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Potential systematic error in laboratory experiments on microbial leakage through filled root canals: an experimental study

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Abstract

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Aim To assess the routes of bacterial leakage in a commonly used two-chamber model designed to evaluate root fillings.

Methodology Fifty-one intact human mandibular premolars with fully developed roots were used. They were left completely intact ($n = 23$), or were accessed, instrumented and either left open ($n = 5$) or root filled with gutta-percha and AH Plus ($n = 23$). All teeth were sealed between two chambers using sticky wax. The apical root aspects were left uncovered. The upper chamber was seeded with *Enterococcus faecalis*. An enterococci-selective broth was used in the lower chamber. Leakage was assessed for 120 days and compared using survival statistics ($\alpha < 0.05$). Subsequently, roots were trans-sected, stained using a 'live' DNA stain (Syto59) and inspected using confocal laser scanning microscopy. An *E. faecalis*-specific RNA probe was used for fluorescence *in situ* hybridization (FISH).

Results Leakage started to occur from day 56, with further occurrence essentially identical between root filled teeth and intact counterparts ($P = 0.71$). All the trans-sections showed fluorescence related to Syto59 between the cementum layer and the sticky wax. Fluorescence was also observed between the root filling and the tubular dentine, whilst it was absent at the interface between root filling and sclerotic dentine. Secondary dentinal tubules, i.e. lateral branches connecting the main counterparts, contained fluorescent material. FISH revealed that Syto59 exclusively stained *E. faecalis*.

Conclusions The current experimental method proved to be unsuitable to compare root fillings. Histology revealed interesting observations regarding the relationship of dentine structure and bacterial leakage, which warrant further investigation.

Keywords: bacteria, dentine, leakage, root canal.

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Introduction

A recent systematic survey of the literature has raised questions regarding the validity of observations made on microbial leakage through filled root canals assessed in a two-chamber model (Rechenberg *et al.* 2011). It was found that in none of the published studies on microbial leakage through root canals filled with a permanent filling material, appropriate negative controls were explicitly installed to exclude leakage through routes other than the filled root canal. Moreover, the commonly observed early microbial leakage in two-chamber models contrasts with histological observations made in teeth that had been properly root filled using gutta-percha and a sealer and were then exposed to the oral microbiota *in situ*

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without a coronal filling for extended time periods (Ricucci & Bergenholtz 2003, Ricucci *et al.* 2009). The reason for this discrepancy remains unknown. In view of the systematic lack of sufficient and appropriate negative controls, a possible explanation is that in the laboratory set-up, microorganisms penetrate not through the filled root canal but rather through other routes, such as potential gaps between the sealing material used to separate the chambers and the outside of the root.

The goal of the current investigation was to trace the routes of bacterial leakage through human root filled teeth in a commonly used two-chamber model, designed to evaluate root fillings. First, the hypothesis that leakage occurs no later through the seal separating the chambers than through the root canal filled with gutta-percha and sealer was tested. Subsequently, the routes of leakage in the root filled specimens were traced histologically using molecular techniques.

Materials and methods

Preparation of teeth

Fifty-one human intact mandibular premolars with fully developed roots extracted for periodontal reasons were selected from the department's collection of teeth. To make sure that each premolar had one single root canal, teeth were radiographed in two planes (Digora; Soredex, Tuusula, Finland) and inspected for cracks and the absence of caries using a dissecting stereomicroscope (Leica Wild M3Z; Wild, Heerbrugg, Switzerland). The periodontal ligament was removed carefully using a periodontal curette. Teeth were immersed in 2.5% NaOCl for 10 min in an ultrasonic bath to remove any periodontal ligament remnants. Subsequently, the teeth were numbered consecutively and randomly assigned with the help of a computer algorithm to an experimental group ($n = 23$), a negative ($n = 23$) and a positive control group ($n = 5$). Whilst the teeth in the negative control group were left untouched, the pulp chambers of the remaining 28 premolars were accessed using a diamond-coated bur. Canals were instrumented in a crown-down manner to two-thirds of the expected working length using ProFile (Dentsply Maillefer, Ballaigues, Switzerland) size 60, 0.04 taper to size 45, 0.04 taper rotary instruments at 250 rpm in a torque-controlled motor (Tecnika; ATR, Pistoia, Italy). A size 10 K-File (Dentsply Maillefer) was inserted into the root canal until the tip was just visible beyond the apex. Working length was determined by subtracting 1 mm from this length. Root canals were then prepared to ProFile size 45, 0.04 taper at working length. Apical patency was maintained by inserting a size 10 K-file 1 mm beyond the apical constriction. Canals were irrigated with 1 mL of a 1% NaOCl after each instrument. After instrumentation, canals were irrigated with 5 mL of 17% EDTA followed by 5 mL of sterile saline. Subsequently, the crowns were cut horizontally using a diamond disc under water-cooling so that all specimens had a uniform canal length of 19 mm.

