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Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set

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The design and evaluation of a set of universal primers and probe for the amplification of 16S rDNA from the Domain *Bacteria* to estimate total bacterial load by real-time PCR is reported. Broad specificity of the universal detection system was confirmed by testing DNA isolated from 34 bacterial species encompassing most of the groups of bacteria outlined in *Bergey's Manual of Determinative Bacteriology*. However, the nature of the chromosomal DNA used as a standard was critical. A DNA standard representing those bacteria most likely to predominate in a given habitat was important for a more accurate determination of total bacterial load due to variations in 16S rDNA copy number and the effect of generation time of the bacteria on this number, since rapid growth could result in multiple replication forks and hence, in effect, more than one copy of portions of the chromosome. The validity of applying these caveats to estimating bacterial load was confirmed by enumerating the number of bacteria in an artificial sample mixed *in vitro* and in clinical carious dentine samples. Taking these parameters into account, the number of anaerobic bacteria estimated by the universal probe and primers set in carious dentine was 40-fold greater than the total bacterial load detected by culture methods, demonstrating the utility of real-time PCR in the analysis of this environment.

Keywords: real-time PCR (TaqMan), detection of bacteria, universal probe, rDNA copy number, carious dentine

INTRODUCTION

Culture dependent methods for enumerating bacterial numbers are known to be biased since bacteria can only be cultivated if their metabolic and physiological requirements can be reproduced *in vitro*. These techniques may take several days to yield a result and therefore are inappropriate in situations where rapid diagnostic decisions are required. Where complex fastidious microbial communities are under investigation, such as the variety of microbial habitats in the oral cavity, enumerating bacteria by traditional microbial culturing techniques may also produce erroneous results (Dymock *et al*., 1996; Kroes *et al*., 1999).

Abbreviations: ANGIS, Australian National Genomic Information Service; 6-FAM, 6-carboxyfluorescein; RTF, reduced transport fluid; TAMRA, 6-carboxy-tetramethylrhodamine; C_T, threshold cycle; T_m, melting temperature of DNA; t_d, bacterial doubling time.

Fluorescence-based methods can also be used to enumerate bacteria. In the food and biotechnology industries, for instance, the automated counting of pure cultures by flow cytometry is well established (Veal *et al*., 2000). However, most bacteria are optically too similar to resolve from each other or from debris using flow cytometry, without artificially modifying the target bacteria using fluorescent labelling techniques such as fluorescent antibodies or fluorescent dyes (Veal *et al*., 2000; Attfield *et al*., 1999). Differences in bacterial cell size, coaggregation of bacteria and the presence of different contaminating matrices (e.g. mud, food, dental plaque, dentine) can also make meaningful counting difficult, if not problematic, by interference with direct, or fluorescence, microscopy (Veal *et al*., 2000).

Rapid enumeration of bacteria can also be achieved by using a variety of molecular approaches (Ward *et al*., 1990; Amann *et al*., 1995; Wintzingerode *et al*.,

1997; Hugenholtz *et al*., 1998). Primers with broad interspecies specificity have been designed to amplify 16S rDNA by PCR and have been used to determine bacterial numbers in complex communities (Wilson *et al*., 1990; Relman *et al*., 1992; Greisen *et al*., 1994; Marchesi *et al*., 1998; Klausegger *et al*., 1999; Suzuki *et al*., 2000). A majority of these studies, however, report the use of more than a single set of primers to detect the bacteria of interest. Other techniques, such as competitive PCR (Blok *et al*., 1997; Rupf *et al*., 1999), are labour-intensive and require the analysis of results from multiple reactions for each test sample. In contrast, real-time PCR, such as the TaqMan system developed by Applied Biosystems, relies on the release and detection of a fluorescent signal following the cleavage of a fluorescent labelled probe by the 5′-exonuclease activity of *Taq* polymerase. In the intact state, the fluorescent signal on the probe, such as 6-carboxyfluorescein (6-FAM), is quenched by the close proximity on the probe of a second dye, 6-carboxy-tetramethylrhodamine (TAMRA). The release of the fluorescent dye during each round of amplification allows for the rapid detection and quantification of DNA without the need for post-PCR processing, such as gel electrophoresis and radioactive hybridization (Heid *et al*., 1996). In addition, the in-built 96-well format greatly increases the number of samples that can be simultaneously analysed.

In theory, conserved regions of 16S rDNA should provide the means for detecting and enumerating complex bacterial populations by real-time PCR, provided a universal probe can be constructed. However, the final determination of bacterial load by real-time PCR in a multi-species population will be influenced by the variation in the number of rRNA operons in a given species (Farelly *et al*., 1995). Bi-directional replication can further increase the numbers of a given rRNA operon, depending on the number of replication forks and the location of the rRNA operon relative to the origin of replication. The number of replication forks is directly related to the generation time, t_d, which in turn depends on the metabolic status of the bacteria at the time of sampling (Neidhardt *et al*., 1990; Klappenbach *et al*., 2000). Not knowing the exact number of copies of 16S rRNA operons in any given species at the time of sampling represents the main limitation to the absolute determination of bacterial numbers by real-time PCR based on 16S rDNA. However, in a variety of complex environmental, industrial and health-related situations in which multi-species populations are sampled along with impurities, or where the bacteria are internalized within a matrix, other methodologies are likely to be far less sensitive or precise.

