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Microflora in teeth associated with apical periodontitis: a methodological observational study comparing two protocols and three microscopy techniques

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Abstract

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Aim The aim of this study was to compare two protocols to examine bacterial colonization in teeth associated with chronic apical periodontitis with acute episodes (ap), using light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Methodology Nine root samples (seven teeth) were processed using either Eastman Dental Institute (EDI) (n = 4 teeth/4 roots) or Zurich (n = 3 teeth/5 roots) protocols. The roots were sectioned longitudinally; one root portion was viewed with SEM, descriptively dividing its length into apical, middle and coronal; semi-thin and ultra-thin transverse sections were viewed under LM and TEM from each third of the other root portion. Each root was therefore examined using all microscopy techniques. Observations of bacterial presence, description and distribution within the root canal lumen and root dentine were systematically recorded using pre-determined criteria.

Results The Zurich technique gave a more predictable division of the root, but the surface was slightly smeared and demineralization was incomplete. The Eastman Dental Institute (EDI) approach appeared to provide better ultrastructural detail. Bacteria were detected in eight of the nine roots. Bacterial biofilms were commonly seen adhering to the root canal surface, containing various cellular morphotypes: rods, cocci, filaments and spirochaetes. Bacteria were more evident apically than coronally, associated with the canal wall but were more commonly evident coronally than apically within the dentinal tubules. Polymorphs (PMNs) were found in all the root thirds, especially apically, often numerous and walling off the bacterial biofilm from the remaining canal lumen.

Conclusions Both protocols had merits and de-merits. The combination of microscopy techniques offered complementary views of intra-radicular bacterial colonization. The perception of confinement of the host/microbial interface at the apical foramen is not entirely correct; PMNs may be found even in the coronal third of root canals containing necrotic pulp tissue.

Keywords: intra-radicular bacteria, microscopy, protocols, root canal.

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Introduction

The role of a polymicrobial infection of the root canal system in apical periodontitis is well established (Kakehashi *et al.* 1965, Sundqvist 1976, Fabricius *et al.* 1982, Tani-Ishii *et al.* 1994) but deep insights into the ecology and physiology of the bacterial

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colonization remain elusive. Much of the current knowledge of intra-radicular infection stems from *in vivo* and *ex vivo* culture studies of sampled bacteria; such approaches tend to bias the revealed micro-flora (Akpata 1976, Kumar *et al.* 2002). The picture of bacterial diversity is influenced by many factors, including growth conditions, sub-culture strategy and the nature of bacterial identification (Rolph *et al.* 2001, Kumar *et al.* 2002, Munson *et al.* 2002, Gulabivala 2004). The number of detected and identified taxa per tooth has increased from 1 to 12 cultured varieties up to 20 phylotypes using culture-independent techniques, with estimates of actual numbers up to 90 (Rolph *et al.* 2001, Munson *et al.* 2002). Whilst, the known diversity of the microflora has increased with improved culture techniques and culture-independent techniques, direct microscopy suggests, as indeed it did even in the time of Miller (1894) that a proportion of the flora still remains uncultured. Furthermore, the process of sampling disturbs insights about the intimate and intricate relationships between bacteria and their abiotic environment (Nair 1987).

Microscopically, bacterial strains are evident as cocci, rods, filamentous or spiral morphotypes and have been shown in a landmark paper to exist mainly in a biofilm lining the root canal wall in the root apex (Nair 1987). This paper provided the first real insight into the morphological distribution of the root canal flora in the *root apex* and its association with the host response. Study of the excellent photo-micrographs provides visual evidence to support the predicted ecological and physical spatial relationships between bacteria (Sundqvist 1992).

Different microscopy techniques possess different properties and propensities to reveal the inherent 'truth' about the bacterial distribution and its structure. Light microscopy (LM) remains a useful base-line technique to provide an overall perspective but lacks resolution to reveal finer details. In contrast, transmission electron microscopy (TEM) possesses the high resolution to reveal ultra-structural details, losing something of the perspective as a trade-off. Hence, Nair used the approach he described as correlative LM and electron microscopy studies to decipher both aspects. Although not using the term 'biofilm', he provided the first real detailed description of the root canal biofilm within root apices, as it related to the aetio-pathogenesis of apical periodontitis. The structure of the microflora within the *entire* tooth, as it relates to approaches to treatment has been little studied.

The validity of the observations made by microscopy rests on the assumption that the processing stages have accurately preserved the anatomical structures and that the imaging system possesses the means and resolution to highlight the relevant features. Knowledge of imaging principles is essential but empirical studies are also necessary to reveal the true *in situ* potential of microscopy techniques. Distortion of tissues and translocation of structural components are possible but need to be minimized or else recognized as artefacts. Detection of such artefacts may not be straightforward but is an important element in the critical appraisal of findings. To this end, the nature of sample fixation and processing may also influence results.

The aim of this methodological observational study was to compare different tooth processing protocols and microscopy (LM, SEM and TEM) techniques to examine bacterial colonization within the coronal, middle and apical thirds of roots associated with apical periodontitis.

Materials and methods

Sample collection and storage

The material for this study consisted of extracted human teeth with radiographically evident periapical lesions (and associated acute episodes) and an absence of periodontal disease or previous pulpal therapy. The teeth were carefully extracted by General Dental Practitioners with minimal pumping motion (Kapalas *et al.* 2001, 2002) and immersed into tubes containing 3% glutaraldehyde (Agar Scientific, Stanstead, UK) in 0.1 mol L^{-1} sodium cacodylate (Agar Scientific) after de-coronation with a sterile diamond bur. The sample teeth were stored at 4 °C to provide a total fixation period of 1 week. Informed consent had been obtained from the patients prior to inclusion in the study pool; seven teeth meeting the above criteria were selected for the study.

Processing for microscopy

Two methods of sample processing were used: (i) the EDI protocol (Vrahopoulos 1989), which involved demineralization *after* embedding; and (ii) the Zurich protocol (Nair 1987), which involved demineralization *before* embedding. The seven selected teeth were randomly assigned to the two processing groups; EDI protocol (n = 4 teeth/4 roots with apical periodontitis) and Nair protocol (n = 3 teeth/5 roots/4 roots with

apical periodontitis). An overview of the key stages in the two processing protocols is shown in Fig. 1.

