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The evolving new understanding of endodontic infections

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The science of oral microbiology is in a period of change from the era of bacterial cultivation to an era of molecular genetic methods and techniques. Already a significant body of new knowledge exists with regard to the oral flora in health and disease. Inevitably, this new knowledge has led to a better understanding of many oral diseases. In endodontics, the prevailing concepts are still to a great extent based on the results of the classical cultivation studies. However, a few groups have started to use molecular methods, and a new understanding of endodontic infections is presently evolving. Thus, the root canal infection clearly is more complex than revealed by cultivation methods alone, and both previously unidentified and uncultivable microorganisms have been detected by molecular methods. A reasonable estimate at present is that the infected root canal contains, not less than 10, but rather between 10 and 50 bacterial species which coincide well with the number of bacterial species normally found in a dental plaque sample and at different sites in the oral cavity. A further interesting finding in the studies using molecular techniques is that the microbiota of the infected root canal appears to be very similar to the flora of the periodontal pocket in patients with active periodontal disease. With regard to infection of periapical lesions in patients with asymptomatic apical periodontitis, electron microscopic and molecular methods have confirmed our cultivation findings that this is a common occurrence. Mature biofilms have been demonstrated on the external surfaces of root tips and in the form of sulfur granules within periapical granulomas. As in dental plaque, *Actinomyces* species appear to have a special role as scaffold builders in the development of sulfur granules. Other bacteria are then attracted to the site and a multibacterial granule (biofilm) develops. In addition, *in situ* hybridization studies show a variety of different bacteria and bacterial morphotypes in periapical lesions. With DNA–DNA hybridization between 11and 39 bacterial species have been recognized in the lesions, again confirming that in patients with active disease, the microbiotas of endodontic and periodontal infections are very similar. Thus, the recent findings demonstrate and confirm that the periapical endodontic lesion is not as hostile to microorganisms as many have thought. As clinicians we have to understand and accept that an infection might not be limited to the root of the tooth, but include the periapical lesion as well.

Introduction

It has been known for more than a century that bacteria may colonize the root canal (1). The importance of bacteria as an etiological factor for pulpal and periapical inflammation as expressed in the literature has varied over the years. However, striking evidence for the role of infection came in the 1960s when it was shown that pulp necrosis and apical periodontitis would not develop in germ-free animals when the pulp was exposed to the oral cavity (2). In humans it has been shown that apical periodontitis with bone resorption will develop only if the necrotic pulp becomes infected (3, 4). Finally, it is known that bacteria isolated from root canals of teeth with apical periodontitis will cause apical periodontitis when inoculated in the root canals of other teeth (5). When reisolated, these bacteria are shown to be the inoculated organisms and thus have the capacity to establish themselves and survive in the root canal and exert pathological influence on periapical tissues.

If the root canal is exposed to the oral cavity, bacteria will accumulate in the pulp chamber and the canal. The normal oral microflora dominated by facultative anaerobic organisms will be present in the root canal. Many strains of obligate anaerobic bacteria will be present as well, but usually in small numbers. However, if the access opening to the root canal is sealed off after the oral flora has been allowed to colonize the root

canal system, a selective bacterial growth begins (6). Already after 7 days, 50% of the cultivable flora is anaerobic bacteria, and soon some 90% of the bacteria may be anaerobic. In the apical area of the root canal where the oxygen tension is the lowest, the predominance of anaerobic bacteria may be even greater than in the main root canal (7). In studies using aerobic and anaerobic culturing, usually 1–6, or occasionally as many as 10 bacterial species are recovered from the root canal (4, 8). From the canal, the bacteria may enter the tubules of the root dentin. One must therefore understand that the term *root canal infection*, which is commonly used, in reality means infection of the root canal system with the main canal, lateral canals, and apical deltas, as well as infection of the root dentin. The periapical lesion, on the other hand, traditionally has been held to be free from bacteria (9–12).

Microbiota of the oral cavity

The oral cavity contains one of the most concentrated accumulations of microorganisms in the human body, and more than 700 different species have been detected so far (13). Some 50% of the oral species are uncultivable or have not yet been cultivated (14), and culture-independent techniques are now being used successfully to better assess the bacterial diversity of the oral cavity in health and disease. The results of these studies are important, both as a background for understanding endodontic infections, and to emphasize the need to redefine the endodontic microflora with the use of molecular methods.

Oral biofilms

The bacteria of the healthy oral cavity, the bacteria of a carious cavity as well as the bacteria associated with periodontal disease and as we shall see, endodontic infections, are seen to reside within biofilms. Biofilms are the preferred method of growth for many and perhaps most species of bacteria. This method of growth provides a number of advantages to colonizing species. A major advantage is the protection the biofilm provides to colonizing species from competing microorganisms, from environmental factors such as host defense mechanisms and potentially toxic substances such as antiseptics or antibiotics. Biofilms also can facilitate processing and uptake of nutrients, cross-feeding (one species providing nutrients for another), removal of potentially harmful metabolic products (often by utilization by other bacteria) as well as the development of an appropriate physicochemical environment (such as a properly reduced oxidation reduction potential) (15).

Biofilms are composed of microcolonies of bacterial cells that are non-randomly distributed in a matrix. The matrix mainly consists of exopolysaccharides, proteins, salts and cell material in an aqueous solution. Bacteria can produce several different polysaccharides depending on the presence of necessary substrates. The extracellular materials can be degraded and utilized by the bacteria in the biofilm, and many microorganisms can both synthesize and degrade the exopolysaccharides. Adhesion to a surface is the essential first step in the development of a biofilm. In the mouth, bacteria can attach to a wide variety of surfaces, including the soft tissue, the teeth and dental materials, but also to other bacteria, cells and collagen fibers. Many bacterial species have surface structures such as fimbriae and fibrils that aid in their attachment to different surfaces. Examples of this are *Actinomyces* species that comprise a major segment of the microbiota attached to the tooth and may be thought of as part of a scaffolding structure of dental plaque (15). Other bacteria have surface proteins that aid both in the initial attachment to a surface and in the cell-to-cell attachment in building a three-dimensional structure. For this the synthesis of exopolysaccharides is important as well.

Oral biofilms are complex, and may comprise 30 or more bacterial species (13, 15, 16). This may be due to the fact that most oral bacteria may adhere to other oral bacteria. Bacteria or microcolonies of bacteria in the biofilm communicate with one another when the cell density is sufficient. This communication, which is referred to as quorum sensing, has the potential to influence the structure of the biofilm by encouraging the growth of species beneficial to the biofilm and discouraging the growth of competitors. Also, the physiological properties of bacteria in the biofilm may be altered through quorum sensing. For example, expression of genes for antibiotic resistance may provide protection for the bacterial community, and it is estimated that bacteria grown in a biofilm have a 1000–1500 times greater resistance to antibiotics than planktonically grown bacteria (15, 17). On the whole, the cell-to-cell communication and transfer of genetic information within biofilms enable the bacteria to change in response to their environment, and the

biofilm structure provides an effective defense against host protective mechanisms and antimicrobial agents.

Bacteria detach from an established biofilm and may colonize new sites. This appears to occur by a nearly continuous detachment of single cells (erosion), by sporadic detachments of large groups of cells (sloughing), or by a process whereby large pieces of biofilm are shed. What might be clinically very important is that, in contrast to the single bacteria, a detached cell cluster may have the same protection from the host defense systems as the biofilm from which it was shed. *In vitro* studies have also shown movement of intact biofilm structures across a surface (15). This may have implications for the colonization of a site as well. It is our opinion that a thorough knowledge of biofilm biology is essential for the understanding of endodontic infections. For a more in depth presentation of the topic, the readers are referred to an extensive overview article by Socransky and Haffajee (15).

The healthy oral cavity

In a recent study using a PCR-based technique (13), nine different sites in the oral cavity of five clinically healthy subjects were analyzed (dorsum of the tongue, lateral sides of the tongue, buccal epithelium, hard and soft palate, surfaces of the teeth, subgingival plaque, maxillary anterior vestibule, and tonsils). Over 60% of the species detected were uncultivable phylotypes. All sites possessed about 20–30 different species or phylotypes except the anterior vestibule that had three to nine species. Some bacteria were common to all sites whereas others appeared to be site specific. For example, *Streptococcus sanguis*, *S. gordonii*, *Abiotrophia defectiva*, and species of *Actinomyces* preferentially colonized the tooth surface. And interestingly, many of the bacteria associated with disease, such as *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Treponema denticola* (18), were not detected in this study.

Carious cavities

In a PCR-based study of the flora of carious cavities, 224 bacterial species and phylotypes were detected, 60% of which were uncultivable (16). Sixty-two new species were detected. Based on the findings of this study, it appears that species other than *Streptococcus mutans*, e.g. *Lactobacillus*, *Bifidobacterium* and *Atopobium*, play an important role in caries production. Also, defined species are involved in the initiation of the disease, e.g. species of *Actinomyces* and non-*mutans* streptococci.

Periodontal disease

Marginal periodontitis, the periodontal pocket and subgingival plaque have been extensively studied since the pioneering investigation by Waerhaug in 1957. Thus, a significant body of knowledge on the infection of periodontal disease has emerged over the years, especially since culture-independent molecular genetic techniques were developed and used (14, 19, 20). Most aspects of this body of knowledge have a direct bearing on the understanding of endodontic infections as well.

The diversity and nature of subgingival plaque in health and disease have been studied by Paster et al. (14) using a PCR-based technique. Sixty percent of the 2.522 clones that were studied fell into 132 species, and 70 of these were identified from multiple subjects. About 40% of the clones were novel phylotypes. Many species or phylotypes were found only in subjects with disease, and a few were detected only in healthy subjects. The predominant subgingival microflora consisted of 347 species or phylotypes. It was estimated that there are 68 additional unseen species for a total estimate of 415 species in subgingival plaque.

