm using diamond-coated ultrasonic tips (Satelec, Paris, France) [5, 8]. The canal retrograde cavities were dried using paper points, and the specimens were randomly divided into eight groups as follows:

Group 1 (N = 10): The 3-mm retrograde cavities were filled with MTA (ProRoot; Dentsply Tulsa Dental, Johnson City, TN, USA) mixed according to the manufacturer's instructions [31–33].
Group 2 (N = 10): The 3-mm retrograde cavities were filled with IRM (Dentsply, Germany) mixed according to the manufacturer's instructions [8, 9].
Group 3 (N = 10): The 3-mm retrograde cavities were filled using Biodentine (Septodont, France) mixed according to the manufacturer's instructions.
Group 4 (N = 5) (positive control): The prepared roots were left without retrograde filling.
Group 5 (N = 5) (negative control): The teeth were left without retrograde preparation and filling, and the entire root surface including the apical portion was covered with two layers of nail varnish [34].
Group 6 (N = 5) (negative control MTA): same as group 1 but without following bacterial contamination
Group 7 (N = 5) (negative control IRM): same as group 2 but without following bacterial contamination
Group 8 (N = 5) (negative control Biodentine): same as group 3 but without following bacterial contamination

The retrograde materials were allowed to set for 24 h at 37 °C and 100% humidity.

The experimental model

Two coats of nail varnish [34] were applied to the surfaces of all teeth excluding the resected apical portion in order to prevent bacterial leakage through lateral canals or other discontinuities in the cementum [35]. All roots were mounted using a model as described previously [36]. In brief, all roots were inserted in Eppendorf plastic tubes of 1.5 mL volume

(20-mL disposable scintillation vials—Sigma-Aldrich Co., St. Louis, MO) and then inserted into a glass vial (Sigma-Aldrich Co., St. Louis, MO, USA) through the opening of the rubber cap, so it fitted tightly inside the glass vial. The junctions between the root, the Eppendorf, and the rubber cap were sealed with cyanoacrylate adhesive (Krazy Glue, Columbus, OH, USA).

The system was then sterilized overnight using ethylene oxide gas [24] and then placed in a 9-mL sterile glass flask containing 4 mL of Brain Heart Infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA), so that approximately 2 mm of the root apex was immersed in the broth [37].

Bacterial contamination of the model

A growth medium for streptomycin-resistant T2 strain, *E. faecalis* bacteria (EF) (ATCC® 29212™), was prepared by mixing 18.5 g of BHI with 500 mL of distilled water. The suspension was autoclaved. In order to prevent contamination by additional bacterial species, 0.5 mg/mL streptomycin sulfate (Sigma-Aldrich Co., St. Louis, MO, USA.) was added. EF is resistant to 0.5 mg/mL streptomycin sulfate [38].

Each specimen was filled from the coronal part of the root canal with the freshly prepared bacterial suspension and incubated at 37 °C and 100% humidity. The bacterial suspension was replaced with a fresh preparation every 24 h, and the total incubation period was 21 days.

Preparation of samples for evaluation

After 21 days of incubation, the presence of turbidity in the BHI broth of each sample was recorded [39]. The specimens were embedded in a self-cure acrylic repair material (UNIFAST Trad, GC America), and the apical 3-mm segment of each specimen containing the root-end preparation and filling was cut perpendicular to the long axis of the root under water cooling with a diamond saw rotating at 500 rpm (Isomet, Buehler Ltd., Lake Bluff, IL, USA). This cut resulted in two slabs of the root, coronal, and apical, of 1 mm thickness each [40].

The samples were stained using LIVE/DEAD BacLight Bacterial Viability Kit L-7012 (Molecular Probes, Eugene, OR, USA), containing separate vials of the two component dyes (SYTO 9 and propidium iodide in 1:1 mixture) for staining of the biofilm. The excitation/emission maxima for these dyes were 480–500 nm for the SYTO 9 stain and 490–635 nm for propidium iodide [41].

Confocal microscopy evaluation

Immediately after the staining procedure, fluorescence from the stained bacteria was observed under a confocal laser scanning microscope (CLSM) (Leica TCS SP5, Leica Microsystems CMS GmbH, Germany). Single-channel imaging and simultaneous dual-channel imaging were used to display green and red fluorescence [25–27].

The CLSM images of the bacterial biofilm were acquired at a resolution of 1024 × 1024 pixels and analyzed by the LAS AF software (version 2.6.0.7266; Leica Microsystems CMS GmbH). The specimens were observed using a ×4 lens. The mesial, distal, buccal, and lingual areas of the specimens were evaluated by the software as follows:

1. The size of fluorescent staining within the evaluated areas, as calculated by the software
2. The viability of the colonized bacteria evaluated as the proportion of live and dead bacteria: the values of green fluorescence (live cells) and red fluorescence (dead cells)
3. The depth of bacterial colonization into the dentinal tubules was measured and recorded considering the canal wall as the starting point [40].

