

Fluorescence *in situ* hybridization (FISH) for direct visualization of bacteria in periapical lesions of asymptomatic root-filled teeth

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Whether micro-organisms can live in periapical endodontic lesions of asymptomatic teeth is under debate. The aim of the present study was to visualize and identify micro-organisms within periapical lesions directly, using fluorescence *in situ* hybridization (FISH) in combination with epifluorescence and confocal laser scanning microscopy (CLSM). Thirty-nine periapical lesions were surgically removed, fixed, embedded in cold polymerizing resin and sectioned. The probe EUB 338, specific for the domain *Bacteria*, was used together with a number of species-specific 16S rRNA-directed oligonucleotide probes to identify bacteria. To control non-specific binding of EUB 338, probe NON 338 was used. Alternatively, DAPI (4',6'-diamidino-2-phenylindole) staining was applied to record prokaryotic and eukaryotic DNA in the specimens. Hybridization with NON 338 gave no signals despite background fluorescence of the tissue. The eubacterial probe showed bacteria of different morphotypes in 50% of the lesions. Rods, spirochaetes and cocci were spread out in areas of the tissue while other parts seemed bacteria-free. Bacteria were also seen to co-aggregate inside the tissue, forming microcolonies. *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* and treponemes of phylogenetic Group I were detected with specific probes. In addition, colonies with *Streptococcus* spp. were seen in some lesions. A number of morphotypes occurred that could not be identified with the specific probes used, indicating the presence of additional bacterial species. CLSM confirmed that bacteria were located in different layers of the tissue. Accordingly, the FISH technique demonstrated mixed consortia of bacteria consisting of rods, spirochaetes and cocci in asymptomatic periapical lesions of root-filled teeth.

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INTRODUCTION

It has been established that bacteria play the major role in the aetiology of apical periodontitis (Kakehashi *et al.*, 1965; Sundqvist 1976). In most instances endodontic infections respond well to local antimicrobial therapy of the root canal. When the root canal is properly instrumented, disinfected and obturated, follow-up studies show a treatment success rate of 80–90% in teeth with apical periodontitis (Kerekes & Tronstad 1979; Byström *et al.*, 1987). It has been generally accepted that bacteria causing endodontic infection of

asymptomatic teeth are present in the root canal system, whereas the periapical lesion is free of bacteria. The defence systems mobilized by periapical inflammation are believed first to eliminate the bacteria that invade the periapex. In long-standing infections with a more or less permanently established microflora in the root canal, the host defences appear to be less effective, and findings have been made suggesting that micro-organisms can survive outside the root canal system, on the root surface and/or in the periapical lesions of asymptomatic root-filled teeth (Happonen *et al.*, 1985; Tronstad *et al.*, 1987, 1990; Iwy *et al.*, 1990; Wayman *et al.*, 1992; Gatti *et al.*, 2000; Sunde *et al.*, 2000a, b). Since tissue invasion is considered a major virulence factor of endodontic bacteria and complete

Abbreviations: CLSM, confocal laser scanning microscopy; DAPI, 4',6'-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; FITC, fluorescein isothiocyanate.

healing of the periapical lesion is the ultimate goal in the treatment of apical periodontitis, the question whether extraradicular infection exists is of both clinical and academic interest.

In situ hybridization has proved to be a useful method for detection and identification of bacteria within their natural environment (for a review see Moter & Göbel, 2000). Recently, fluorescence *in situ* hybridization (FISH) has been applied to detect bacteria in tissue embedded in cold polymerizing resin (Moter *et al.*, 1998a; Hammer *et al.*, 2001). In these plastic sections, visualization of bacteria and excellent histological conservation were achieved. Furthermore, confocal laser scanning microscopy (CLSM) has been established as a valuable tool for obtaining high-resolution images and three-dimensional reconstructions of a variety of biological samples (Wagner *et al.*, 1994; Manz *et al.*, 1995; Moter *et al.*, 1998a; Wecke *et al.*, 2000).

The aim of the present study was to visualize and identify bacteria directly within periapical lesions by means of FISH in combination with epifluorescence and CLSM.