EDI protocol

Longitudinal splitting of the roots

The roots were grooved longitudinally using an ultra-fine diamond disc (Metrodent, Huddersfield, UK) along the narrowest surface of the root in a fume cupboard (Labcaire, Clevedon, UK). The root was then firmly pressed into unset lab putty (Optosil® and Xantopren®; Heraeus Kulzer, Hanau, Germany) and allowed to set. Splitting of the root was completed using an osteotome, exposing the pulp canal space in both sections. The section containing more hard tissue was used for the LM and TEM examination, whilst the other half was used for SEM examination.

Processing for SEM

The root halves allocated for SEM examination were dehydrated in a graded series of alcohol (20%, 50%, 70%, 90% and 3× 100% for 10 min each), placed in hexamethyldisilazane (HMDS) (TAAB Laboratories Ltd, Reading, UK) for 5 min, then removed and left on filter paper for 2–3 h for the HMDS to evaporate. The samples were attached to aluminium SEM stubs (Agar Scientific) using carbon conducting cement (Neubauer Chemikalien, Munster, Germany) and sputter-coated with gold/palladium in a Polaron E5000 Sputter Coater (Quorum Technologies Ltd, Newhaven, UK).

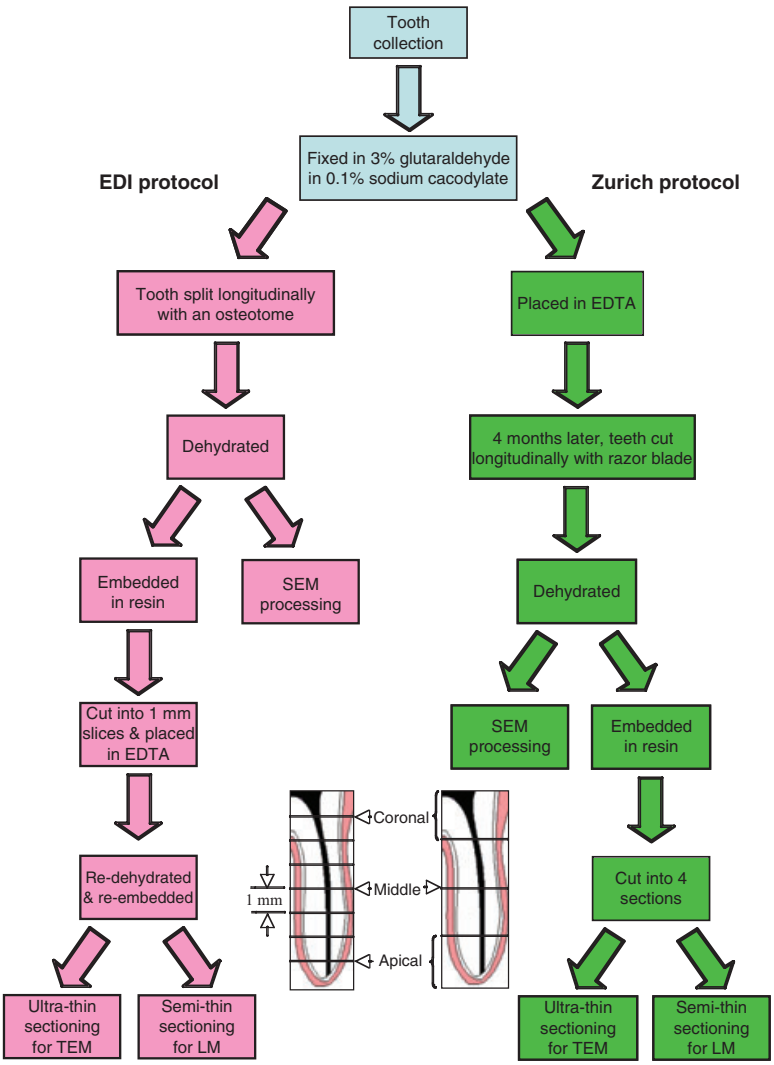

Figure 1 Flow chart showing the succession of stages for each processing protocol.

The specimens were viewed in a Cambridge Stereoscan 90B (LEO Electron Microscopy Ltd, Cambridge, UK) operating at 15 kV and digital images were captured using I-SCAN 2000 software (ISS Group, Manchester, UK).

Processing for LM and TEM

The root halves allocated for LM and TEM were dehydrated in a graded series of alcohol (20%, 50%, 70% and 3× 90% for 10 min each) and infiltrated with LR White resin (The London Resin Company, London, UK). This was performed in stages as follows: initial immersion in LR White resin and 90% alcohol (ratio of 1 : 1) for 2 h at 4 °C; immersion in pure fresh LR White for 30 min at 4 °C; immersion in fresh LR White overnight (10–12 h) at 4 °C and the following morning, for 1 h, at 4 °C. The sections were embedded in tinfoil containers (Buyrite UK Ltd, Aldershot, UK) containing 20 mL of LR White and 30 μL LR White accelerator. Air was excluded from the setting process using parafilm (Agar Scientific) over the exposed resin mix, which was polymerized for 1 h in the freezer, then overnight at 4 °C and then removed to warm up to room temperature.

The embedded roots were sliced transversely using a high-speed diamond saw (Exact, Aberdeen, UK) into 1-mm thick sections. The slices were decalcified in 0.15 mol L^{-1} EDTA in specimen tubes for 3–8 weeks at room temperature on a tissue rotator at 2 rpm (TAAB, Rotator type N; Agar Scientific). The EDTA solution was changed every 2–3 days until the dentine could be easily cut with a single edge carbon steel razor blade (Agar Scientific). The slices were dehydrated again and re-embedded in LR White resin as described above.

Sectioning for LM

Semi-thin sections of 0.5 and 1 μm were cut with a Diatome (Diatome AG, Biel, Switzerland) diamond knife on an ultramicrotome (Reichert UltracutE; Cambridge Instruments, Cambridge, UK). These were stained with toluidine blue and used to check sample orientation before proceeding with LM and TEM. Slides were viewed on an Olympus BX50 optical microscope (Olympus, Southall, UK).

Sectioning for TEM

Ultra-thin sections (90–100 nm) were cut using the same technique, and collected on either carbon-formvar coated copper 200 mesh grids (Agar Scientific) or gold 400 mesh grids (Agar Scientific). The sections were then stained on the grid with 0.4% (w/v) uranyl acetate in absolute alcohol for 5 min, followed by 5 min in Reynold's (1963) lead citrate. Sections were examined on a TEM (100CXII; JEOL, Welwyn Garden City, UK) operating at 80 kV and images were recorded onto Kodak 4 EM film (TAAB Laboratories Ltd).

