Ultrastructural Examination of Failed Molar Retreatment with Secondary Apical Periodontitis: An Examination of Endodontic Biofilms in an Endodontic Retreatment Failure

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Abstract
A light and electron microscope examination of the resected root tip of a failing endodontically re-treated lower molar was examined. The tooth had been initially treated 10 years ago and then re-treated 2 years ago. The resected root tip was sectioned axially, and thin sections were examined through the entire length of the specimen. Thin sections were examined with a transmission electron microscope. The thin sections were randomly chosen along the isthmus areas between the mesiobuccal and mesiolingual canals. Our findings suggest that a complex, variable, multispecies biofilm was present the entire length of the specimen. (J Endod 2009;35:1303–1309)

Key Words
Apical periodontitis, biofilm, endodontic failure, endodontic retreatment, ultrastructural

Primary apical periodontitis is one of the most common bacterial diseases of humans, affecting as many as 60% of all individuals worldwide at some point in their life span (1). It has been established conclusively that the cause of the disease stems from the microbial colonization of the root canal space by oral microorganisms, leading to subsequent periapical breakdown from the spread of these organisms, or their toxins, into the periodontal ligament space and bone (2–4). Of the more than 700 species of bacteria thought to be present in the mouth (5), it appears only a limited number of species are capable of surviving and persisting in the root canal environment, and an even smaller number are capable of surviving endodontic treatment regimens whose sole purpose is directed toward eliminating them (6–10).

Contemporary research has also tried to make a distinction between primary apical periodontitis and secondary apical periodontitis (6–12). The distinction proposed is that in primary apical periodontitis (disease present before any treatment) the organisms are predominately gram-negative obligate or facultative anaerobes that are multispecies in character, whereas in secondary disease (post-treatment disease) the organisms are facultative gram-positive organisms of a very limited species distribution and are perhaps even monoinfections (11–13).

There is much recent work that demonstrates that the most likely disease model for endodontics is a biofilm model, just as it is in periodontal disease, and that although acute disease might be caused by planktonic organisms, the source for all these organisms is from a preexisting and ever-present biofilm (14–19). By using combined light and electron microscopy, Nair et al (18–20) have shown conclusively that in both primary and secondary apical periodontitis, the organisms colonizing the root canal space are growing in verdant biofilms.

One might suspect that the organisms capable of surviving treatment rendered 10 years ago and then re-treated to a very high standard with 30 days of Ca(OH)2 would show a less verdant biofilm structure, given the extreme nutrient scarcity in such cases, and that such a biofilm would consist of only a very small number of species. Certainly the culturing model and the literature predict that (21). This work attempts to test that viewpoint by carefully examining one such case in great detail.

Materials and Methods
The patient was a 39-year-old woman in good general health with no significant medical history. She presented in 2005 with periodic spontaneous pain and tenderness at tooth #30. Clinical tests revealed #30 was mildly tender to percussion. It had a periapical radiolucency associated with the distal root and widening of the periodontal ligament at the apex of the mesial root. There were no significant probing depths and no evidence of a sinus tract. The adjacent teeth were non-tender and responded normally to cold. According to the patient, the original root canal treatment was performed in about 1995 and had “bothered her on and off” over the years.

After discussing the options of retreatment, surgery, or extraction, the patient made the decision to re-treat. Access was made through her metal-ceramic crown, and gutta-percha was removed from 3 canals. There was an untreated distobuccal...
The 4 canals were prepared with a combination of rotary and hand instruments, all to size 45. Sodium hypochlorite (approximately 5%) was used for irrigation throughout. At the end of the appointment, 17% ethylenediaminetetraacetic acid (EDTA) was used for 1 minute to remove the smear layer. Two percent chlorhexidine was used as the final irrigant, also for 1 minute. Working lengths were determined with a combination of an electronic apex locator (Root ZX; J. Morita, Tokyo, Japan) and paper points. All canals were patent. There were

Figure 1. Timeline of treatment. (A) Preoperative radiograph; original treatment in 1995. (B) Calcium hydroxide placement. (C) Final obturation. (D) Recall radiograph with lesion on mesial root. (E, F) Apical surgery radiographs.