The question then is which of these many species are the causative agents of marginal periodontitis. Important in this regard is the method developed by Socransky et al. (19) for hybridizing large numbers of DNA samples against large numbers of DNA probes on a single support membrane, the so-called checkerboard DNA–DNA hybridization technique. This technique does not require bacterial viability although initially the probe bacteria must be cultured. It detects species in low proportions, and amplification of the DNA is not necessary for identification. This technique has been used to examine over 13 000 subgingival plaque samples from 185 adult subjects (15). The study compared the microbiotas of healthy and diseased sites, actively progressing lesions and non-progressing lesions, as well as successfully and unsuccessfully treated sites after different forms of periodontal therapy. Three species, *Actinobacillus actinomycetemcomitans*, *P. gingivalis* and *B. forsythus*, were strongly associated with periodontal disease status, disease progression and unsuccessful therapy. As such, these species were designated as periodontal pathogens at the 1996 World Workshop on Periodontology (21). Moreover, it was

confirmed that the associations of bacteria in a mixed infection are not random (18, 22). Six closely associated groups or complexes of bacterial species were recognized. A red complex consisting of *B. forsythus*, *P. gingivalis* and *T. denticola* is one group found in patients with active disease. An orange complex including *Campylobacter rectus*, *Campylobacter showae*, *Eubacterium nodatum*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *P. nigrescens* and *Peptostreptococcus micros* may be important for disease progression as well. A third, blue group includes the *Actinomyces* and a fourth, green group consists of *Capnocytophaga* species, *A. actinomycetemcomitans*, *Eikenella corrodens* and *Campylobacter concisus*. The streptococci make up the fifth, yellow group and the sixth purple group comprises *Veillonella parvula* and *Actinomyces odontolyticus*. The dominant species both supragingivally and subgingivally are *Actinomyces*, but significantly higher counts, proportions and prevalence of red and orange complex species are found in the samples from the periodontitis subjects. It is concluded that *B. forsythus*, *P. gingivalis* and *T. denticola* have a decisive influence on the progression of disease in patients with active marginal periodontitis. In addition, other putative periodontal pathogens including *F. nucleatum* subsp. *vincentii*, *C. rectus* and *P. intermedia* have been found to be more prevalent in periodontitis patients than in well-maintained subjects (23).

Endodontic infections

Root canal infection

The crown of the tooth is the main portal of entry for bacteria into the root canal space (24). This is readily understood in teeth with pulp exposures or carious lesions. In teeth with intact crowns it may be more difficult to see how the bacteria reach the root canal. However, in reality an 'intact' crown rarely exists. Teeth have dentin exposed by abrasion, erosion and attrition, or by scaling and root planing during prophylaxis and treatment of periodontal disease. Enamel–dentin cracks commonly occur and become filled with plaque and bacteria (25). They are almost bound to run at an angle with the dentinal tubules so that a single crack may lead to the exposure of a large number of tubules. Another possible pathway of infection of the necrotic pulp and root canal space is the apical foramina and accessory canals through a hematogenous spreading of bacteria. This appears highly likely considering the fact that bacteremia is a commonly occurring phenomenon in man (26, 27). However, the evidence for this is at present inconclusive.

The infection of the root canal ultimately leads to liquefaction necrosis of the pulp (24). The bacteria form biofilms, first and foremost on the root canal walls, but also in conjunction with tissue remnants in the canal (Fig. 1a). Bacteria may also enter the tubules of the root dentin (Fig. 1b). Especially, this seems to be the case when the root cementum has been removed by caries, abrasion or external root resorption so that the tubules have become exposed to the oral cavity or the periodontal ligament (28). There are also suggestions that bacteria found in the dentinal tubules are special and unique to the oral cavity, allegedly because of the restricting environment of the tubules (29).

There is no study as of yet on the flora of the root canal like those of Paster et al. (14) and Aas et al. (13) on the flora of the periodontal pocket and various sites of the oral cavity. Still a number of investigations on root canal infection using culture-independent, molecular techniques have appeared in recent years, clearly showing that the root canal flora is much more complex than revealed by cultivation methods alone (30–34). Munson et al. (35) used cultural and molecular analyses to determine the microflora in aspirate samples collected from five infected root canals. Sixty-five taxa were identified, of which 26 were found by the molecular method alone. A mean of 20.2 taxa was found in each sample. A new species of *Dialister* was the only organism present in all samples. Twenty-seven novel taxa were detected. In a similar study, Rolph et al. (36) found 44% of the samples to be positive by culture while 68% were positive by PCR. They conclude that molecular techniques can detect the presence of bacteria in endodontic infections when culture techniques yield a negative result, and can be used to identify a wider range of endodontic-infection-related bacteria including the presence of previously unidentified or uncultivable ones. Thus, unidentified bacteria were detected in these studies, and bacteria difficult to culture such as *Prevotella tannerae* (37), *Actinomyces radicidentis* (38), *Olsenella* spp. (35, 39), *Dialister pneumosintes* (40), *Treponema maltophilum* (33, 41), *T. amylovorum*, *T. medium* and *T. lecithinolyticum* (33) were found in infected root canals for the first time, or were detected in higher numbers than previously described.

In a recent study by our group (42), the root canals of 30 teeth with asymptomatic apical periodontitis were

