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Quantitative Microbiological Study of Human Carious Dentine by Culture and Real-Time PCR: Association of Anaerobes with Histopathological Changes in Chronic Pulpitis

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The bacteria found in carious dentine were correlated with the tissue response of the dental pulps of 65 teeth extracted from patients with advanced caries and pulpitis. Standardized homogenates of carious dentine were plated onto selective and nonselective media under anaerobic and microaerophilic conditions. In addition, real-time PCR was used to quantify the recovery of anaerobic bacteria. Primers and fluorogenic probes were designed to detect the total anaerobic microbial load, the genera *Prevotella* and *Fusobacterium*, and the species *Prevotella melaninogenica*, *Porphyromonas endodontalis*, *Porphyromonas gingivalis*, and *Micromonas* (formerly *Peptostreptococcus*) *micros*. The pulpal pathology was categorized according to the cellular response and degenerative changes. Analysis of cultured bacteria showed a predominance of gram-positive microorganisms, particularly lactobacilli. Gram-negative bacteria were also present in significant numbers with *Prevotella* spp., the most numerous anaerobic group cultured. Real-time PCR analysis indicated a greater microbial load than that determined by colony counting. The total number of anaerobes detected was 41-fold greater by real-time PCR than by colony counting, while the numbers of *Prevotella* and *Fusobacterium* spp. detected were 82- and 2.4-fold greater by real-time PCR than by colony counting, respectively. Real-time PCR also identified *M. micros*, *P. endodontalis*, and *P. gingivalis* in 71, 60, and 52% of carious samples, respectively. Correlation matrices of the real-time PCR data revealed significant positive associations between *M. micros* and *P. endodontalis* detection and inflammatory degeneration of pulpal tissues. These anaerobes have been strongly implicated in endodontic infections that occur as sequelae to carious pulpitis. Accordingly, the data suggest that the presence of high levels of these bacteria in carious lesions may be indicative of irreversible pulpal pathology.

The microbial populations involved in dental caries are known to be highly complex and variable and have not yet been fully identified, although key organisms are generally recognized to be associated with disease progression. The bacteria involved in caries initiation and early caries development, particularly the mutans group streptococci and lactobacilli, have been well documented (34). As the lesion progresses, there is a transition from predominantly facultative gram-positive bacteria in early caries to anaerobic gram-positive rods and cocci and gram-negative rods in deep carious lesions (15). Previous research has associated the presence of anaerobic gram-negative rods, such as *Fusobacterium*, *Prevotella*, and *Porphyromonas*, with symptomatic teeth (11, 19), infected pulps (38), and periapical abscesses (29, 35), whereas anaerobic gram-positive cocci, such as peptostreptococci, have been associated with apical infections (10).

While it is well recognized that bacteria and their products play a major role in dental caries and associated pulpal inflammation (5), attempts to correlate clinical signs and symptoms with pulpal histopathology or to relate the presence of specific bacteria in root canal infections with clinical symptoms have been unsuccessful (7, 27). These limitations may be a natural characteristic of complex polymicrobial infections (caries, apical periodontitis) or the result of an incomplete microbial profile of the environment.

Few studies have analyzed the microbiology of deep carious dentine or examined the relationship between the microflora of dentine caries and the histopathology of chronic pulpitis. Attempts to culture anaerobic bacteria from carious dentine result in a significant underestimation of the numbers of bacteria present (23, 26). In contrast, molecular techniques have the potential to produce reliable means of quantifying bacterial DNA and therefore bacterial numbers. One method of achieving this is real-time PCR, a cyclical enzymatic reaction in which two synthetic oligonucleotide primers and a fluorogenic probe hybridize to the nucleotide base sequences specific for the target organism within a sample. By monitoring the release of fluorescence with each PCR cycle, the progress of the reaction can be recorded in real time and the amount of DNA in the sample can be quantified, thus allowing the number of a given bacterial species to be enumerated (14).

In this study we report on the enumeration of bacteria isolated from carious dentine by both colony counting and real-time PCR and correlate the number and type of bacteria with pulpal pathology.

MATERIALS AND METHODS

Source of carious dentine. Sixty-five vital carious teeth were obtained and the carious dentine was collected as described previously (23), in the manner approved by Central Sydney Area Health Service Ethics Review Committee, Sydney, New South Wales, Australia (reference no. 6/96). The unrestored teeth with

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coronal dentine caries were selected on the basis of clinical diagnostic tests which indicated reversible pulpitis (pain and heightened sensitivity to hot and cold stimuli), but without obvious exposure of the pulp tissue and with periodontal pocket depths of less than 4 mm.

Determination of numbers of CFU. The carious dentine from each tooth was individually weighed, and a standard solution of 10 mg (wet weight) of dentine per ml of reduced transport fluid (RTF) (30) was prepared at 37°C in an anaerobic chamber. The carious dentine fragments were dispersed in RTF by first vortexing the fragments for 20 s and then homogenizing by hand in a 2-ml glass homogenizer for 30 s. Serial dilutions (10^{-3} to 10^{-6}) of these suspensions were prepared in RTF; and 100-μl samples were plated in duplicate and incubated either in an anaerobic chamber for up to 2 weeks or, for microaerophilic conditions, in an anaerobic jar with a CO_2 gas pack (Oxoid, Basingstoke, United Kingdom) at 37°C for 48 h. The total microbial load per milligram (wet weight) of dentine was determined by measurement of the number of CFU on Trypticase soy agar (Oxoid) containing 1 μg of menadione ml^{-1}, 0.5 μg of hemin ml^{-1}, 400 μg of L-cysteine ml^{-1}, and 5% horse blood (Amyl Media, Kings Langley, New South Wales, Australia) under both anaerobic and microaerophilic conditions (U.S. Department of Health and Human Services, 1982).