Zurich protocol

Demineralization

The roots were placed in 0.15 mol L^{-1} EDTA and 0.5% glutaraldehyde (Agar Scientific) in specimen tubes on a tissue rotator at 2 rpm. Initially, the EDTA solution was replaced every 2–3 days over 3 months, and then changed everyday for the remaining 1 month. Progress was checked by carefully inserting a single-edged carbon steel razor blade (Agar Scientific) into the dentine, taking care not to penetrate to the root canal.

Longitudinal cutting of the roots

After approximately 4 months in EDTA, the roots were demineralized sufficiently to allow, gentle, controlled, longitudinal cutting of the roots. At this point, one-half of each root was randomly designated for SEM and the other half for LM and TEM.

The root associated with the periapical lesion was used from each tooth, except for tooth R6, a molar, from which all three roots were used for comparison, although only two were radiographically associated with periapical lesions (R6 a, b – Table 1).

The root halves designated for SEM were dehydrated to 100% ethanol, immersed in HMDS and allowed to dry as for the EDI protocol, handling with greater care because of the demineralization. Those samples due for TEM examination were dehydrated to 90% ethanol and embedded in LR White resin in the same manner as the initial part of the EDI protocol without the necessity for demineralization and re-embedding.

Selection of fields of view for both protocols

Scanning electron microscopy

The entire root half was first examined under low magnification. Then, starting coronally, the root was examined horizontally millimeter by millimeter, using the μbar on the image as a guide. At each millimeter level, the site of examination was magnified to ×5000. This horizontal scanning was repeated at the next adjacent apical level until the entire root canal had been traversed. Observations were made on this basis and representative photographic images were recorded

Table 1 Summary of viewable fields for each protocol (EDI/Zurich) and the presence/absence of bacteria by microscopy technique, tooth, root and segment

Protocol	Root no.	Root portion	Periapical lesion visible on radiograph	SEM		LM		TEM	
				Lumen	Dentinal tubules	Lumen	Dentinal tubules	Lumen	Dentinal tubules
EDI protocol	R1	Coronal	✔	×	×	×	×	×	×
		Middle	✔	×	×	×	×	×	×
		Apical	✔	o	o	×	×	×	×
	R2	Coronal	✔	✔	×	o	o	o	o
		Middle	✔	✔	✔	o	o	o	o
		Apical	✔	o	o	✔	×	✔	×
	R3	Coronal	✔	×	×	o	o	o	o
		Middle	✔	✔	×	✔	×	–	–
		Apical	✔	×	×	o	o	o	o
	R4	Coronal	✔	✔	×	✔	✔	✔	✔
		Middle	✔	✔	×	✔	✔	✔	✔
		Apical	✔	✔	✔	✔	×	✔	×
Zurich protocol	R5	Coronal	✔	✔	✔	✔	✔	✔	✔
		Middle	✔	✔	×	✔	✔	✔	✔
		Apical	✔	✔	×	✔	×	✔	×
	R6A	Coronal	✔	✔	×	✔	✔	✔	✔
		Middle	✔	✔	×	✔	✔	–	–
		Apical	✔	✔	×	✔	×	✔	×
	R6B	Coronal	✔	×	×	✔	✔	✔	✔
		Middle	✔	×	×	✔	✔	–	–
		Apical	✔	✔	×	✔	×	-	–
	R6C	Coronal	×	o	o	✔	✔	✔	✔
		Middle	×	×	×	✔	✔	–	–
		Apical	×	×	×	×	×	×	×
	R7	Coronal	✔	✔	×	✔	✔	✔	✔
		Middle	✔	✔	×	✔	✔	–	–
		Apical	✔	✔	×	✔	✔	✔	✔

✔, bacteria detected; ×, Bacteria not detected; –, insufficient demineralization; o, canal not visible.
R6A, root; from tooth 6; root A.

or when bacterial colonization patterns worthy of note were discerned.

Light microscopy

For the EDI protocol, 1-μm sections were cut from the most coronal, middle and most apical slices of the root. The Zurich protocol involved cutting the whole embedded root into four equal corono-apical portions and then 1-μm thick sections were cut from the coronal, apical sections and from either of the two middle sections (Fig. 1). Stained sections were examined to verify presence of the canal in the section; upon confirmation 5–7 sections of either 0.5 or 1 μm were cut and examined at ×200, ×400 and ×1000 (oil immersion) magnifications. Representative photographs were taken at both low and high magnification for maintaining perspective and obtaining the highest resolution.

Transmission electron microscopy

The LM findings informed the further sectioning for TEM for both protocols. Two sections were cut and examined from the same sites as for the LM sections for each third of the root. The sections were initially examined at the lowest magnification for perspective before zooming in at higher magnifications. Photographic images were recorded at a number of magnifications to illustrate findings.

Comparison between EDI and Zurich protocols

The EDI and Zurich protocols were subjectively compared using the following measures:

1 *Ease of processing*. This was judged by the ability to split or section the root in a controlled manner to view the root canal and its contents, as well as the time taken for complete processing of the roots;

2 *Accuracy of findings*. Note was made of actual or apparent artefacts, distortion or evident bacterial translocation.

Analysis of findings

Observational data were collected as systematically as possible to build a coherent picture of the intraradicular infection, in particular highlighting any common, surprising or unusual findings. An attempt was made to record presence or absence and density of bacteria as objectively as possible to enable comparison. Simple descriptive statistics were used to analyse the findings.

Results

Comparison of processing protocols

The principal difference between the processing for the two protocols was the length of time to progress from unfixed sample to SEM/TEM examination. The Zurich protocol was several weeks longer than the EDI protocol because of longer decalcification times. However, once demineralization was complete, the Zurich protocol allowed more controlled and accurate bisecting of the root, than the less predictable root splitting required for the EDI protocol. Table 1 summarizes the viewable fields for each protocol (EDI/Zurich) and the presence/absence of bacteria by microscopy technique, tooth, root and segment.

Comparison of techniques by SEM

From each root, one-half was prepared for SEM, four from the EDI protocol and five from the Zurich protocol. The tooth structure and root canal contents observed in samples processed using the two protocols were similar (Fig. 2) although it was noted that in some of the Zurich samples the dentine surface had a 'smeared' appearance (Fig. 3). As a result of the more accurate dividing of the root with the Zurich protocol, there were more root portions, 14 of 15, in which the root canal was visible as opposed to 10 of 12 with the EDI protocol. In both of the EDI samples without a visible canal, this occurred in the important apical portion.