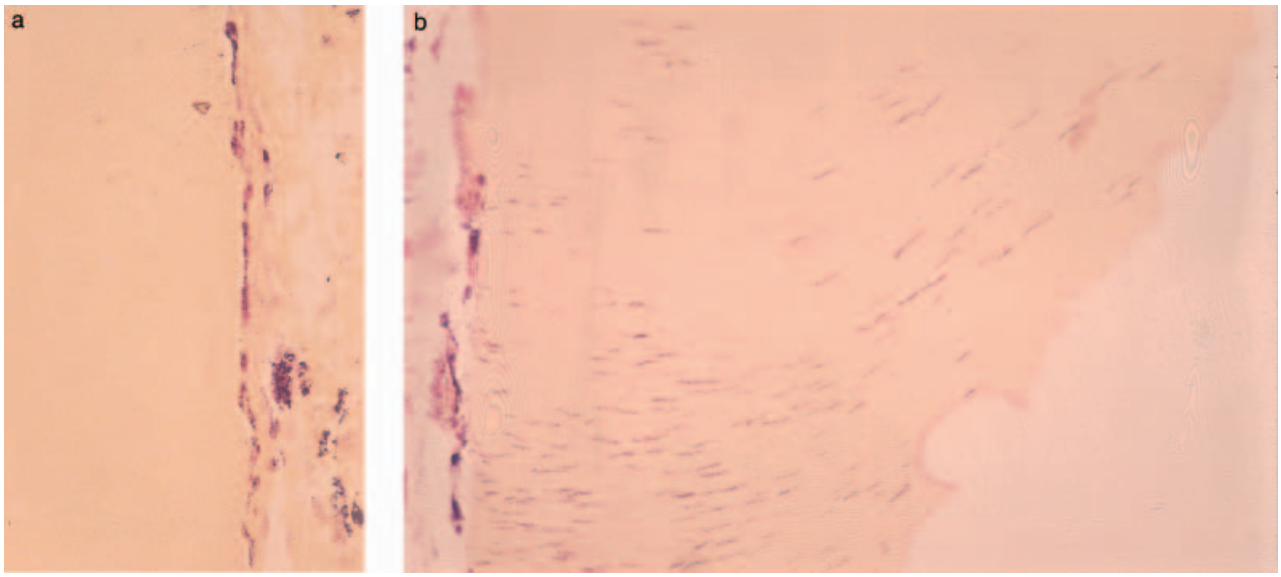


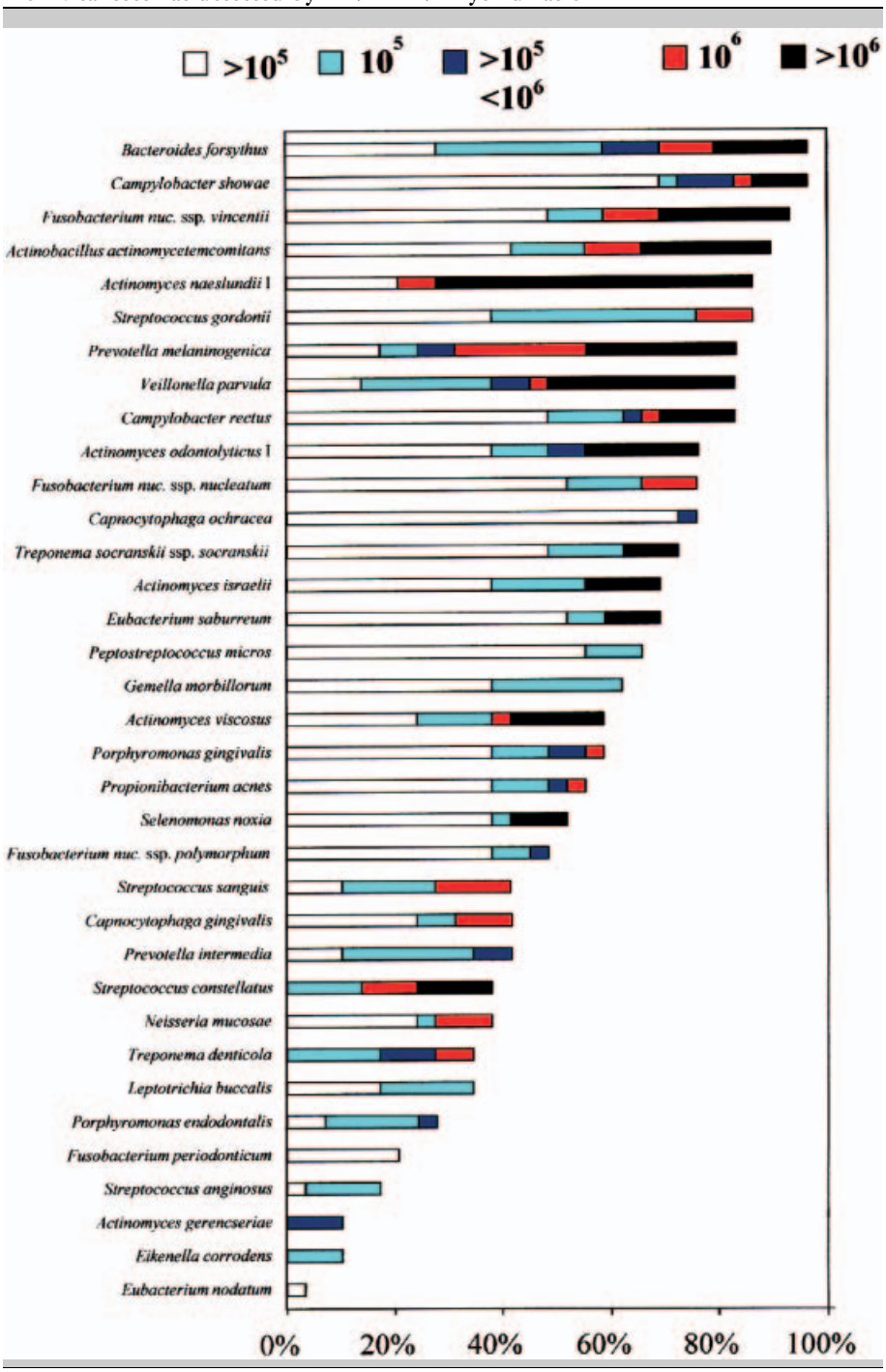
Fig. 1. Necrosis of the dental pulp. (a) Microorganisms have formed biofilms on the root canal wall and in necrotic tissue in the canal. (b) Bacteria on root canal wall and in adjacent dentinal tubules. Brown and Brenn stain.

evaluated for the presence of 40 commonly occurring microorganisms in endodontic and periodontal infections using whole genomic DNA probes and checkerboard DNA–DNA hybridization (18). The DNA of 35 bacteria was detected with a range of 5–31 species (Table 1). Five probes were negative for all root canals. For the sake of comparison, bacterial samples from the root canals of the same teeth were cultured aerobically and anaerobically and 42 microorganisms with the normal range of 0–6 species per canal were recovered. Clearly, there will have to be more uncultivable bacteria than were detected with our 40 probes. Thus, a reasonable estimate at present might be that an infected root canal contains, not less than 10, but rather between 10 and 50 bacterial species. This is in good agreement with the findings by Munson et al. (35), and interestingly, coincides well with the number of bacterial species normally found in a dental plaque sample (15) and at different sites in the oral cavity (13).

Considering the large number of microorganisms in the oral cavity, the microbial composition of the root canal flora will have to vary. However, given the results of the studies mentioned above, it becomes clear that the microbiota of the root canal is very similar to the microbiota of the periodontal pocket *in patients with active periodontal disease* (18). Thus, in our study using the checkerboard DNA–DNA hybridization technique (42), *B. forsythus*, *C. showae*, *F. nucleatum* ssp. *vincentii* and *A. actinomycetemcomitans* were present in more than 90% of the root canals. Other designated periodontopathogens like *P. gingivalis* (60%), *C. rectus* (80%), *P. intermedia* (50%), *Selenomonas noxia* (60%), *P. micros* (70%), *Treponema socranskii* (70%), and *T. denticola* (40%) were commonly present as well. The 'red complex' bacteria, *B. forsythus*, *P. gingivalis* and *T. denticola*, which are known to have a decisive influence on the progression of disease in patients with active marginal periodontitis (18), were jointly present in 40% of the root canals. *Porphyromonas endodontalis* which in combination with other root canal bacteria is known to cause transmissible infection in guinea-pigs (43), was recovered from 30% of the canals. Interestingly, combinations of the same endodontic bacteria, but without *P. endodontalis*, did not cause transmissible infections in the same experiments. Still these and other apparent non-infective bacteria may play an important role in maintaining the root canal infection by providing growth factors for the principal pathogens and by synthesizing and degrading extracellular material in the biofilm (15).

The associations of bacteria in mixed infections are not random. With regard to oral bacteria, this is best known from the studies on dental plaque summarized above, recognizing six rather distinct bacterial groups or complexes (18). Clearly, the root canal is different from the periodontal pocket, and at present it is not known whether the findings in plaque are totally valid for endodontic infections. However, there are also important similarities between the two sites in that the biofilm in both instances is formed by oral bacteria onto hard

Table 1. Bar chart of type and frequency of bacteria from the root canal of 30 asymptomatic, non-vital teeth as detected by DNA–DNA hybridization



The length of the bars indicates the percentage of the canals colonized. From (42). Reproduced with permission from the International Union of Microbiological Societies.

tissues of the tooth, dentin in the root canal and cementum or (mostly) dentin in the periodontal pocket. The recent studies cited above using molecular techniques find the same bacteria in the infected root canal as in the periodontal pocket in patients with active periodontal disease. Also, it is well known that in established root canal infections where Gram-negative species are numerically important, *F. nucleatum* plays a dominating role (44). This species coaggregates with most oral bacteria, including strains of *P. gingivalis*, *T. denticola*,

A. actinomycetemcomitans, *P. intermedia*, *Eubacterium* species, and *Actinomyces* species. The important red and orange complexes contain most of these coaggregating species. It appears, therefore, that in patients with active disease, the microbiotas of the root canal and the periodontal pocket are very similar and much more than what was understood in the era of cultivation studies.

Microorganisms are also frequently found in the root canals of root-filled teeth with apical periodontitis, i.e. in teeth where the disease has persisted or emerged after the treatment is completed (45). However, in these teeth the flora is different from the flora of untreated teeth with root canal infection in that it is heavily dominated by facultative anaerobic species. Enterococci, streptococci and staphylococci are frequently isolated organisms under these conditions (46–49), and fungi, enteric and environmental organisms like *Pseudomonas aeruginosa* have been isolated as well (34, 50–52). Clinically, this is important in that a different antimicrobial regime might be called for in the retreatment of teeth with post-treatment disease than would normally be used in primary treatment of root canal infections.

Extraradicular infection

Bacteria in perapical lesions

As mentioned in the introduction, the traditional opinion has been that in teeth with asymptomatic apical periodontitis, the infecting microorganisms are harbored in the root canal system and in tubules of the root dentin, whereas the periapical lesion is free of bacteria. Probably, the defense systems mobilized by periapical inflammation at first will eliminate the bacteria from the root canal that invade the periapex. However, in long-standing infections with a fairly permanently established microflora in the root canal, the host defenses are less effective, and microbial invasion of the periapical lesion may take place (53). Thus, Happonen et al. (54) by means of immunocytochemical methods have demonstrated the presence of *Actinomyces* spp. and *Propionibacterium propionicum* in asymptomatic periapical lesions refractory to endodontic treatment. Further studies have confirmed that these bacteria may survive in the granulation tissue outside the root canal (55, 56). Our group then demonstrated with anaerobic cultivation that also other anaerobic and facultative anaerobic bacteria are able to survive in periapical inflammatory lesions of asymptomatic teeth (57, 58). *Prevotella* and *Porphyromonas* spp. as well as Gram-positive anaerobic rods and cocci were commonly found as were enteric bacteria and *P. aeruginosa*. Yeasts were occasionally recovered as well.

Biofilm on root surfaces

The cultivation findings were supported by observations in a scanning electron microscopic study (59). Ten root tips were removed during surgical treatment of root-filled teeth with post-treatment disease, five teeth with the diagnosis of asymptomatic apical periodontitis and five teeth with the diagnosis of apical periodontitis with fistula. To the naked eye, the surgically removed root tips appeared denuded. When

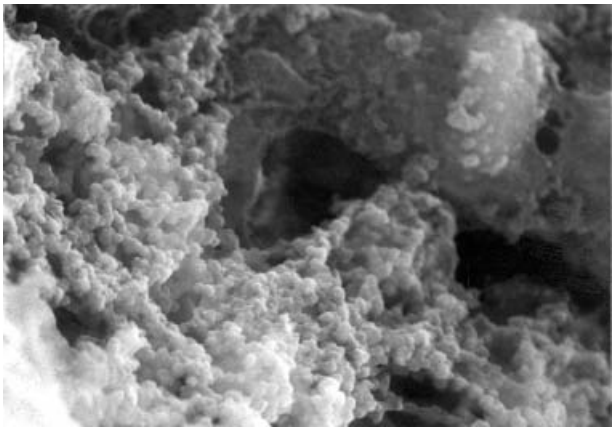


Fig. 2. Scanning electron micrograph of bacterial biofilm on surface of root tip within periapical lesion of root-filled tooth with asymptomatic apical periodontitis. The biofilm is dominated by cocci and short rods in an extracellular matrix. From (59).