A more detailed study of isolates representing the five microbial genera *Streptococcus*, *Lactobacillus*, *Actinomyces*, *Prevotella*, and *Fusobacterium* was subsequently undertaken on the basis of previous studies (15, 19). Mitis salivarius agar (Oxoid) and Rogosa agar (Oxoid) were used to study the growth of streptococci and lactobacilli, respectively, under microaerophilic conditions (95% N_2, 5% CO_2). Cadmium fluoride acriflavin tellurite (CFAT) agar containing 13 μg of cadmium sulfate ml^{-1}, 85 μg of sodium fluoride ml^{-1}, 1.2 μg of neutral acriflavin ml^{-1}, 2.5 μg of potassium tellurite ml^{-1}, and 5% horse blood was used for the selective isolation of *Actinomyces* spp. and related gram-positive filamentous organisms under anaerobic conditions (40). Kanamycin and vancomycin blood agar (KVA) containing 100 μg of kanamycin ml^{-1}, 7.5 μg of vancomycin ml^{-1}, 1 μg of menadione ml^{-1}, 0.5 μg of hemin ml^{-1}, 400 μg of L-cysteine ml^{-1}, and 5% horse blood was used for the identification of the obligate anaerobic *Prevotella* spp. (6). Crystal violet-erythromycin (CVE) agar containing 1 μg of crystal violet ml^{-1}, 4 μg of erythromycin ml^{-1}, and 5% horse blood was used for the isolation of *Fusobacterium nucleatum* under anaerobic conditions (36). An initial group of 20 specimens was examined to identify the main colony forms on selective media. One CFU of each type that was noted under a stereomicroscope was subcultured, Gram stained, and assessed with biochemical diagnostic test kits (RapID ANA II [Innovative Diagnostic Systems, Atlanta, Ga.]; Rapid ID 32 Strep, API 50 CH, and API 50 CHL medium for *Lactobacillus* spp. [bioMérieux, Marcy-l'Ètoile, France]). To confirm the accuracy of testing, *Streptococcus mutans* LT11 (31), *Lactobacillus acidophilus* ATCC 4356 (Institute of Dental Research Culture Collection, Westmead Centre for Oral Health, Westmead, New South Wales, Australia), and *Actinomyces israelii* ATCC 12102, *Prevotella melaninogenica* ATCC 25845, and *F. nucleatum* ATCC 25586 (American Type Culture Collection, Rockville, Md.) were used as controls. Following identification, plates for each of the clinical samples were examined and the numbers of each main colony type were recorded. The unused dispersed carious dentine samples were frozen at −80°C for subsequent PCR analyses.

Isolation of DNA from bacterial cultures and carious dentine. Reference bacteria were cultured to the late exponential phase, harvested by centrifugation (14,000 × *g* at 18 to 20°C for 2 min), washed, and resuspended in 10 mM phosphate buffer (pH 6.7) containing 1 mg of lysozyme ml^{-1}, 1 mg of mutanolysin ml^{-1}, and 5 mM $ZnCl_2$. After incubation at 60°C for 30 min, DNA was extracted and purified with a QIAamp DNA Mini Kit (QIAGEN, Clifton Hill, Victoria, Australia) according to the instructions of the manufacturer. The DNA concentration (A_{260}) and purity (A_{260}/A_{280}) were measured.

To extract DNA from the anaerobic bacteria present in homogenized carious dentine, frozen suspensions were thawed on ice and 80-μl samples were combined with 100 μl of ATL buffer (QIAGEN) and 400 μg of proteinase K (QIAGEN). The samples were vortexed for 10 s prior to incubation at 56°C for 40 min, with vortexing every 10 min to lyse the cells. Following the addition of 200 μg of RNase (Sigma), the samples were incubated for a further 10 min at 37°C before the DNA was finally purified with a QIAamp DNA Mini Kit according to the instructions of the manufacturer.

Design of probes and primers for real-time PCR. The GenBank database was searched for 16S rRNA gene (rDNA) sequences of the bacteria of interest. The sequences were aligned by using the Genetics Computer Group program PILEUP (Wisconsin Package, version 8, 1994), accessed through the Australian National Genomic Information Service (ANGIS; http://www.angis.org.au). Regions of identity were assessed manually and were then checked for possible cross-hybridization with other bacterial genes by using the database similarity search program BLAST (2), also accessed through ANGIS. Species-specific probe and primer sets were designed from the variable regions of the 16S rDNAs of *Porphyromonas endodontalis*, *Porphyromonas gingivalis*, *P. melaninogenica*, and *Micromonas* (formerly *Peptostreptococcus*) *micros*, whereas a conserved region in the 16S rDNA was chosen for the design of the *Prevotella* and *Fusobacterium* genus-specific probe and primer sets (Table 1).

