

Microbiota of Periapical Lesions Refractory to Endodontic Therapy

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The periapical microbiota of 36 teeth with refractory apical periodontitis was investigated. None of the teeth had responded to conventional endodontic or long-term (> 6 months), calcium-hydroxide treatment. Eight patients had received antibiotics systemically. After anaerobic culture, a total of 148 microbial strains were detected among 67 microbial species. One of the 36 lesions was culture-negative. Approximately half (51.0%) of the bacterial strains were anaerobic. Gram-positive species constituted 79.5% of the flora. Facultative organisms, such as *Staphylococcus*, *Enterococcus*, *Enterobacter*, *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas*, *Bacillus*, or *Candida* species were recovered from 27 of the lesions (75%). Sulfur granules were found in 9 lesions (25%). In these granules *Actinomyces israelii*, *A. viscosus*, *A. naeslundii*, and *A. meyeri* were identified. Other bacterial species, both Gram-positive and Gram-negative, were detected in the granules as well. Two sulfur granules did not contain *Actinomyces*. Scanning electron microscopy demonstrated rod- and spirochete-like cells in the granules, and transmission electron microscopy revealed organisms with copious amounts of extracellular material. Outer membrane vesicles were also seen. Some of the granules were calcified. This study demonstrated a wide variety of microorganisms, particularly Gram-positive ones, in the periapical lesions of teeth with refractory apical periodontitis.

It has been shown that microorganisms from the root canal of the tooth can invade periapical endodontic lesions of asymptomatic teeth and establish an infectious disease process extraradicular (1–5). The infection is usually polymicrobial, comprising anaerobic and facultative bacteria known from studies on the microflora of the root canal (6) and the periodontal pocket (7). In most instances, endodontic infections respond well to conventional root canal therapy. When the root canal is properly instrumented, dis-

infected, and obturated, follow-up studies show a success rate of teeth with apical periodontitis of 80% to 90% (8, 9). Still, this means that 10% to 20% of periapical lesions do not respond to local treatment of the tooth. It is not known whether the lack of response of refractory periapical lesions is due to the inaccessibility of the extraradicular microorganisms or to the presence of a microbiota, which is different from that normally found in endodontic infections. It has been shown that extraradicular bacteria may form colonies or aggregates where they are surrounded by extracellular material (10). Sometimes the bacterial aggregates have the form of granules with diameters up to 3 to 4 mm. These granules often have a bright, yellow color, and because of this, in older literature are referred to as sulfur granules (11). There are also indications that the flora of refractory lesions may be atypical. Thus, the root canal flora of root-filled teeth, where the treatment has failed, has been shown to differ markedly from the flora of infected root canals of untreated teeth (12–14).

The aim of this study was to recover and 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 to be optimal but without effect on the periapical lesions as evaluated clinically and radiographically over time.

MATERIALS AND METHODS

Endodontic Treatment

This study comprised 36 patients (20 men) with a mean age of 50 yr (± 17). The patients were receiving treatment at the Graduate Endodontic Clinic, Dental Faculty, University of Oslo, Oslo, Norway. The treatment was performed within a period of 6 yr (from May 1993 until February 1999). Deep periodontal pockets or endo-perio-like lesions in conjunction with the teeth were not present.

The patients had received the diagnosis of asymptomatic apical periodontitis (26 patients) or apical periodontitis with fistula (10 patients) and were undergoing the standard nonsurgical, endodontic treatment of the department, i.e. complete instrumentation of the root canal in the first visit and a period of 2 to 3 weeks with calcium hydroxide in the root canal, before being examined again at a second visit. Common for the patients included in this study was that the local endodontic treatment was ineffective, i.e. peri-

