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Assessment of periradicular microbiota by DNA-DNA hybridization

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Abstract – In the present study the "checkerboard" DNA-DNA hybridization technique was used to identify bacteria in periapical endodontic lesions of asymptomatic teeth. Thirty-four patients with root-filled teeth and apical periodontitis were divided into two groups, each containing 17 patients. In Group 1, a marginal incision was performed during surgery to expose the lesion, and in Group 2, a submarginal incision was applied. The gingiva and mucosa were swabbed with an 0.2% chlorhexidine gluconate solution prior to surgery. Bacterial DNA was identified in all samples from the two groups using 40 different whole genomic probes. The mean number (±SD) of species detected was 33.7±3.3 in Group 1 and 21.3±6.3 in Group 2 ($P<0.001$). The majority of the probe-detected bacteria were present in more lesions from Group1 than from Group 2. The differences were most notable for *Campylobacter gracilis, Porphyromonas endodontalis, Propionibacterium acnes, Capnocytophaga gingivalis, Fusobacterium nucleatum* ssp. *nucleatum, Fusobacterium nucleatum* ssp. *polymorphum, Prevotella intermedia, Treponema denticola, Streptococcus constellatus* and *Actinomyces naeslundii* I. Bacterial species such as *Actinobacillus actinomycetemcomitans* and *Bacteroides forsythus* were detected in more than 60% of the lesions from both groups. Also, *P. endodontalis* was abundant in periapical tissue. The data supported the idea that following a marginal incision, bacteria from the periodontal pocket might reach the underlying tissues by surgeon-released bacteremia. The study provided solid evidence that bacteria invade the periapical tissue of asymptomatic teeth with apical periodontitis. The detection of much more bacteria with the "checkerboard" DNA-DNA hybridization method than has previously been recovered by anaerobic culture indicated that the endodontic (and periodontal) microfloras should be redefined using molecular methods.

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Key words: apical periodontitis; DNA-DNA hybridization; endodontic infection; extraradicular infection

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Most of the flora in endodontic infections is anaerobic (1, 2). An inherent problem with anaerobic cultivation techniques for bacterial recovery is that species difficult to cultivate or present in amounts below the detection limit for cultivation will not be identified. It is estimated that approximately 50% of the oral flora is uncultivable (3, 4). It is likely that at least some members of this uncultivable part of the flora are involved in disease and may account for treatment failure. During the last decade molecular genetic methods relying on species-specific oligonucleotides have been used to identify bacteria in clinical samples without the need for isolation or biochemical tests for their identification. Studies carried out at the epigenetic and genetic levels have provided dramatic new insights into relationships that exist between procaryot-