Statistical evaluation

The results were evaluated statistically as follows: *t* test was used to compare the proportion of live and dead bacteria with different retrograde filling materials and to compare the stained areas at the buccal/lingual/mesial/distal areas. One-way ANOVA was used to evaluate the size of fluorescent staining within the evaluated areas and the depth of bacterial colonization into the dentinal tubules with the various retrograde filling materials, as well as to compare the sizes of stained areas of the coronal and apical slabs. $p < 0.05$ was considered as statistically significant.

Results

No fluorescence and no turbidity were observed in all negative control groups, and fluorescence and turbidity were found in all the specimens of the positive control group.

The stained areas were significantly larger in the buccal and lingual directions compared to the mesial and distal directions, in all groups ($p < 0.05$) (Fig. 1).

When comparing the different retrograde filling materials (Fig. 2), there were no significant differences in the sizes of fluorescent staining within the evaluated areas (dead and live bacteria combined) ($p > 0.05$). However, there were significantly more dead bacteria than live bacteria in the IRM and Biodentine groups, and there were significantly more live bacteria than dead bacteria in the MTA group ($p < 0.05$).

The minimal and maximal colonization depths into the dentinal tubules were 210 and 1643 μm, respectively, with a mean of 755 μm. No significant differences were found regarding the depth of bacterial colonization into the dentinal

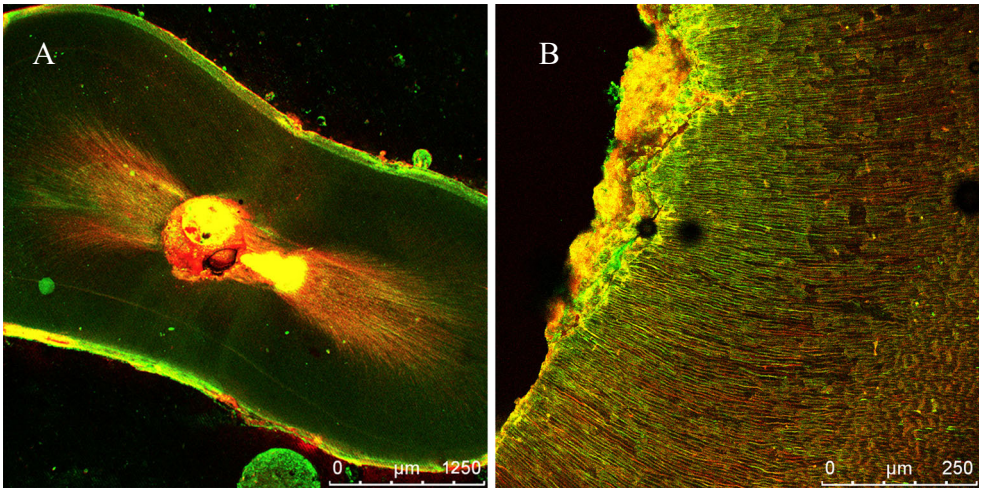

Fig. 1 Confocal laser scanning microscopy (CLSM) images of the bacterial colonization of the dentin. The infected dentin was stained with LIVE/DEAD BacLight Bacterial Viability Kit and analyzed by the LAS AF software. **a**, **b** Two different microscope fields in which vital (*green*) and dead (*red*) bacteria inside the dentinal tubules are clearly visible. The magnification of some tubules (**b**) shows the presence a coccoidal structure in numerous branches in the radicular dentin, a butterfly-like appearance seen on the root cross sections (**a**) that occurs as a result of increased sclerosis along the tubules located on the mesial and distal sides of the canal lumen

tubules between the evaluated materials (MTA, IRM, Biodentine) ($p > 0.05$). Table 1 presents the depths of bacterial colonization into the dentinal tubules for the different groups.

There were no significant differences between the coronal and apical slabs in any of the evaluations ($p > 0.05$).

Discussion

It had been argued that the main goal of root-end management during surgical endodontic treatments is to prevent the invasion of bacteria and their by-products from the root canal