TABLE 1. Sequences of oligonucleotide primers and probes

Bacterium detected and primer or probe	Sequence (5′ → 3′)[a]	T_m (°C)[b]
Universal[c]		
Forward	TCCTACGGGAGGCAGCAGT	59.4
Reverse	GGACTACCAGGGTATCTAATCCTGTT	58.1
Probe	CGTATTACCGCGGCTGCTGGCAC	69.9
Fusobacterium[d]		
Forward	AAGCGCGTCTAGGTGGTTATGT	58.8
Reverse	TGTAGTTCCGCTTACCTCTCCAG	58.6
Probe	CAACGCAATACAGAGTTGAGCCCTGCATT	69.9
Prevotella		
Forward	CCAGCCAAGTAGCGTGCA	58.1
Reverse	TGGACCTTCCGTATTACCGC	58.5
Probe	AATAAGGACCGGCTAATTCCGTGCCAG	68.8
P. melaninogenica		
Forward	GTGGGATAACCTGCCGAAAG	58.1
Reverse	CCCATCCATTACCGATAAATCTTTA	58.3
Probe	CAAATCTGATGCCGTCATCGAAGACTATGC	69.4
P. endodontalis		
Forward	GCTGCAGCTCAACTGTAGTCTTG	58.1
Reverse	TCAGTGTCAGACGGAGCCTAGTAC	58.6
Probe	CATTCCGCATACCTTCGGTCTCCTCTAGC	69.6
P. gingivalis		
Forward	TCGGTAAGTCAGCGGTGAAAC	58.8
Reverse	GCAAGCTGCCTTCGCAAT	58.7
Probe	CTCAACGTTCAGCCTGCCGTTGAAA	68.8
M. micros		
Forward	AGTGGGATAGCCGTTGGAAA	58.1
Reverse	GACGCGAGCCCTTCTTACAC	58.5
Probe	ACCGCATGAGACCACAGAATCGCA	68.6

[a] All probes were labeled at the 5′ end with FAM and at the 3′ end with TAMRA.
[b] The melting temperature (T_m) of DNA was determined with Primer Express software version 1.0; (Applied Biosystems).
[c] See reference 23 for further details.
[d] The *Fusobacterium*-specific primer and probe set designed for the detection of *F. nucleatum* would also detect *F. periodonticum*, *F. alocis*, and *F. simiae* if they were present in a sample.

All primer and probe sets were subjected to the guidelines established by Applied Biosystems (Foster City, Calif.) by using Primer Express software. Database searches showed that each set was specific for the target bacterium. In the case of *Fusobacterium*, the primer and probe set designed would allow the detection of *F. nucleatum* along with *Fusobacterium periodonticum*, *Fusobacterium alocis*, and *Fusobacterium simiae.* Once they were designed, the probes and primers were synthesized by Applied Biosystems. The oligonucleotide probes were labeled with the fluorescent dyes 6-carboxyfluorescein (FAM) at the 5′ end and 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end (Table 1). The design of the universal probe and primers has been described previously (23).

Specificities of primers and probes for real-time PCR. The bacteria used to test the specificities of the probes and primers included *S. mutans* LT11 and *Streptococcus sanginis* (formerly *S. sanguis*) ATCC 10556 (American Type Culture Collection) grown at 37°C in brain heart infusion broth (Oxoid) under 95% N_2–5% CO_2; *F. nucleatum* ATCC 25586, *Fusobacterium necrophorum* ATCC 25286, *A. israelii* ATCC 12102, and *Actinomyces naeslundii* ATCC 12104 (American Type Culture Collection) grown at 37°C in brain heart infusion broth in an anaerobic chamber (85% N_2, 5% CO_2, 10% H_2); and *P. gingivalis* ATCC 33277, *P. melaninogenica* ATCC 25845, *Prevotella loescheii* ATCC 15930, *M. micros* ATCC 33270, and *Peptostreptococcus anaerobius* ATCC 27337 (American Type Culture Collection) grown at 37°C in an anaerobic chamber in CDC broth (1% Trypticase peptone and 1% Trypticase soy broth [Difco, Becton Dickinson, Md.],

1% yeast extract [Oxoid], 5 mg of NaCl ml^{-1}, 400 μg of L-cysteine ml^{-1} [Sigma Chemical Co., St. Louis, Mo.]) containing 5 μg of hemin ml^{-1} (Sigma), 2 μg of menadione ml^{-1} (Sigma), and 2% horse serum (Commonwealth Serum Laboratories, Parkville, Victoria, Australia). *P. endodontalis* ATCC 35406 (American Type Culture Collection) was also grown in an anaerobic chamber by the method of Zerr et al. (39). *L. acidophilus* ATCC 4356 and *Lactobacillus rhamnosus* ATCC 7469 (Institute of Dental Research Culture Collection) were grown at 37°C in MRS broth (Oxoid) under 95% N_2–5% CO_2. Each probe and primer set was also checked for its ability to recognize the human DNA supplied in the Beta-Actin Detection Kit (Applied Biosystems) and was found to be negative.

The specificities of the probe and primer sets for their target DNA were tested in duplicate with the TaqMan Universal PCR Master Mix in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). For *P. endodontalis* the specificity of the primer and probe set was checked by using the TaqMan PCR Core Reagent Kit (Applied Biosystems) since no PCR product was detected with the TaqMan Universal PCR Master Mix. Each real-time PCR was carried out in a 25-μl volume containing 100 nM each forward primer, reverse primer, and probe and between 10 and 100 pg of template DNA μl^{-1}. The real-time PCR conditions were set at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Data analysis used Sequence Detection software (version 1.6.3), supplied by Applied Biosystems.