Translocation of root canal contents as a result of processing was sometimes observed with both protocols. On the cut (Zurich – Fig. 3) or fractured (EDI – Fig. 6) dentine surface, this could be clearly discerned as superficial cells and debris (Fig. 3) but within the canal this was less easy to identify.

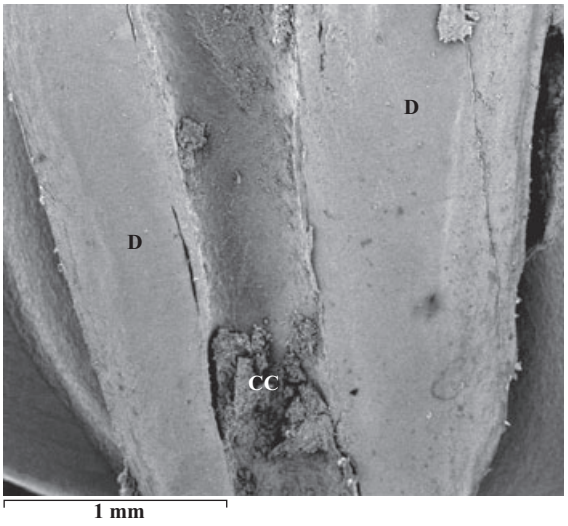

Figure 2 R5 (Zurich protocol) SEM low magnification LS root showing dentine (D), the root canal and cellular material (CC) (μbar represents 1 mm).

Bacterial cell morphology (rods, cocci and filaments) was easily distinguished with SEM (Fig. 4), but only at the sample surface, and the presence of a thick extracellular matrix masked underlying bacteria. It was, however, possible to discern the relative thickness of

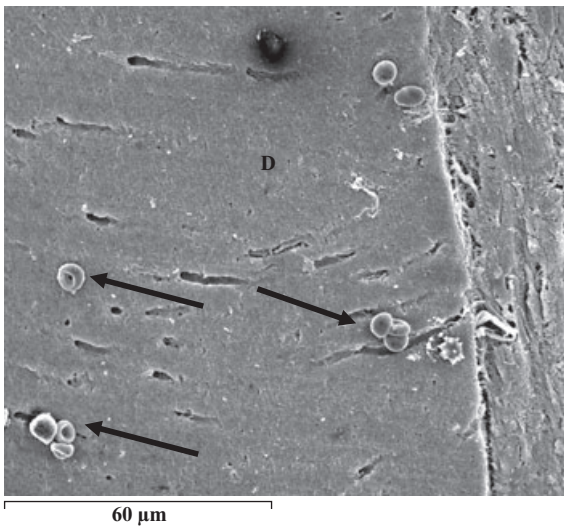

Figure 3 R5 (Zurich protocol) SEM showing the smeared dentine and some translocated RBCs (arrows) (μ bar represents 60 μm).

the bacterial layer in some instances where a fortuitous cut through the thickness of the biofilm revealed the inner topography (Fig. 5). The appearance of the bacterial biofilm within the canal seemed similar for both protocols and the relationship between the bacterial biofilm and the canal anatomy was clear (Fig. 5).

The SEM examination detected bacteria within the canal in seven of nine roots, and 16 of 27 root portions. In only three roots were bacteria observed in the dentine tubules, two from the EDI protocol (Fig. 6) and one from the Zurich protocol, although the slight smearing of the dentine made examination more difficult.

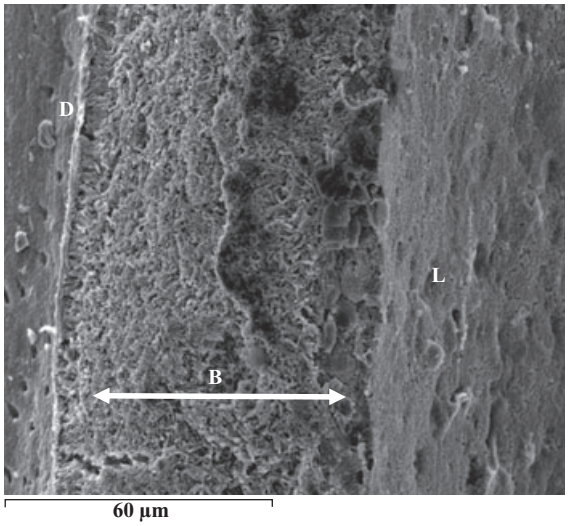

Figure 5 R5 (Zurich protocol) SEM apical section through dentine (D) and biofilm (B) within the canal lumen (L) (µ bar represents 60 µm).

Comparison of techniques by LM

Light microscopy provided the best overall perspective of the root canal, enabling larger areas to be observed at low magnification (Fig. 7). There was little difference between the two protocols in terms of the type of information gained from the samples, providing details of the structure and distribution of bacterial biofilms and cells, and also an indication of the bacterial morphology, although care should always be taken interpreting cross-sections of cells. It was evident from the LM observation of all three portions from root R1 that this was, in fact, a vital pulp.

However, a difference between the protocols was noted, a consequence of the splitting of the roots with the EDI protocol, in which the whole canal was within the SEM portion and therefore no canal could be found in the LM samples. In all the Zurich samples, the lumen was present in the LM sections whereas in two of the roots processed by the EDI method there was no visible lumen in two of the three portions. The dentine tubules were easily visible in all the LM sections and, in 12 of 23 portions, were observed to contain bacteria, even

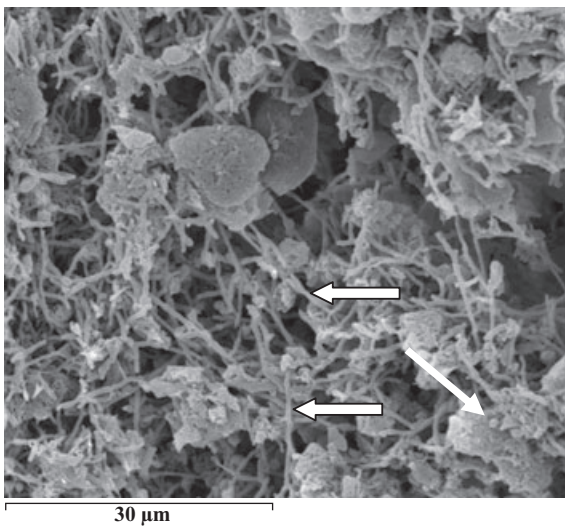

Figure 4 R4 (EDI protocol) SEM middle section showing bacteria morphotypes, filaments (F) and cocci (arrows) (µ bar represents 30 µm).