Root filling procedure

Root canals from the experimental group were dried with sterile paper points and filled by an endodontic resident (DKR) using the continuous wave of condensation technique (Buchanan 1994). In brief, heat (System B; SybronEndo, Orange, CA, USA) and Schilder pluggers (Dentsply Maillefer) that could be inserted to 16 mm were selected. Gutta-percha master cones (Autofit GT.04; SybronEndo, Glendora, CA, USA) were then adjusted at full working length so that they fitted snugly. The sealer (AH Plus; Dentsply DeTrey, Konstanz, Germany) was applied using the master cone. After down-packing the master cones at 200 °C using a heat plugger and hand condensation, a three-incremental back-filling procedure was performed using a heated gutta-percha injection apparatus (Obtura II; Obtura Spartan, Fenton, MO, USA). Each back-filling procedure was followed by vertical compaction until the full 19 mm was filled. Positive controls were left unfilled. All groups were mounted in the test set-up.

Test set-up

An experimental system similar to a published set-up (Barthel *et al.* 2000) was used (Fig. 1). Five-millilitre pipetter tips (Quality Range; Synmedic AG, Zürich, Switzerland) were cut off at the narrow end individually for each specimen so that the rim fitted 1 mm apically from the root equator. Subsequently, the root was sealed in the pipetter tip using sticky wax (Belladi brown, Art. No. 3702; Rolf Ammann Zahnärztliche Produkte, Altnau, Switzerland), so that the most apical 3 mm remained free of the sealant. Round holes with a diameter of 9 mm were punched into the lids of 10-mL glass vials (Faust Laborbedarf, Schaffhausen, Switzerland). Pipetter tips with inserted teeth were snugly fitted into the holes of the lids, so that the tooth root extended into the vial. Pipetter tips were sealed to the

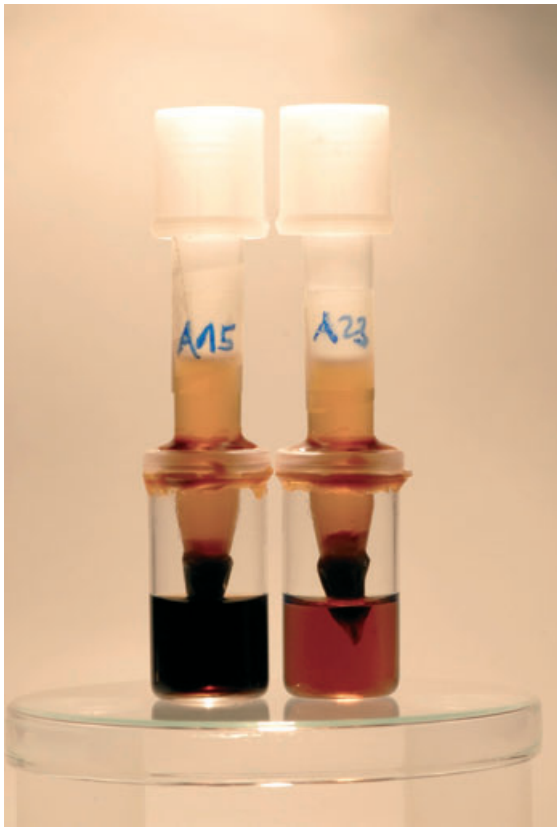

Figure 1 Two-chamber model used in the current study. A nonspecific nutrient broth was filled into the upper chamber, an enterococci-selective broth into the lower chamber. Note the blackening of the broth in the lower chamber of the specimen to the left, indicating bacterial leakage. The chambers were separated using sticky wax. According to Rechenberg *et al.* (2011), the apical 3 mm of the root was left uncovered in test (instrumented and root filled teeth) and control specimens (intact teeth), so that leakage through the sticky wax cuff could be controlled.