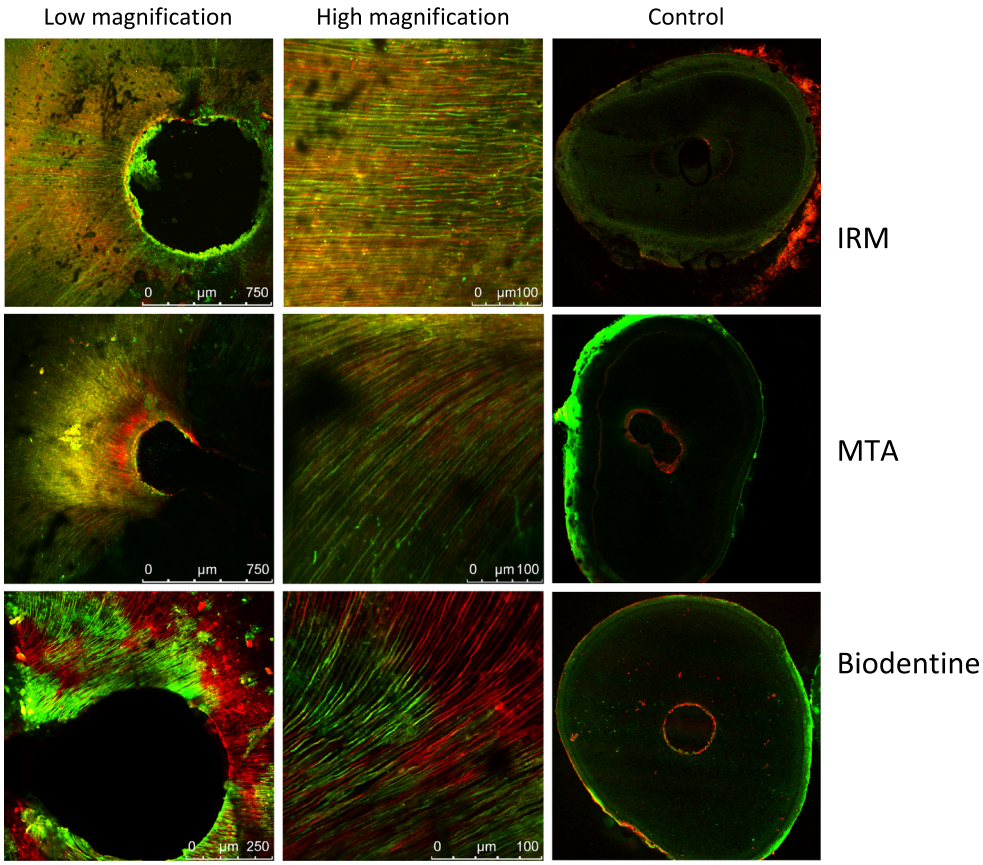

Fig. 2 Confocal laser scanning microscopy (CLSM) images of experimental and control specimens after 21 days. Only the dentinal tubules of the experimental groups are infected

Table 1 Depth of bacterial colonization into the dentinal tubules (μm)

	Number	Mean	Std. deviation	Std. error	95% Confidence interval for mean		Minimum	Maximum
					Lower bound	Upper bound		
Biodentine	7	625.35	295.01	111.50	352.51	898.19	210.50	911.18
IRM	8	673.53	308.00	108.90	416.04	931.03	456.00	1355.00
MTA	7	976.39	352.18	133.11	650.68	1302.11	591.00	1643.50
Total	22	754.56	341.17	72.74	603.30	905.83	210.50	1643.50

IRM intermediate restorative material, *MTA* mineral trioxide aggregate

system into the periradicular tissues [4, 5, 8, 9]. While the penetration of live bacteria into periapical tissues following the surgery may be important in some cases (e.g., extraradicular infections or periapical abscesses [42, 43]), the main clinically relevant concern is that of bacterial colonization and infection of the dentin and the filling-dentine interfaces following the surgery [44, 45]. Bacterial colonization of the root canal may cause an inflammatory reaction when bacterial by-products such as endotoxins or exotoxins gain access to the periradicular tissues [46, 47].

Bacterial colonization of dentin is an active process mediated by cell division and availability of nutrients. Following bacterial colonization of the dentin, the dentinal tubules may eventually become a safe haven for bacteria [48]. Gram-positive and facultative anaerobes are the most frequently isolated bacteria from root canal-treated teeth with persistent intra-radicular infections. Among them, *E. faecalis* is prevalent [49]. In the root canal environment, *E. faecalis* bacteria play an important role in bacterial biofilm formation. Therefore, *E. faecalis* biofilms are considered as an appropriate model for evaluating root canal bacterial colonization [50–54]. *E. faecalis* is a non-motile, facultative anaerobic bacterium [55], known to be highly recalcitrant due to its ability to withstand alkaline conditions and glucose starvation. Thus, it is prone to cause persistent infections [56, 57].

Peters et al. [58] argued that bacteria in the dentinal tubules are entombed beneath the root canal filling and will eventually die. However, microbiological and histological studies demonstrated the growth of isolated islands of biofilms between an existing root canal filling and dentin walls [59, 60], and into the dentinal tubules [27, 48].

An ideal root-end filling material should prevent bacterial colonization and ensuing leakage of bacterial by-products into the periradicular tissues [44]. Understanding of the pathological process following endodontic surgery requires an experimental model, which should enable not only to assess the ability of the root-end filling to prevent bacterial migration through the filled root end but also to track and quantify the microbial colonization within the root canal space, at the filling-dentine interfaces and in the dentinal tubules [22–24].