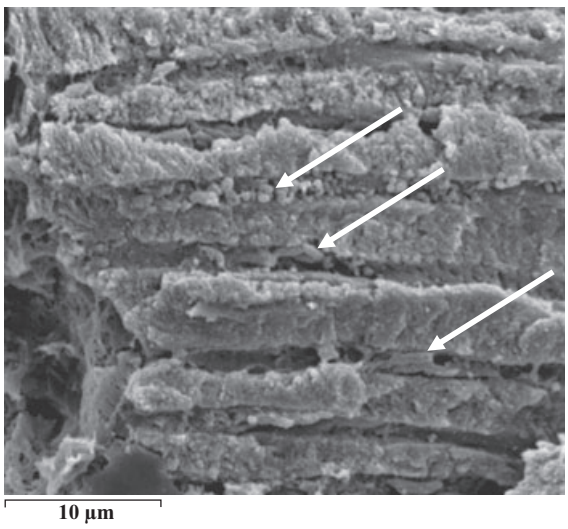

Figure 6 R4 (EDI protocol) SEM apical section showing bacteria (arrows) within the dentine tubules (µ bar represents 10 µm).

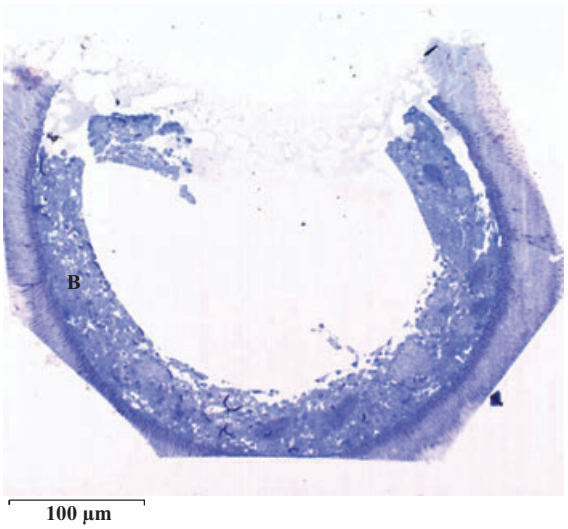

Figure 7 R5 (Zurich protocol) light microscopy (LM) apical section showing the overall view. The canal wall has a thick biofilm (B) with the luminal part containing some poorly visible amorphous substance (µ bar represents 100 µm).

when the bacterial film was sparse (Fig. 8). In 10 of these, bacteria were found in the LM sections where SEM had not found them, and in one, the opposite occurred.

In some samples, it was observed that polymorphs (PMNs) and some RBCs formed a layer several cells thick over the bacterial biofilm (Fig. 9). This was observed in two teeth and was most prominent in the apical segments but less so in the middle and coronal segments. In one root, a second bacterial biofilm (although less dense) could be observed on the luminal aspect of the PMN layer (Fig. 9), thus a layer of PMNs was sandwiched between two bacterial biofilms.

Comparison of technique by TEM

As the same samples were used for both the semi-thick LM and ultra-thin TEM sections, the reported absence of a root canal in both was due to inadequate demineralization. However, in five of the 15 root portions processed by the Zurich technique, although the dentine was demineralized sufficiently for the LM sectioning, it was insufficient for the ultra-thin sectioning and therefore these were not viewed by TEM. In four of the five cases, this was a middle portion of the root (Table 1).

In most cases, TEM provided similar information to LM except that TEM conferred the considerable advantage over the other techniques in the detail of visual information available on the cells and bacteria. The TEM of the biofilm in Figs 10 and 11 showed the close arrangement and morphology of the cells, including spirochaetes. Furthermore, the PMNs in this Zurich processed sample (Fig. 11) appeared to be 'leached' of

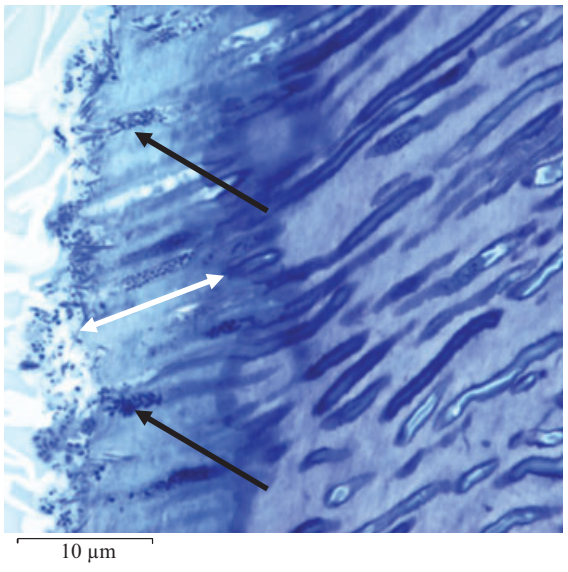

Figure 8 R4 (EDI protocol) LM middle section showing bacteria (arrows) within the dentine tubules (µ bar represents 10 µm).

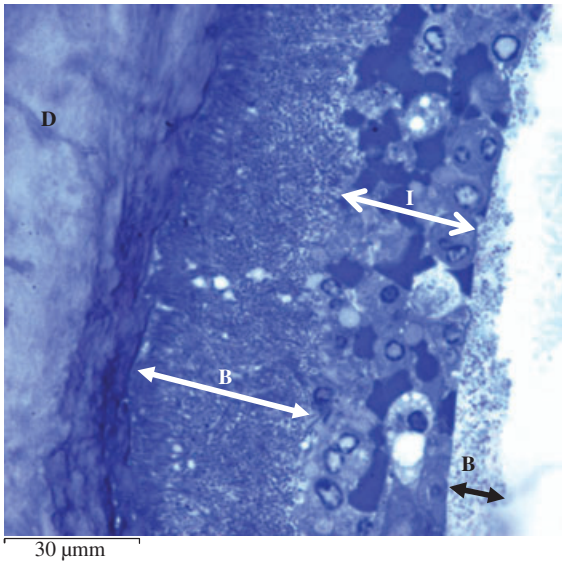

Figure 9 R5 (Zurich protocol) LM apical section showing bacterial biofilm (B) adherent to the canal surface and walled in by PMNs and RBCs (I) beyond which there is a further biofilm (µ bar represents 30 µm).

cytoplasmic contents, whereas in EDI samples the immune cells appeared healthy (Fig. 12).