apical exudation persisted or fistulas did not close. An attempt was then made to improve the local antimicrobial treatment by using long-term calcium hydroxide therapy (15). After a thorough clinical and radiographic examination of the unresponsive teeth, making sure that there were no untreated canals, fractures, or other obvious reasons for the failing treatment, it was verified that the root-canal instrumentation was complete. The root canals were then rinsed with sodium hypochlorite and EDTA, and a paste of calcium hydroxide and saline was condensed into the canals. The access cavities were filled bacteria-tight with zinc oxide-eugenol cement. After 3 weeks, the patients were examined again. There had been no episodes of pain or swellings, but none of the fistulas had closed. The calcium hydroxide was then removed, and the root canals were irrigated with sodium hypochlorite and EDTA. New calcium hydroxide was packed and sealed into the canals, this time for a period of 3 months. At the examination after 3 months, there was no clinical or radiographic evidence of improvement. The clinical procedures described were then repeated, and the calcium hydroxide was renewed for a second 3-month period. At the next examination, i.e. after 6 months and 3 weeks of calcium hydroxide treatment, one patient had a slight palatal swelling, another patient complained of apical tenderness, and a third had ample exudation from the root canal when the calcium hydroxide paste was removed. These patients received systemic, antibiotic treatment (phenoxymethyl-penicillin). None of the fistulas had closed. Five of the patients with fistulas received systemic, antibiotic treatment (one patient phenoxymethyl-penicillin, one patient amoxicillin, and three patients amoxicillin plus metronidazole) but without apparent clinical effect. The remaining patients were asymptomatic but showed no evidence of treatment effect. It was concluded that the apical periodontitis did not respond to the nonsurgical treatment, and the patients were given the diagnosis, refractory apical periodontitis. The teeth were then root-filled and the patients were scheduled for surgical treatment (apicoectomy).

Sampling of Periapical Lesions

Bacterial samples were taken from the periapical lesion immediately after reflecting the flap. The gingiva and mucosa of the field of operation were swabbed thoroughly by using sterile gauze soaked in an 0.2% chlorhexidine gluconate solution. The tongue and lips were held back, and care was taken to keep the suction tip away from the area to avoid contamination during the microbial sampling. After a marginal incision, a full-thickness flap was reflected and the periapical lesion was exposed. At least three sterile endodontic paper points were inserted into the lesion toward the root tip. In addition, the periapical lesion was immediately removed by using sterile curettes. The paper points and periapical lesions were placed in glass vials, containing 10 ml of prereduced anaerobically sterilized (PRAS), VMGA III transport medium (12).

Sulfur granules, if present, were collected and transferred to vials as described above and/or to glass tubes that contained 2.5% glutaraldehyde/0.1 M phosphate buffer for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). All samples were brought to the Institute of Oral Biology, Dental Faculty, University of Oslo for microbiological culture and electron microscopy.

Microbiological Assessment

CULTIVATION

The sealed tubes with the microbiological samples were agitated in a whirly mixer (Labinco, Breda, The Netherlands) for 10 s. The sulfur granules were crushed in a sterile mortar before being seeded. Serial 10-fold dilutions of the transport fluid were made in one-fourth strength PRAS Ringer's solution, supplemented with 0.05% L-cysteine free base (Sigma, St. Louis, MO). A VPI Anaerobic Culture System (Bellco, Vineland, NJ) was used to flush the tubes continuously with an anaerobic gas mixture (90% N₂, 5% CO₂, 5% H₂). Each dilution, while kept in the Anaerobic Culture System, was pipetted in volumes of 0.1 ml onto lactose agar plates (Biocar Diagnostics, Beauvais, France), TSBV agar plates, Sabouraud dextrose agar plates (Biocar Diagnostics), and prereduced trypticase soy agar plates supplemented with 5% defibrinated whole human blood, 5 mg/l of hemin, and 0.5 mg/l of menadione. The plates were incubated anaerobically (90% N₂, 5% CO₂, 5% H₂) in evacuation jars (Anoxomat System, WS9000, Mart, The Netherlands) at 37°C and opened after 14 days for microbiological examination.