Figure 2. (A) Submitted specimen. (B) Decalcified and sectioned. (C) Gutta-percha in mesiobuccal and mesiolingual canal and isthmus. (D) Typical area sampled. (E) Isthmus tissue, thick section. (F) Higher magnification of area in (E).
no internal cracks evident under the surgical operating microscope. At the end of the appointment, Ca(OH)2 (Ultracal; Ultradent Products, Salt Lake City, UT) was injected into all the canals, and a lentulo spiral was used to ensure it was all the way to working length. A radiograph confirmed this. Cavit (3 M ESPE, Minneapolis, MN) was used to temporarily close the access opening.

Eleven days later, the patient had a flare-up. Initially she had pain, which lessened as she developed swelling. Clindamycin 300 mg 4 times

**Figure 3.** (A) Semi-thin histologic section showing area of interest. (B) TEM of area (A) showing varied biofilm. (C) Higher magnification of small insert area from (B) showing multiple phenotypes within a complex biofilm. (D) Higher magnification of larger insert in (B) showing complex biofilm community. Note that none of these communities are evident from the histologic semi-thin section, and there is no evidence of any neutrophilic infiltration.

**Figure 4.** (A) Semi-thin area of interest. (B) TEM of biofilm from (A) with no leukocyte infiltration evident. (C) Random thin section of area near mesiobuccal canal gutta-percha. (D) TEM of same area (C) demonstrating dentin, pulpal remnant, and complex biofilm. Note that none of these biofilms are evident from the light microscope histologic sections.
a day was prescribed. The swelling resolved after a few days, and she remained asymptomatic until her obturation appointment about 2 weeks later.

At the obturation appointment the Ca(OH)2 was removed, some additional instrumentation was performed, and the same irrigants were used plus a final rinse with alcohol to help dry the canals.

Gutta-percha cones (Diadent .06 taper; Diadent Corp, Vancouver, BC, Canada) were fit and condensed by using a warm vertical technique in combination with Kerr EWT Sealer (Sybron Dental Technologies, Orange, CA). The porcelain margins at the occlusal edge of the access cavity were etched with 10% hydrofluoric acid (Ultradent), and the access opening was restored with composite (BuildIT; Pentron Clinical

**Figure 5.** Variety of bacterial morphotypes. (A) Unusual segmented gram-negative bacterial organism. (B) Dentin, pulp (tissue remnants), and biofilm bacteria in matrix. (C) Gram-negative bacteria with unusual cellular inclusions. (D) Biofilm bacteria apparently in the process of shedding cell membrane fragments.

**Figure 6.** Isthmus area pulp tissue (box insert on left). TEM of same specimen on the left. (A) Dentin. (B) Pulp. (C) Biofilm bacteria in a thick glycocalyx. (D) Multiplying gram-negative and gram-positive bacteria.
Technologies, Wallingford, CT; and Tetric Ceram; Ivoclar Vivadent, Amherst, NY) and a 4th generation dentin bonding system (Optibond; Sybron Dental Technologies).

In 2007, approximately 30 months later, the patient presented with spontaneous pain and tenderness at tooth #30. There were no significant probings and no swelling or sinus tract. The distal lesion was greatly reduced in size, but the mesial root now exhibited radiolucency. The patient was presented with the options of apical surgery or extraction. She chose surgery. Her husband, who is a physician, had already ordered clindamycin for her. A cone beam computed tomography scan was ordered because of the proximity of the mandibular canal and mental foramen.

About a week later the surgery was performed. A buccal full-thickness flap was reflected, and an osteotomy was made through the buccal plate. Because of the nerve proximity the root was resected several millimeters coronal to the apex, and the root end was removed in one piece and placed immediately in modified Karnovsky’s fixative and submitted (Fig. 1).

A mesial root-end preparation was made with ultrasonics, and a retrofilling of mineral trioxide aggregate (Dentsply International, York, PA) was placed. The flap was closed with 4 gut sutures, which were removed after 2 days. The patient has presented twice for follow-up appointments at 5 and 12 months. She was symptom-free, but healing was incomplete.