lids using sticky wax. The lid–pipetter tip–tooth units were sterilized in ethylene oxide and then aerated for 2 days in a sterile environment. All the following steps were performed under sterile conditions in a microbiological safety cabin (SFE 120EN; Skan AG, Allschwil, Switzerland). After autoclaving, the glass vials were filled with 7 mL of sterile enterococci-selective bile esculin azide broth (Enterococcosel Broth; Difco, Benton Dickinson Co., Sparks, MD, USA). The lids containing the pipetter tips and teeth were reattached to the glass vials. Each pipetter tip was filled with 5 mL of an overnight culture of *Enterococcus faecalis* ATCC 29212 in fluid universal broth (FUM, Gmür & Guggenheim 1983), which had previously been adjusted to an optical density of 1.0 at 550 nm by dilution with FUM. The opening of the pipetter tips was covered with sterile plastic caps to impede evaporation and prevent contamination of the FUM. The whole set-up was moved to an incubator in ambient air at 37 °C. The medium (4.9 mL of the 5 mL) was exchanged with fresh sterile FUM under aseptic conditions every 2 weeks to ensure viability of the cells. Purity of growth in the upper chamber was checked at these times by cultivation on Columbia blood agar (Oxoid, Basingstoke, UK) and subsequent observation of colony morphology and cellular characteristics using a phase-contrast microscope (Leitz Dialux 22; Leica, Basel, Switzerland) at 1000× magnification.

Leakage was assessed by two investigators by checking whether the bile esculin azide broth in the lower chamber turned black (Fig. 1). Blackening is related to hydrolysis of the esculin, which is a sign of enterococcal metabolism (Facklam 1973). Purity of growth in the lower chamber was checked as described previously.

Staining of trans-sections

After 120 days, all roots from the experimental group were gently removed from the test set-up and prepared for confocal laser scanning microscopy (CLSM). Specimens including the wax seal were embedded in epoxy resin (Stycast 1266; Emerson & Cuming, Westerlo, Belgium) and sectioned (Isomet low speed saw; Buehler, Lake Bluff, IL, USA) under water-cooling. Sections were obtained from the most apical third of the sticky wax-covered root aspect, perpendicular to the long axis of the root, at a thickness of 0.6 mm. Trans-sections were DNA-stained by incubation at 15 °C in the dark for 60 min with a 0.1% solution of a DNA stain (Syto59; Invitrogen AG, Basel, Switzerland) in physiological saline.

After staining, the trans-sections were washed in 0.9% NaCl and embedded in 50 μL of Mowiol (Calbiochem-Novabiochem Corp., San Diego, CA, USA), which was prepared as described elsewhere (Thurnheer *et al.* 2006). Specimens were thus connected to borosilicate chamber slides (Lab-Tek; Nunc GmbH & Co KG, Langenselbold, Germany) and stored at room temperature in the dark for at least 6 h prior to CLSM analysis.

Fluorescence *in situ* hybridization (FISH)

To confirm that the stained cells were *E. faecalis*, random trans-sections were additionally stained with

FISH. A custom-synthesized *E. faecalis*-specific 16S rRNA probe (Efae470, GATACCGTCAGGGGACGTTC) labelled at the 5′-end with Cy3 was purchased from Microsynth (Balgach, Switzerland). The probe was used at a final concentration of 5 ng mL^{-1} in the presence of 30% formamide in the hybridization buffer. Prior to hybridization, sections were covered for 60 min at 37 °C with protectRNA RNase inhibitor (Sigma-Aldrich, diluted 1 : 500 in 0.9% NaCl; Gmür & Lüthi-Schaller 2007). Trans-sections were prepared for FISH as described (Thurnheer *et al.* 2004). In brief, trans-sections were treated with lysozyme (5 min) followed by pre-hybridization (15 min, 46 °C) and hybridization (120 min, 46 °C). After washing, trans-sections were embedded in 50 μL of Mowiol (Calbiochem-Novabiochem Corp.) and stored at room temperature in the dark for at least 6 h prior to CLSM analysis.