Detection of individual and mixed bacterial DNAs by real-time PCR. Once the specificities of the primer and probe sets had been established, optimization of these sets was undertaken to select the concentrations that provided the most efficient amplification of the target DNAs. The ability to detect specific bacteria was verified (in triplicate) both with the DNA of individual organisms and by mixing approximately equal amounts of DNA (3 to 4 pg μl^{-1}) extracted from *F. nucleatum*, *P. melaninogenica*, *P. endodontalis*, *P. gingivalis*, and *M. micros*. The optimized concentrations of the forward primer, the reverse primer, and the fluorogenic probe in the 25-μl reaction volume were 300, 300, and 200 nM, respectively, for *F. nucleatum*; 100, 300, and 200 nM, respectively, for *P. melaninogenica*; 100, 200, and 175 nM, respectively, for *P. endodontalis*; 100, 100, and 150 nM, respectively, for *P. gingivalis*; and 200, 200, and 100 nM, respectively, for *M. micros*. The *Prevotella* genus-specific primer and probe set and the universal primer and probe set were also optimized such that the concentrations of the forward primer, the reverse primer, and the fluorogenic probe were 300, 300, and 175 nM, respectively, for the universal set and 300, 600, and 200 nM, respectively, for the *Prevotella* genus-specific set. A standard curve based on *P. melaninogenica* DNA (829 fg to 8.29 ng) was used to determine bacterial numbers.

Sensitivity of detection of bacterial DNA by real-time PCR. The sensitivity of real-time PCR in detecting DNA was determined in duplicate with DNA extracted from *F. nucleatum*, *P. melaninogenica*, *P. gingivalis*, *P. endodontalis*, and *M. micros* by using the appropriate homologous DNA as a standard (ranges, 2.0 fg to 2.0 ng, 82.9 fg to 8.29 ng, 3.6 fg to 3.6 ng, 24.1 fg to 2.41 ng, and 77.2 fg to 7.72 ng, respectively), as well as from the genus *Prevotella* and total bacteria, by using *P. melaninogenica* DNA as the standard (range, 82.9 fg to 8.29 ng).

Enumeration of anaerobic bacteria in carious dentine by real-time PCR. Purified DNA from carious dentine was used with optimized concentrations of each probe and primer set and an appropriate standard to separately enumerate the total anaerobic bacterial load and the load of the specific individual anaerobic species present in the carious dentine (see above). All analyses were performed in triplicate, and the mean ± standard error of the mean was calculated. Positive controls consisted of approximately 4 pg of homologous bacterial DNA μl^{-1}, and negative controls consisted of sterile H_2O.

Calculation of bacterial cell numbers by real-time PCR. The amount of anaerobic bacterial DNA measured by real-time PCR was converted to theoretical cell numbers to allow comparison with the CFU data. In order to achieve this, the real-time PCR data were optimized by using standard curves derived with DNA extracted from the anaerobic species being enumerated; in the case of the *Prevotella* genus-specific and the universal primer and probe sets, however, *P. melaninogenica* DNA was selected as the standard on the basis of its reported prevalence in carious dentine (19). However, as accurate quantification by real-time PCR also requires knowledge of the size of the genome and the copy number of 16S rDNA within a cell (9, 23) and since this information is unknown for most oral anaerobes, it was necessary to assume that the genome size of all anaerobes was similar to that of *P. gingivalis* (2.2 Mb; The Institute for Genomic Research Microbial Database [http://www.tigr.org]) and that each cell therefore contains 2.37 fg of DNA.

Processing of dental pulp tissues. Immediately following sampling of the carious dentine, the teeth were removed from the anaerobic chamber and the pulp tissue was retrieved, processed, and sectioned as described previously (19), with the exception that tissue fixation was done for 24 h at 4°C in 4% paraformaldehyde in phosphate-buffered saline. All pulp tissue sections were stained with 1% toluidine blue, coded to avoid subsequent examiner bias, and initially examined at low magnification (×50) before selected areas were chosen for further examination at a higher magnification (×312). Each tissue sample was viewed through a graticule eyepiece that divided the section into a series of fields. Depending on the cross-sectional area of the pulp tissue, between 11 and 261 fields were examined per tissue section. Thirty sections were examined for each pulp tissue section.

TABLE 2. Bacteria detected in carious dentine by colony counting

Bacterium	No. of CFU (mg of dentine)$^{-1}$[a]		
	Range	Median	Mean ± SEM
Anaerobic	1.4×10^5–3.7×10^7	5.5×10^6	$(7.3 \pm 0.9) \times 10^6$
Microaerophilic	2.5×10^4–4.7×10^6	8.4×10^5	$(1.2 \pm 0.1) \times 10^6$
Prevotella	0.0–(5.1×10^6)	1.1×10^5	$(4.7 \pm 1.3) \times 10^5$
P. melaninogenica	0.0–(5.1×10^6)	1.0×10^5	$(4.3 \pm 1.3) \times 10^5$
F. nucleatum	0.0–(1.0×10^6)	7.8×10^4	$(1.8 \pm 0.3) \times 10^5$
Actinomycetes	0.0–(5.3×10^6)	7.4×10^4	$(2.3 \pm 0.9) \times 10^5$
Lactobacilli	0.0–(1.9×10^7)	5.2×10^5	$(1.0 \pm 0.3) \times 10^6$
Streptococci	0.0–(3.1×10^6)	1.7×10^5	$(3.7 \pm 0.7) \times 10^5$

[a] Data collected for 65 samples.