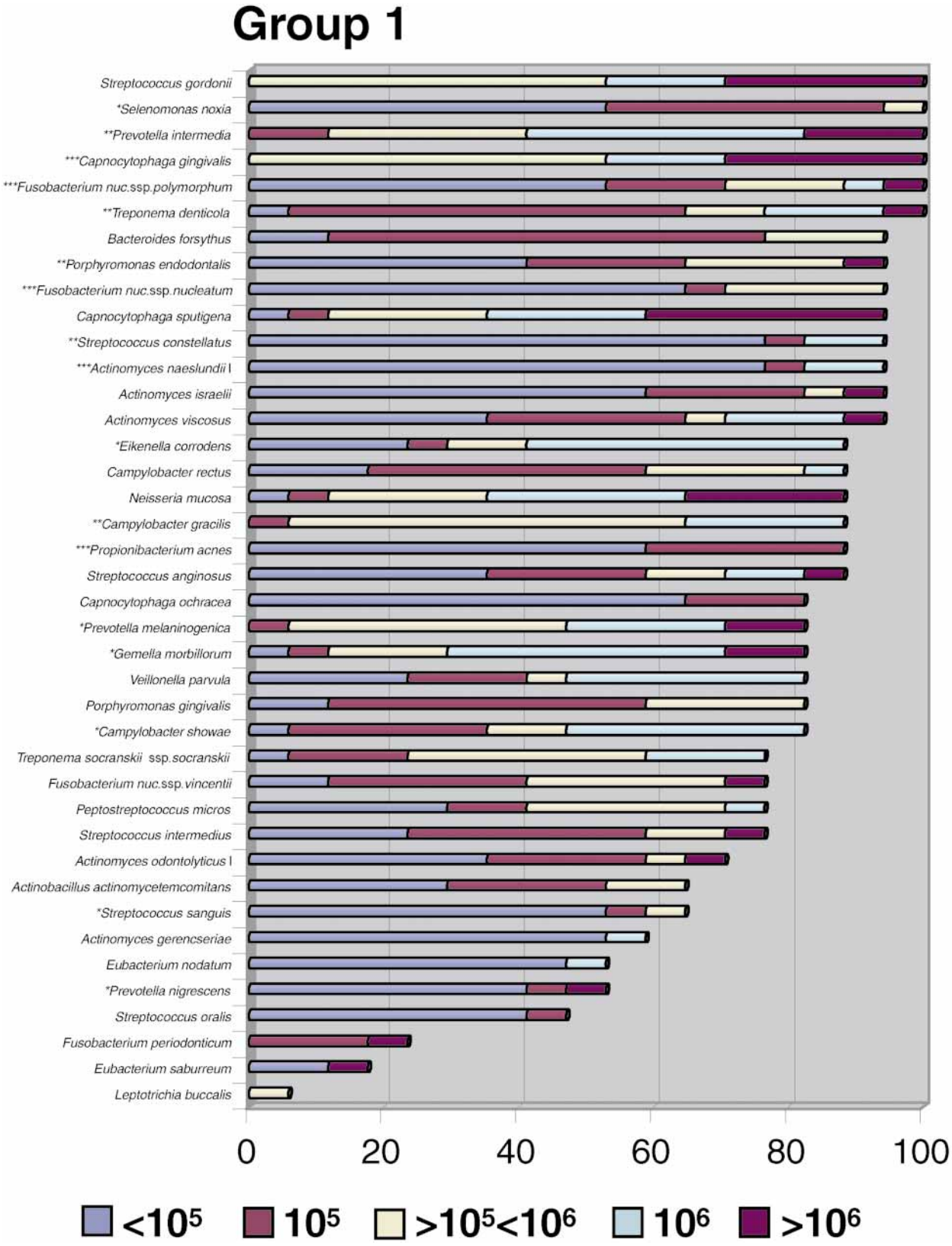
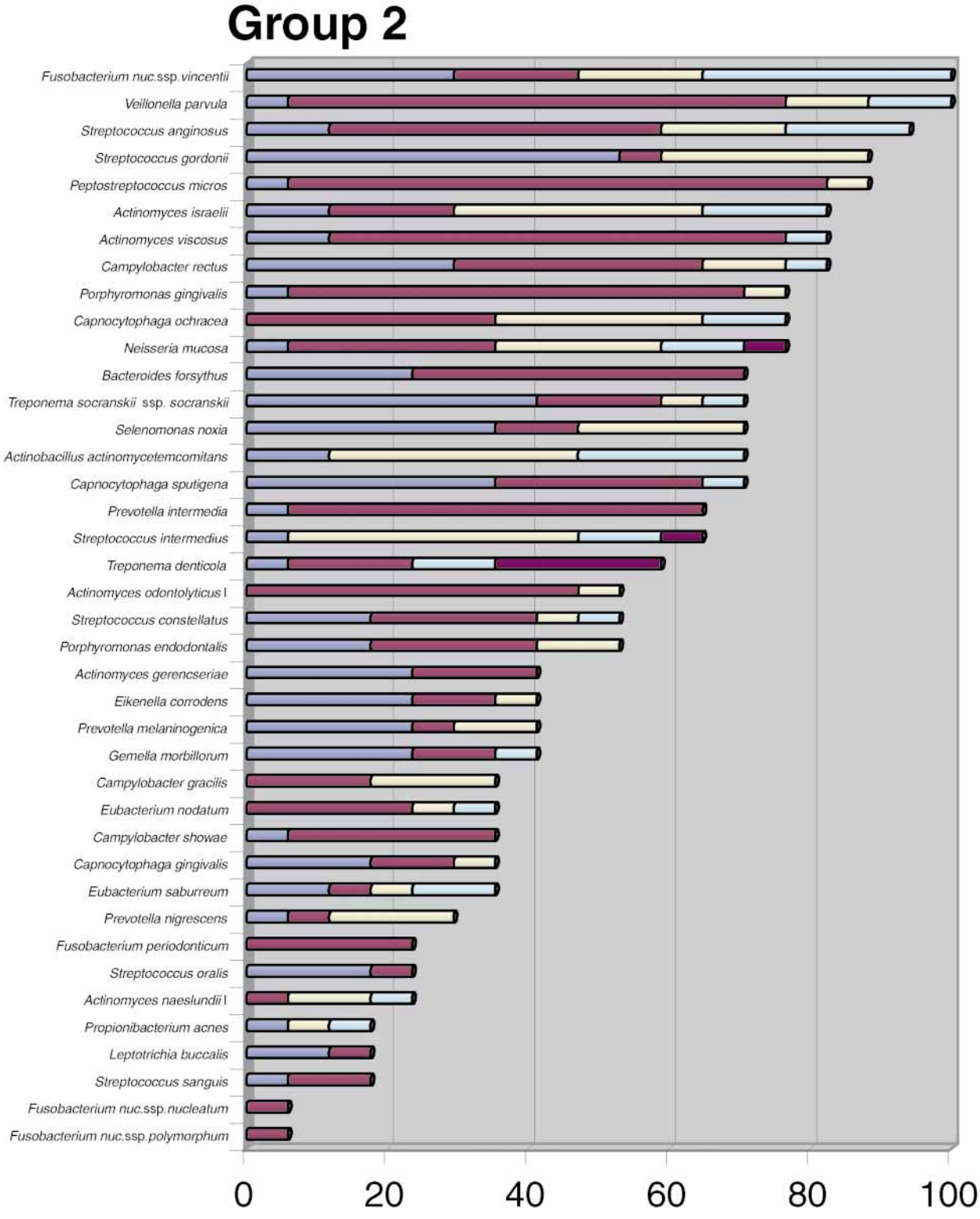