METHODS

Patients. The material came from two groups of patients: Group 1 was collected in Oslo and contained 20 patients (9 men) with a mean age of 47 years (± 11). Group 2 was collected in Bochum and included 19 patients (11 men) with a mean age of 53 years (± 22). The patients from both groups received surgical endodontic treatment of teeth with apical periodontitis. Two surgeons performed the surgery in Oslo and one surgeon did the surgery in Bochum. All teeth were asymptomatic. Sinus tracts or endo-perio-like lesions in conjunction with the teeth to be treated were not present at the time of surgery. Radiographs showed that the teeth referred for treatment were root-filled and had periapical radiolucencies with diameters between 4 and 15 mm. Each patient received treatment of one tooth only. The patients were treated with apicectomies. In Group 1 a submarginal incision was applied during surgery, while a marginal incision was made in Group 2 (Sunde *et al.*, 2000a).

Processing of tissue specimens. The enucleated periapical lesions from the 39 patients were washed with sterile saline, fixed directly with 3.7% (v/v) formaldehyde in PBS (pH 7.4) and kept at 4 °C. The embedding procedure, utilizing cold polymerizing resin (Technovit 8100; Kulzer), was performed according to the manufacturer's instructions. The specimens were washed overnight in PBS

containing 6.8% (v/v) sucrose, dehydrated in acetone for 1 h and infiltrated with the plastic solution (Technovit 8100 base-liquid with hardener I) for at least 6 h. After adding hardener II, the periapical lesions were aligned properly in the wells of Histoform S (Kulzer), sealed with cover foil and placed in the refrigerator. After polymerization, histoblocs were mounted on the specimens using Technovit 3040. The blocks were stored at 4 °C prior to sectioning.

The blocks were sectioned on a rotary microtome (Medim, Type DDM 0036) using steel knives with hard metal edges. Tissue sections (3 µm) were straightened on sterile water, placed on silanized slides [Super Frost (Medim); 3-aminopropyltrimethoxysilane (Sigma)] and stored at 4 °C. Three tissue sections from different parts of each lesion were hybridized. The sections were examined prior to the FISH procedure for autofluorescence.

Oligonucleotide probes. Probe EUB 338, which is complementary to a portion of the 16S rRNA gene conserved in the domain *Bacteria*, was used to visualize the entire bacterial population in the specimens (Amann *et al.*, 1990). The group-specific probes TRE I, TRE II and TRE IV (Choi *et al.*, 1997; Moter *et al.*, 1998b) were applied to the detection of most phylotypes of oral treponemes of Group I (including *Treponema vincentii* and *Treponema medium* as cultivable members), Group II (including *Treponema denticola*) and Group IV (including *Treponema maltophilum* and *Treponema lecithinolyticum*), respectively. For the demonstration of *Streptococcus* spp. a genus-specific probe, STREP, was used (Trebesius *et al.*, 2000). For visualization of *Fusobacterium* spp. a genus-specific probe, FUSO, was designed, and species-specific probes were designed for *Actinobacillus actinomycetemcomitans* (ACTACT), *Tannerella forsythensis* [B(T)AFO], *Porphyromonas gingivalis* (POGI), *Prevotella intermedia* (PRIN) and *Veillonella parvula* (VEPA) (Table 1). All probes have been deposited in ProbeBase (Loy *et al.*, 2003); an online resource for rRNA-targeted oligonucleotide probes where probe difference alignments are available (<http://www.microbial-ecology.de/probebase/index.html>).

To evaluate their specificity in FISH, all specific oligonucleotide probes were tested against *Treponema vincentii* (ATCC 35580), *Treponema denticola* (ATCC 35405), *Treponema maltophilum* (ATCC 51939^T), *Treponema socranskii* subsp. *socranskii* (ATCC 35536^T), *Treponema socranskii* subsp. *buccale* (ATCC 35534^T), *Fusobacterium nucleatum* subsp. *nucleatum* (ATCC 25586^T), *Porphyromonas gingivalis* (ATCC 33277^T), *Prevotella intermedia* (ATCC 25611^T), *Veillonella parvula* (ATCC 10790^T), *Veillonella dispar* (ATCC 17748), *Actinobacillus actinomycetemcomitans* (ATCC 43718), *Campylobacter rectus* (ATCC 33238^T), *Capnocytophaga gingivalis* (MCCM 00858), *Streptococcus viridans* (clinical isolate), *Haemophilus influenzae* (clinical isolate) and *Tannerella forsythensis* (formerly *Bacteroides forsythus*) (ATCC 43037^T).