Preliminary identification of pure cultures was based on aerotolerance, colony and cell morphology, colony pigmentation, and Gram-staining of cells. Enzymatic/biochemical profiling relied on commercial diagnostic kits designed for identification of a multitude of different microorganisms (API, bioMérieux, Marcy-l'Etoile, France). Anaerobic isolates were identified by means of the Rapid ID 32 A kit, using 32 standardized and miniaturized enzymatic tests. Streptococcal identification was performed with the Rapid ID 32 Strep kit, applying 32 standardized and miniaturized enzymatic tests, and staphylococcal identification was based on the API STAPH kit with 26 standardized and miniaturized biochemical tests. Identification of nonenteric facultative Gram-negative rods was performed with the API 20 NE kit, applying 8 conventional biochemical tests and 12 assimilation tests. The identification of enterobacteria and other facultative, anaerobic, Gram-negative rods was based on the rapid ID 32 E kit, using 32 standardized and miniaturized enzymatic tests. The identification of yeasts was made with the ID 32C kit that applies 32 standardized and miniaturized assimilation tests. The preparation and incubation of the kits were carried out according to the manufacturer's recommendations. Reading of the kits occurred automatically in an ATB reader (API, bioMérieux). The results of the reactions, transferred into a numerical code, were treated in a database system for identification (API Plus, bioMérieux).

SCANNING AND TRANSMISSION ELECTRON MICROSCOPY

In four cases, the sulfur granules were investigated by SEM and in three cases by TEM. After dehydration in ethanol, the granules intended for SEM were critically point dried with carbon dioxide as the transitional fluid. The dried specimens were attached to metal stubs with silver paste and sputter-coated with gold/palladium, thickness 30 nm, in a vacuum evaporator. Coated samples were examined in a scanning electron microscope (30 ESEM; Philips, Eindhoven, The Netherlands). In addition, the granules were examined by energy dispersive X-ray analysis (EDXA), using the same microscope furnished with an X-ray analyzer.

The biopsies for TEM were fixed for 24 h at room temperature and then stored in 0.1 M of phosphate buffer with ruthenium red until preparation. Postfixation was performed in 1% osmium tetroxide for 2 h at 4°C. After fixation, the blocks were rapidly dehydrated in a graded series of acetone solutions and embedded in Vestopal W. Ultrathin sections were cut on a Leica Ultra-Cut microtome. The sections were treated with uranyl acetate for 15 min, followed by lead citrate for 3 min. They were examined in a Philips CM 120 TEM microscope (Philips).

RESULTS

Thirty-five of the 36 periapical lesions yielded microbial growth. The culture of one lesion was negative. Of the 35 lesions positive for growth, 33 were polymicrobial, whereas two yielded only one bacterial species. A total number of 148 microbial strains were detected from 67 different microbial species (Table 1). Approximately half of the bacterial strains (51.0%) were anaerobic. The number of microbial species isolated from each lesion was between 1 and 11, with a mean number of 4.1 ± 2.5 . Of the bacteria isolated, 79.5% were Gram-positive.

Twenty-seven (75%) of the 36 lesions contained organisms such as *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas*, *Enterococcus*, *Enterobacter*, or *Candida* species; *Staphylococcus* species, coagulase-positive or coagulase-negative, were detected in 22 (61.1%) of the 36 periapical samples. In 3 of these patients, 2 to 4 different *Staphylococcus* species were recovered (36.1%). *Bacillus* species were isolated from 7 (19.4%) of the 36 samples, *Pseudomonas*, *Stenotrophomonas*, and *Sphingomonas* species in 4 (11.1%), *Enterococcus* species in 3 (8.3%), and *Enterobacter cloacae* in 1 lesion (2.8%). *C. albicans* was recovered from 2 (5.6%) of the 36 patients. No pure culture of yeasts was detected.

All the periapical samples from the eight patients having taken antibiotics before surgery yielded microbial growth. In five of these patients, *Enterococcus faecalis*, *Staphylococcus* species, *Pseudomonas* species, *E. cloacae*, and/or *C. albicans* were found.

There was no statistical difference ($p < 0.05$, Fisher exact test) in the occurrence of microbial species between patients with the diagnosis of asymptomatic apical periodontitis (26 patients) and those with the diagnosis of apical periodontitis with fistula (10 patients).