Confocal laser scanning microscopy

The presence of viable cells was inspected by a DMI6000B inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a He-Ne laser, an Ar laser, and a TCS SP5 computer-operated confocal laser scanning system. Filters were set to 540–580 nm for Cy3 and 620–700 nm for Syto59, and confocal images were obtained using a 63× oil immersion objective (numeric aperture 1.4). Image acquisition was performed in 8-line average mode. Overviews were merged from multiple images using the tile scan mode of the microscope software (LAS AF 2.1.0; Leica). Data were further processed using Imaris™ 6.4.0 software (Bitplane AG, Zürich, Switzerland). Syto59-stained areas were depicted in blue. Trans-sections were also inspected macroscopically at appropriate magnification (MZ 16A. Leica); images were captured with a DSLR (DFC420 C; Leica).

Statistical analysis

The duration until a specimen leaked (in days) was recorded as event time. If a specimen did not leak for 120 days, the event time of 120 days was entered into the computer program as a censored variable. Kaplan–Meier survival curves were constructed, and event times between specimens with a root filling versus counterparts with an intact crown were compared using the log-rank test. The alpha-type error was set at 0.05.

Results

The incidence of growth of *E. faecalis* in the lower chamber of the leakage set-up was essentially identical between root filled teeth and counterparts with an intact crown (Fig. 2, $P = 0.71$). The test set-up prevented leakage for 61 days and the control counterpart for 56 days. Positive controls with instrumented unfilled root canals all leaked within 3 days. All the control samples from upper and lower chambers showed pure growth of *E. faecalis* throughout the experiment.

When root filled teeth were embedded in epoxy resin and trans-sectioned, it was noted macroscopically that there was blackened medium (indicating either the presence of viable *E. faecalis* cells or leakage of blackened enterococci-selective broth from the lower chamber) between the cementum layer and the sticky wax in most specimens (Fig. 3). Inspection by CLSM showed that all the teeth had fluorescence related to the DNA stain between the cementum layer and the sticky wax (Fig. 4). Moreover, Syto59-related fluorescence was present in all the root filled specimens between the root filling and the dentine (Fig. 4). This fluorescence was exclusively observed at the root filling to dentine interface and into the dentinal tubules up to 400 μm in a centrifugal direction (Fig. 4). Inter-tubular dentine and the tubules further away from the root canal were

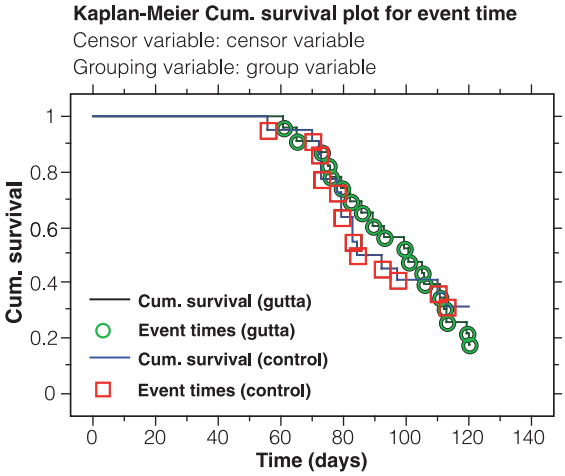

Figure 2 Kaplan–Meier survival curve depicting the occurrence of bacterial leakage (event) between test (Gutta) and control group (intact teeth) over 120 days. Note that leakage occurred almost identically between the two groups, suggesting passage of the enterococci through routes other than the filled root canal.