Table 1. Oligonucleotide probes

Probe	Sequence (5'–3')	Target species	Formamide (% v/v)*
ACAC	TCCATAAGACAGATTC	<i>Actinobacillus actinomycetemcomitans</i>	30
B(T)AFO	CGTATCTCATTTTATCCCTGTA	<i>Tannerella forsythensis</i>	20
POGI	CAATACTCGTATGCCCGTTATTC	<i>Porphyromonas gingivalis</i>	20
PRIN	CTTACTCCCAACAAAAGCAGTTACAA	<i>Prevotella intermedia</i>	20
FUSO	CTAATGGGACGCAAAGCTCTC	<i>Fusobacterium</i> spp.	30
VEPA	TCTAACTGTTTCGAAGAAGGCCTT	<i>Veillonella parvula</i>	30

*Percentage formamide in the hybridization buffer for optimal hybridization conditions in FISH experiments.

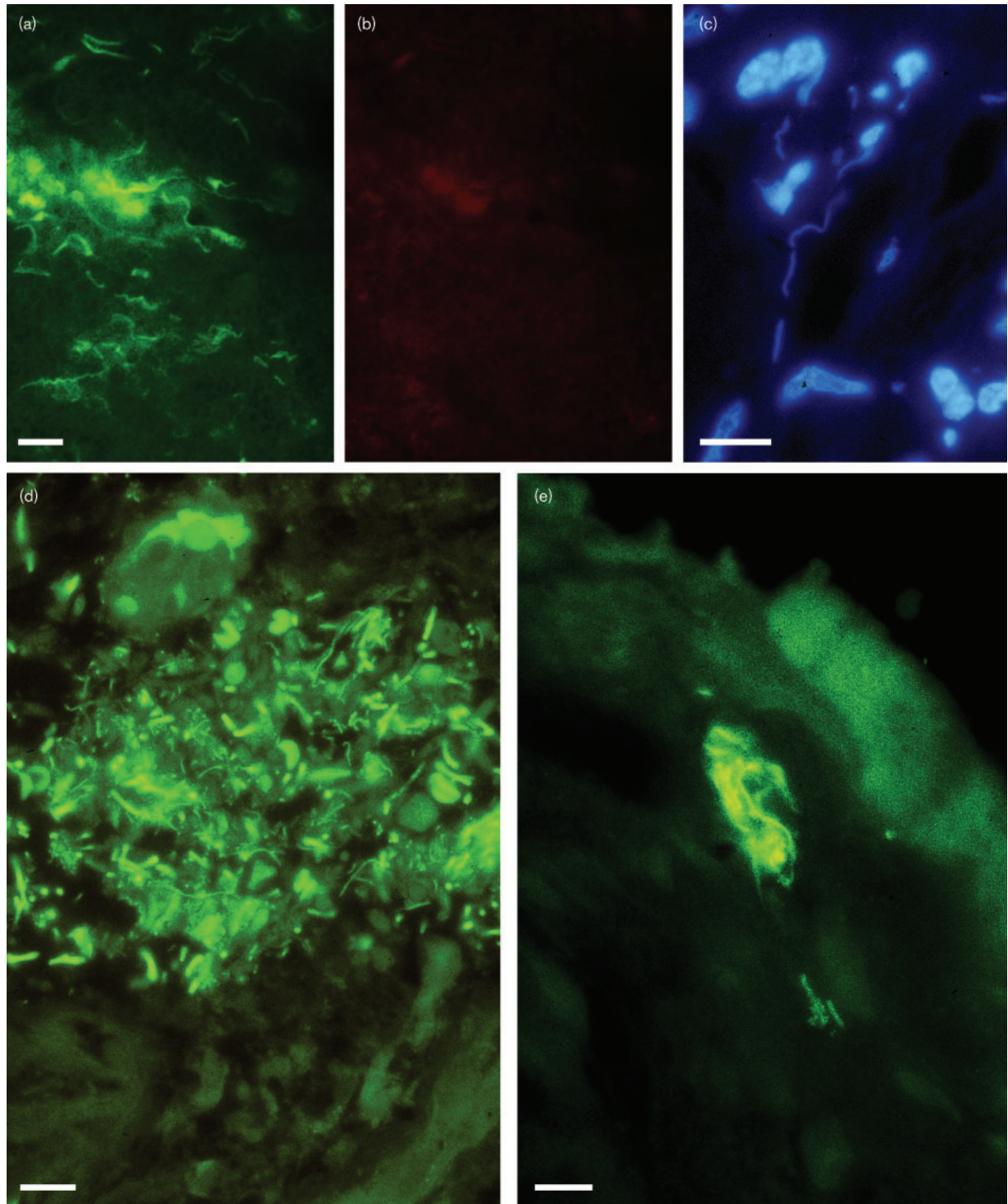


Fig. 1. (a) Simultaneous hybridization with the *Bacteria*-specific probe EUB338-FITC (green) and the complementary control probe NON EUB-Cy3 (red) on a tissue section from a periapical endodontic lesion localized several EUB338 FITC (green)-positive bacteria of different morphotypes spread among lesion cells and fibres. (b) Only background fluorescence of the tissue could be seen with the Cy3 filter on the same tissue section as shown in (a). (c) DAPI staining of a tissue section from a periapical endodontic lesion. Blue signals show staining of host cells and bacterial DNA. (d) FISH on a tissue section from a periapical granuloma using the *Bacteria*-specific probe EUB 338-FITC. A small area of tissue necrosis and EUB 338-detected bacteria of different morphologies can be seen. No bacteria could be detected with specific probes. (e) FISH on a tissue section from a periapical endodontic lesion. Hybridization with the *Bacteria*-specific probe EUB 338-FITC shows a microcolony of co-aggregated bacteria of different morphotypes. No bacteria could be detected with specific probes. Bars, 10 μm .

using fixed cells (Moter *et al.*, 1998a). Control slides with a selection of these fixed bacterial cells were included in every hybridization experiment together with tissue sections.

Besides *A. actinomycetemcomitans*, probe ACTACT also matches *Pasteurella* sp. MCCM02539, a sequence submitted while this work was in progress. Therefore, we could not exclude hybridization with this species. The closest negative control for ACTACT was *Haemophilus influenzae* with a single mismatch as determined by sequencing, that was not detected by the probe. Similarly, probe POGI has none or one single mismatch to the recently published species *Porphyromonas gulae*, isolated from the gingival sulcus of various animal hosts (Fournier *et al.*, 2001). Therefore, we cannot exclude cross-hybridization with this species that so far has not been detected in humans. *V. dispar* was the closest negative control for probe VEPA and yielded only weak signals when hybridized. Probe B(T)AFO detects 13 sequences deposited as *Bacteroides forsythus* in the GenBank and EMBL databases. However, it has seven mismatches to the recently published *Bacteroides* spp. BU063 that is associated with health (Leys *et al.