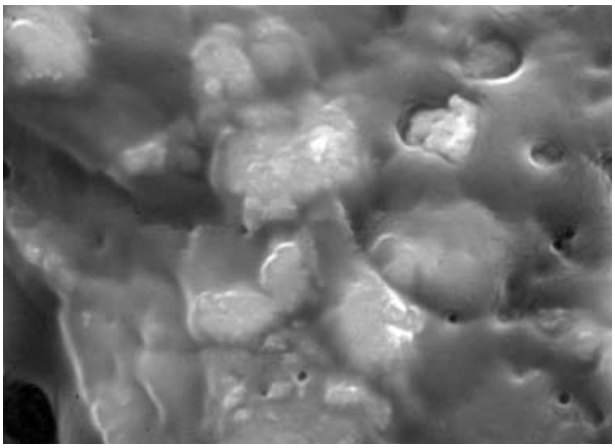


Fig. 3. Scanning electron micrograph of bacterial biofilm adjacent to apical foramen of root-filled tooth with asymptomatic apical periodontitis. Bacterial colonies are recognized within smooth and structureless extracellular material. From (59).

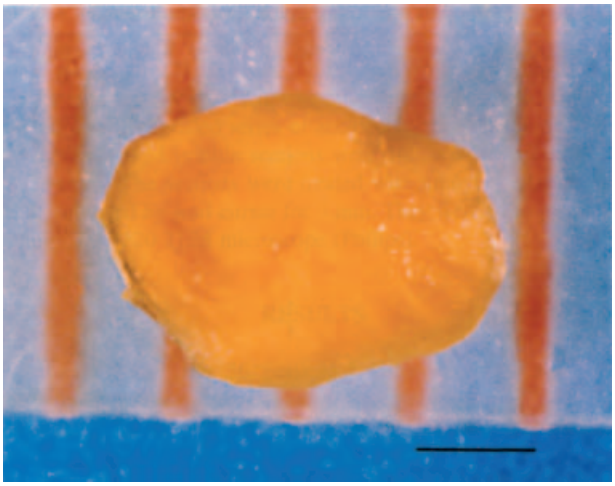


Fig. 4. Sulfur granule from periapical lesion of tooth with refractory apical periodontitis. The granule is soft, yellowish in color and 3–4 mm in diameter. Three additional granules were recovered from the same lesion. From (66). Reproduced with permission from Lippincott, Williams & Wilkins.

examined in the microscope, the root tips were covered by soft tissue with fibers and cells in various stages of degradation. A bacterial plaque or biofilm was seen in areas of the root surfaces between fibers and cells and in crypts and holes (Fig. 2). The biofilm contained varying amounts of an amorphous extracellular material, sometimes making it difficult to distinguish individual bacterial cells. Still the biofilm clearly was dominated by cocci and short rods, but filamentous and fibrillar forms were recognized as well, sometimes with cocci attached to their surfaces. An additional conspicuous finding was a smooth, structureless coating or layer at the apex of the root tip, seemingly adjacent to the apical foramen which was not visible in any of the specimens (Fig. 3). This continuous, smooth layer was seen in nine of 10 specimens and was interpreted as extracellular material of a biofilm, since at higher magnification, a variety of bacterial forms could be recognized in the smooth material. The bacteria had formed colonies and were completely embedded in the structureless film.

Thus, bacteria were observed at the surfaces of all root tips studied. The bacteria were well established and had formed mature biofilms in many areas of the apical root surfaces. As discussed above, a biofilm offers many advantages to its residents like increased resistance to antimicrobial agents (17, 60, 61), an increase in the local concentration of nutrients (61), and an opportunity for genetic exchange (62). In addition, bacteria in biofilms communicate for quorum sensing purposes (63) and produce growth factors across species boundaries (64). By appearance, two different types of biofilms were noted, and it may be speculated that the smooth, structureless, extracellular material of the biofilm outside the apical foramen represents a polysaccharide that acts as a highly effective diffusion barrier, for instance against an antibiotic (61, 65). It is more than likely that the presence of mature biofilms at the root tip is important for maintaining the periapical inflammatory process. Interestingly, no differences were found between the teeth with or without fistulas.

Microbiota of refractory lesions. Sulfur granules

An attempt was made to identify the flora of refractory periapical endodontic lesions, i.e. lesions of teeth with apical periodontitis where the local treatment, including the antibacterial treatment of the tooth, was judged

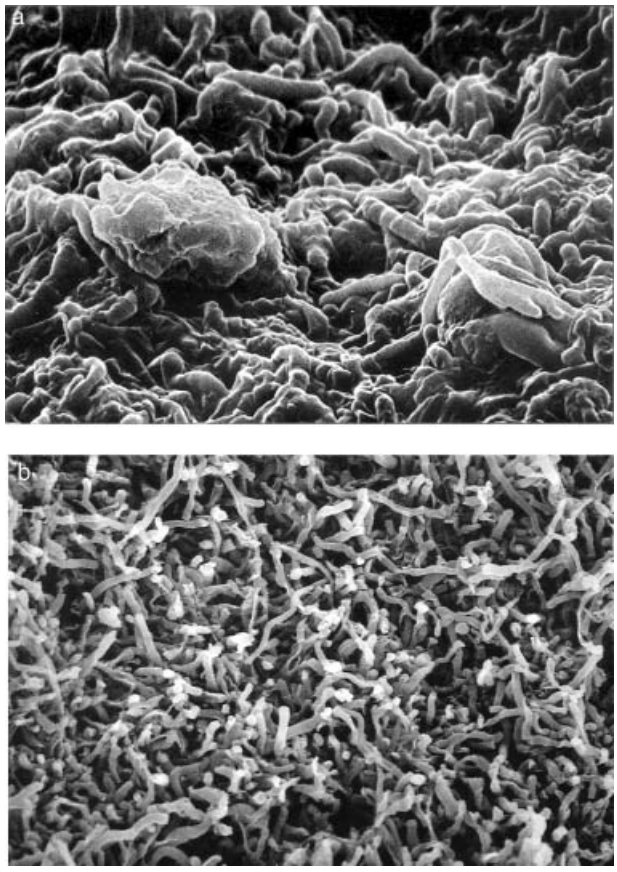


Fig. 5. Scanning electron micrograph of surface area of sulfur granule seen in Fig. 4. Microorganisms that are tightly packed and glued together make up the outer boundary of the granule. Two macrophages are seen, seemingly engulfing bacteria. From (66). Reproduced with permission from Lippincott, Williams & Wilkins.

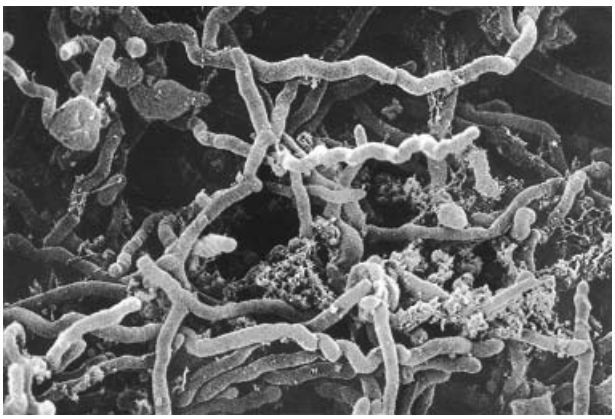


Fig. 6. Scanning electron micrograph of cut surface of sulfur granule seen in Fig. 4. The granule consists of an abundance of bacteria in a biofilm setting. Rod-like organisms are prominent and spiral-formed bacteria are seen (arrow). From (66). Reproduced with permission from Lippincott, Williams & Wilkins.

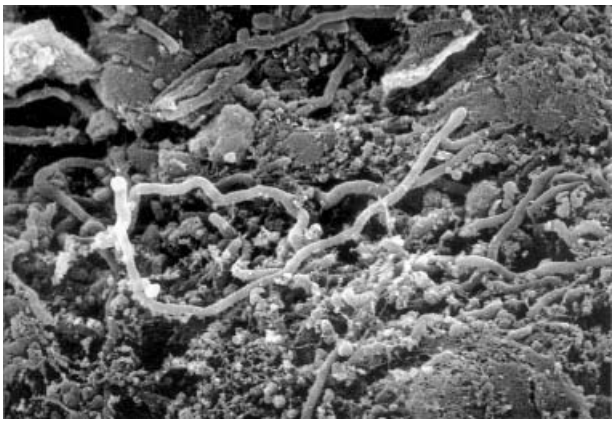


Fig. 7. Scanning electron micrographs of cut surfaces of sulfur granules. (a) In addition to rod-like and spiral-formed bacteria, an amorphous material is seen between the cells. (b) Microorganisms and large amounts of partly calcified extracellular material are present. From (66). Reproduced with permission from Lippincott, Williams & Wilkins.

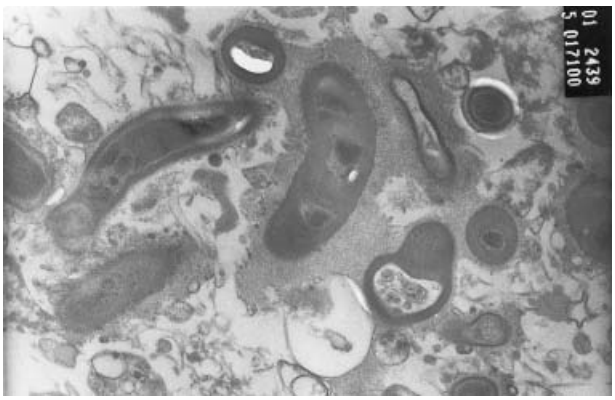


Fig. 8. Transmission electron micrograph from sulfur granule. Gram-positive bacteria are seen. An extracellular material is enveloping several of the bacteria. From (66). Reproduced with permission from Lippincott, Williams & Wilkins.