Periapical Samples with Sulfur Granules

In the periapical lesions from 9 (25%) of the 36 patients, sulfur granules were recovered (Fig. 1). The granules were present in numbers between 2 and more than 10. They varied in color between whitish-grey, bright yellow, brownish, or brownish-green. Some of the granules were soft. Others were hard and appeared calcified to varying degrees.

CULTIVATION

Granules from seven of the nine patients yielded bacteria by culture. The granules from two patients were culture-negative. In all culture-positive cases, three to six microbiotic species were detected (Table 2).

Actinomyces israelii, *A. viscosus*, *A. meyeri*, and *A. naeslundii* were cultured from five of the seven cases positive for growth. In

all these cases, microbes other than *Actinomyces* species were recovered as well: *Propionibacterium acnes*, *P. propionicum*, *Peptostreptococcus prevotii*, *Gemella morbillorum*, *Clostridium sor-delli*, *C. bifermentans*, *Leptotrichia buccalis*, *S. chromogenes*, *S. epidermidis*, *Vibrio metschnikovii*, and *Streptococcus* species. In the two cases not exhibiting *Actinomyces* species, *Aerococcus viridans*, *Bacteroides ureolyticus*, *G. morbillorum*, *Capnocytophaga* species, *Pseudomonas aeruginosa*, *S. warneri*, and *S. oralis* were cultured.

In the two patients in whom no organisms were detected by culture of the sulfur granules, cultivation of the periapical granuloma showed *Sphingomonas paucimobilis* and *S. warneri* in one patient and *Stenotrophomonas maltophilia* in the other. One of these patients had taken amoxicillin plus metronidazole before surgery.

Electron Microscopy

SEM

By SEM, it was seen that the sulfur granules were tightly packed with microorganisms (Fig. 2). Rod-like organisms were prominent (Fig. 3), and spiral-formed bacteria were commonly seen (Figs. 3 and 4). In many of the granules, an amorphous material was present between the bacterial cells (Fig. 4). In those granules that clinically felt hard, this material when examined with EDXA showed high amounts of silicon and low amounts of calcium (Fig. 5). Occasionally, macrophages were observed on the surface of the granules engulfing bacteria (Fig. 2).

TEM

In TEM of the sulfur granules, bacteria with a Gram-positive and Gram-negative cell wall were observed. Outside the cell wall, a slime-like layer was often present. This layer was seen to envelop several bacterial cells (Fig. 6). Outer membrane vesicles were observed in close contact with the bacterial cell wall and were also spread out between cells. Macrophages were seen in some areas, some of them with a number of engulfed bacteria (Fig. 7).

DISCUSSION

Sampling of microorganisms in the periapical area during surgery is difficult because of possible contamination from the indigenous oral microflora. In this study, we used a surgical technique and sampling procedures that proved reliable in a previous methodological study on extraradicular infection (3). It was, therefore, assumed that the bacteria recovered in this study were present in the refractory lesions before surgery.

Long-term calcium hydroxide treatment of the root canal of teeth with apical periodontitis is a time-consuming, but usually efficient, method of root-canal disinfection (15). It is used with considerable success in so-called problem teeth, i.e. teeth with a large periapical lesion, incomplete root formation, progressive external root resorption, and where conventional endodontic treatment has failed. The long-term calcium hydroxide method was used in this study in a serious effort to obtain bacteria-free roots.

The microflora in the refractory cases in this study, being dominated by Gram-positive organisms with approximately equal proportions of facultative and obligate anaerobes, was clearly

TABLE 1. Microorganisms isolated from periapical lesions of 36 patients with refractory apical periodontitis