*, 2002). Therefore, no cross-hybridization with this species would be expected. *Campylobacter rectus* with one mismatch to probe FUSO allowed optimization of the respective probe. Probes B(T)AFO and PRIN had more than three mismatches to the closest available phylogenetic relative and were therefore optimized using the above-mentioned panel of oral species.

All probes used for FISH were synthesized commercially and 5' end-labelled with fluorescein isothiocyanate (FITC) providing a green signal or with fluorochrome Cy3 (indocarbocyanine; Thermo Hybaid Interactiva division) giving a bright orange signal. The group-, genus- and species-specific probes labelled with the Cy3 fluorescent dye were applied simultaneously with the probe EUB 338-FITC. Some samples were tested in parallel with NON 338-Cy3, a probe complementary to EUB 338, in order to control non-specific binding of the EUB 338 probe (Wallner *et al.*, 1993). Alternatively, some samples were stained with DAPI (4',6'-diamidino-2-phenylindole), which detects DNA of bacteria, fungi and host cells. DAPI staining was applied simultaneously with two specific probes, using the Vit-dental system (Vermicon) according to the manufacturer's instructions.

FISH. The hybridization buffer contained 0.9 M NaCl, 20 mM Tris/HCl, pH 7.3, and 0.01% SDS. The stringency was adjusted by varying the formamide concentration from 0 to 30% (v/v), depending on the oligonucleotide probe used. Pre-warmed hybridization buffer (20 µl) was mixed with approximately 5 pmol of the appropriate oligonucleotide probe and carefully applied to the tissue sections. After incubation for 3.5 h in a dark humid chamber at 46 °C, each of the slides were rinsed with sterile double-distilled water, air-dried in the dark and mounted with Citifluor AF 1 (Chemical Laboratory, University of Kent).

Epifluorescent microscopy and CLSM. An epifluorescence microscope (Axioskop; Zeiss) was used to view the bacteria in hybridized sections processed for FISH. The microscope was equipped with a 50 W high-pressure mercury lamp (HB050; Osram) and ×10, ×40 and ×100 objectives (Zeiss). Narrow band filter sets HQ-F41-007 and HQ-F41-001 (AHF; Analysentechnik) were used to analyse the DAPI, FITC and Cy3 signals, respectively at a magnification of ×1000. Photomicrographs were taken using a Kodak Ektachrome HC 400 film. A Zeiss confocal laser scanning microscope equipped with an Ar-ion laser (488 nm) and two HeNe lasers (543 and 633 nm) was used to record optical sections. Image processing was performed with a standard software package delivered with the instrument (Zeiss SLM version 1.6). Reconstructed and processed images were produced on slide film (Kodak Professional, HC 100).