**Tissue Processing**

Immediately after removal, the apical root tip was fixed by immersion for 1 week in modified Karnovsky’s fixative consisting of 2% paraformaldehyde and 2.5% glutaraldehyde buffered in 0.2 mol/L sodium phosphate buffer supplemented with sucrose and adjusted for a pH of 7.2 and an osmolarity of 320 mOsm. Thereafter, the root tip was decalcified for 8 weeks in 0.25 mol/L EDTA and 4% glutaraldehyde supplemented with sucrose. The demineralized apex was sectioned into 4 horizontal disks, and then each horizontal section was further subdivided in half in the coronal-apical direction. The 8 decalcified sections

![Figure 7](image1.png)

**Figure 7.** Bacterial profiles indicating active protein synthesis as well as intact cell walls and cell membranes.

![Figure 8](image2.png)

**Figure 8.** Bacterial blebbing from gram-negative biofilm bacteria. (B) Membrane-enclosed bleb. (C) Higher magnification of bleb.
were then labeled and photographed with a Zeiss surgical microscope (ProErgo, Germany) (Fig. 2).

The decalcified sections were postfixed in 1% OsO4 for 2 hours followed by 0.5% uranyl acetate for 1 hour and then dehydrated in ascending concentrations of ethyl alcohol and embedded in Epon 812 (Ted Pella, Redding, CA).

Serial 2-μm survey sections along the entire root length and random examinations of the isthmus areas in all sections were performed. In all, more than 2000 survey sections were examined from the apical tip to the most coronal section. Two-micrometer survey sections were taken by using glass knives in an ultra microtome, stained with methylene blue, and examined with a Zeiss AxioObserver microscope (Zeiss, Oberkochen, Germany). From those survey sections, areas near the main canal in the isthmus area were selected for transmission electron microscope (TEM) examination. Five-micrometer pyramids were created with an LKB Pyramitome (Diversified Equipment Co, Lorton, VA) TEM at 80 kV at multiple magnifications, and selected sections were photographed by using Kodak 4489 (Eastman Kodak, Rochester, NY) film and scanned by using an Epson 750 (Epson America, Inc, Long Beach, CA) scanner at 1200 dpi. Other than brightness and contrast, no other image processing adjustments have been made to the scanned files.

Results

Light Microscope Examination

Histologically, a mesiobuccal and mesiolingual canal filled with gutta-percha was present the entire length of the specimen. A communicating isthmus was also present the entire length. In the sections examined, several accessory or lateral canals were seen. The histologic assessment of the isthmus tissue showed evidence of pulpal remnants with minimal vascularity and very few neutrophils present. In addition, no obvious bacterial colonies were seen with the light microscope at any level (Figs. 3, 4).

TEM Examination

Because random thin-sections were taken, we were surprised to find bacterial biofilm present in every section examined.

Of particular note in many of the sections examined was the complete absence of any intact neutrophils. Only occasional red blood cells were seen and also very few planktonic organisms. Nearly all bacteria were seen to be encased in a matrix that was many layers thick composed of bacteria in various stages of degeneration, with apoptotic cells, ghost cells, and dividing cells all present in the biofilm. In some sections, bacterial biofilm appeared to reside in the dentinal tubules and in interstitial areas of the dentin or fibrodentin. The varied morphotypes seen in this environment differ considerably from those morphotypes observed in prior studies in which there was not significant nutrient deprivation, and the morphotypes more closely resembled planktonic forms (Fig. 5 A, C).

Another unsuspected finding was that the appearance of the biofilm differed significantly from one area to the other, even areas in very close proximity. Thus the biofilm community present in this tooth was not a homogeneous, uniform biofilm but one of incredible variety and changeability. Many different morphotypes were observed in the matrix, and the composition of the biofilm changed dramatically even between adjacent sections 2 μm away (Fig. 6).

Many of the bacteria appeared to be undergoing a lytic process, and others seemed embedded in a collagenous matrix (Fig. 5B, D). Still many other areas showed bacterial profiles with many ribosomes suggestive of active protein synthesis and secretion (Fig. 7).

In many areas bacterial blebbing was observed. High magnification of these blebs suggests these are cell membrane fragments of the bacterial cell membrane (22, 25) (Fig. 8).

Discussion

There are significant limitations to ultrastructural examinations, especially with regard to biofilm investigations. Biofilms are collections of organisms that exist in a hydrated matrix called extrapolymeric substance (EPS) that is almost completely lost during tissue processing. TEM and scanning electron microscope imaging both occur in a very high vacuum (10E-10 bar), and all water is lost during specimen preparation, so what is seen under the microscope is a very poor substitute for understanding how these organisms are really structured in these communities (24–29). Because the extracellular milieu in biofilms is mostly water and that water is completely lost in processing, we are left to really guess how these cells are actually connected and what complexities these communities might have in their fully hydrated and natural state (24–26). Nevertheless, even with these limitations, ultrastructural studies can help elucidate the structure and composition of endodontic biofilms.