Fig. 1. Stacked bar chart of frequency of bacteria and their concentration as detected with "checkerboard" DNA-DNA hybridization in 17 periapical lesions (Group 1) when a marginal incision was performed (left bar) and in 17 periapical lesions (Group 2) when a submarginal incision was performed. The total length of each bar stack indicates the % of sites colonized. The different colors within each bar indicate the percentage of sites colonized by different levels of the species. The significance of difference between the two groups was evaluated using the Fisher exact test or the chi-square test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



es and have also led to new approaches towards their identification. At the epigenetic level, similarities in homologous translation products, i. e., RNA and proteins, are compared. At the genetic level the primary structures of DNA are compared either by sequencing techniques or by hybridization molecular techniques. The "checkerboard" DNA-DNA hybridization technique facilitates rapid processing of large numbers of clinical samples with respect to a multitude of bacterial species and does not require bacterial viability

(5,6). In addition, the DNA probe method may detect species in low proportions, and amplification of the DNA is not necessary for identification.

In a recent methodological study of extraradicular endodontic infection, bacteria were demonstrated in periapical lesions of asymptomatic teeth (7). In that study two different flap designs were applied during surgery and before sampling. Bacteria were recovered from 70% of the periapical lesions regardless of the surgical technique used. However, it appeared that the use of a marginal incision resulted in either a direct or circulatory translocation of bacteria from the marginal periodontium to the surgically exposed bone surface. When a submarginal incision was made, contamination of the underlying bone was only rarely seen.

The aim of the present study was to further investigate extraradicular endodontic infections using the "checkerboard" DNA-DNA hybridization technique (5) for verification of bacteria in endodontic periapical lesions. A second aim was to investigate if two different flap designs would influence the detection rate of bacterial DNA in periradicular tissue.