RESULTS

Bacteria detected with probe EUB 338

With the universal eubacterial probe EUB 338-FITC, bacteria could be detected by FISH in 10 of the 20 samples investigated in Group 1 and in 10 of the 19 samples examined in Group 2. In all specimens where bacteria were observed, they were seen in localized areas of the lesions, while large areas of the lesions seemed to be bacteria-free. The observed bacteria were located within the tissue and could not be seen at the tissue borders.

Most often rods, cocci and especially spirochaetes of different sizes hybridized with the EUB 338 probe only. These bacteria were often seen spread among cells and fibres in the tissue (Fig. 1 a). Simultaneously, hybridization with the probe EUB 338-FITC and the control probe NON 338-Cy3 showed several bacteria with the FITC filter while no signals could be seen with the Cy3 filter (Fig. 1b).

Bacteria of different morphologies could also be detected with DAPI staining (Fig. 1c).

In three lesions, areas of tissue necrosis were apparent and micro-organisms of different morphologies were observed (Fig. 1d). In some lesions the EUB 338 probe detected co-aggregated bacteria of different morphologies within microcolonies (Fig. 1e). In addition, single colonies of EUB 338-detected cocci were also seen.

A distinct morphotype of large, curved bacterial rods was detected with the EUB 338 probe in several lesions (Fig. 2a).

Bacteria detected with specific probes

Tannerella forsythensis (Fig. 2b), *Prevotella intermedia* and *Porphyromonas gingivalis* (Fig. 2c) were observed in three different lesions. The TRE I probe reacted specifically in one lesion (Fig. 2d). The treponemes were seen spread between eukaryotic cells and fibres in the tissue and some treponemes seemed to migrate around the periapical cells. The probe for the genus *Streptococcus* reacted specifically in three different lesions. In one of these a homogeneous colony with *Streptococcus* spp. was detected, while in two lesions mixed colonies of *Streptococcus* and other cocci detected with the EUB 338 probe, were observed (Fig. 3a–c).

CLSM allowed three-dimensional reconstruction of the tissue and confirmed that the cells observed were bacteria located in different tissue layers (Fig. 4). With this technique we could also see the spatial distribution of the bacteria. The organisms were located between fibres and cells, whereas no bacteria were visible within tissue cells.

DISCUSSION

This is the first investigation of the periapical microflora of asymptomatic apical periodontitis where the FISH and