Microorganism	All patients (n = 36)	No fistula/No antibiotics (n = 23)	No fistula/Antibiotics (n = 3)	Fistula/No antibiotics (n = 5)	Fistula/Antibiotics (n = 5)
<i>Actinomyces israelii</i>	6	6			
<i>Actinomyces meyeri</i>	3	2	1		
<i>Actinomyces naeslundii</i>	5	3		1	1
<i>Actinomyces viscosus</i>	7	5		1	1
<i>Actinomyces</i> species	1		1		
<i>Aerococcus viridans</i>	1	1			
<i>Bacillus cereus</i>	2	2			
<i>Bacillus circulans</i>	1	1			
<i>Bacillus laterosporus</i>	1			1	
<i>Bacillus lentus</i>	1			1	
<i>Bacillus pumilus</i>	1	1			
<i>Bacillus sphaericus</i>	1	1			
<i>Bacteroides ureolyticus</i>	5	4			1
<i>Bifidobacterium</i> species	1	1			
<i>Candida albicans</i>	2	1	1		
<i>Capnocytophaga</i> species	3	2	1		
<i>Clostridium acetobutylicum</i>	1			1	
<i>Clostridium bifermentans</i>	2	2			
<i>Clostridium difficile</i>	2	2			
<i>Clostridium fallax</i>	1	1			
<i>Clostridium sordelli</i>	1				1
<i>Clostridium tyrobutyricum</i>	3	3			
<i>Clostridium</i> species	1		1		
<i>Enterobacter cloacae</i>	1				1
<i>Enterococcus faecalis</i>	2	1			1
<i>Enterococcus</i> species	1	1			
<i>Eubacterium lentum</i>	1			1	
<i>Fusobacterium nucleatum</i>	6	4	1	1	
<i>Gemella haemolysans</i>	1			1	
<i>Gemella morbillorum</i>	6	3	1	1	1
<i>Haemophilus</i> species	1	1			
<i>Lactobacillus</i> species	3	1	2		
<i>Leptotrichia buccalis</i>	1	1			
<i>Leucostonoc</i> species	1		1		
<i>Micrococcus</i> species	2	1			1
<i>Peptostreptococcus micros</i>	2	1	1		
<i>Peptostreptococcus prevotii</i>	1				1
<i>Porphyromonas endodontalis</i>	1	1			
<i>Porphyromonas gingivalis</i>	1			1	
<i>Prevotella buccae</i>	1				1
<i>Prevotella intermedia</i>	2	1		1	
<i>Prevotella oralis</i>	1	1			
<i>Propionibacterium acnes</i>	6	5			1
<i>Propionibacterium granulosum</i>	2		1		1
<i>Propionibacterium propionicum</i>	2	1			1
<i>Pseudomonas aeruginosa</i>	2	2			
<i>Sphingomonas paucimobilis</i>	1				1
<i>Staphylococcus aureus</i>	1			1	
<i>Staphylococcus capitis</i>	2	2			
<i>Staphylococcus chromogenes</i>	1	1			
<i>Staphylococcus epidermidis</i>	9	5		2	2
<i>Staphylococcus hominis</i>	5	2	2	1	
<i>Staphylococcus warneri</i>	2	1			1
<i>Staphylococcus xylosus</i>	2	2			
<i>Staphylococcus</i> species	6	6			
<i>Stenotrophomonas maltophilia</i>	1	1			
<i>Streptococcus anginosus</i>	1	1			
<i>Streptococcus constellatus</i>	1	1			
<i>Streptococcus downei</i>	1	1			
<i>Streptococcus gordonii</i>	2	2			
<i>Streptococcus mitis</i>	2			1	1
<i>Streptococcus oralis</i>	4	2	1		1
<i>Streptococcus sanguis</i>	1	1			
<i>Streptococcus vestibularis</i>	1	1			
<i>Streptococcus</i> species	3	3			
<i>Veillonella</i> species	2	2			
<i>Vibrio metschnikovii</i>	1				1