In this paper, we report the design of a universal probe and primers set which specifically detects 16S rDNA of the Domain *Bacteria* and which is fully compatible with the TaqMan real-time PCR system. We have further characterized the use of this universal probe and primers set in enumerating bacteria with differing t_d and rDNA copy and applied this information to the determination of the anaerobic bacterial load in clinical samples derived from carious dentine where colony counting has, historically, been the preferred option.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* strains JM109 (Yanisch-Perron *et al*., 1985), NM522 (Gough & Murray, 1983) and XL-1 Blue (Stratagene) were available from previous studies. *Staphylococcus aureus* strains ATCC 12600, ATCC 9144, ATCC 12598, BM 10458 and BM 10143, *Staphylococcus epidermidis* strains ATCC 35983 and ATCC 14990, *Staphylococcus haemolyticus* ATCC 29970 and *Staphylococcus haemolyticus* (infiltrative keratitis isolate), *Staphylococcus schleiferi* ATCC 43808, *Pseudomonas aeruginosa* strains ATCC 19660, ATCC 15442, 6294 and 6206, *Pseudomonas fluorescens* (infiltrative keratitis isolate), *Pseudomonas putida* (lens saline isolate), *Pseudomonas stutzeri* (infiltrate isolate), *Pseudomonas alcaligenes* (laboratory isolate), *Pseudomonas* sp. and *Serratia marcescens* ATCC 274 were kindly provided by Dr Mark Willcox, Co-operative Research Centre for Eye Research and Technology, University of New South Wales, Australia. All *Escherichia*, *Staphylococcus*, *Pseudomonas* and *Serratia* species were grown in Luria–Burtani (LB) broth (Miller, 1972) at 37 °C in a shaking incubator. *Streptococcus mutans* LT11 (Tao *et al*., 1993) and *Streptococcus sanguinis* ATCC 10556 (obtained from the American Type Culture Collection, Manassas, VA, USA) were grown at 37 °C in Brain Heart Infusion broth (Oxoid) under 95 % N_2 and 5 % CO_2 (v/v); *Fusobacterium nucleatum* ATCC 25586, *Fusobacterium necrophorum* ATCC 25286, *Actinomyces israelii* ATCC 12102 and *Actinomyces naeslundii* ATCC 12104 were obtained from the American Type Culture Collection and grown at 37 °C in Brain Heart Infusion broth in an anaerobic chamber (85 % N_2, 5 % CO_2, 10 % H_2, by vol.). *Porphyromonas gingivalis* ATCC 33277, *Prevotella melaninogenica* ATCC 25845, *Peptostreptococcus micros* ATCC 33270 and *Peptostreptococcus anaerobius* ATCC 27337 were obtained from the American Type Culture Collection and grown at 37 °C in an anaerobic chamber in CDC broth [1 %, w/v, trypticase peptone and 1 %, w/v, trypticase soy broth (Difco), 1 %, w/v, yeast extract (Oxoid), 5 mg NaCl ml^{-1}, 400 μg L-cysteine ml^{-1} (Sigma)] containing 5 μg haemin ml^{-1} (Sigma), 2 μg menadione ml^{-1} (Sigma) and 2 % (v/v) horse serum (Commonwealth Serum Laboratories). *Porphyromonas endodontalis* ATCC 35406, obtained from the American Type Culture Collection, was also grown in an anaerobic chamber according to the method of Zerr *et al*. (1998). *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus rhamnosus* ATCC 7469 from the Institute of Dental Research Culture Collection (Westmead Centre for Oral Health, Westmead, NSW 2145, Australia) were grown at 37 °C in MRS broth (Oxoid) under 95 % N_2 and 5 % CO_2 (v/v).

Source of carious dentine. Twenty carious teeth were obtained with informed consent from randomly selected patients who presented with pain and requested extraction to relieve their symptoms. Patients were excluded from the study if they reported a history of significant medical disease or antimicrobial therapy within the previous four months. Unrestored teeth with coronal enamel and dentine caries were selected for inclusion in the study on the basis of clinical diagnostic tests which indicated that they were vital, with clinical symptoms of reversible pulpitis (pain and heightened sensitivity to hot and cold stimuli). The study was approved by the Central Sydney Area Health Service Ethics Review Committee, Sydney, Australia (ref. no. 6/96).