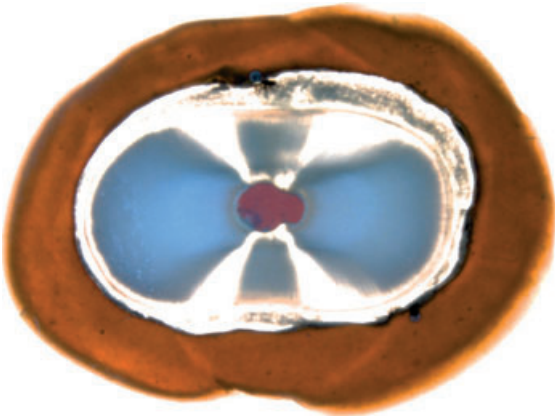

Figure 3 Macroscopic photographic image of a root trans-section that was later assessed under the confocal microscope. This trans-section shows the typical pattern of dentine sclerosis: open tubules are present in the buccal and oral aspects of the root dentine, whilst the inter-proximal aspects are sclerotic. Note the blackened discoloration between the sticky wax cuff and the cementum. The discoloration indicates either the presence of viable *Enterococcus faecalis* cells or leakage of blackened enterococci-selective broth from the lower chamber and therefore demonstrates how loose this interface really was.

not stained with the fluorescent dye in any specimen. Furthermore, it was noted that Syto59-related fluorescence was usually not seen at the root filling to dentine interface in areas with tubular sclerosis (Fig. 5, lower panel). In tubular areas, it was noted that also secondary dentinal tubules, i.e. the lateral branches connecting the main tubules, contained Syto59-stained (fluorescent) material (Fig. 5, upper panels).

Fluorescent *in situ* hybridization with an *E. faecalis*-specific probe in random specimens revealed that the Syto59-stained material was *E. faecalis* (Fig. 6).

Discussion

The current study revealed that sticky wax is not a suitable material to separate chambers if leakage of enterococci through root canals filled with gutta-percha and sealer is compared to a potential control treatment. Furthermore, bacterial leakage in the root canal system occurred mainly through tubular dentine or at the interface between root filling and tubular dentine.

Laboratory tests in endodontics using human teeth theoretically have the advantage over purely artificial set-ups that the clinical situation can be mimicked and parameters of interest can be identified. However, experiments need to be controlled properly to yield meaningful results. It was attempted in the present investigation to control systematic error at multiple levels. In accordance with the suggestion published in a recent systematic review on microbial leakage through filled root canals (Rechenberg *et al.* 2011), the apices of the negative control teeth were not covered with sticky wax, so that in this respect, root filled teeth and control counterparts were similar. This has not explicitly been performed in any previous study on bacterial leakage through root filled teeth (Rechenberg *et al.* 2011). On the contrary, the whole roots of the negative control teeth were covered with the material used to seal teeth between chambers in 57 of 67 published investigations. An enterococci-selective broth was used in the lower chamber of the current set-up to avoid contamination