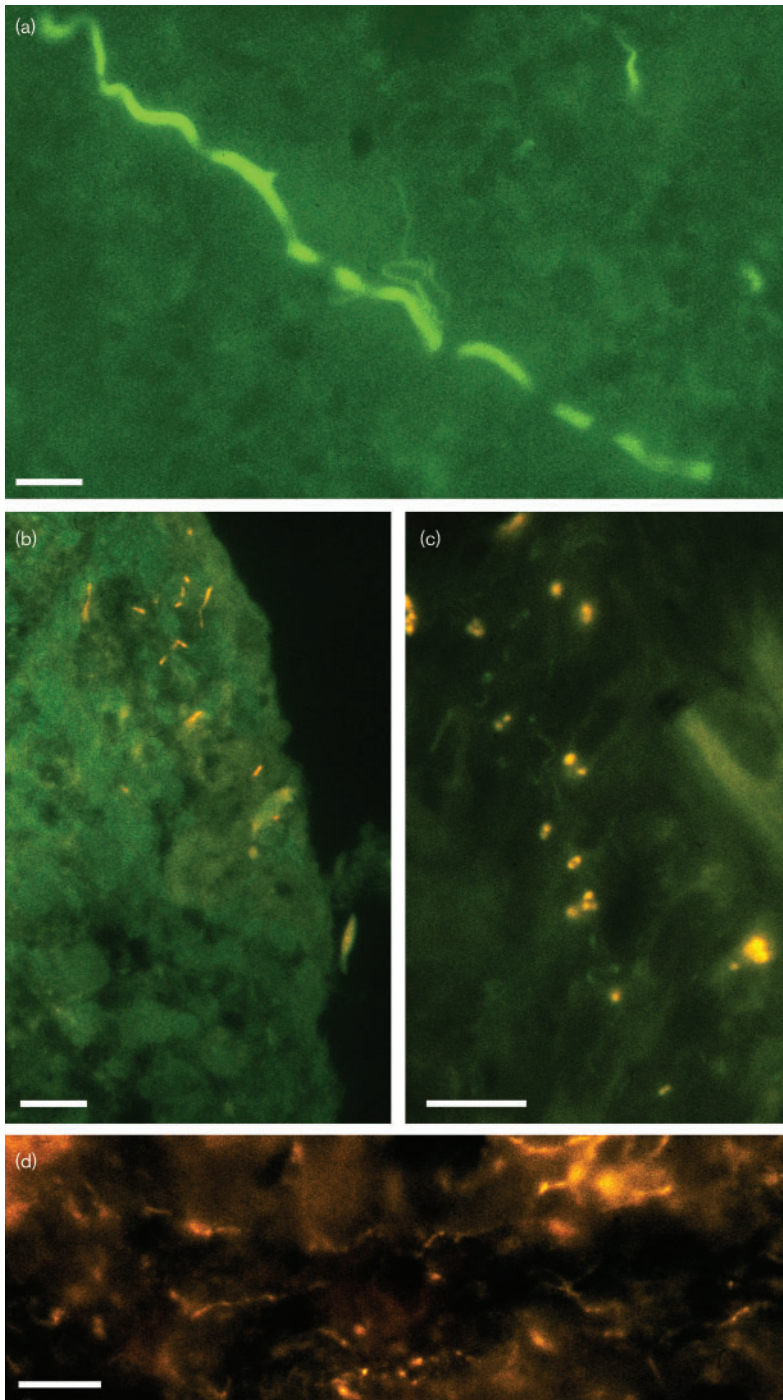


Fig. 2. FISH on tissue sections from a periapical endodontic lesion. (a) Hybridization with the *Bacteria*-specific probe EUB 338-FITC shows a distinct morphotype of a large, curvy bacterium. (b) Simultaneous hybridization with the *Bacteria*-specific probe EUB 338-FITC and the species-specific probe for *Tannerella forsythensis*-Cy3 localizes *Tannerella forsythensis* cells (orange) in a small area of the tissue. (c) Simultaneous hybridization with the *Bacteria*-specific probe EUB 338-FITC and the species-specific probe for *Porphyromonas gingivalis*-Cy3 localizes *Porphyromonas gingivalis* cells (orange) in a small area of the tissue. (d) Hybridization with group-specific probe TRE I-Cy3. TRE I cells (red) seen with the Cy3 filter are spread between lesion cells and fibres. Bars, 10 µm.

CLSM techniques have been used. FISH, which turned out to be a powerful method for visualizing micro-organisms in their natural environment, demonstrated bacteria in 50 % of the periapical lesions. The method was additionally improved by using CLSM, which allowed optical sectioning, three-dimensional reconstruction and therefore exact localization and observation of the spatial distribution of bacteria in different layers of the periapical lesion and around periapical cells.

By using the control probe NON 338 and DAPI staining, non-specific binding could be excluded, which gave confidence to the results achieved, especially when unusual and large morphotypes of bacteria were observed. In addition, autofluorescence was excluded by viewing the sections prior to the FISH procedure. No bacteria were observed at the borders of the lesions, indicating that contamination had been prevented. This was consistent with the results of a previous methodological study of