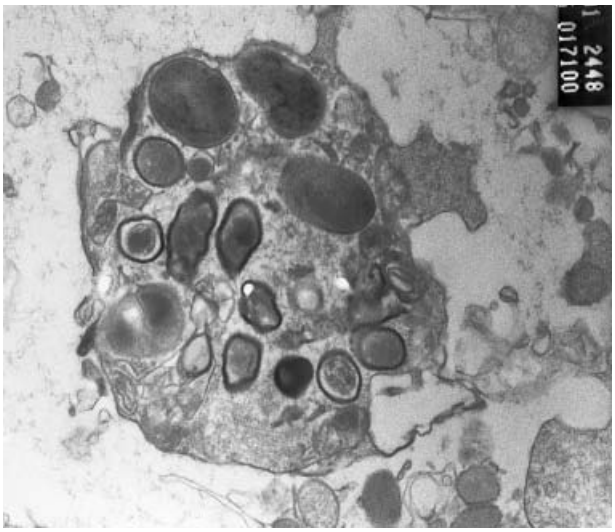


Fig. 9. Transmission electron micrograph from sulfur granule. A macrophage with a variety of engulfed bacteria is seen. From (66). Reproduced with permission from Lippincott, Williams & Wilkins.

to be optimal, but did not affect healing of the periapical lesions as evaluated clinically and radiographically over time (66). The periapical flora of these teeth was clearly different from the flora that normally responds to treatment. It was highly dominated (80%) by Gram-positive organisms and 75% of the refractory lesions contained *Streptococcus*, *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas*, *Enterococcus*, *Enterobacter* or *Candida* species. Interestingly, the flora of the refractory lesions was very similar to the root canal flora of root-filled teeth undergoing retreatment where Gram-positive organisms dominate (5, 46, 47, 49, 51, 67). Of clinical interest was also the fact that the organisms persisted in patients who had taken antibiotics systemically before sampling.

A conspicuous finding in this study was the presence of so called sulfur granules in the periapical granulation tissue in nine of 36 lesions (25%) (Fig. 4). The granules had a diameter of up to 3–4 mm, and in the scanning electron microscope it was seen that they were tightly packed with microorganisms (Figs 5 and 6). Rod-like bacteria were prominent (Fig. 6) and spirochete-like organisms were commonly seen (Figs 6 and 7). In many granules an amorphous extracellular material was

present between the cells, giving the granules the appearance of a biofilm (Fig. 7). Some granules felt hard to touch and the extracellular material then appeared partly mineralized (Fig. 7b). In the transmission electron microscope an extracellular material was seen as well, often enveloping several bacterial cells (Fig. 8). Outer membrane vesicles were observed in close contact with bacterial cell walls and were also spread out between cells. Macrophages were present, some of them with a number of engulfed bacteria (Figs 2 and 9). Granules from seven of the nine patients yielded bacteria by culture, and in the culture-positive granules, three to six microbiotic species were detected. *Actinomyces israelii*, *A. viscosus*, *A. meyeri* and *A. naeslundii* were cultured from five of the seven granules positive for growth. In all these granules, microbes other than *Actinomyces* were recovered as well: *Propionibacterium acnes*, *P. propionicum*, *Peptostreptococcus prevotii*, *Gemella morbillorum*, *Clostridium sordelli*, *C. bifermentas*, *Leptotrichia buccalis*, *Staphylococcus chromogenes*, *S. epidermidis*, *Vibrio metchnikovii*, and *Streptococcus* species. In the two granules that did not exhibit *Actinomyces* species, *Aerococcus viridans*, *Bacteroides ureolyticus*, *G. morbillorum*, *Capnocytophaga* species, *P. aeruginosa*, *S. warnerii*, and *S. oralis* were cultured. In the two patients where no bacteria were detected by culture of the sulfur granules, cultivation of the periapical granuloma showed *Sphingomonas paucimobilis* and *S. warnerii* in one patient and *Stenotrophomonas maltophilia* in the other.

It may appear that *Actinomyces* species have a special role also in the development of the sulfur granules in periapical granulomas. As in dental plaque, these organisms apparently are pioneer bacteria that build scaffolds so that other bacteria are attracted and may establish themselves at the site. A biofilm then develops, in these instances in the form of granules in the tissue. The occurrence of strict anaerobic bacteria such as spirochetes in the sulfur granules suggests that the granules may contain microenvironments with a low reduction–oxidation potential (68). Findings from studies using miniature electrodes have shown that oxygen can be completely consumed in the surface layers of a biofilm, leading to anaerobic niches in its deeper layers (17). In sulfur granules from cervicofacial and thoracic actinomycosis, *A. israelii* is recovered in about 90% of the cases (69). We found *A. israelii* as well, but in addition three other *Actinomyces* species were recovered from the granules of the periapical lesions. Also, four sulfur granules did not show the presence of any *Actinomyces* species. However, there is no evidence that sulfur granules may form without the participation of these organisms. Although great care was taken, it is conceivable that they were not recovered by our cultivation procedures, or simply, they may have died in the biofilm.

In teeth where endodontic therapy has been compromized or grossly inadequate, for instance after repeated but inadequate antibiotic treatment, multiple openings and closings of the root canal, or inadequate periapical surgical treatment, the presence of enteric and environmental bacteria and yeast is especially conspicuous (58). In such instances organisms like *Escherichia coli*, *Bacteroides fragilis*, *P. aeruginosa*, *Enterobacter*, *Clostridium*, *Proteus* and *Klepsiella* species and yeast are recovered. The presence of these and other mainly nonoral microorganisms suggests that blood-born infection of the periapical lesion may take place.

Are microbiological samples from periapical lesions unavoidably contaminated?

A number of studies using cultivation techniques now had become available, all supporting our findings (70–72). Still the results met with skepticism, and contamination during sampling was regarded as a likely reason for the positive cultures. The question of whether bacterial samples from periapical lesions routinely are contaminated by the indigenous oral flora was then addressed by our group in a methodological study (73). Because of the importance of this study, it will be cited here in some detail.

Thirty patients referred for surgical treatment of root-filled teeth with asymptomatic apical periodontitis were devided into two groups, Group 1 and Group 2, each containing 15 patients. The patients were treated with apicoectomies, and in Group 1, a marginal incision was made to expose the periapical lesion, and in Group 2, a submarginal incision was made. Before incision, the gingiva and mucosa were washed with 0.2% chlorhexidine gluconate. Bacterial samples were taken from the mucosa before reflecting the flap, and from the exposed alveolar bone and the periapical lesion immediately after. All samples were cultured anaerobically on all-purpose and selective media.

In Group 1, 12 of 15 patients (80%) yielded bacteria from their mucosal samples despite the chlorhexidine wash (Table 2). Bacterial growth was observed in all samples from the alveolar bone (100%) while the

Table 2. Microorganisms isolated from mucosa, alveolar bone and periapical lesion in 15 patients following *marginal* surgical incision

N	Mucosa	Alveolar bone	Periapical lesion
1	*Streptococcus oralis*	*Capnocytophaga* sp.	No growth
		Actinomyces naeslundii	
		Actinomyces viscosus	
2	No growth	*Capnocytophaga* sp.	*Actinomyces naeslundii*
		Fusobacterium nucleatum	*Actinomyces* sp.
			Streptococcus oralis
			Streptococcus bovis
3	*Streptococcus parasanguis*	*Staphylococcus capitis*	*Staphylococcus chromogenes*
	Actinomyces naeslandii	***Actinomyces naeslundii***	
	Actinomyces israelii	*Vibrio metschnikovii*	
	Actinomyces viscosus		
4	*Gemella morbillorum*	*Leuconostoc* sp.	No growth
	Prevotella oralis	*Actinomyces viscosus*	
	Streptococcus parasanguis	*Streptococcus oralis*	
	Streptococcus mitis	***Streptococcus mitis***	
5	No growth	*Streptococcus intermedius*	*Staphylococcus epidermidis*
			Peptostreptococcus sp.
			Fusobacterium nucleatum
6	*Streptococcus oralis*	*Clostridium tyrobutyricum*	No growth
		Actinomyces meyeri	
		Fusobacterium nucleatum	
		Eubacterium limosum	
		Prevotella intermedia	
7	No growth	*Staphylococcus hominis*	*Propionibacterium acnes*
		Streptococcus sp.	*Staphylococcus epidermidis*
			Staphylococcus capitis
			Clostridium difficile
			Porphyromonas endodontalis
			Fusobacterium nucleatum
8	***Streptococcus mitis***	***Streptococcus mitis***	*Fusobacterium nucleatum*
		Streptococcus oralis	*Peptostreptococcus magnus*
		Clostridium tyrobutyricum	*Actinomyces* sp.
		Staphylococcus warneri	*Porphyromonas endodontalis*
9	*Streptococcus oralis*	*Staphylococcus capitis*	*Fusobacterium nucleatum*
		Gemella morbillorum	*Actinomyces naeslundii*
		Gemella haemolysans	*Clostridium tyrobutyricum*
		Eubacterium lentum	*Streptococcus mitis*
		Micrococcus luteus	*Peptostreptococcus prevotii*

Table 2. Continued

N	Mucosa	Alveolar bone	Periapical lesion
		Actinomyces odontolyticus	***Actinomyces odontolyticus***
			Capnocytophaga sp.
10	*Streptococcus sanguis*	*Streptococcus mitis*	No growth
	Streptococcus sp.	*Actinomyces naeslundii*	
11	*Streptococcus sanguis*	*Capnocytophaga* sp.	*Staphylococcus epidermidis*
	Actinomyces naeslundii		
	Streptococcus oralis		
12	*Streptococcus sanguis*	*Capnocytophaga* sp.	*Eubacterium lentum*
		Streptococcus parasanguis	*Prevotella intermedia*
		Staphylococcus sp.	*Peptostreptococcus* sp.
		Staphylococcus epidermidis	
		Propionibacterium acnes	
13	*Streptococcus oralis*	*Bacteroides ureolyticus*	*Gemella morbillorum*
	Streptococcus mitis	*Staphylococcus epidermidis*	*Peptostreptococcus magnus*
		***Veillonella* sp.**	***Veillonella* sp.**
			Fusobacterium nucleatum
14	*Streptococcus oralis*	*Eubacterium lentum*	*Streptococcus constellatus*
		Gemella morbillorum	
		Prevotella intermedia	
		Actinomyces israelii	
		Actinomyces odontolyticus	
15	*Actinomyces viscosus*	*Bacteroides ureolyticus*	*Staphylococcus xylosus*
	Streptococcus sanguis	*Streptococcus* sp.	