Material and methods

Microbiological sampling

This study comprised 34 patients referred for surgical-endodontic treatment of teeth with apical periodontitis. All teeth were asymptomatic. Fistulous tracts or endo-perio-like lesions related to the teeth to be treated were not present. Radiographically, it was evident that the patients referred for treatment had root-filled teeth with periapical radiolucencies of diameters varying between 4 and 15 mm. Each patient was referred for the treatment of one tooth.

Local anesthesia (xylocaine 1:50.000 adrenaline) was used either as maxillary infiltration or by mandibular block. All surgery was performed by the same operator (POL). The patients were treated with apicoectomies. The tongue and lips were held back and the field of operation was swabbed thoroughly prior to surgery with sterile gauze soaked in an 0.2% chlorhexidine gluconate solution. Two different flap designs were used. In Group 1, which comprised 17 patients (15 women), with a mean age of 54.2±11.4 years, a marginal incision with one vertical releasing incision was made. A full-thickness muco-periosteal flap was then reflected, starting from the vertical incision in an attempt to avoid contamination of the underlying bone by microorganisms from the gingival sulcus. In Group 2, which comprised 17 patients (10 women) with a mean age of 48.4±10.1 years, a submarginal incision in the attached gingiva with one vertical releasing incision was made. A full-thickness muco-periosteal flap was then reflected without involving the sulcular area of the periodontium. The periapical lesions were removed using a sterile curette and immediately placed in vials containing 10 mL of pre-reduced anaerobically sterilized (PRAS) dental transport medium (Anaerobe Systems, Morgan Hill, CA, USA). Within 2 hours all samples were brought to the Department of Oral Biology, Faculty of Dentistry, University of Oslo. In the microbiological laboratory the sealed tubes with the tissue samples from the periapical lesions were agitated on a whirly mixer (Cenco, Breda, the Netherlands) for 10 s to release bacteria.

"Checkerboard" DNA-DNA hybridization

The "checkerboard" method of Socransky et al. (5) was used for DNA-DNA hybridization. Each sample was pipetted in volumes of 0.1 mL and transferred to separate Eppendorf tubes. Half a milliliter of 0.5 M NaOH was added to each sample and the samples were frozen (−20°C). All samples were placed in a water bath (100°C) and boiled for 5 min. They were then neutralized using 0.8 mL of 5 M ammonium acetate. The released DNAs from the 34 periapical samples in the two groups together with DNA standards extracted from 10^5 and 10^6 cells of each of 40 bacterial test species (Fig. 1 and 2) were placed into the lanes of a Minislot (Immunetics, Cambridge, MA, USA) and then deposited on a nylon membrane (Boehringer Mannheim, Mannheim, Germany). The membranes were fixed by baking at 68°C for 30 min followed by exposure to ultraviolet light for 30 s. Transport medium without samples were included as controls. The membrane with the fixed DNA was placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90° to the channels of the device. Each channel was used as a hybridization chamber for separate DNA probes. The membranes were prehybridized at 42°C for 1 h in 50% formamide, 5 × SSC (1×SSC=150 mM NaCl, 15 mM sodium citrate, pH 7.0), 10% casein (Sigma, St. Louis, MO, USA), 5×Denhardt's reagent, 25 mM sodium phosphate (pH 6.5) and 10 mg/mL yeast RNA (Boehringer Mannheim). Digoxigenin-labeled, whole genomic probes and hybridization buffer containing 45% formamide, 5×SSC, 1×Denhardt's reagent, 20 mM sodium phosphate (pH 6.5), and 10 mg/mL yeast RNA, 20 ng/mL labeled probe, 10% dextran sulfate (Sigma) and 10% casein were placed in individual lanes of the Miniblotter. The whole apparatus was covered with Saran wrap and transferred to a sealed plastic bag. The membranes were hybridized overnight with gentle shaking at 42°C. They were washed in a plastic dish to remove loosely bound probe. To detect hybrids, the membranes were blocked with 10% casein in maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) and then incubated with anti-digoxigenin antibody conjungated with alkaline phos-

phatase (Boehringer Mannheim), diluted 1:20 000 in maleic buffer. Signals were detected chromogenically using NTB/BCIP tablets (Boehringer Mannheim) overnight.