With frequent use of reference slides, the tissue fields were assigned to one of four categories with a particular tissue appearance, as described previously by Massey et al. (19). These categories were (i) minimal inflammatory change, which consisted of an essentially normal tissue pattern with minimal inflammatory infiltrate and soft tissue disturbance; (ii) soft tissue degeneration, which consisted of some abnormal connective tissue architecture with changes including thickening of basement membranes through to replacement of tissue by hyaline-affected material often infiltrated with diffuse calcification; (iii) hard tissue degeneration, which consisted of hard tissue changes with evidence of dystrophic calcification; and (iv) inflammatory degenerative change, which consisted of tissues showing widespread infiltration of acute and/or chronic inflammatory cells with abscess formation and necrotic changes.

Most sections showed evidence of a number of different types of pathology. However, an index representing the dominant pathological category was derived for each sample by determining the category most frequently identified within the graticule fields.

Statistical analyses. Nonparametric methods of statistical analysis were applied, as preliminary scrutiny indicated that the data were markedly skewed. Wilcoxon signed-rank statistics, the Kruskal-Wallis test, analysis of variance, *t* tests, and correlation matrices were applied to test differences, including those between the real-time PCR and the colony counting methods of enumeration of the bacteria and between various specificity tests and the interspecies relationships between pathological category and bacterial load. All calculations made use of the program package S-PLUS (MathSoft Engineering & Education, Inc., Cambridge, Mass.), obtained through the Mathematical and Information Sciences Division of the Commonwealth Scientific and Industrial Research Organisation, Sydney, New South Wales, Australia.

RESULTS

Bacteria cultured from carious dentine samples. The number of CFU per milligram of carious dentine showed considerable variability, as did the specific genera detected in individual samples (Table 2). Hundredfold differences in total microbial loads were apparent between samples, while the loads of specific genera and species in the carious dentine varied by up to 4 orders of magnitude between individual teeth. The data were noticeably skewed, with the mean values for specific bacteria or groups greater than the median in each case (Table 2). Anaerobic bacteria were isolated from all samples. The numbers of colonies cultivated on nonselective plates were approximately sixfold greater than the numbers grown under microaerophilic conditions. Analysis of the numbers of CFU showed a predominance of gram-positive bacteria, with *Lactobacillus* spp. being cultivated in the greatest numbers on the selective media. Gram-negative organisms were also

TABLE 3. Specificities of primers and probes for detection of 16S rDNA by real-time PCR in homologous sample or as part of a mixture of DNAs

Bacterium	Amt of DNA detected (mean ± SEM %)[a]	
	Homologous DNA	Mixed DNA
Prevotella	100.0 ± 5.8	98.1 ± 10.7
P. melaninogenica	100.0 ± 13.8	105.2 ± 19.0
Fusobacterium[b]	100.0 ± 3.5	100.6 ± 4.3
M. micros	100.0 ± 2.5	103.6 ± 1.8
P. endodontalis	100.0 ± 8.0	92.4 ± 8.9
P. gingivalis	100.0 ± 0.5	100.6 ± 2.7

[a] The data were determined from triplicate assays with *P. melaninogenica* DNA in the range of 829 fg to 8.29 ng as the standard. No significant differences between the estimate of the amount of DNA when it was the sole source or was part of a mixture of the five DNAs were found by the *t* test.

[b] The *Fusobacterium*-specific primer and probe set designed for the detection of *F. nucleatum* would also detect *F. periodonticum*, *F. alocis*, and *F. simiae* if they were present in a sample.

present in significant numbers, with the *Prevotella* spp. being the largest of the anaerobic group (Table 2).

Five different colony morphologies were identified on CFAT medium. Biochemical analysis indicated that these were *A. israelii*, *Actinomyces odontolyticus*, *A. naeslundii*, and *Bifidobacterium* and *Propionibacterium* species. Seven main colony forms were evident on KVA medium and were identified as *P. melaninogenica*, *Prevotella intermedia*, *Prevotella buccae*, *Prevotella oris*, *Prevotella corporis*, and *Capnocytophaga* and *Lactobacillus* species. Of these, the black-pigmented *Prevotella* spp. were isolated in the greatest numbers. Four colony types were evident on CVE plates, with three being identified as *F. nucleatum* and one being identified as *Leptotrichia buccalis*.

Four main colony types were distinguishable on mitis salivarius agar. Biochemically these resembled *S. mutans*, *S. sanguinis*, *Streptococcus salivarius*, and *Streptococcus anginosus. Lactococcus*, *Leuconostoc*, and *Enterococcus* species were also present. Nine colony forms of lactobacilli were readily discernible on Rogosa agar. These were shown to be the numerically dominant organisms *L. acidophilus* (five different colony morphologies), *L. rhamnosus*, *Lactobacillus paracasei*, *Lactobacillus fermentum*, and *Lactobacillus plantarum*.

Colony counting showed that, of the 65 carious teeth, 63 (97%) were positive for *F. nucleatum* and *Streptococcus* spp., 62 (95%) were positive for *Lactobacillus* spp., 59 (91%) were positive for *Prevotella* and *Actinomyces* spp., and 57 (88%) were positive for *P. melaninogenica*.