When LM detected bacteria in the dentinal tubules, this was confirmed by TEM, except for those samples that were not sufficiently demineralized. In many of these samples, there was an apparent attachment of some bacteria to the collagen (Fig. 13) that must have been present before demineralization and may indicate exposed or available collagen epitopes within the canal.

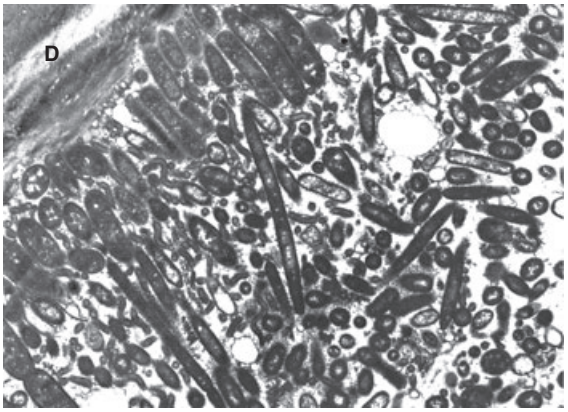

Figure 10 R5 (Zurich protocol) transmission electron microscopy (TEM) apical section showing bacterial biofilm (B) extending from the canal surface with palisading of the bacterial cells. The initial attachment to the canal dentine wall (D) appears to be due to filamentous morphotypes with coccal forms further out towards the canal lumen (TEM ×5000).

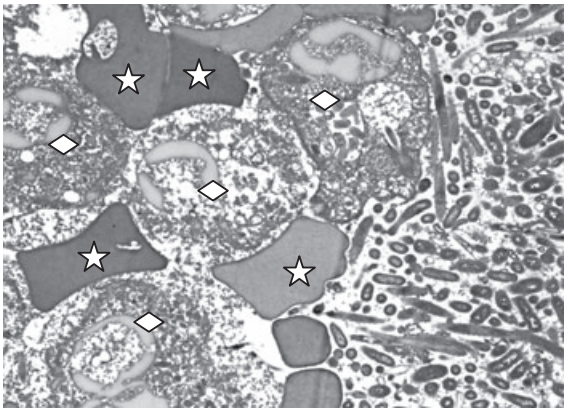

Figure 11 R5 (Zurich protocol) TEM apical section showing the layer of PMNs (♦) and RBCs (*) covering the bofilm. Note the loss of cellular contents from the PMNs (TEM ×2700).

Summary of observations

The Zurich technique allowed examination of the root canal in most SEM samples, all LM sections but only half of the TEM sections. In contrast, for the EDI technique, most of the canals were visible in the SEM, but only three-quarters could be used for LM and TEM. Generally, the correlation between LM and TEM was good but SEM provided rather different information. When bacteria were detected in the canal using LM or TEM, their presence was not always found in the SEM samples, although this may reflect the use of different halves of the root canal for each type of technique.

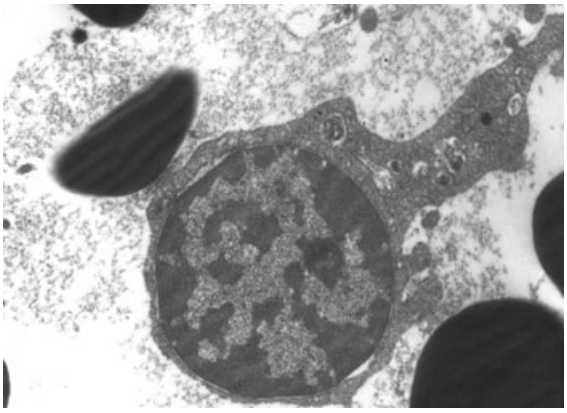

Figure 12 R2 (EDI protocol) TEM coronal section a healthy inflammatory cell, probably a lymphocyte, within the canal lumen (TEM ×6700).

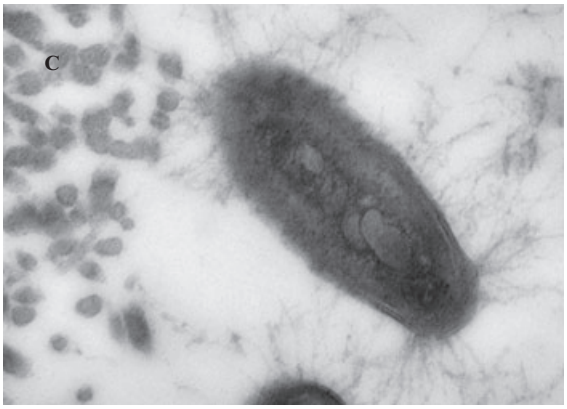

Figure 13 R4 (EDI protocol) TEM middle section showing the apparent attachment of a bacterium to the collagen fibres (C) (TEM ×40 000).

Bacteria were detected in eight of the nine roots examined, including root R6C, apparently not associated with a periapical lesion, but bacteria were not found in root R1, which although positive for presence of periapical lesion, was found to be a vital pulp. The pooled data from all microscopy techniques (Table 1) showed that in the bacteria-positive teeth, bacteria were detected in the canal lumens in all the root segments except 3 (R3 coronal and apical; R6c apical). In contrast, they were less frequently detected in the dentinal tubules, especially in the apical portions, and detection was more accurate by LM and TEM techniques.

The pattern of bacterial distribution, both in the canal lumen and on the canal walls, varied enormously both from root to root and within each root. Continuous biofilms were only evident in teeth with grossly carious exposures and continuous communication with the oral environment. The structure, thickness and morphotypic composition varied considerably. The bacterial biofilm was mainly evident on the canal wall with interspersed bacterial aggregates in what seemed to be residual necrotic pulp tissue. Sometimes bacterial cells seemed to be present in the canal lumen in apparent isolation (perhaps planktonic forms). In teeth with intact pulp chambers, the canal lumen appeared empty but was filled with some amorphous material (Fig. 7). In some teeth, the relative abundance of detectable bacteria was greater coronally, usually associated with carious crowns. Rarely did the middle portion have the greater abundance but, in teeth with intact pulp chambers, the relative abundance of bacteria was greater in the apical segments. Each tooth seemed to have its own variation of infection pattern.

The patterns of colonization of the dentinal tubules appeared to follow relatively more predictable but nevertheless variable behaviour. Dentinal tubules usually appeared to be colonized as a continuation of the canal wall infection, although the diversity of morphotypes were more restricted. Other instances showed variable colonization of adjacent tubules sometimes with highly dense colonization of the tubules in the predentine with reducing density of colonization further away from the canal lumen into the dentine.