Previous ultrastructural studies have attempted to narrow the focus of ultrastructural investigation to areas thought likely to contain concentrations of organisms. It is a well-established fact that a light microscope examination of specimens frequently fails to identify organisms that are known to be present, even when using special bacterial stains to aid in identification. Identifying bacteria within a biofilm is even more problematic than identifying colonies of planktonic organisms because biofilm bacteria are encased in a polysaccharide matrix.

Previous studies have narrowed the areas investigated to areas in which there is an obvious infiltration of neutrophils as seen in the light microscope during the survey sections (19, 20). Our approach was different because part of the biofilm disease model is that mature biofilms effectively hide themselves from immune surveillance and that biofilm organisms might be present where there is no neutrophilic concentration of organisms (26, 27). For this reason, random samples were taken from isthmus areas. In every sample taken, biofilm organisms were present, suggesting that limiting areas of investigation to only where neutrophils are observed might result in missing significant biofilm populations.

Another striking finding was the morphologic diversity displayed and how different these organisms appeared compared with prior published studies (18–20). TEM examination of biofilm bacteria in their natural habitat reveals them to be significantly different morphologically than their planktonic cousins or even than biofilm bacteria that are in environments with greater access to nutrients. The extent and significance of this morphologic diversity need further investigation. The authors want to emphasize that although there is an apparent wide morphologic diversity present in this case, ascribing pathogenicity because of this diversity would be an error.

The observation of bacterial blebbing is also of interest. Bacterial blebbing or membrane vesicle formation is a known mechanism that growing gram-negative bacteria use to establish a colonization niche, transmit virulence factors, modulate host defense and response, and even kill other bacteria. These vesicles can critically impact disease because of their high inflammatory capability. These vesicles typically contain lipopolysaccharides of the cell membrane, adhesins, toxins, proteases, alkaline phosphatase, and other immunomodulatory compounds and are one of the primary ways in which gram-negative bacteria interact with prokaryotic and eukaryotic cells in their environment (22, 30).
In the present study, we saw both gram-positive and gram-negative organisms surviving in an extremely harsh and nutrient-deficient environment that had existed for more than a decade. These organisms appear to be in the biofilm mode of growth, which is a consortium of species in a complex matrix in which cell metabolism is low, exopolymERIC substance (EPS) is high, and the arrangement of organisms is highly variable from region to region. In addition, they do not appear ultrastructurally similar to their planktonic cousins, and it appears that multiple stressors have induced sublethal and structural changes, resulting in organisms that would probably not be culturable, but that are still persistent and viable, a hallmark sign of a biofilm. These sessile, embedded microbes have drastically down-regulated rates of cell division, giving them a persistence that enables survival in extremely harsh environments (23, 27, 31, 32). Factors affecting the survival of bacteria in this hostile environment include not only nutrient deprivation but immune attack by host cells, osmotic stress, chemical insult, oxidative stress, hostile pH changes, desiccation, autochthonous microbial toxins, bacteriophage attack, and mechanical physical injury. However, all of these stressors have been shown to be ineffective in eliminating bacteria within natural biofilms, and many studies have been published that document the incredible persistence of biofilm bacteria despite such stressors. Once nutrient availability is reestablished, for example, if the apical or coronal seal allows exposure to serum, these organisms can alter their gene expression to accommodate to their new environment (23, 31–35).

Conclusion

The biofilm community in this one case appears to have been incredibly adaptive and persistent, despite a hostile environment that would easily kill planktonic organisms. Ultrastructurally they appear far different from their planktonic brethren, and it will take further investigations to fully understand all the different forms they might take. A fuller understanding of the complex natural life cycle of bacteria will enable us to gain a better understanding of chronic bacterial infection and its role in endodontic failure. This experiment attempts to show from an ultrastructural viewpoint that the microbial communities that exist within the endodontic space for decades after treatment can thrive and prosper there with no apparent nutrient source and that their communities consist of diverse, multispecies biofilms.

References