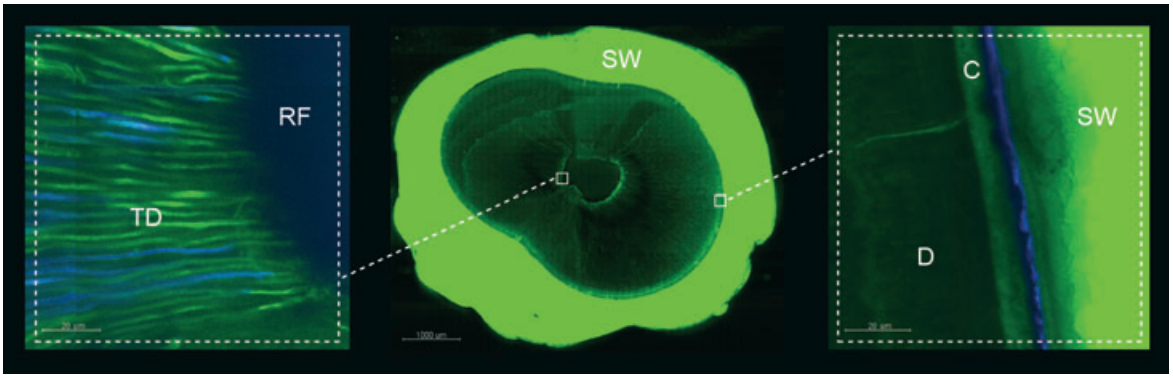

Figure 4 Confocal micrograph depicting a whole root trans-section. The overview micrograph (middle panel) is a maximum intensity projection (MIP) of 12 merged optical sections captured within a 80-μm layer (*z*-axis) inside the trans-section. Close-ups reveal Syto59-related fluorescence in tubular dentine next to the root filling (left), but also between sticky wax and cementum. C, cementum; D, dentine; RF, root filling; SW, sticky wax; TD, tubular dentine.

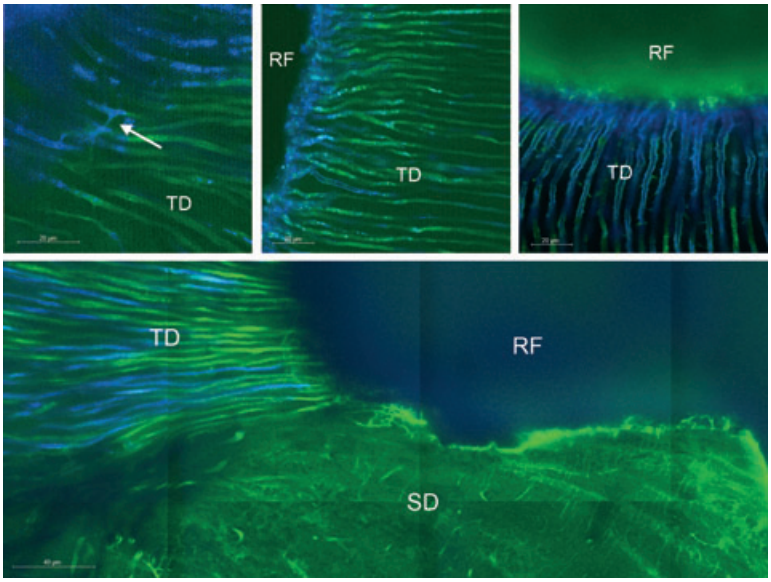

Figure 5 Upper panels: confocal micrographs at higher magnification from different root filled specimens revealing the apparent abundance of fluorescence related to the 'live' DNA stain used in this study (Syto59) in dentinal tubules, including the secondary tubules (lateral branches) that can run in the long axis of the root (arrow). Lower panel: merged optical section overview of a root filled specimen. Note that there is no Syto59-related fluorescence in the sclerotic aspect of the dentine or at the interface between sclerotic dentine and root filling, whilst the tubular dentine is stained in the tubule *lumina*. RF, root filling; SD, sclerotic dentine; TD, tubular dentine.

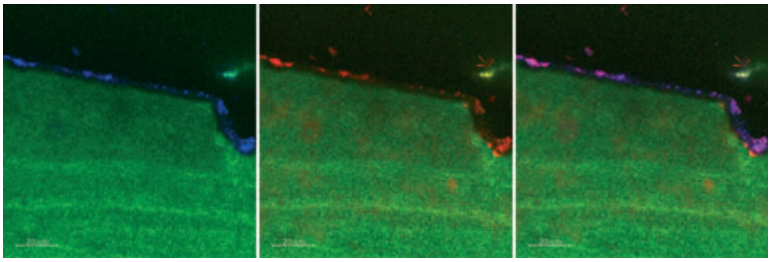

Figure 6 Interface between a root and sticky wax. Total DNA (Syto59) stained in blue, Cy3-labelled FISH probe Efae470 stained in red. Superimposed images on right panel. Note that the purple areas result from Syto59-stained DNA and the specific signal for *Enterococcus faecalis*, revealing that Syto59 exclusively stained *E. faecalis*.