A dentin infection model has particular significance in studying apical periodontitis [61]. Microbiological, histological, and microscopic techniques have been used to study the presence of bacteria inside root canals and within the dentinal tubules [62]. Previous *ex vivo* studies attempted to evaluate leakage in the presence of root-end filling using different models such as the dye penetration model. They reported that the leakage pattern may be related to various factors such as the root-end resection angle and the exposure of dentinal tubules [63–65]. However, these studies were limited since they were using indirect models, incapable of evaluating the actual routes of bacterial penetration and colonization. The traditional two-chamber model of bacterial leakage also suffers from significant inherent shortcomings such as uncertainty as to the real routes of the bacterial colonization in the experimental groups, and the absence of proper histological controls [66]. Unlike the dye models or the two-chamber model of bacterial leakage, in the current study, the actual routes of microbial colonization were traced histologically, and positive and negative histological controls were used to confirm the adequacy of the experimental model. No fluorescence or turbidity was observed in the negative control group, while fluorescence and turbidity were found in all specimens of the positive control group, thus ensuring the adequacy of the experimental model.

Several microscopic techniques have been used to evaluate the bacterial colonization of dentin, including stereomicroscopy [67], scanning electron microscopy (SEM) [68, 69], transmission electron microscopy (TEM) [70], and confocal laser scanning microscopy (CLSM) [25–27]. The use of the CLSM technique, which has been described and applied in previous studies [25–27, 40, 71], is considered as useful as the traditional microbiological histological standard electron microscopy, and as PCR-based techniques for the identification of viable bacteria in dentinal tubules [27]. Furthermore, the use of CLSM, along with the live/dead staining method, provides information about both the extent of the dentin infection and the vitality of bacteria in the infected dentinal tubules in situ [25–27]. To our knowledge, this is the first study that evaluated the bacterial colonization in the apical part of extracted human teeth following root-end resection and filling, using CLSM.

In the present study, we found that the bacterial colonization was more extensive in the bucco-lingual direction compared to the mesio-distal direction. This finding is in concordance with a previous study [72]. The reason for it may be related to a phenomenon called the "butterfly effect," a butterfly-like appearance seen on root cross sections that results from increased sclerosis along the tubules located on the mesial and distal sides of the canal lumen. This effect is common in the single-rooted teeth of humans in a wide range of ages [73, 74].

No significant differences in the bacterial colonization area were found between the coronal and apical slabs ($p > 0.05$). The distance between the two sections was approximately 1 mm, which may explain the lack of difference. Further studies are needed in order to evaluate the bacterial colonization at different levels of the root canal.

The depth of bacterial penetration into the dentinal tubules was not affected by the type of root-end filling (MTA, IRM, or Biodentine). The maximal depth was 1643 μm and the mean was 755 μm. Peters et al. [69] evaluated the depth of penetration of bacteria into the root dentin of teeth with periapical lesions and reported that in more than half of the infected roots, bacteria were present in the deep dentin close to the cementum. They attributed their results to the fact that anaerobic culturing of dentin is a more sensitive method to detect these bacteria than histology. In that context, CLSM seems to be a favorable technique to evaluate bacterial colonization in the dentinal tubules since it allows to assess both viable and dead bacteria; thus, it is capable of assessing the true extent of the bacterial penetration into the dentinal tubules.

In the present study, the viability of the colonized bacteria was affected by the type of root canal filling material: more live bacteria were found in the MTA group in comparison to the IRM and Biodentine groups. In another study comparing IRM and MTA, it was shown that MTA had an antibacterial effect on some of the facultative anaerobic bacteria and no effect on any of the strict anaerobic bacteria, while IRM had antibacterial effects on both types of the tested bacteria [75].

The antibacterial properties of retrograde filling materials were previously assessed [76, 77]. Slutzky et al. [76] have shown that IRM was antibacterial against *E. faecalis* immediately after setting and sustained this ability for at least 1 day. Chong et al. in 1994 [77] demonstrated the same effects in retrograde fillings. According to its manufacturer, Biodentine holds antibacterial properties due to alkalization of the environment, and also due to its high pH that exerts a clear inhibitory effect on microorganisms. In addition, the alkaline change leads to the disinfection of adjacent hard and soft tissue structures [18–20, 78–80].

Conclusions

Under the limitations of an *ex vivo* model, the current study demonstrated that following root-end filling, bacteria may colonize within the root canal space at the filling-dentine interfaces and penetrate deep into the dentinal tubules. This colonization is not homogenous, favoring the bucco-lingual aspect of the root. The viability of the colonized bacteria may be affected by the type of root-end filling material.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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