Immediately after extraction, each tooth was placed in a container of reduced transport fluid (RTF; Syed & Loesche, 1972) and transferred to an anaerobic chamber at 37 °C containing 85 % N_2, 5 % CO_2 and 10 % H_2 (by vol.). Superficial plaque and debris overlying the carious lesion were removed and the surface rinsed several times with RTF. Using sterile sharp excavators, all the softened and carious dentine was collected as small fragments from each tooth. Sampling was completed within 20 min of tooth extraction.

Determination of c.f.u. in carious dentine. The carious dentine extracted from each tooth was individually weighed and a standard suspension of 10 mg wet wt dentine (ml RTF)$^{-1}$ was prepared at 37 °C in an anaerobic chamber (see above). The dentine fragments were homogeneously dispersed in RTF by first vortexing for 20 s and then homogenizing by hand in a 2 ml glass homogenizer for 30 s. Samples (100 µl) of 10^{-3}–10^{-6} serial dilutions of these suspensions were prepared in RTF and plated in duplicate onto Trypticase Soy agar (Oxoid) containing 2 µg menadione ml^{-1}, 5 µg haemin ml^{-1}, 400 µg L-cysteine ml^{-1} (Sigma) and 5 % (v/v) horse blood (Amyl Media) (US Department of Health and Human Services – Centres for Disease Control, 1982). The plates were incubated at 37 °C in an anaerobic chamber containing 85 % N_2, 5 % CO_2 and 10 % H_2 (by vol.) for 14 d and the number of c.f.u. counted to determine the total microbial load (mg wet wt dentine)$^{-1}$. The unused dispersed carious dentine samples were frozen at −80 °C.

Determination of viable bacteria from *in vitro* cultures. Viable cell counts of cultures of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were determined by plating 100 µl of a 10^{-6} dilution of the appropriate culture grown in LB broth on LB agar plates and counting the colonies after aerobic incubation at 37 °C for 24 h.

Extraction of DNA from bacterial cultures. DNA was isolated from individual bacterial species either by using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions, or by using a freeze–boil method. In the latter instance, bacterial cells from a 250 µl aliquot of culture were obtained by centrifugation (14000 ***g***, 2 min, 18–20 °C) and resuspended in 45 µl 10 mM phosphate buffer, pH 6·7, prior to freezing at −20 °C. The frozen cells were then heated in a boiling water bath for 10 min.

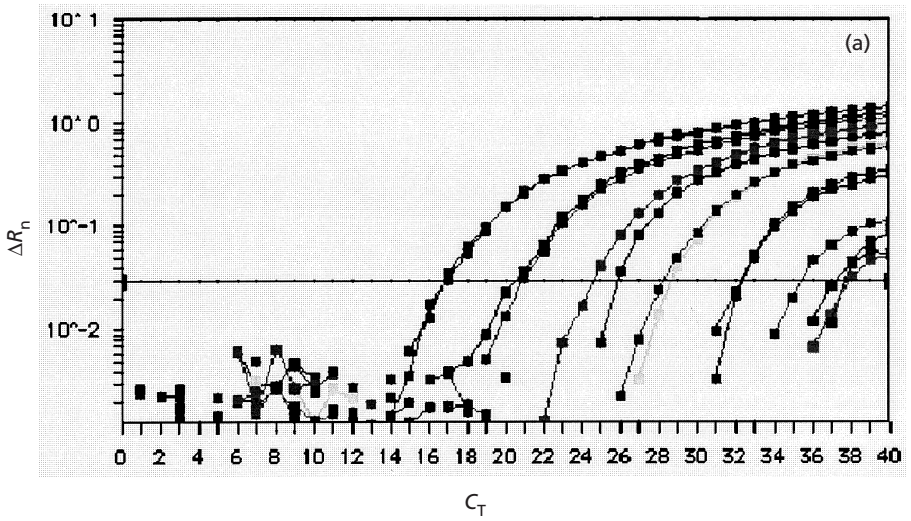
Extraction of anaerobic bacterial DNA from carious dentine. Frozen suspensions of homogenized carious dentine were thawed on ice and 80 µl samples removed and combined with 100 µl ATL buffer (Qiagen) and 400 µg proteinase K (Qiagen). The samples were vortexed for 10 s and then incubated at 56 °C for 40 min with periodic vortexing for 10 s every 10 min to allow complete lysis of the cells. Following the addition of 200 µg RNase (Sigma), the samples were incubated for a further 10 min at 37 °C. DNA free of contaminating RNA was then purified using the QIAamp DNA Mini Kit, according to the manufacturer's instructions.

Sources of other bacterial DNA. DNA from *Legionella pneumophila* serogroup 4 ATCC 33156, serogroup 5 ATCC 33216, serogroup 6 ATCC 33215, serogroup 1 Knoxville-1 ATCC 33153 and Philadelphia-1, as well as *Legionella anisa*, *Legionella bozemanii* (now *Fluoribacter bozemanae*) serogroup 2, *Legionella londiniensis*, *Legionella* (now *Tatlockia*) *maceachernii* and *Legionella waltersii* was kindly provided by Mr Rodney Ratcliff, Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, SA, Australia. DNA from *Mycobacterium tuberculosis* H37RV was kindly provided by Mr Greg James, Microbiology Laboratory, Westmead Hospital, NSW, Australia.