The observation regarding the presence and close association of PMNs and sometimes RBCs with bacterial aggregates and films was not consistent. It was more prominent in some teeth than others and, where present, was always observed in the apical portions and frequently in the middle and coronal portions. In one tooth, a second bacterial biofilm, although less dense, was observed on the luminal aspect of the PMN layer, implying that this was not a result of tooth preparation.

Discussion

The prime purpose of this study was to evaluate the utility of different microscopy techniques and protocols to gain visual insights into the presence, distribution and structure of bacterial colonization in teeth associated with apical periodontitis, regardless of the clinical condition of the tooth; the intention was to use a wide selection of tooth conditions meeting selection criteria to evaluate the breadth of morphotypic bacterial diversity. Many studies on the microflora of infected roots have used teeth with gross caries, presumably because of their easier availability (Nair 1987, Baumgartner & Falkler 1991, Sen *et al.* 1995). An additional clinical parameter in this study was the acute presentation of the selected teeth. A subsidiary but important aim was to compare two tooth processing protocols. The importance of this aspect is that the validity of microscopy observations rest on accurate preservation of the *in situ* anatomical structures and relationships. Distortion of tissues or translocation of structural components may obscure the 'truth', and they correctly need to be recognized as artefacts. The problem for the observer is to be able to distinguish real from artefact without a 'positive control'. To be able to do so requires a good appreciation of what is to be expected based on understanding of biology, familiarity with the technical aspects of the procedures and critical interpretation. Purely morphological studies are able to give morphological insight but cannot enable dissection of the relationships and roles of bacterial species and their interaction with host cells. Such insights may only be obtained in the future through *in situ* labelling studies, which require the preservation or exposure of target cell surface, structural or chemical elements (Lam *et al.* 2000, Tan *et al.* 2000); the preserving protocols therefore become important. The purpose of this study was not to explore the effect of protocols on such cell surface targets but to evaluate the effect of such protocols on normal structural viewing, in the first instance. Studies on *in situ* hybridization will be reported separately. Another key factor is that each microscopy technique requires an independent section; the same section may not be viewed by all techniques. Absolute comparison between microscopy techniques is therefore impossible and relative comparison reliant on viewing adjacent sections that are thin enough to

represent, more-or-less, the same structures. This was more easily possible for LM/TEM views than for SEM, because, of necessity, the opposite halves were viewed and these could theoretically have different bacterial colonization, particularly in teeth with intact pulp chambers.

Some key features of difference between the protocols bear discussion. Previous microscopy studies have split their test teeth using a technique similar to the EDI protocol (Lin & Langeland 1981, Molven *et al.* 1991, Sen *et al.* 1995) but none commented on its inherent problem of unpredictability; a feature mostly reduced by practicing on spare rather than sample teeth. Furthermore, the lack of comment may reflect that the studies could select from both halves, whereas in the present study, the portion with the larger canal component was reserved for LM and TEM, theoretically compromising that used for SEM. Numerous approaches have been used to split teeth and alternative methods have been reported (Rapp 1985) but without tested consensus. The Zurich protocol was favoured for its more predictable cutting of the demineralized root compared with the splitting of mineralized tissues in the EDI protocol. The predictable cuts in the Zurich protocol were, however, associated with an apparently smeared appearance of the dentine, in contrast to the rougher fractured surface produced by the EDI protocol (Fig. 3 vs. Fig. 6); the significance of this feature is unknown although it may affect an appraisal of dentine tubule content. It should be added that such a feature was absent in the published material from the Zurich laboratory and could be a feature of adaptation in another laboratory.

A further putative advantage of the Zurich protocol is that the natural morphological relationships and conjunction of the structures would be less likely to be disturbed. In contrast, in the EDI protocol, the various washings of the pre-split and open canal surface prior to sputter-coating could potentially result in translocation of 'loose' structures such as planktonic bacteria or pulp debris (Nair 1987, and personal communication) (Fig. 3). Translocated debris and bacteria are sometimes evident in publications using the SEM (Sen *et al.* 1995). In general though, the SEM views did not seem much distorted or different between the protocols in this study. Furthermore, the enmeshed and matted appearance of the bacterial biofilm was confirmed by the different microscopy techniques and appeared to suggest that at least this feature of the bacterial colonization remained preserved regardless of the protocol used.

The careful and slow approach used by Nair (1987) to demineralize the test specimens is laudable and is most likely to yield accurate images representing the 'truth', nevertheless, within the time constraints imposed in this study, the slower process resulted in several middle root segments remaining un-demineralized and therefore un-viewable (Table 1). The counter-argument against the Zurich protocol was that the more aggressive demineralization, albeit slower, and associated long fixation periods, may damage surface antigens (Hobot & Newman 1991) and probe targets for *in situ* hybridization (Binder 1992).

It would be intuitively expected that each microscopy technique with its own unique characteristics would yield different perspectives on the objects under scrutiny; each hopefully yielding unique accuracy in some way so that they together complement findings to build a more accurate overview. The findings of this study confirm these expectations and potential, whilst at the same time highlighting the advantages and disadvantages of each microscopy technique.

The SEM, with its propensity for revealing surface topography was generally useful for deciphering detail over the entire canal surface, whilst retaining contextual perspective at lower magnifications; this also enabled the proportion of the surface colonized to be estimated. The technique was also useful for describing cell morphotypes but by the same token, surface coverage with cells or extra-cellular matrices precluded revelatory insight into biofilm structure and relationships.

The LM provided an excellent overview of the collective bacterial colonization and its variation from site to site within the selected section, particularly on the canal wall. Its main limitation is the level of magnification and resolution necessary to determine inter-cellular and cellular-abiotic relationships. Furthermore, morphotypic differentiation was relatively gross and lacked discriminatory detail.

Transmission electron microscopy was the most discriminating technique for providing fine detail of the microflora and its relationship to adjacent structures, as well as cell-to-cell contacts. Furthermore, the internal cellular morphology was also most clearly seen by TEM.

It is evident that correlative studies using LM and TEM provide the best conjunction, as reported by Nair (1987). Furthermore, the combination with SEM provides further insights but the processing required is different from that for LM and TEM and may be more prone to distortion of surface detail.

The pictures of bacterial quantity and density were broadly comparable between the microscopy techniques (Table 1), confirming the utility of using adjacent serial sections for LM and TEM. However, differences were sometimes apparent, both between sections, and by microscopy technique, the latter mainly because of SEM sections, which would by definition have viewed geographically different locations from those viewed by LM or TEM.