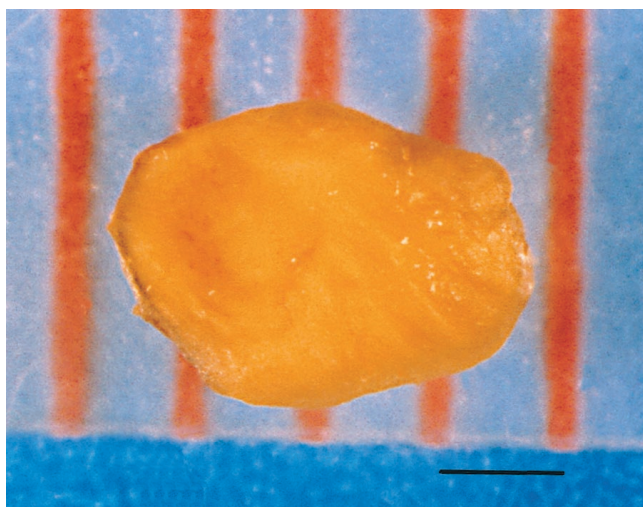


FIG 1. Sulfur granule recovered from refractory periapical endodontic lesion. The granule was soft, yellowish in color, and 3 to 4 mm in diameter. Three additional sulfur granules were recovered from the same lesion. Bar = 10 mm.

TABLE 2. Microorganisms isolated from sulfur granules recovered from periapical lesions of nine patients with refractory apical periodontitis

Patient no.	Antibiotic	Microorganism
1		<i>Actinomyces israelii</i> <i>Clostridium bifermentans</i> <i>Propionibacterium acnes</i>
2	Fenoxymethylpenicillin	<i>Actinomyces viscosus</i> <i>Clostridium sordelli</i> <i>Gemella morbillorum</i> <i>Peptostreptococcus prevotii</i> <i>Propionibacterium acnes</i> <i>Vibrio metschnikovii</i>
3		<i>Actinomyces israelii</i> <i>Actinomyces viscosus</i> <i>Leptotrichia buccalis</i> <i>Staphylococcus epidermidis</i>
4		<i>Actinomyces meyeri</i> <i>Actinomyces naeslundii</i> <i>Staphylococcus chromogenes</i>
5		<i>Aerococcus viridans</i> <i>Bacteroides ureolyticus</i> <i>Gemella morbillorum</i>
6		<i>Actinomyces israelii</i> <i>Propionibacterium propionicum</i> <i>Streptococcus species</i>
7		<i>Capnocytophaga species</i> <i>Pseudomonas aeruginosa</i> <i>Staphylococcus warneri</i> <i>Streptococcus oralis</i>
8		None*
9	Amoxicillin-metronidazole	None*

* Microorganisms were recovered from corresponding periapical lesion.

different from that which we have recently found in asymptomatic apical periodontitis, applying the same experimental techniques (3). On the other hand, it was similar to the root canal flora of root-filled teeth with radiographically verified apical periodontitis

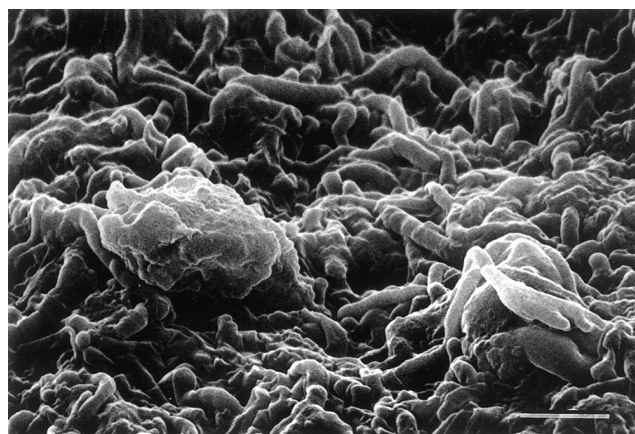


FIG 2. SEM of surface area of sulfur granule seen in Fig. 1. Microorganisms that are tightly packed and glued together make up the outer boundary of the granule. Two macrophages are seen, seemingly engulfing bacteria. Bar = 5 μ m.



FIG 3. SEM of cut surface of sulfur granule seen in Fig. 1. The granule consists of an abundance of bacteria. Rod-like organisms are prominent and spiral-formed bacteria are seen (arrow). Bar = 5 μ m.

(12–14). Thus, 75% of the refractory periapical lesions contained *Staphylococcus*, *Bacillus*, *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas*, *Enterococcus*, *Enterobacter*, or *Candida* species. These organisms even persisted in five of eight patients who had taken antibiotics systemically before surgery.