Identical species strains in the same patient are given in bold. From (73).

periapical lesions gave bacterial growth in 11 of 15 patients (73%). Microorganisms cultivated from the mucosa differed in all instances from the microorganisms recovered from the periapical lesions. In three patients, identical species were cultivated from the mucosa and the exposed bone within the same patient; however, the biochemical/enzymatic profiles of these species were different. In two patients, *A. odontolyticus* and *Veillonella* sp. were cultivated from both the exposed alveolar bone and the periapical lesion and the biochemical/enzymatic profiles of these two species strains were identical (Table 2). In Group 2, bacteria were cultured from the mucosa in 11 of 15 patients (73%) (Table 3). Three samples from the alveolar bone (20%) and 10 from the periapical lesions (67%) gave positive growth. The predominant cultivable bacteria were anaerobic. Again, the microorganisms cultivated from the mucosa differed in all instances from the bacterial species recovered from the exposed bone and the periapical lesions. In one patient, *P. acnes* was isolated from both the exposed bone and the periapical lesion. The biochemical and enzymatic profiles of these strains were different. Thus, only two microorganisms, *A. odontolyticus* in one patient and *Veillonella* sp. in a second patient, were cultivated from both the exposed alveolar bone and the periapical lesion and had identical biochemical profiles (Table 3).

Contamination of the periapical lesion during reflection of the flap and the microbiological sampling procedures was, therefore, a very minor or rather a

Table 3. Microorganisms isolated from mucosa, alveolar bone and periapical lesion in 15 patients following *submarginal* surgical incision

N	Mucosa	Alveolar bone	Periapical lesion
1	*Streptococcus oralis*	No growth	*Staphylococcus capitis*
			Staphylococcus epidermidis
			Staphylococcus hominis
			Staphylococcus xylosus
			Streptococcus constellatus
			Pseudomonas sp.
			Clostridium difficile
			Bacillus cereus
			Propionibacterium acnes
			Enterococcus sp.
2	No growth	No growth	*Propionibacterium acnes*
3	*Gemella morbillorum*	No growth	No growth
	Actinomyces viscosus		
	Streptococcus sp.		
4	No growth	No growth	No growth
5	*Staphylococcus warneri*	No growth	*Clostridium bifermentans*
	Actinomyces viscosus		*Staphylococcus epidermidis*
	Veillonella sp.		*Propionibacterium propionicum*
			Propionibacterium acnes
6	*Streptococcus mitis*	***Propionibacterium acnes***	***Propionibacterium acnes***
		Peptostreptococcus magnus	*Staphylococcus epidermidis*
			Bacteroides ureolyticus
7	*Actinomyces viscosus*	*Capnocytophaga* sp.	*Prevotella oralis*
	Streptococcus mitis		*Actinomyces naeslundii*
8	*Actinomyces israelii*	*Veillonella* sp.	*Actinomyces naeslundii*
9	*Actinomyces viscosus*	No growth	No growth
	Actinomyces israelii		
	Veillonella sp.		
10	*Anaerobiospirillum succiniproducens*	No growth	No growth
	Propionibacterium granulosum		
	Staphylococcus sp.		
11	No growth	No growth	No growth
12	*Streptococcus mitis*	No growth	*Propionibacterium granulosum*
			Staphylococcus epidermidis
13	No growth	No growth	*Porphyromonas endodontalis*
			Bacteroides ureolyticus
			Fusobacterium nucleatum
			Clostridium sp.
14	*Veillonella* sp.	No growth	*Staphylococcus* sp.
	Actinomyces viscosus		*Streptococcus anginosus*
	Streptococcus mitis		*Prevotella intermedia*
			Prevotella denticola
			Peptostreptococcus micros
15	*Clostridium bifermentans*	No growth	*Bacillus* sp.
	Streptococcus mitis		*Gemella haemolysans*

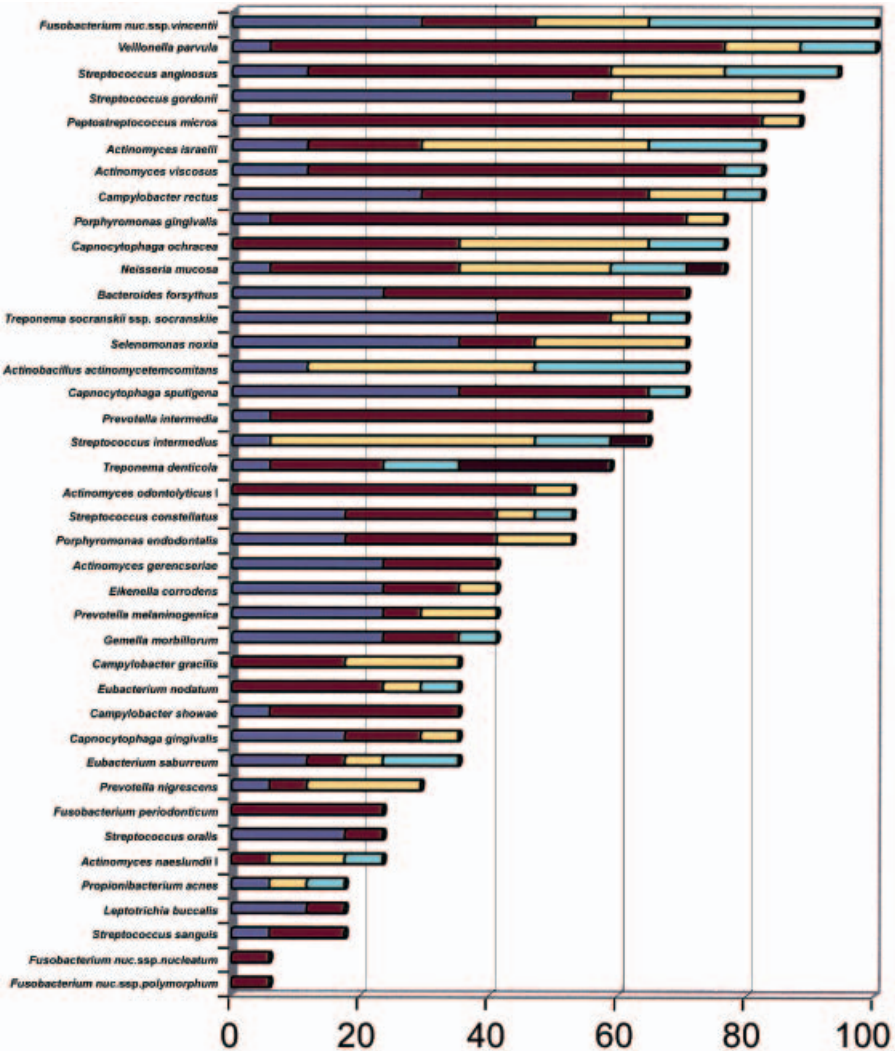
Identical species strains in the same patient are given in bold. From (73).

non-existing problem in the above study. Still, one must be aware that a translocation of bacteria from the sulcus into the surgical site may occur when a marginal incision is made. The findings support the results of our previous cultivation studies (57, 58) in that microorganisms were recovered from the periapical lesions in 21 of 30 patients. By cultivation, the lesions yielded between one and 10 bacterial species and about two-thirds were anaerobes.

Checkerboard DNA–DNA hybridization

As discussed above, an inherent problem with anaerobic cultivation techniques is that fastidious species or species present below the detection limit for cultivation will not be identified. Sixty percent of the oral flora has been found to be uncultivable (13). As with the root canal flora, there is no study as of yet on the flora of periapical endodontic lesions like the study of Paster et al. (14) on the flora of the periodontal pocket. However, a few studies on extraradicular endodontic infections using culture-independent, molecular techniques have been reported (74–76). The findings of these studies held together with the electron microscopic findings summarized above have added greatly to our understanding of the complexity of the extraradicular flora (59, 66). Our group has performed a study using the checkerboard DNA–DNA hybrid-

Table 4. Bar chart of type and frequency of bacteria as detected with DNA–DNA hybridization in 17 periapical lesions of asymptomatic teeth following submarginal incision and sampling from lesions



The length of the bars indicates the percentage of lesions colonized. From (75).

ization technique developed by Socransky et al. (19) (75).

Thirty-four patients referred for surgical treatment of root-filled teeth with asymptomatic apical periodontitis were devided into two groups, Group 1 and Group 2, each containing 17 patients. The patients were treated with apicoectomies, and in Group 1, a marginal incision was made to expose the periapical lesion, and in Group 2, a submarginal incision was made. The treatment procedures outlined in the methodological study summarized above (73) were carefully followed. The 40 DNA probes used by Socransky et al. (18) in their studies of the periodontal flora were used in our study as well. Bacterial DNA was identified in all samples from the two groups. Sterile transport medium without sample gave no hybridization signals. The number of species per lesion varied between 26 and 39 (mean 33.7 ± 3.3) in Group 1 (marginal incision) and between 11and 34 (mean 21.3 ± 6.3) in Group 2 (submarginal incision) (Table 4), confirming the results of our methodological study (73) that an apparent circulatory translocation of bacteria to the periapical lesion may occur when the sulcus is included in the flap.