DNA sequence analysis and design of the universal primers and probe. The designed probe and primers set were based on regions of identity within 16S rDNA following the alignment of sequences from most of the groups of bacteria outlined in *Bergey's Manual of Determinative Bacteriology* (Holt *et al*., 1994). The 16S rDNA sequences (GenBank accession no. in parentheses) from *Bacteroides forsythus* (AB035460), *Porphyromonas gingivalis* (POYRR16SC), *Prevotella melaninogenica* (PVORR16SF), '*Cytophaga baltica*' (CBA5972), *Campylobacter jejuni* (CAJRRDAD), *Helicobacter pylori* (HPU00679), *Treponema denticola* (AF139203), *Treponema pallidum* (TRPRG16S), *Leptothrix mobilis* (LM16SRR), *Thiomicrospira denitrificans* (TDE243144), *Neisseria meningitidis* (AF059671), *Actinobacillus* (now *Haemophilus*) *actinomycetemcomitans* (ACNRRNAJ), *Haemophilus influenzae* (HIDNA5483), *Escherichia coli* (ECAT1177T), *Salmonella typhi* (STRNA16), *Vibrio cholerae* (VC16SRRNA), *Coxiella burnetii* (D89791), *Legionella pneumophila* (LP16SRNA), *Pseudomonas aeruginosa* (PARN16S), *Caulobacter vibrioides* (CVI009957), *Rhodospirillum rubrum* (RR16S107R), *Nitrobacter winogradskyi* (NIT16SRA), *Wolbachia* sp. (WSP010275), *Myxococcus xanthus* (MXA233930), *Corynebacterium diphtheriae* (CD16SRDNA), *Mycobacterium tuberculosis* (MTRRNOP), *Streptomyces coelicolor* (SC16SRNA), *Actinomyces odontolyticus* (AO16SRD), *Bacillus subtilis* (AB016721), *Staphylococcus aureus* (SA16SRRN), *Listeria monocytogenes* (S55472), *Enterococcus faecalis* (AB012212), *Lactobacillus acidophilus* (LBARR16SAZ), *Streptococcus mutans* (SM16SRNA), *Clostridium botulinum* (CBA16S), *Peptostreptococcus* (now *Micromonas*) *micros* (PEP16SRR8), *Veillonella dispar* (VDRRNA16S), *Fusobacterium nucleatum* (X55401), *Chlamydia trachomatis* (D89067) and *Mycoplasma pneumoniae* (AF132741) were aligned using the GCG program PILEUP (Wisconsin Package Version 8, 1994) accessed through the Australian National Genomic Information Service (ANGIS, http://www.angis.org.au).

The Primer Express Software provided by Applied Biosystems was of limited value in determining a universal probe and primers set as the primary selection criterion of the software is the length of the amplicon (50–150 bp). The use of this software resulted in a series of best fit suggestions for the universal probe and primers set, leading to unsatisfactory sequence homology for many of the bacterial genera. As a result, the regions of identity within the 16S rDNA had to be assessed manually, with the Primer Express Software being limited to checking for primer–dimer or internal hairpin configurations, melting temperature (T_m) and percentage G + C values within possible primer/probe sets. The final chosen set, including the forward primer, 5′-TCCTACGGGAGGCAGCAGT-3′ (T_m, 59·4 °C), the reverse primer, 5′-GGACTACCAGGGTATCTAATCCTGTT-3′ (T_m, 58·1 °C) and the probe, (6-FAM)-5′-CGTATTACCGCGGCTGCTGGCAC-3′-(TAMRA) (T_m, 69·9 °C), complied with six of the eight guidelines set by Applied Biosystems for the design of primers and probes. These included T_m of the DNA being between 58 and 60 °C for the primers and 68 and 70 °C for the probe; the G + C content being between 30 and 80 mol %; no runs of more than three consecutive Gs in either the primers or the probe; no G on the 5′ end of the probe; and the probe selected from the strand with more Cs than Gs. The primers and probe set only deviated from the ideal in that the last 5 nt of the 3′ end of the forward primer contained more than two GCs and that the amplicon of 466 bp (based on that generated between residues 331 and 797 on the *Escherichia coli* 16S rRNA gene) exceeded the 50–150 bp that was recommended.