Statistical analyses

The data were analyzed with the aid of SigmaStat for Windows, and examined for normality and equal variance. The difference between the number of species between the two groups was sought using the Mann-Whitney rank sum test. Significant differences between the two groups for each species were sought using the chi-square test or the Fisher exact test. A *P*-value less than 0.05 was regarded as statistically significant.

Results

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 in Group 1 where a marginal incision had been made, whereas samples from Group 2, where a submarginal incision was applied, contained 11–34 species per lesion. The mean number (±SD) of species was 33.7±3.3 in Group 1 and 21.3±6.3 in Group 2 ($P<0.001$). The prevalence and concentrations of the 40 probe-detected bacteria in the 34 periapical samples are seen in Fig. 1. Each of the 40 bacteria was detected in both groups. In Group 1, 36 of the 40 bacteria were detected in 50% of the lesions, while in Group 2, 22 of the 40 bacteria were present in 50% of the lesions. The majority of the bacteria were present in more lesions from Group 1 than from Group 2. The differences were most notable for *Campylobacter gracilis, Porphyromonas endodontalis, Propionibacterium acnes, Capnocytophaga gingivalis, Fusobacterium nucleatum* ssp. *nucleatum, Fusobacterium nucleatum* ssp. *polymorphum, Prevotella intermedia, Treponema denticola, Streptococcus constellatus* and *Actinomyces naeslundii. Actinobacillus actinomycetemcomitans, F. nucleatum* spp. *vincentii, Eubacterium saburreum, Peptostreptococcus micros, Veillonella parvula, Streptococcus anginosus* and *Leptotrichia buccalis* tended to be present in higher numbers in lesions of Group 2 than in Group 1, but the differences were not significant. *A. actinomycetemcomitans* and *Bacteroides forsythus* were identified in more than 60% of the lesions with no significant difference between the two groups.

P. endodontalis was identified in 94.1% of the lesions in Group 1 and in 52.9% of the lesions in Group 2. Species present in high numbers ($\geq 10^6$ cells) in Group 1 (in more than three lesions) were *P. intermedia, Capnocytophaga sputigena, Gemella morbillorum, Eikenella corrodens, Neisseria mucosa, C. gingivalis, Streptococcus gordonii, Prevotella melaninogenica, Campylobacter showae, C. gracilis, V. parvula, Actinomyces viscosus* and *T. denticola.* Species present in high numbers ($\geq 10^6$ cells) in Group 2 (in more than three lesions) were *A. actinomycetemcomitans, F. nucleatum* spp. *vincentii* and *T. denticola.* The five species most frequently detected in Group 1 were *S. gordonii, Selenomonas noxia, P. intermedia, C. gingivalis, F. nucleatum* ssp. *polymorphum* and *T. denticola.* In Group 2 the five species most frequently detected were *F. nucleatum* ssp. *vincentii, V. parvula, S. anginosus, S. gordonii, P. micros* and *A. israelii.*