Pseudomonas, enteric rods, *Candida*, *Staphylococcus*, and *Enterococcus* species have also been detected in the subgingival flora of patients with refractory adult periodontitis (16–18). These organisms can be conspicuous in patients receiving prolonged chemotherapy against infectious diseases. It is, therefore, conceivable that they have intrinsic resistance to antimicrobial substances as such. A good example is enterococci, which show intrinsic resistance to some antibiotics, e.g. penicillin (19). Also, *E. faecalis* has been shown in vitro to resist the antibacterial effect of calcium hydroxide (20, 21). Several of these organisms have adapted over time to live in many different environments. Their numbers, rapid fluctuations, and amenability to genetic change probably give them tools for adaptation (19) that sometimes outpace what we can generate in the clinic in trying to keep up with them.

Another interesting aspect in this context is that extracellular material has been reported to surround bacterial aggregates outside

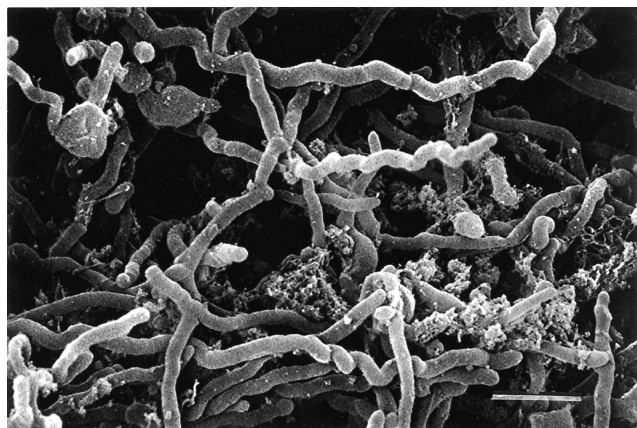


FIG 4. SEM of cut surface of sulfur granule. In addition to mainly rod-like and spiral-formed bacteria, an amorphous material is seen between the cells. In this granule, the extracellular material was not calcified (EDXA). Bar = 5 μ m.

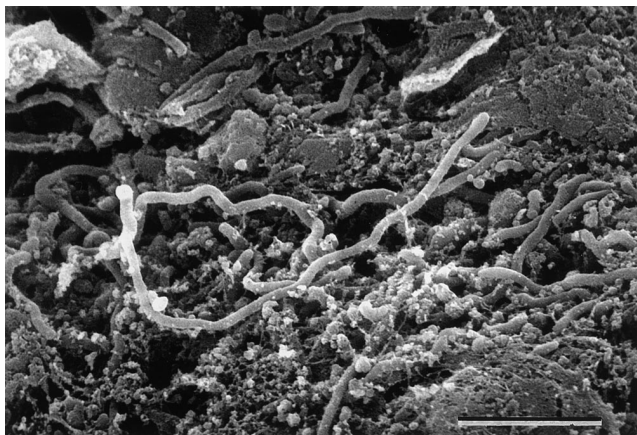


FIG 5. SEM of cut surface of sulfur granule. Microorganisms and large amounts of mainly calcified extracellular material are seen. The material was high in silicon and low in calcium (EDXA). Bar = 5 μ m.

the root canal (10). The lower metabolic rates of sedentary cells in this biofilm-like structure may make them less susceptible to antimicrobial substances; the members of the biofilm bacteria might be better equipped to pump out antibiotics before they can cause damage; or biofilm-dwellers may produce fewer proteins that are targeted by conventional antibiotics (22). Furthermore, biofilm-bacteria exchange DNA much more readily than do free-floating bacteria, which might accelerate the transfer of antibiotic resistance genes in the periapical biofilm.

A noteworthy finding of this study was the presence of sulfur granules in 9 (25%) of the refractory periapical lesions. The high occurrence suggested that sulfur granules can be significant in maintaining apical periodontitis.