The universal probe and primers were checked for possible



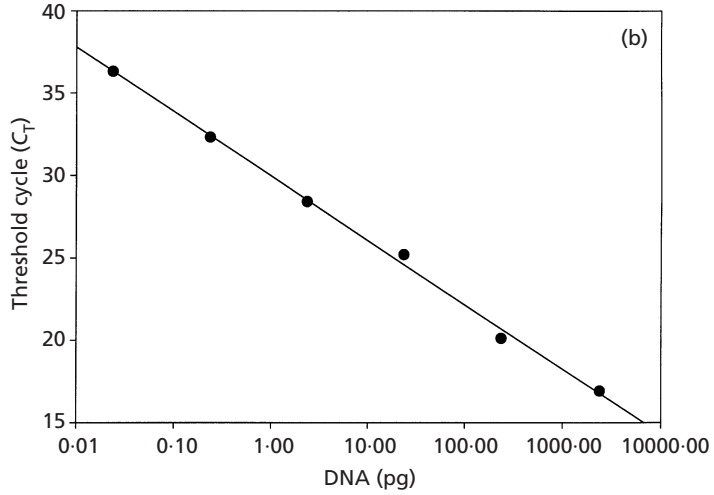


Fig. 1. (a) Sensitivity of the universal probe and primers set in detecting *Escherichia coli* rDNA. Purified *Escherichia coli* DNA was used as the template in quantities of 2380 pg, 238 pg, 23·8 pg, 2·38 pg, 238 fg and 23·8 fg, representing C_T values in the range 16·9–35·3 where the intercept of the magnitude of the fluorescence signal (ΔR_n) with the horizontal threshold line in bold represents the C_T value for a given sample. The fluorescence signal at C_T 37·7 corresponds to the no-template-control and represents bacterial DNA contamination in the commercially supplied reagents. (b) Relation between the threshold cycle and the apparent amount of *Escherichia coli* DNA using TaqMan real-time PCR. Each point represents an amount of *Escherichia coli* DNA corresponding to the C_T value using the universal probe and primers set. The correlation coefficient of the straight line, R^2, was 0·994.

cross-hybridization with bacterial genes other than 16S rDNA as well as genes from *Eucarya* and *Archaea* using the database similarity search program BLAST (Altschul *et al.*, 1990) accessed through ANGIS. The BLAST search results showed only one significant hit – that of a specific breast cancer cell line (BT029) which was detected only by the reverse primer. However, the universal primers did not amplify the human DNA sample supplied by Applied Biosystems in their Beta-actin Detection Kit probe set, thus confirming the specificity of the probe and primers set for the 16S rDNA of the Domain *Bacteria*.

PCR conditions. Amplification and detection of DNA by real-time PCR were performed with the ABI-PRISM 7700 Sequence Detection System (Applied Biosystems) using optical grade 96-well plates. Duplicate samples were routinely used for the determination of DNA by real-time PCR, except in the case of carious dentine where the DNA was amplified in triplicate and mean values calculated. The PCR reaction was performed in a total volume of 25 µl using the TaqMan Universal PCR Master Mix (Applied Biosystems), containing 100 nM of each of the universal forward and reverse primers and the fluorogenic probe, except for the determination of the predominantly anaerobic bacterial load in carious dentine where 300 nM of the forward and reverse primers and 175 nM of the fluorogenic probe were used with the TaqMan PCR Core Reagents Kit. The reaction conditions for amplification of DNA were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data analysis made use of Sequence Detection Software version 1.6.3 supplied by Applied Biosystems.

DNA standards used for determining bacterial number by real-time PCR. *Escherichia coli* DNA was generally used as the standard for determining bacterial number by real-time PCR. However, to determine the effect of variations in rDNA copy number as well as the multiplying effect of the t_d on the calculation of bacterial number, DNA standards were also prepared from two rapidly growing aerobic bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, with t_d *in vitro* in the order of 20–50 min and two slow-growing obligate oral anaerobes, *Prevotella melaninogenica* and *Porphyromonas endodontalis*, with t_d *in vitro* in the order of 5–15 h. Standard graphs were always prepared from data accumulated at the same time as the test samples to act as internal controls.

Relative estimation of bacteria in an artificial *in vitro* mixture and in carious dentine. To determine the validity of using the

Table 1. Representative bacterial species detected by real-time PCR using the universal probe and primers set