by other taxa. In the upper chamber, a nonselective, nondiscolouring broth was chosen to prevent false-positive results from the leakage of dye molecules rather than bacteria. Purity of growth was checked throughout the experiment. Furthermore, a DNA stain (Syto59) that is actively transported into the cell and thus almost exclusively stains viable cells (Neumann *et al.* 2000) was used to trace bacterial leakage. This prevented false-positive results obtained from the staining of dead bacterial cells. Moreover, root trans-sections rather than longitudinal sections were used to trace the presence of viable bacteria. In a two-chamber model, leakage occurs from the crown towards the apex. If bacteria had passed beyond a specific trans-section, they had to be visible somewhere within this trans-section. In longitudinal sections, there would have been a high likelihood to miss the root aspect that was permeated by the enterococci. The application of CLSM detects fluorescence related to DNA stains up to 100 μm from the surface of a dentine specimen and thus highlights bacterial clusters. Consequently, single bacteria that were 'smeared' on the trans-sectional surfaces during sawing and processing were not an issue. Nevertheless, the current study was still a laboratory experiment, and results cannot be extrapolated to the *in situ* situation. The water column of the broth in the upper chamber exerted a pressure that is not found in the oral cavity. The bacteria under investigation were kept in monoculture in a broth, a situation that differs greatly from the situation *in situ*. As has been pointed out (Ricucci & Bergenholtz 2003), percolation of nutrient broth into the dentine could create a microenvironment in the root canal that may be more favourable to support microbial viability than

the corresponding microenvironment found *in situ*. Furthermore, root canal filling materials show some autofluorescence in CLSM, impairing their accurate assessment. Therefore, the current methodology cannot conclusively clarify all relations between bacterial leakage, root canal filling materials and tooth structures.

There has been some debate over the question whether root fillings are indeed as insufficient as has been believed based on *in vitro* experiments. Whilst most laboratory studies found microbial leakage through filled root canals to occur within weeks (Rechenberg *et al.* 2011), histological observations on unrestored, adequately root filled teeth that were extracted from patients have indicated otherwise (Ricucci & Bergenholtz 2003, Ricucci *et al.* 2009). The reason for this discrepancy, at least under the current laboratory conditions, is most likely related to the inadequate seal between the two chambers in the *in vitro* model. Sticky wax (a combination of bees wax, paraffin and resins) is not a good sealant (Barthel *et al.* 2001). In this experiment, a blackened discoloration between the sticky wax cuff and the cementum was noticed during sectioning the specimens in their apical third. The discoloration indicates either the presence of viable *E. faecalis* cells or leakage of blackened enterococci-selective broth from the lower chamber and most notably demonstrates how loose this interface really was (Fig. 3). Sticky wax was used to separate the two chambers in 23 of the 67 recently reviewed studies on microbial leakage through filled root canals (Rechenberg *et al.* 2011). Expecting sticky wax to be a relatively poor sealant, this material was chosen solely in this investigation, to highlight the possibility of a systematic error in such experiments. Even if a better sealant such as silicone or epoxy resin was used, it still remains questionable whether that could channel microbial leakage exclusively through the root canal system. The reason for this is that the most commonly used root canal sealers are silicone or epoxy resin based. It thus remains questionable why leakage should occur through the much smaller interface between sealing material and dental hard tissue inside the root canal rather than that on the outer root surface. Based on all the methodological pitfalls related to microbial leakage studies, it would appear reasonable to not use such methodology to compare different root filling materials.

The current results also suggest that gutta-percha and AH Plus root fillings are more bacteria-tight than was thought previously, based on ill-controlled tests. However, it must be conceded that even with the current approach, it cannot be known for certain that leakage occurred exclusively through routes outside the canal (bacteria were, after all, also seen in the root dentine of the trans-sections). Nevertheless, it is *very likely* that bacteria transgressed via that route rather than the root canal. First and foremost, leakage in the current system occurred similarly (meaning within a limited time frame) in all specimens, including the teeth that were intact (Fig. 2). The use of survival statistics to evaluate leakage results has been questioned (Wu 2008). Whilst it is true that this approach is of debateable value to compare the sealing ability of two or more root filling materials, it was applied here to visualize the almost identical occurrence of leakage between root filled teeth and intact counterparts. This finding alone suggests that leakage did not occur through the filled root canals, but mainly through other routes. In addition, some intact teeth were also sectioned and processed for CLSM. These showed only bacteria between the outer root surface and sticky wax, but not inside the root (data not shown).