As in the root canal, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Campylobacter* and *Treponema* species were commonly detected. The red complex bacteria *B. forsythus*, *P. gingivalis* and *T. denticola* were present in 70% of the lesions, and *P. endodontalis* was present in 50%. Of Gram-positive anaerobes, *Actinomyces*, *Propionibacterium*, *Peptostreptococcus* and *Eubacterium* species were frequently detected, and facultative *Actinomyces*, *Streptococcus*, *Enterococcus* and *Staphylococcus* species were present as well. Our findings were fully confirmed by the results of a parallel checkerboard DNA–DNA hybridization study carried out by a different group using the same 40 DNA probes (74). The probe-detected bacteria are well known from studies on periodontal infection (18), root canal infection (34, 36, 42) and periapical infection (57, 58, 66, 73), and it is again confirmed that in patients with active disease, the microbiotas of endodontic and periodontal infections are very similar.

Fluorescence in situ hybridization

The visualization of mature bacterial biofilm on the external surfaces of root tips and in the form of sulfur granules in periapical granulomas has aided us in understanding the nature of extraradicular infection. Recently, a fluorescence *in situ* hybridization (FISH) method has been developed whereby bacteria may be detected and even identified in the tissue of their natural environment (for a review, see (77)). In 3 μm thick formalin-fixed, plastic-embedded tissue sections excellent conservation and visualization of bacteria has been achieved (77, 78). Our group has carried out a study using the FISH technique to visualize and, as much as possible with the probes available, identify bacteria directly within periapical lesions of asymptomatic root-filled teeth (76). The sections from the lesions were examined in a confocal laser scanning microscope that has become a valuable tool for obtaining high-resolution images and three-dimensional reconstructions of a variety of biological samples (78–81). A probe, EUB 338, which is specific for the domain *Bacteria* was used to visualize the entire bacterial population in the specimens (82). In addition a number of group-specific, genus-specific and species-specific probes were applied. All probes have been deposited in ProbeBase, an online resource for rRNA-targeted oligonucleotide probes (83). In order to assess the specificity of the probes, control slides with known, fixed bacterial cell cultures were included in every hybridization experiment with tissue sections.

With the universal probe EUB 338, bacteria were observed in 20 of 39 periapical lesions (Fig. 10). The bacteria were present in localized areas of the lesions, whereas large areas appeared to be free of bacteria (Fig. 11). The observed bacteria were always located within the tissue and no bacteria were seen at the borders of the lesions. Hybridization with the EUB 338 probe showed a variety of different bacterial morphotypes, cocci, rods, spiral and spindle shaped. A distinct morphotype of a large curved bacterium was seen in several sections (Fig. 12), resembling the spirochete-like organism of 140 μm observed in the root canal (84). Often organisms with different morphologies were seen to coaggregate forming small ecosystems in the tissue (Fig. 13). Monocolonies of cocci were also seen, and the probe for the genus *Streptococcus* reacted specifically in three different lesions. One of these colonies was a homogenous colony with *Streptococcus* spp. whereas two colonies were mixed with *Streptococcus* and other cocci detected with the EUB 338 probe (Fig. 14). Rods and especially spirochete-like organisms were present between cells and fibers in the tissue. The TRE 1 probe reacted specifically in one lesion,

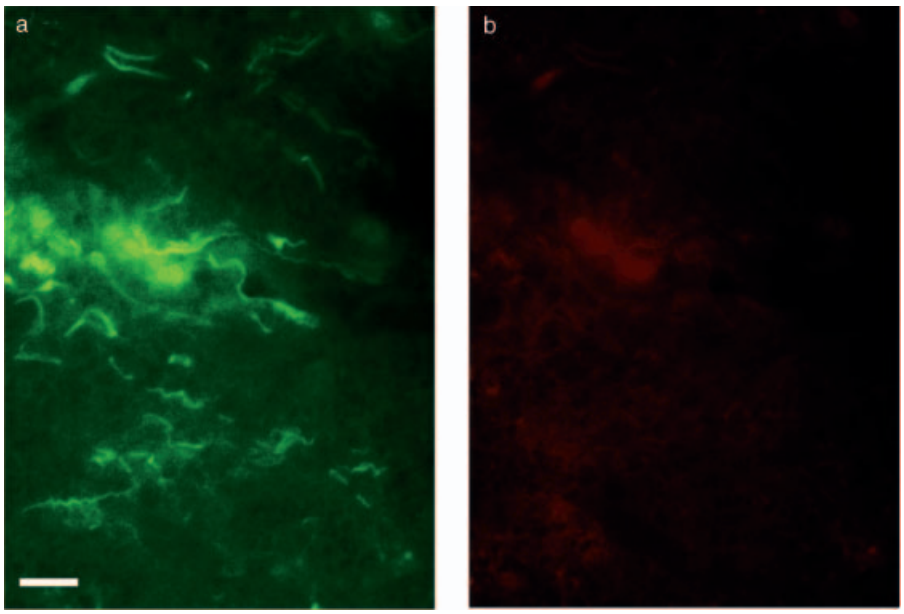
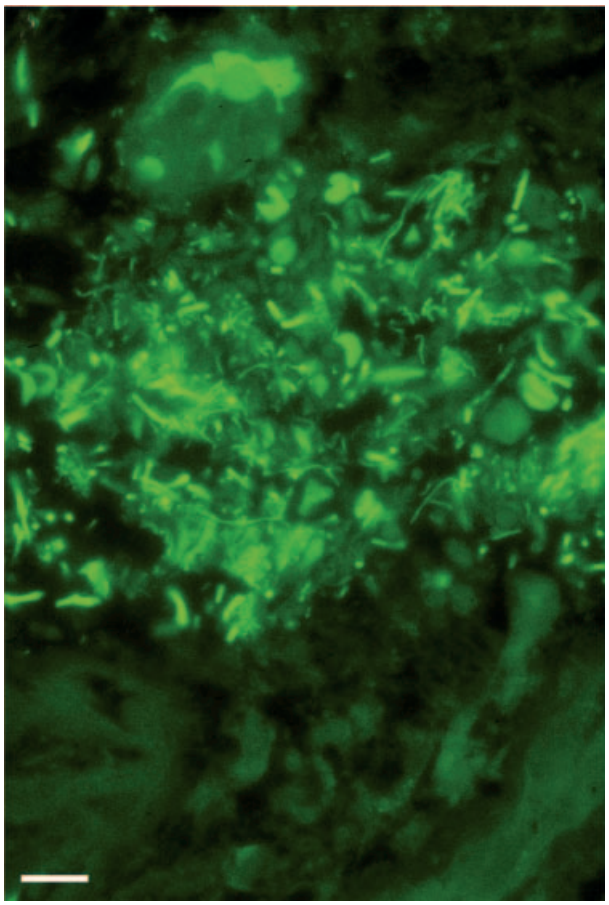


Fig. 10. (a) Fluorescence *in situ* hybridization of section from periapical granuloma using the probe EUB 338 which is specific for the domain *Bacteria*. A number of bacteria of different morphotypes are visualized in the tissue. Bar, 10 μm. (b) The same section as in (a) exposed to complimentary control probe, NON EUB-Cy3 (red). Only slight background fluorescence of the tissue is seen. From (76). Reproduced with permission from SGM.



indicating the presence of *Treponema vincentii* and/or *T. vincentii*-related organisms, indicating a role for these organisms in endodontic infections. Otherwise no specific signals could be obtained with the treponeme-specific probes, emphasizing the considerable genetic diversity of this group of organisms (85,86). Hybridization with probes for *B. forsythus*, *P. gingivalis* and *P. intermedia* gave positive signals in three different lesions (Fig. 15). This finding is consistent with the results of previous studies on endodontic infections using molecular techniques (30, 74, 75, 87).

The FISH technique turned out to be a powerful method for visualization of mixed populations of microorganisms in their natural environment. The signal intensity of the bacteria was very bright, indicating a high amount of rRNA in the cells. This is evidence of physiologic activity at the time of fixation (88, 89), and also made it easy to recognize and distinguish the various morphotypes present in the lesions. The method was additionally improved by

Fig. 11. Fluorescence *in situ* hybridization using the *Bacteria*-specific probe EUB 338 on section from periapical granuloma. A large number of bacteria of different morphotypes is present in limited area of the tissue. Bar, 10 μm. From (76). Reproduced with permission from SGM.

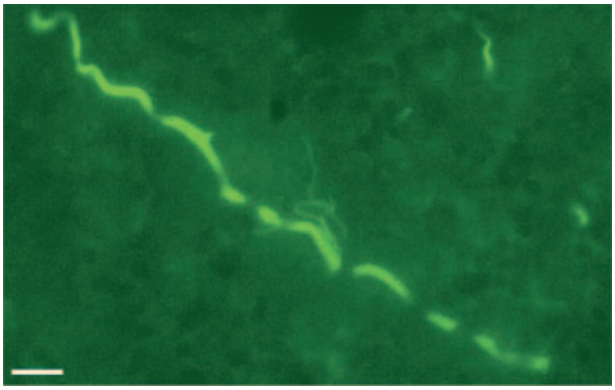


Fig. 12. Fluorescence *in situ* hybridization using the *Bacteria*-specific probe EUB 338 on section from periapical granuloma. A distinct morphotype of large curved bacterium reminding of large spirochete-like organism (140 μm) previously observed in the root canal (13) is seen in the tissue. Bar, 10 μm. From (76). Reproduced with permission from SGM.