Bacterial species*	C_T†
Gram-negative aerobic bacteria	
Pseudomonas aeruginosa ATCC 19660	18·14
Pseudomonas aeruginosa ATCC 15442	18·46
Pseudomonas aeruginosa 6294	18·26
Pseudomonas aeruginosa 6206	19·67
Pseudomonas fluorescens	19·19
Pseudomonas putida	22·35
Pseudomonas stutzeri	18·87
Pseudomonas alcaligenes	19·33
Pseudomonas sp.	19·52
Legionella pneumophila Knoxville-1 ATCC 33153	21·93
Legionella pneumophila serogroup 4 ATCC 33156	21·34
Legionella pneumophila serogroup 5 ATCC 33216	20·08
Legionella pneumophila serogroup 6 ATCC 33215	21·19
Legionella pneumophila Philadelphia-1 ATCC 33152	25·18
Legionella anisa	24·02
Legionella bozemanii serogroup 2 (now *Fluoribacter bozemanae*)	21·46
Legionella londiniensis	20·50
Legionella (now *Tatlockia*) *maceachernii*	22·97
Legionella waltersii	21·96
Gram-negative facultative anaerobic bacteria	
Escherichia coli JM109	19·86
Escherichia coli NM522	28·22
Escherichia coli XL-1 Blue	26·95
Serratia marcescens ATCC 274	20·96
Gram-negative anaerobic bacteria	
Porphyromonas gingivalis ATCC 33277	23·50
Porphyromonas endodontalis ATCC 35406	22·05
Prevotella melaninogenica ATCC 25845	20·48
Fusobacterium necrophorum ATCC 252	23·15
Fusobacterium nucleatum ATCC 25586	21·05
Gram-positive bacteria	
Staphylococcus aureus ATCC 12600	16·15
Staphylococcus aureus ATCC 9144	29·57
Staphylococcus aureus ATCC 12598	27·41
Staphylococcus aureus BM 10458	26·32
Staphylococcus aureus BM 10143	27·20
Staphylococcus epidermidis ATCC 35983	17·88
Staphylococcus epidermidis ATCC 14990	22·27
Staphylococcus haemolyticus ATCC 29970	21·14
Staphylococcus haemolyticus	22·28
Staphylococcus schleiferi ATCC 43808	22·29
Streptococcus sanguinis ATCC 10556	17·05
Streptococcus salivarius ATCC 25975	20·27
Streptococcus gordonii ATCC 10558	20·03
Streptococcus mutans LT11	18·78
Peptostreptococcus anaerobius ATCC 27337	22·36
Peptostreptococcus (now *Micromonas*) *micros* ATCC 33270	22·83
Gram-positive asporogenous bacteria	
Lactobacillus acidophilus ATCC 4356	20·73
Lactobacillus rhamnosus ATCC 7469	24·53
Actinomycetes	
Actinomyces naeslundii ATCC 12104	24·32
Actinomyces israelii ATCC 12102	26·38
Mycobacterium tuberculosis H37RV	26·00

* Groups of bacteria based on *Bergey's Manual of Determinative Bacteriology* (Holt *et al*., 1994).

† DNA was either extracted from equivalent volumes of cultured bacteria or obtained from independent sources and diluted to be within the range of the threshold cycle (C_T) of the standard graph (C_T vs [*Escherichia coli* DNA]). The data are the means of duplicate determinations. Variation in duplicates was $\leqslant 3{\cdot}7\,\%$, except where underlined where the duplicates varied between 6·0 and 11·8 %.

universal probe and primers set to estimate the total number of bacteria in a mixed culture, three bacteria, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, were grown separately *in vitro* to late-exponential–early-stationary phase and equal volumes of the three cultures (2 ml) mixed together. The number of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* c.f.u. at stationary phase was determined by serial dilution on agar plates and compared with the relative bacterial load determined by real-time PCR using the universal probe and primers set and *Escherichia coli* DNA as the standard.

The total number of c.f.u. obtained from carious dentine samples were determined by serial dilution on agar plates in an anaerobic chamber as described above and compared with the relative bacterial load determined by real-time PCR using the universal probe and primers set and *Prevotella melaninogenica* ATCC 25845 DNA as the standard.

RESULTS AND DISCUSSION

Sensitivity of the universal probe and primers in detecting *Escherichia coli* rDNA

TaqMan technology determines the PCR cycle at which the increase in fluorescence of the reporter dye reaches a threshold cycle (C_T) (Fig. 1a). C_T is proportional to the log of the amount of target DNA and hence the log of the number of bacteria in the sample, provided there is only one copy of the reported sequence within the genome. Our standard graph was based on *Escherichia coli* rDNA, where one *Escherichia coli* cell theoretically equates to the detection of 4·96 fg DNA, provided the seven copies of rDNA in each copy of the chromosome (Farelly *et al*., 1995) are not taken into consideration (Fig. 1b). Using *Escherichia coli* as a standard, we consistently detected between 238 fg (corresponding to 48 *Escherichia coli* cells) and 2·38 ng *Escherichia coli* DNA (corresponding to $4{\cdot}8 \times 10^5$ *Escherichia coli* cells). This we designated as the apparent amount of DNA and the apparent number of *Escherichia coli* cells. It should be noted that at extreme high and low C_T values, a two-

fold error in the estimation of the relative amount of DNA can occur. C_T values below 0·1 pg and above 1000 pg DNA should therefore be avoided in calculating the amount of DNA in a sample, provided alternative dilutions are practical (Fig. 1b).