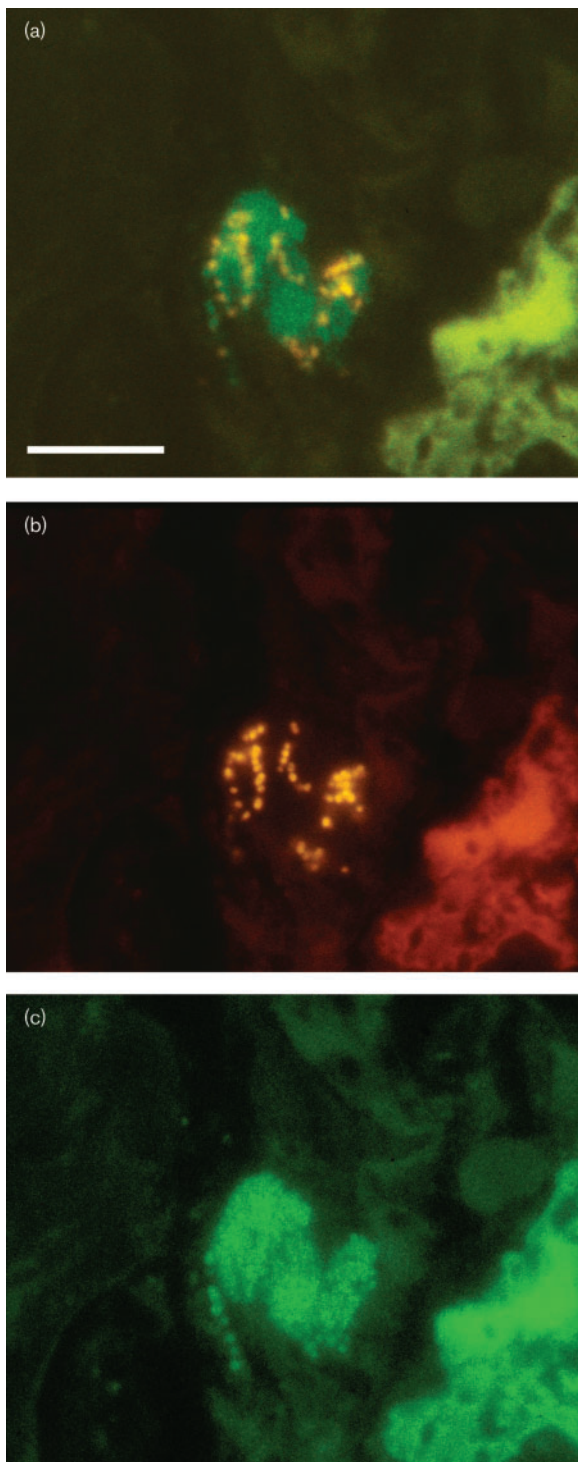


Fig. 3. (a) FISH on a tissue section from a periapical granuloma. Simultaneous hybridization with the *Bacteria*-specific probe EUB 338-FITC and the genus-specific probe for *Streptococcus*-Cy3 shows a mixed colony of streptococci (orange) and other cocci detected with the EUB 338 probe (green). Calcified tissue can be seen in the bottom right corner. (b) The same tissue section as in (a) seen with the Cy3 filter showing only streptococcal cells. (c) The same tissue section showing all cocci detected with the EUB 338 probe as seen with the FITC filter. Bar, 10 μ m.

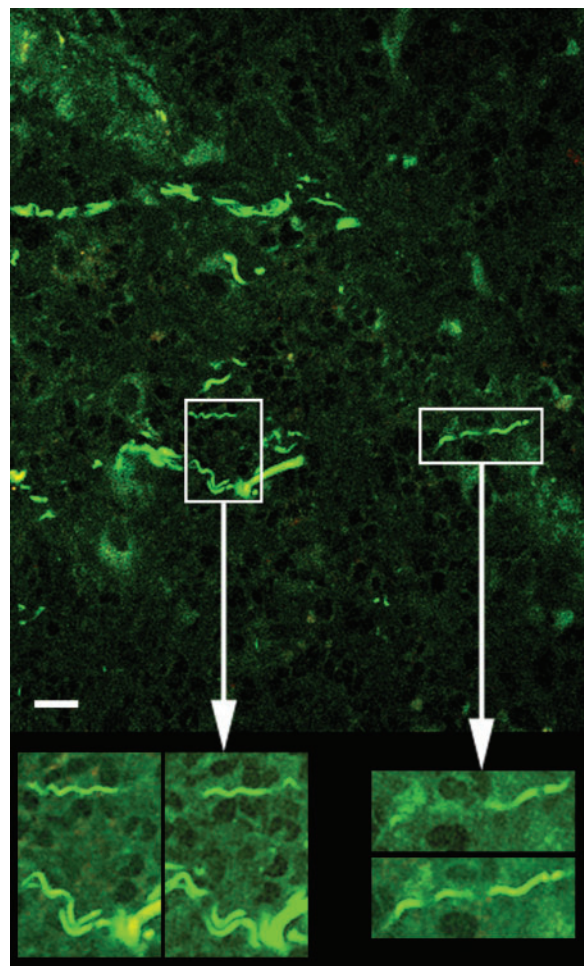


Fig. 4. FISH on a tissue section from a periapical granuloma. Bacteria were detected with the EUB 338 probe as shown by CLSM. Analysis of different optical sections using CLSM allows localization of spirochaete- and rod-like bacteria within the tissue (white frames). Bar, 10 μ m.

extraradicular infection where our surgical technique proved to be adequate for microbial sampling of periapical lesions (Sunde *et al.*, 2000a).