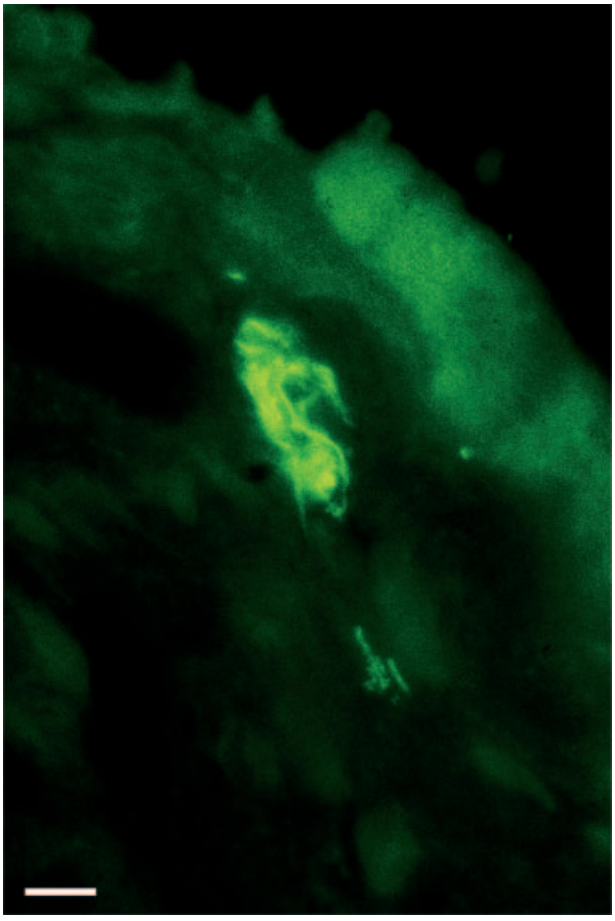


Fig. 13. Fluorescence *in situ* hybridization using the *Bacteria*-specific probe EUB 338 on section from periapical granuloma. A microcolony of coaggregating bacteria of different morphotypes is seen in the tissue. Bar, 10 μm. From (76). Reproduced with permission from SGM.

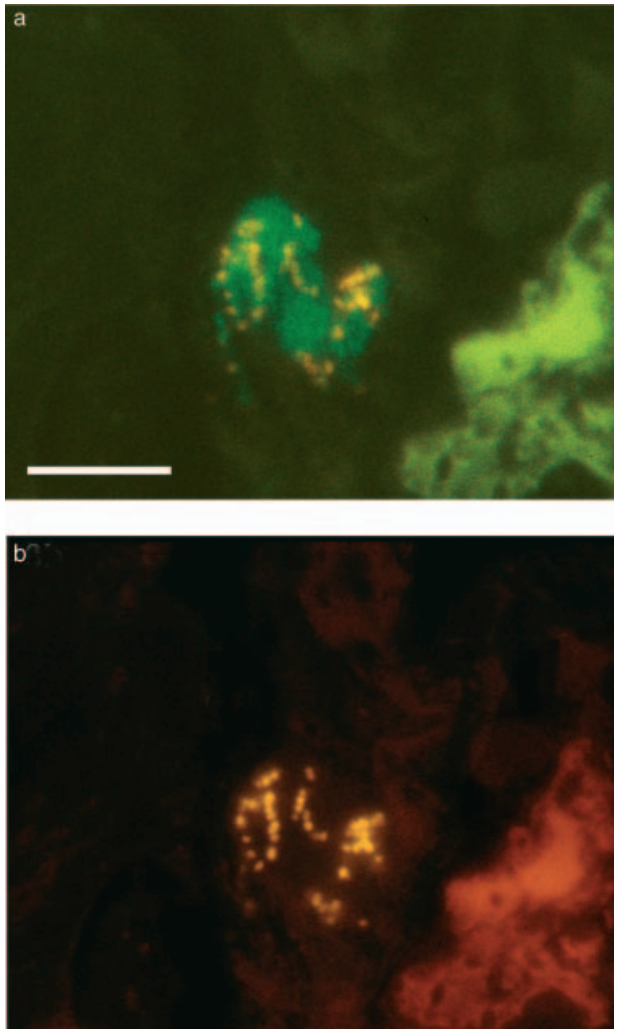


Fig. 14. Fluorescence *in situ* hybridization on section from periapical granuloma. (a) Simultaneous hybridization with the *Bacteria*-specific probe EUB 338 and the genus-specific probe for *Streptococcus* shows a mixed colony of streptococci (orange) and other cocci (green). Bar, 10 μm. (b) The same section as in (a) seen with the *Streptococcus*-specific probe showing only the streptococcal cells. From (76). Reproduced with permission from SGM.

examining the specimens in the confocal laser scanning microscope. This microscope allowed three-dimensional observation and thereby exact localization and visualization of the spatial distribution of the bacteria in different layers of the sections as well as between and around components of the tissue. With the development of additional specific probes, the method may become even more important for the visualization and especially identification of the microorganisms of extraradicular infections.

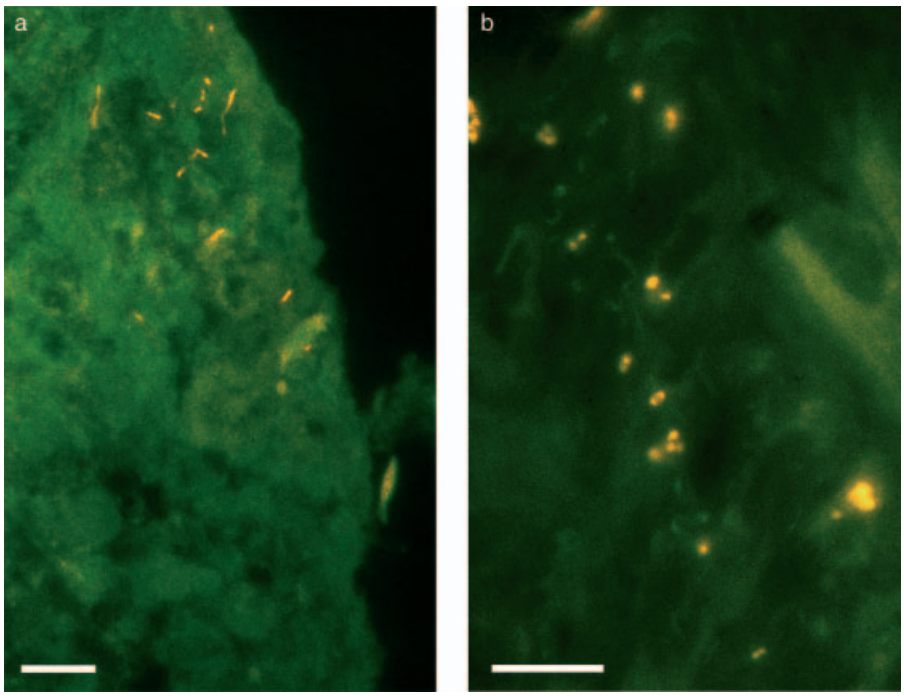


Fig. 15. Fluorescence *in situ* hybridization on section from periapical granuloma. (a) Simultaneous hybridization with the *Bacteria*-specific probe EUB 338 and the species-specific probe for *Bacteroides forsythus* demonstrates the presence of *B. forsythus* cells (orange) in an area of the tissue. Bar, 10 μm. (b) Simultaneous hybridization with the *Bacteria*-specific probe EUB 338 and the species-specific probe for *Porphyromonas gingivalis* shows the presence of *P. gingivalis* (orange) in an area of the tissue. Bar, 10 μm. From (76). Reproduced with permission from SGM.

Concluding remarks

More than 700 different bacterial species, of which over 50% have not yet been cultivated, have been detected in the oral cavity. The breadth and diversity of the oral flora in health and disease are continuously being investigated, and with the molecular methods that have become available in recent years, great progress has been made in understanding the nature of oral infections and assessing the organisms associated with disease. With regard to endodontic infections, to a great extent we are still in the era of bacterial cultivation, although a few groups have taken up molecular methods in their work. Thus, a new understanding of endodontic infections is slowly evolving due to the results of molecular and electron microscopic studies. Many more bacteria are found with hybridization studies than with cultivation, and interestingly, the flora of endodontic infections appears to be very similar to the flora of the periodontal pocket in patients with active periodontal disease. Even the numbers of infecting organisms in mature periodontal and endodontic biofilms are similar in that about the same number of bacterial species is found in infected root canals and in periapical lesions as in plaque samples from patients with active periodontal disease.

Extraradicular infection is a common occurrence in asymptomatic teeth with apical periodontitis. This has now been verified with bacterial cultivation, checkerboard DNA–DNA hybridization, FISH and electron microscopic demonstration of mature bacterial biofilm at the surfaces of root tips and in the form of granules inside the lesions. It has been difficult to gain acceptance for these new findings in that the periapical granuloma has been regarded as a very hostile environment for bacterial growth and survival (49, 90, 91). This has been an unfortunate misunderstanding. Most of the microorganisms recovered from the periapical lesions are known to adapt over time to live in many different environments, and their numbers, rapid fluctuations and amenability to genetic change give them effective tools for adaptation (92). Also, bacteria have a variety of strategies to avoid engulfment and degradation by phagocytes, facilitating proliferation and spread in host tissues (93). Moreover, they have a number of strategies for overcoming host innate and adaptive immune responses (53), and in fact can establish life-long chronic infections in their hosts

(94, 95). Also, evidence that recently has become available suggests the involvement of herpes viruses in the etiopathogenesis of apical periodontitis (96, 97). The viruses may cause the release of tissue destructive cytokines and the initiation of cytotoxic and immunopathologic events. The immune impairment and tissue changes resulting from the herpes virus infection may then aid bacteria in invading and surviving in the periapical lesion. Thus, the periapical lesion may not be as hostile to bacteria as many have thought (98), and as clinicians we have to understand and accept that an infection might not be limited to the root canal, but include the radiolucent periapical lesion as well.

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