Detection in the apparent range of 4·8–48 cells was limited by contamination from bacterial DNA in the commercially supplied reagents. The degree of contamination varied with different kits of the TaqMan Universal PCR Master Mix and TaqMan PCR Core Reagents Kit. This contamination is thought to be present in either the enzyme preparation or the chemical reagents used for PCR (Bottger, 1990; Schmidt *et al*., 1991; Corless *et al*., 2000; Lyons *et al*., 2000), an observation verified in this current study by the presence of rDNA in reagent mixes and negative controls containing no added *Escherichia coli* DNA (Fig. 1a). To minimize this problem we tested different lot numbers of TaqMan Universal PCR Master Mix and TaqMan PCR Core Reagents Kit supplied by Applied Biosystems and purchased only those with minimum contaminating DNA. Although 40 cycles are theoretically available for the reaction, contamination of reagents manifest in the no-template-control restricted the sensitivity of the reaction to C_T values below 33–38 cycles, depending on the batches of reagents used.

Broad-range detection of bacterial species by the universal probe and primers set

To determine the ability of the universal probe and primers set to detect a broad range of bacteria, samples of DNA extracted from 49 different strains representing 34 different species from the major groups of bacteria listed in *Bergey's Manual of Determinative Bacteriology* (Holt *et al*. 1994), were subjected to real-time PCR using the probe and primers set. All of the selected species were detected within a C_T range of 17·05–34·00 (Table 1). For each species there was little variance in the value of $2{\cdot}00 \times 10^2$ (range $1{\cdot}98 \times 10^2$–$2{\cdot}06 \times 10^2$) *Escherichia coli*-equivalent bacteria (pg DNA)$^{-1}$ when *Escherichia coli* DNA was used as a standard, indicating that the source of DNA was not influencing the level of detection and that the probe and primers set was equally efficient in detecting the DNA irrespective of the species from which it was extracted. Only in the case *Micromonas* (formerly *Peptostreptococcus*) *micros* was there a mismatch in identity between the probe and primers set and the 16S rDNA. This constituted a single nucleotide deletion in the 16S rDNA compared with the 5′ end of the forward primer. This sequence discrepancy was clearly tolerated during real-time PCR detection of *Micromonas* (formerly *Peptostreptococcus*) *micros* DNA (Table 1).

Although larger than the 150 bp limit set in the Applied Biosystems protocol, our 466 bp amplicon was clearly uniformly successful in detecting a wide range of bacteria. Corless *et al*. (2000) have recently reported a universal probe and primers set as a tool for the rapid detection of bacteria by real-time PCR. However, our analysis of their forward and reverse primers and probe showed multiple mismatches with most of the dental pathogens, including *Bacteroides forsythus*, *Porphyromonas gingivalis* and *Prevotella melaninogenica*, and one or more mismatches with many other bacteria at the 5′ as well as the 3′ ends. Similarly, the universal probe and primers set described by Lyons *et al*. (2000) for the detection of total bacteria in dental plaque has one or two mismatches within the probe for the 16S rDNA of *Staphylococcus aureus*, *Campylobacter jejuni*, *Helicobacter pylori*, *Wolbachia* sp., *Micromonas* (formerly *Peptostreptococcus*) *micros*, *Fusobacterium nucleatum*, *Mycoplasma pneumoniae*, *Leptothrix mobilis* and *Thiomicrospira denitrificans*, and their reverse primer has no corresponding 16S rDNA sequences in the databases for numerous Gram-positive and Gram-negative bacteria. Furthermore, their probe and primers set exceeds a number of the guidelines set by Applied Biosystems for TaqMan technology. Their probe was selected from the strand with more Gs than Cs, which could affect the amount of fluorescence measured. The T_m of 48·4 and 35·4 °C for their forward and reverse primers, respectively (calculated from their reported sequences using Primer Express Software), contrasts with the 58–60 °C set by Applied Biosystems and necessitated the authors to use additional annealing steps at 52 °C for 1 min and 72 °C for 2 min to allow for the elongation of their longer amplicon (727 bp). This extended the total reaction time by at least 70 min when using the ABI-PRISM 7700 Sequence Detection System for real-time PCR. The total time taken for quantification was further increased by the requirement for an initial PCR reaction.

Effect of the source of standard DNA on the measurement of relative DNA concentration

To confirm that a DNA standard other than that of *Escherichia coli* should result in a difference in the relative amount of DNA detected due to variations in rDNA copy number and the effect of the t_d on this number, the relative amounts of DNA from the rapidly growing aerobic bacteria *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* were compared with the slow-growing obligate oral anaerobes *Prevotella melaninogenica* and *Porphyromonas endodontalis*. In each instance the relative amount of DNA was estimated by real-time PCR using each of the five DNAs as standards and compared with the amount of DNA determined at A_{260} (set at 100 %). It would be expected that comparison of like DNA by real-time PCR with the known amount of added DNA would be approximately 100 %. In two instances this was not the case. For both *Pseudomonas aeruginosa* and *Prevotella melaninogenica* approximately twice the amount of DNA was detected. This was due in part to the fact that the relative amounts of DNA were calculated by Sequence Detection System version 1.6.3 software supplied by Applied Biosystems based upon the arbitrary placement of the horizontal threshold line used to