In most cases, bacteria were detected with the eubacterial probe EUB 338 only. The organisms were spread between tissue cells and fibres in the lesion or were co-aggregated, forming microcolonies. The bright signal intensities of the bacteria indicated a high amount of rRNA, which is evidence for physiological activity of the cells at the time of sampling (Kemp *et al.*, 1993; Wallner *et al.*, 1993).

In all lesions where bacteria were observed, they seemed to colonize localized parts of the lesions while other parts seemed to be bacteria-free. Co-aggregation and accumulation of bacteria may suggest that a synergistic interaction is taking place between the organisms involving use of food chains and consorted degradation of complex host and bacterial exopolymers. Positive interactions between

different species occur in the periodontal pocket (van Winkelhoff *et al.*, 1987; Socransky *et al.*, 1998) and in the root canal (Fabricius *et al.*, 1982; Sundqvist 1992), and it is likely that positive interactions also exist between bacteria living in periapical lesions.

In one lesion, Group I treponemes were observed. *Treponema vincentii* and/or *Treponema vincentii*-related organisms, which are Group I treponemes, have been associated with human periodontal diseases (Choi *et al.*, 1994; Moter *et al.*, 1998b; Willis *et al.*, 1999; Dewhirst *et al.*, 2000), but have to our knowledge not previously been reported in the root canal or in periapical lesions. In a previous epidemiologic study on oral treponemes, the parallel use of oligonucleotide probes specific for cultivable and as-yet-uncultivable organisms showed a great discrepancy between cultivable and as-yet-uncultivable treponemes of Group I, II and IV (Moter *et al.*, 1998b), suggesting the presence of novel yet unknown organisms at a high frequency. In the present study several spirochaete-like organisms of different sizes were detected with the universal probe EUB 338, but no specific signals could be obtained with the treponeme-specific probes. The explanation may be that the probes did not enable detection of all treponemes present, emphasizing the considerable genetic diversity of this group of organisms (Choi *et al.*, 1994; Dewhirst *et al.*, 2000). Previously, our group has described a large uncultivable spirochaete-like organism of 180 µm inside the root canal (Dahle *et al.*, 1993), and in a previous study with transmission and scanning electron microscopy spiral-form bacteria were commonly seen in 'sulfur granules' from periapical lesions (Sunde *et al.*, 2002). The detection of these strict anaerobic bacteria suggested that both the root canal and periapical lesion contain highly reduced microenvironments. Because spirochaetes are difficult or even sometimes seemingly impossible to cultivate, their prevalence in endodontic infections is probably underestimated.

Other noteworthy observations in the present study were the positive signals obtained from *Porphyromonas gingivalis*, *Prevotella intermedia* and *Tannerella forsythensis* appearing in three different lesions. These organisms have been associated with periodontal disease (Ashimoto *et al.*, 1996; Socransky *et al.*, 1998), similar to oral treponemes. Their presence in periapical lesions is consistent with recent observations from the root canal and periapical lesions based on molecular methods (Conrads *et al.*, 1997; Gatti *et al.*, 2000; Sunde *et al.*, 2000b; Jung *et al.*, 2001; Roças *et al.*, 2001).

Streptococci were observed in microcolonies in several lesions. They are considered important pathogens in dental caries and have often been isolated from the root canal and periapical lesions.

Most of the probes used did not show specific reaction with any of the observed bacteria. One possibility of non-detection is interference from elastin, collagen and blood cells causing bright autofluorescence. This could have

decreased the signal-to-noise ratio and it cannot be excluded that some specific fluorescent signals were masked, particularly from small spirochaetes.

It is also possible that some strict anaerobic bacteria may not be detected by the FISH technique due to their low metabolic activity and the small number of rRNA copies, resulting in low signal intensity and false negative results. However, many of the detected micro-organisms seem to be not yet cultured isolates or are not so-called oral pathogens.

In conclusion, direct visualization of bacteria with the FISH technique provided additional support to the notion that bacteria are present in periradicular tissue of asymptomatic teeth with apical periodontitis, that the bacteria here constitute a consortium of different species and that some of them are probably as yet uncultivable. This technique may also have a potential value in elucidating the aetiology/pathogenesis of extraradicular infection. However, more FISH studies combined with epifluorescence and CLSM should be done with a battery of specific probes for visualization and identification of additional bacteria, including uncultivable ones. No doubt, the endodontic microflora needs to be revisited.

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