Specificities and sensitivities of primer and probe sets in detecting bacterial DNA by real-time PCR. The universal probe and primer set is known to detect members of all anaerobic bacterial groups (23), while the *Prevotella*- and *Fusobacterium*-specific probe and primer sets and the probe and primer sets specific for *P. melaninogenica*, *P. endodontalis*, *P. gingivalis*, and *M. micros* detected the DNA of the target organism, with no cross-reactivity being evident.

Given amounts of DNA from *Prevotella* or the five different selected species could be accurately quantified by real-time PCR whether the DNAs were present as the sole source of DNA in a sample or were present as part of a mixture of all five DNAs (Table 3). This indicated that quantification of DNA from a particular species or genus was not affected even though DNAs from a variety of other species were present. Sensitivity testing showed that the estimated detection limit for the universal probe and primer set was 43 bacteria, while the estimated detection limits for the probe and primer sets specific for *Prevotella*, *Fusobacterium*, *P. melaninogenica*, *P. endodontalis*, *P. gingivalis*, and *M. micros* were 173, 1, 39, 8, 3, and 16 bacteria, respectively.

Enumeration of anaerobic bacteria in carious dentine by real-time PCR. The theoretical number of anaerobic bacteria per milligram (wet weight) of dentine was estimated by real-time PCR with bacterial DNA extracted from the 65 samples (Table 4). As noted with the data obtained by determination of the numbers of CFU, considerable variabilities in the number and nature of the microflora were apparent, with up to a 52-fold difference in the total anaerobic microbial load detected between samples. In samples in which they were detected, the number of *Prevotella* and *Fusobacterium* organisms varied by up to 4 orders of magnitude, the number of *M. micros* and *P. endodontalis* organisms varied by at least 3 orders of magnitude, and the number of *P. gingivalis* organisms varied by 2 orders of magnitude.

When comparative data were available, the number of samples positive for a specific bacterium increased and the number of bacteria estimated by real-time PCR was greater compared with the number determined by counting CFUs. The estimated number of anaerobic bacteria was 41-fold greater when the number was measured by real-time PCR; for *Prevotella* and *Fusobacterium*, these values were 82- and 2.4-fold greater, respectively. The proportion of samples positive for *Fusobacterium* increased from 97% by colony counting to 100% by real-time PCR. Similarly, the proportion of samples positive for *Prevotella* increased from 91 to 97%. In contrast, greater numbers of *P. melaninogenica* were observed by counting of the numbers of colonies following culture of the samples than by real-time PCR. For all except five samples, however, the inability to detect the presence of a given species by real-time PCR was confirmed by colony counting. For the five exceptions, the numbers of CFU identified in the samples as *P. melaninogenica* by biochemical tests could not be enumerated by real-time PCR.

A Wilcoxon signed-rank statistic was used to compare the number of bacteria determined by colony counting with that determined by real-time PCR. The results showed a significant difference ($P < 0.0001$) between the two methods for the counts of total anaerobic bacteria and *Prevotella*, but no dif-

TABLE 4. Bacteria detected in carious dentine by real-time PCR

Bacterium	No. of cells mg of dentine^{-1}[a]		
	Range	Median	Mean ± SEM[c]
Total anaerobic[b]	2.1×10^7–1.1×10^9	2.6×10^8	$(3.0 \pm 0.3) \times 10^8$
Prevotella	0.0–(2.0×10^8)	1.6×10^7	$(3.9 \pm 0.7) \times 10^7$
P. melaninogenica	0.0–(3.5×10^6)	7.6×10^4	$(2.6 \pm 0.7) \times 10^5$
Fusobacterium	4.8×10^2–5.7×10^6	7.2×10^4	$(4.2 \pm 1.1) \times 10^5$
M. micros	0.0–(1.6×10^7)	3.6×10^5	$(1.5 \pm 0.5) \times 10^6$
P. endodontalis	0.0–(1.3×10^6)	6.8×10^3	$(8.4 \pm 3.7) \times 10^4$
P. gingivalis	0.0–(2.5×10^4)	1.0×10^3	$(3.9 \pm 1.0) \times 10^3$

[a] Data collected for 65 samples.

[b] Detected with the universal primer and probe set (23).

[c] Determined from triplicate measurements.

TABLE 5. Distribution of genera and species in each histopathological category, as determined by colony counting

Bacterium	No. of CFU (mean ± SEM)[a]			
	Minimal inflammatory change	Soft tissue degeneration	Hard tissue degeneration	Inflammatory degenerative change
Prevotella	$(5.5 \pm 2.2) \times 10^5$	$(5.0 \pm 2.9) \times 10^5$	$(1.0 \pm 0.5) \times 10^5$	$(4.7 \pm 1.5) \times 10^5$
F. nucleatum	$(1.7 \pm 0.4) \times 10^5$	$(1.7 \pm 0.6) \times 10^5$	$(2.1 \pm 1.4) \times 10^5$	$(1.9 \pm 0.6) \times 10^5$
Actinomycetes	$(3.6 \pm 2.0) \times 10^5$	$(1.1 \pm 0.2) \times 10^5$	$(5.2 \pm 2.8) \times 10^4$	$(2.0 \pm 0.6) \times 10^5$
Lactobacilli	$(1.4 \pm 0.7) \times 10^5$	$(7.3 \pm 2.8) \times 10^5$	$(9.1 \pm 4.0) \times 10^5$	$(6.7 \pm 1.4) \times 10^5$
Streptococci	$(5.1 \pm 1.5) \times 10^5$	$(1.8 \pm 0.6) \times 10^5$	$(3.2 \pm 1.3) \times 10^5$	$(3.5 \pm 0.9) \times 10^5$

[a] Data collected for 65 samples, which were tested in triplicate.

ference between the two was found for *Fusobacterium* ($P = 0.109$) and *P. melaninogenica* ($P = 0.238$).