Bacteria were not detected in one root apparently associated with a periapical lesion, otherwise, a variety of morphotypes were found in all canal segments consisting of cocci, rods, filaments, spiral forms and yeasts. The existence of a periapical lesion associated with an inflamed but vital pulp is not a novel finding. The present study found that of all the roots which could be examined fully, only one had fewer bacteria apically than coronally. In the other roots (including those with intact pulp chambers), there was a transition from the coronal segment to the apical segment, of greater relative bacterial abundance apically. This contrasts with other work (Shovelton 1964), where the sample was also made up of both open and closed pulp systems. There was a lack of consistency in the middle root segments, where some roots had fewer bacteria than either the coronal or apical segments and others where the bacterial abundance formed a continuous transition from coronal to apical. The distribution could potentially be explained by abundance of nutritive sources coronally and apically. A carious exposure may allow seepage of salivary components from the coronal aspect, forming a diffusion gradient towards the apex. Once the bacteria are established apically, the stimulation of inflammation apically may then play a part in deriving nutrition from the inflammatory serum exudate (Khot *et al.* 2004). The relative scarcity of bacteria in the middle segment could be explained by its farthest location from opposing sources of nutrition (coronal or apical); it being the lowest point on two opposing gradients. The evidence of dividing bacterial cells in the middle segments suggests the presence of sufficient nutrients in this part of the canal at some point. In some species, such as staphylococci, divided cells may remain joined for some time after division.

The patterns of bacterial distribution in the canal lumen and on canal walls varied. Some teeth had discontinuous biofilms together with variable density and layers of cells, whilst others had thick continuous, dense biofilm layers. The structure, thickness and morphotypic composition also varied considerably but the species diversity of the flora may only be speculated upon without *in situ* hybridization. Some niches in the root canal seemed apparently more suited to biofilm growth than others, although the main bacterial colonization seemed to be on the canal walls; that within the lumen, in the middle and coronal segments, seemed more scattered. It is not known whether these bacteria, apparently 'floating' without attachment represent planktonic phenotypes or are biofilm phenotypes attached to a 'surface' of degrading tissue that is invisible in the chosen microscopy technique. Each tooth seemed to have its own variation of infection, corroborating the findings of various culture and culture-independent studies (Sundqvist 1976, Rolph *et al.* 2001, Munson *et al.* 2002). The impression in some teeth was that, indeed this was a nutrient-depleted environment but in others, the canal system appeared to be nutrient-rich with active bacterial growth and propagation. It is possible that acute apical symptoms may be due to such rapid and proliferative bacterial growth rather than because of specific species. Associations between species and acute symptoms although often made, have not proved fruitful, because the presence of the same species can be confirmed in asymptomatic teeth. The answers may lie in strain variation.

Yeast cells were detected in 3/7 (43%) teeth in this study, a value that fits within the range previously reported: by microscopy, 8–40% (Molven *et al.* 1991, Sen *et al.* 1995); by culture, 5–55% (Slack 1975, Egan *et al.* 2002); and by molecular detection, 21% (Baumgartner *et al.* 2000). Yeasts have been implicated in failed cases, raising the suggestion that reduction of bacteria during treatment may allow yeasts to overgrow and predominate in the low-nutrient environment (Sundqvist 1992).

Bacterial invasion of dentinal tubules was predominantly seen in the coronal and middle root segments; in contrast Sen *et al.* (1995) reported dentinal tubule invasion in the middle and apical root segments. The presence of bacteria on inter-tubular dentine casts some doubt on the SEM findings. The findings in the apical root segments are consistent with reports of fewer dentinal tubules in this region (Mjör *et al.* 2001). Dentinal colonization was heaviest in the pre-dentine and mainly confined towards the canal lumen end of tubules than the cementum; in agreement with some (Shovelton 1964, Nair 1987) but contradicting others (Peters *et al.* 2001). The finding of apparent bacterial association or attachment to dentine collagen (Fig. 13) would appear to be *in situ* confirmation of the suggestion previously made by Love & Jenkinson (2002).

Another interesting finding in the present study was the presence of PMNs in all thirds of the roots with necrotic pulps; the finding was particularly surprising in the coronal segments but would be consistent with pus exudation into the canal. Nair (1987) had previously reported PMNs amongst bacteria in the apical sections of roots associated with apical periodontitis, but these were described as isolated wandering cells. Their presence was explained by virtue of chemotactic signals from intra-canal bacteria. The extensive presence of PMNs in the root canals of teeth associated with apical periodontitis (with acute episodes), apparently strategically attempting to 'wall off' the bacterial biofilm adherent to the canal wall was unexpected and unique in the endodontic published literature. The observation which was consistent between LM/TEM techniques and different teeth and roots, alters the perception of the root canal ecology in such acute cases. First, it implies the presence of sufficient moisture or a water-saturated medium through which they can propel themselves to such distances into the canal. Second, there should be sufficient nutrients to allow them to migrate and survive in such locations ('technically' beyond the viable part of the body), bearing in mind their short life-span [3–4 days (Taussig 1984)]. Third, it changes the perception of the host/microbial interface as being confined to the apical foramen. Clearly, at least one branch of this host/microbial interaction is capable of extending into the length of the necrotic, infected canal associated with symptoms. Given the short life span of PMNs, the growth of a bacterial biofilm on the luminal aspect of the layer of PMNs suggests a very dynamic ecological niche in such a tooth. The relative equality between the protocols, at least in terms of quality of viewable sections, opens the doors towards use of microscopy with immuno-labelling to further dissect the root canal ecology and the dynamics of infection. The PMNs would appear to play an important role in apical periodontitis and perhaps apical healing.

Conclusions

The Zurich protocol was more predictable than the EDI protocol in creating longitudinal sections and possibly bacterial detection by microscopy but the quality of observed sections seemed equivalent. Each microscopy technique provided a unique perspective and together allowed complementary synthesis of the presence and morphological distribution of bacteria within roots. Each tooth presented a unique pattern of bacterial infection but all exhibited bacterial biofilms on canal walls; 8/9 roots showed bacteria. Bacteria in the canal lumen were often associated with other structures but sometimes appeared 'free-floating'. In general, bacteria appeared more abundant apically than coronally but dentinal tubule colonization was more common in coronal and middle thirds. PMNs were often found 'walling off' bacterial biofilm along the entire length of the root canal wall, although they were in higher numbers apically. The findings provide interesting insights into the nature of host/microbial interaction and the ecology of infected root canals.

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