Discussion

In the present study, DNA-DNA hybridization was used to detect bacteria in periapical lesions of teeth with asymptomatic apical periodontitis. Up to now almost all studies on the endodontic microflora have been based on cultural methods. It has been estimated that there are 500 different bacterial species in marginal periodontitis (8). About 50% of this flora (250 species) is not cultivable (Paster, 1999; personal communication). Actually, 200 of these uncultivable or previously unrecognized species have now been detected in a variety of periodontal diseases by molecular methods. There is every reason to believe that a fairly similar number of uncultivable bacterial species remains undetected in the root canal and probably also in inflamed periapical tissue. Actually, this was suggested by the results of our "checkerboard" study. With this molecular method a large number of bacteria was detected periapically in asymptomatic teeth with apical periodontitis. This was in contrast to a similar study using anaerobic culturing where the normal low number of bacterial species (<10) was recovered (7). It is noteworthy that the same types of periapical lesions were sampled in the two studies, that the same sampling technique was used, and that the same surgeon applied identical operative procedures in both studies. Moreover, our findings were in agreement with those of a parallel "checkerboard" DNA-DNA hybridization study carried out by a different group in a different laboratory where much more bacteria were detected in periapical lesions than have previously been recovered by anaerobic culture (9). Another parallel to these observations is when DNA is extracted from all microbial cells present in pus from abscesses (10). 16S rRNA was amplified by PCR using universal primers and sequences were compared to those in databases of known oral microorganisms. The comparisons have shown that unculturable and novel species are present in every sample and that they may comprise over 25% of the microflora. A typical example of what is lost during culturing is spirochetes. In acute necrotizing ulcerative gingivitis and certain forms of marginal periodontitis, spirochetes comprise over 50% of the bacterial flora. Spirochetes are also present in the root canal (11). Although the number of recognized cultivable oral tre-

ponemes has been extended to 10, approximately 50 additional currently uncultivable spirochetes have been detected (Paster, 1999; personal communication). To us this indicates that our current knowledge of the endodontic as well as the periodontal microflora is unsatisfactory, and that we will have to redefine these microfloras with the use of molecular methods.

Another noteworthy finding in this study was that much more bacteria were detected in periapical lesions after a marginal incision than after a submarginal incision was made. Similar findings were made by Gatti et al. (9). In the previous methodological study of extraradicular infection it was suggested that a circulatory translocation of bacteria from the marginal periodontium to the periapical area might occur when a marginal incision is made (7). This hypothesis was strengthened by the present results in that not only were many more bacteria recovered following a marginal incision than a submarginal incision, but the periapical flora was different as well, depending on which surgical technique was used. Thus, with the marginal incision the periapical lesion flora was dominated by organisms such as *S. gordonii, S. noxia, P. intermedia, C. gingivalis, F. nucleatum* ssp. *polymorphum* and *T. denticola*. After the submarginal incision, organism such as *F. nucleatum* ssp. *vincentii*, *V. parvula*, *S. anginosus*, *S. gordonii, P. micros* and *A. israelii* were most predominant. Most of the probe-detected bacteria were well known from studies on root canal infection (1, 2, 12) and periapical infection (7, 13, 14). However, the high prevalence of *A. actinomycetemcomitans*, *B. forsythus* and *P. endodontalis* in Group 2 was unexpected. *A. actinomycetemcomitans* is a recognized periodontopathogen (15), but has not frequently been detected in the root canal, and to our knowledge, not in periapical lesions. *B. forsythus* is another well known periodontopathogen (15), and was recently identified for the first time in infected root canals through 16S rRNA sequencing (16). The high prevalence of *B. forsythus* in periapical tissue was in agreement with findings by Gatti et al. (9) and indicated that this organism is a common endodontic pathogen. Abundance of *P. endodontalis* in periapical tissue has to our knowledge not previously been reported.

The high detection of microbial DNA from periapical lesions was similar to what we have seen over the years in our microbial diagnostic service based on samples from root canals (unpublished results). The fact that the tubes with transport media without samples gave no signals also gave confidence to the technique. Studies based on whole genomic probes may sometimes be disturbed by cross reactions. However, Socransky et al. (17), using an identical set up for probe preparation and probe selection conclude that cross reactivity is not a major problem in interpreting the results. One limitation of our study, though, was the fact that it was not possible to decide whether the detected DNA came from viable or non-viable bacteria. If the DNA came from non-viable bacteria some may dispute the clinical importance of this finding. However, any DNA whether "dead" or "alive" would come from an organism which under no circumstances should be in the periapical tissue, which is supposed to be sterile. In any case, it is likely that organisms detected in high concentrations would be important in inducing periapical inflammation.

In conclusion, the findings of the present investigation provided solid evidence that bacteria invade the periapical tissue in asymptomatic teeth with apical periodontitis.

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