A. israelii is reported to be the most common isolate in sulfur granules from cervicofacial and thoracic actinomycosis, being recovered in approximately 90% of the cases (23). Other bacteria have also been cultured from actinomycotic lesions (23). In the periapical sulfur granules, four different *Actinomyces* species were recognized: *A. israelii*, *A. viscosus*, *A. meyeri*, and *A. naeslundii*. In all these cases, a wide spectrum of other bacteria was detected in addition to *Actinomyces*. Even spirochete-like cells were seen in



FIG 6. TEM from sulfur granule. Gram-positive bacteria are seen. An extracellular material is enveloping several of the bacteria. Bar = 5 μ m.

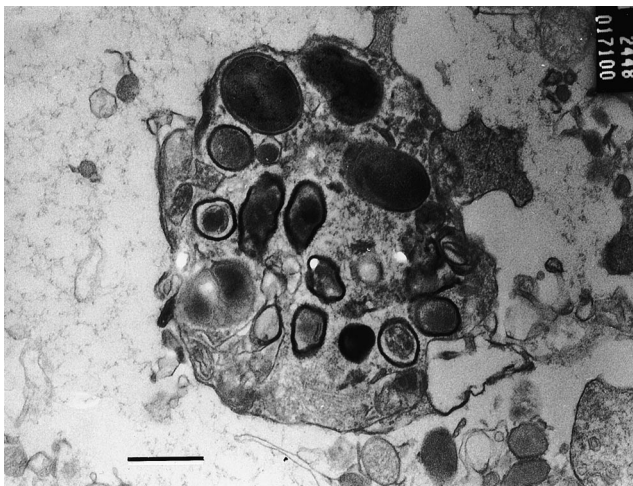


FIG 7. TEM from sulfur granule. A macrophage with a variety of engulfed bacteria is seen. Bar = 5 μ m.

SEM pictures. Spirochetes, which are found in the root canal (24), have to our knowledge not previously been detected in sulfur granules. Accordingly, the microbiota of the sulfur granules seemed to be less specific than generally considered. Most of the organisms in the granules were simultaneously present in the periapical lesions. It is, therefore, likely that the microbiota of the refractory lesions contributed to that of the sulfur granules.

The presence of strict anaerobic bacteria in the sulfur granules suggested that they contained microenvironments with a low redox potential. Probably, the facultative organisms consumed oxygen by their growth, stimulating proliferation of strict anaerobes. The electron microscopic studies demonstrated that phagocytosis of granule microorganisms occurred. However, phagocytosis may have been impeded due to the existing anaerobic environment. Some of the organisms of the granules exhibited copious amounts of extracellular material. One bacterium detected here, *P. aeruginosa*, is known to produce a thick, alginate capsule (25). Clinical isolates of *P. aeruginosa* can also have an external polysaccharide layer, referred to as slime layer, a mucoid substance or glycocalyx composed of repeating sugar chains (25). This layer is antiphago-

cytic, represents an impermeable layer to certain antibiotics, and reduces susceptibility to opsonizing antibodies.

In several of the sulfur granules, outer membrane vesicles were seen. These vesicles can serve as adhesins, attaching to and interacting with bacterial cells, even of different species, and to extracellular matrixes (26). Outer membrane vesicles may also bind antimicrobial substances, e.g. disinfectants, thereby providing bacterial resistance to these substances (26).

Many of the sulfur granules were calcified. The mineral source for this calculus may have been periapical exudates. However, streptococci and other bacteria have been reported to form extracellular as well as intracellular mineral deposits (27). Extracellular bacteria may, therefore, have contributed to calcification of the granules as well.

This study demonstrated that a wide variety of microorganisms, comprising facultative and anaerobic bacteria as well as yeasts, remained in refractory periapical endodontic lesions after long-term, root-canal treatment with calcium hydroxide (and systemic-antibiotic treatment). In this flora, 79.5% of the strains were Gram-positive. *Staphylococcus*, *Enterococcus*, *Enterobacter*, *Bacillus*, *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas*, and *Candida* species were detected in 27 (75%) of 36 lesions. The flora was different from that of asymptomatic apical periodontitis recently described (3). Sulfur granules, containing *Actinomyces* species and other bacteria, were detected in 9 lesions (25%), and many of the granules were calcified.

We are indebted to Renate Hars and Steinar Stølen for skillful assistance with TEM and SEM, respectively.

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