Pulpal histopathology. For descriptive purposes, histopathological sections of the 65 pulps were divided into four groups on the basis of the dominant pathology, although the majority of sections showed evidence of more than one type. Based on this assignment of a dominant pathology, 43% exhibited an essentially normal histology (a category i pattern), with only focal areas exhibiting inflammatory infiltration or soft tissue disturbances. Soft tissue degeneration (category ii) was evident in 29% of the samples. These pulps showed evidence of pulpal fibrosis and changes to the microvasculature characterized by vessel narrowing and basement membrane thickening, with hyaline deposits often containing diffuse calcifications. Hard tissue degeneration (category iii) changes, characterized by dystrophic calcifications as the dominant pathology, were apparent in 11% of pulpal specimens. The remaining 17% displayed inflammatory degenerative change (category iv), characterized by a mononuclear infiltrate of primarily plasma cells, with smaller numbers of macrophages and lymphocytes. Evidence of abscess formation and areas of necrosis were also noted in category iv samples (19).

Interbacterial associations within carious dentine and associations with the histopathology of pulpitis. The relation between pairs of microbial species present in carious dentine was determined by using Pearson's correlation, while any association between multiple species was determined by using the Bonferroni adjustment, preset at a significance level of 0.05. Correlations were determined for all of the data for the bacteria and for individual histopathological categories (Tables 5 and 6). While the correlation coefficients (r) were low, significant multiple associations between the numbers of CFU of *Prevotella* and *F. nucleatum* ($r > 0.356$) and between the numbers of CFU of lactobacilli and streptococci ($r > 0.356$) were noted in carious dentine. Analyses of the different anaerobic bacteria (by real-time PCR) indicated further associations between *Prevotella*, *P. melaninogenica*, and *Fusobacterium* ($r > 0.346$) and between *Fusobacterium*, *M. micros*, and *P. endodontalis* ($r > 0.346$).

Multivariate analyses were also performed on the number of bacteria determined by colony counting or real-time PCR and the histopathological data (Tables 5 and 6). Irrespective of the method used to enumerate the bacteria in carious dentine, no significant relationship was apparent between the total number of anaerobic or microaerophilic bacteria and the histopathological category. For culture data, there was also no significant relationship between the specific genera and/or species of bacteria present and the histopathological category except in the case of the minimal inflammatory change category (category i), for which the analyses indicated an association between *F. nucleatum* and *Prevotella*, as well as between streptococci and lactobacilli. However, analyses of the anaerobic bacteria detected by real-time PCR indicated significant multiple associations involving *Fusobacterium* in combination with *P. endodontalis*, *M. micros*, and/or *Prevotella* with the first three histopathological categories (Table 7). In the inflammatory degenerative change category (category iv), only one significant microbial association was observed, and that was the one between *P. endodontalis* and *M. micros* (Table 7), although a high but nonsignificant correlation ($r = 0.839$) also existed between the anaerobes *Fusobacterium* and *P. endodontalis*.

DISCUSSION

The results of the present study confirm previous reports that the largest group of isolates from carious dentine is the facultative gram-positive rods, even though *Lactobacillus* spp. were isolated at lower frequencies than in previous studies (11, 15, 17, 19). The finding of significant numbers of gram-negative anaerobic bacteria, however, has previously been reported only by Massey et al. (19). Other studies noted few or no gram-negative rods (8, 11, 15, 17) or did not investigate these bac-

TABLE 6. Distribution of genera and species in each histopathological category, as determined by real-time PCR

Bacterium	No. of bacteria (mean ± SEM)[a]			
	Minimal inflammatory change	Soft tissue degeneration	Hard tissue degeneration	Inflammatory degenerative change
Prevotella	$(3.9 \pm 1.0) \times 10^7$	$(4.5 \pm 1.5) \times 10^7$	$(1.6 \pm 1.1) \times 10^7$	$(4.0 \pm 1.7) \times 10^7$
Fusobacterium[b]	$(5.3 \pm 2.2) \times 10^5$	$(3.2 \pm 1.7) \times 10^5$	$(3.2 \pm 1.9) \times 10^5$	$(4.0 \pm 2.1) \times 10^5$
M. micros	$(9.3 \pm 2.8) \times 10^5$	$(1.1 \pm 0.9) \times 10^6$	$(4.4 \pm 2.6) \times 10^5$	$(4.9 \pm 2.9) \times 10^6$
P. endodontalis	$(1.3 \pm 0.8) \times 10^5$	$(4.8 \pm 3.9) \times 10^4$	$(9.7 \pm 4.4) \times 10^3$	$(7.4 \pm 4.5) \times 10^4$
P. gingivalis	$(5.0 \pm 2.3) \times 10^3$	$(3.0 \pm 1.0) \times 10^3$	$(3.8 \pm 3.0) \times 10^3$	$(3.5 \pm 1.0) \times 10^3$

[a] Data collected for 65 samples, which were tested in triplicate.
[b] The *Fusobacterium*-specific primer and probe set designed for the detection of *F. nucleatum* would also detect *F. periodonticum*, *F. alocis*, and *F. simiae* if they were present in a sample.