Interestingly and perhaps even more importantly, the current study suggested that leakage of microorganisms through filled root canals, if it does occur, might not predominantly happen at the immediate interface between root filling and canal wall, but rather through tubular aspects of dentine. Observations in clinical cases of teeth that had been root filled but not restored (Ricucci & Bergenholtz 2003, Ricucci *et al.* 2009) are in agreement with the current report. In these teeth, bacteria were found mainly in tubular, i.e. coronal, aspects of the root. It should be noted in this context the coronal aspects of teeth contain more patent tubules than the apical counterparts (Paqué *et al.* 2006). The reason for this is twofold: tubular sclerosis, i.e. the occlusion of dentinal tubules by calcium–phosphate precipitates, is an age-related physiological phenomenon, which occurs in an apical-to-coronal direction (Nalbandian *et al.* 1960, Vasiliadis *et al.* 1983a). Furthermore, the apical root canal walls of adults can be covered by a nontubular fibrodentine layer (Schroeder & Scherle 1988). Sclerotic and fibrotic aspects of root dentine represent a natural impasse for bacterial penetration. Whilst this can be deduced from classic publications on dentine structure (Vasiliadis *et al.* 1983b) and root canal wall infection (Shovelton 1964), the current communication would be the first to explicitly demonstrate the correlation between dentine structure and infection. These findings also suggest why infected root dentine of old individuals contains

less microorganisms than that of young counterparts (Kakoli *et al.* 2009). It was also observed in the present investigation that in teeth affected by root caries that bacteria penetrated not only through primary but also through secondary dentinal tubules. This is in line with observations in human root caries (Schüpbach *et al.* 1990). Secondary tubules are lateral branches connecting the main tubules. In teeth with vital pulps, secondary tubules contain communicating side branches of odontoblastic processes that divert at a 90° angle. Consequently, secondary tubules run along the long axis of the root and may be an avenue of root dentine contamination in root filled teeth. If bacteria really permeate through secondary tubules along the long axis of the root, then the concept of filling root canals may have to be reconsidered. In that case, clinical approaches should emphasize more on sealing of the tubular dentine. This may be especially important in teeth of children and young adults, which contain tubular dentine up to the apex.

The current observations on the relation between dentinal structures and bacterial infection may also have consequences regarding appropriate tests to compare root filling materials. In laboratory model systems, roots were often shortened to a uniform length to standardize the canal length. However, decapitated teeth have exposed dentinal tubules. This could introduce bias. If some teeth are shortened to expose the dentine and others are not, leakage could differ between these specimens. Based on the current observations, it would furthermore appear that bacterial leakage through root canals filled with current materials may simply take too long for two-chamber test setups to be properly controlled. Fluid transport through filled roots (Wu *et al.* 1993) or capillary flow porometry (De Bruyne *et al.* 2005), on the other hand, may also be linked to patent secondary tubules, and may thus be more reliable, quicker and more reproducible tests than a two-chamber model with bacteria as markers of leakage. This, however, needs to be further investigated. Future studies should compare the sealability of different materials used to separate two-chamber leakage models. Furthermore, the bacterial passage through different types of dentine should be investigated and how age-related changes in dentine structure influence this phenomenon (Kakoli *et al.* 2009).

Conclusions

Under the current conditions, bacterial leakage occurred through the sticky wax seal separating the two chambers of a standard laboratory set-up and through the root canal filled with gutta-percha and an epoxy resin sealer. Bacteria permeated tubular aspects of dentine in root filled teeth, whilst sclerotic or atubular dentine and the interface between sealer and dentine devoid of patent tubules remained bacteria-tight. Bacterial leakage through secondary dentinal tubules (i.e. lateral branches connecting the main tubules) was observed. This suggests that bacteria do not necessarily penetrate filled roots at the immediate interface between root filling material and the canal wall, but also deeper in the (tubular) dentine.

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