TABLE 7. Correlations between anaerobic bacteria detected by real-time PCR and histopathological categories for carious dentine

Histopathological category	Microbial associations	r^a
Minimal inflammatory change	*Fusobacterium*[b], *P. endodontalis*	>0.518
	Fusobacterium, *Prevotella*	>0.518
Soft tissue degenerative change	*P. endodontalis*, *M. micros*	>0.618
	Fusobacterium, *M. micros*	>0.618
	Fusobacterium, *Prevotella*	>0.618
Hard tissue degenerative change	*Fusobacterium*, *Prevotella*	>0.907
Inflammatory degenerative change	*P. endodontalis*, *M. micros*	>0.779

[a] Correlation coefficients (*r*) for multiple comparisons were obtained by using the Bonferroni adjustment, preset at a significance level of 0.05.

[b] The *Fusobacterium*-specific primer and probe set designed for the detection of *F. nucleatum* would also detect *F. periodonticum*, *F. alocis*, and *F. simiae* if they were present in a sample.

teria (21). In the earliest of these studies, this may have been due to difficulties in the isolation of anaerobes (17) or the use of an inadequate anaerobic environment (8). Similar concerns have also been reported in the area of wound infection, in which the potential role of anaerobes has been neglected (4).

A number of studies in endodontic and periodontal microbiology have compared culture methodologies with PCR for the detection of specific bacteria, with a consensus that bacterial detection by PCR is more sensitive and more reliable and has a better ability than culture of the same samples to recognize species (1, 20, 22, 25). The finding in the present study of significant increases in the numbers of *Prevotella* spp. and the total anaerobic load in carious dentine following bacterial enumeration by real-time PCR is in agreement with the previous observations. The reported inability to cultivate *P. gingivalis* or *P. endodontalis* from carious dentine is pertinent to this argument (19), even though the latter species can be identified in dentine caries by immunohistological staining with species-specific antisera (24). Both of these species were readily detected by real-time PCR in 52 and 60% of samples, respectively. *M. micros* was also detected by real-time PCR in 71% of carious dentine samples. Both *M. micros* and *P. endodontalis* have been associated with endodontic pathologies following pulpal necrosis related to advanced coronal caries (13, 29), and *P. gingivalis* has been implicated in periodontal disease (32).

Despite the associations between pulpal inflammation and specific anaerobic bacteria, as deduced by real-time PCR, the anaerobic bacteria detected represented, on average, only 12% of the total anaerobic microflora present in the carious dentine. It is therefore necessary to exercise some caution in assigning important positive or negative correlations by using only a minor percentage of the species present in the carious dentine samples. Further studies are required to determine the nature and the number of these other species. With this proviso in mind, correlations could still be made between the histopathological changes in the pulp and those anaerobic bacteria detected by real-time PCR. The results obtained demonstrated that the presence of similar groups of bacteria was positively associated with most categories of pulpal pathology, including the minimally inflamed category (category i). Despite the presence of predominantly normal pulp tissue in this group, there was frequent evidence of other forms of pathology in the tissue sections. The variations in pulpal pathology noted at the time of extraction might represent differences in the timing of disease progression, acute or chronic phases of development, host responses, or the virulence of bacteria at specific sites.

Analysis of the real-time PCR data indicated an association of *Fusobacterium* with one or more of the organisms *P. endodontalis*, *Prevotella* spp., and *M. micros* in all but the most severe category of pulpal inflammation. Similar associations have been reported in infected root canals (16, 28); and species of "*Bacteroides*" (*Prevotella* and *Porphyromonas*), *Fusobacterium*, and *Peptostreptococcus* have been strongly linked to periapical tissue destruction (29). Synergistic anaerobic infections in animal models have also demonstrated the pathogenic potentials of both *Prevotella* and *Porphyromonas* (18), particularly when they are associated with *F. nucleatum* (3) and *M. micros* (33). Although the significance of *Prevotella* and *Porphyromonas* in carious dentine is not fully understood, a correlation between the *Prevotella* species present in dentine caries and advanced inflammatory pulpal change has been reported (19). Furthermore, the presence of this group of anaerobes has consistently been associated with both acute and chronic anaerobic wound infections in humans (4). It would therefore appear that this group of organisms plays a significant role in anaerobic infections in diverse locations.

A discrepancy in the current study was the finding that the number of CFU of *P. melaninogenica* exceeded the number enumerated by real-time PCR. This anomaly could be accounted for by incorrect biochemical identification of the colonies. Difficulty in identifying *P. melaninogenica* in clinical samples with commercial biochemical test kits has been noted elsewhere (12). It is possible that some of the colonies identified as *P. melaninogenica* could have been *Prevotella tannerae*, a recently described black-pigmented *Prevotella* previously identified incorrectly as *Prevotella intermedia* and *Prevotella nigrescens* (37).

In conclusion, to our knowledge this is the first report of a study that has identified and enumerated a number of potentially important anaerobic bacteria in carious dentine by real-time PCR. This information not only confirms the results of other studies but also extends these findings by suggesting associations between specific anaerobic species and pulpal pathology.

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