Table 2. Effect of species-specific DNA standards on the relative estimation of DNA concentration using the universal probe and primers set for real-time PCR

Bacterium	Relative amount of DNA (%)*					
	A_{260}†	*Staphylococcus aureus* DNA std	*Escherichia coli* DNA std	*Pseudomonas aeruginosa* DNA std	*Porphyromonas endodontalis* DNA std	*Prevotella melaninogenica* DNA std
Staphylococcus aureus	100	106	145	294	1231	2278
Escherichia coli	100	46	96	139	550	1304
Pseudomonas aeruginosa	100	48	96	139	456	669
Porphyromonas endodontalis	100	8	17	9	108	201
Prevotella melaninogenica	100	5	11	10	68	122

* The species-specific standard DNA graphs (C_T vs [DNA]; cf. Fig. 1) were generated from *Escherichia coli* DNA within the range 238 fg–2·38 ng, from *Pseudomonas aeruginosa* DNA within the range 25 fg–2·5 ng, from *Staphylococcus aureus* DNA within the range 27·5 fg–2·75 ng, from *Prevotella melaninogenica* DNA within the range 1·12 pg–112 ng and from *Porphyromonas endodontalis* DNA within the range 240 fg–24 ng. The mean values of duplicate determinations are shown. Variation between duplicates was $\leqslant 2{\cdot}7\,\%$, except where underlined where the values for the *Escherichia coli*, *Prevotella melaninogenica* and *Pseudomonas aeruginosa* DNA standards varied by 4·8, 10·5 and 15·9 % respectively.

† The concentration of DNA was determined spectrophotometrically and normalized to 100 % prior to diluting in the range of 100- to 1000-fold for determination by real-time PCR.

determine the C_T (cf. Fig. 1a). The horizontal threshold line was therefore adjusted to bring these two values as close to 100 % as possible and the relative amount of DNA recalculated (Table 2).

As expected, variation in the relative amount of DNA was observed when the standard DNA differed from that of the species being evaluated (Table 2). However, significant error (> threefold) was only observed when the fast-growing aerobic bacteria were compared with the DNA standards of the slow-growing obligate anaerobes (over estimation) or conversely, when the obligate anaerobes were compared to the DNA of the fast-growing aerobes (under estimation) (Table 2).

The data in Table 2 allowed an estimation of the ratio of the number of copies of the 16S rRNA operons in the different species. A mean ratio of 20:10:9:1:1 (to the nearest integer) for the copy numbers in *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Porphyromonas endodontalis* and *Prevotella melaninogenica*, respectively, fitted the data. This implied that the fast-growing aerobes, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* possessed approximately twice the known chromosomal complement of 16S rRNA operons. The data also predicted that the obligate anaerobes possess only one or two 16S rRNA operons per chromosome. The exact copy numbers are currently unknown.

These results demonstrate that failure to compare DNA from similar groups of bacteria possessing similar growth rates readily leads to an under or over estimation of the amount of DNA by one order of magnitude. Our analyses, however, show that if the ratio of the estimated amount of DNA measured against a rapidly growing bacterium, such as *Staphylococcus aureus*, to that measured against a slow-growing bacterium, such as *Prevotella melaninogenica*, is $<1{\cdot}0$, then the number of bacteria in the sample should be estimated using the DNA extracted from the fast-growing bacterium. If the ratio is $>1{\cdot}0$, the alternative standard DNA from the slow-growing bacterium should be used. In practice, this may simply require reference to a standard curve where the DNA is derived from a bacterium considered to represent the predominant species in the sample. Others, however, have come to different conclusions. For instance, Lyons *et al*. (2000) found no difference in the number of rDNAs per bacterial cell for *Haemophilus* (formerly *Actinobacillus*) *actinomycetemcomitans*, *Porphyromonas gingivalis*, *Escherichia coli* and group G streptococci and therefore assumed that the mean number of 16S rDNA operons in each bacterial cell was similar in all dental plaque samples. Thus, they made no attempt to compensate for differences in 16S rDNA copy number.

Comparison of viable cell numbers and the relative estimation of bacteria in an artificial *in vitro* mixture using real-time PCR

To determine the validity of using the universal probe and primers set to estimate the total number of bacteria in a mixed culture, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were grown separately and equal volumes of the three cultures mixed together. The number of bacteria was similar irrespective of whether the estimation was made using real-time PCR or colony counting (Table 3), despite the fact that the number of copies of the 16S rRNA operons in a single chromosome of *Escherichia coli* is seven while

Table 3. Enumeration of bacterial cell numbers by viable cell count and real-time PCR

Bacterial culture	Viable cell count [cells (ml culture)$^{-1}$]*	Relative estimation of cell numbers by real-time PCR [cells (ml culture)$^{-1}$]†
Escherichia coli	$6{\cdot}5 \times 10^8$	$6{\cdot}7 \times 10^8$
Pseudomonas aeruginosa	$3{\cdot}3 \times 10^9$	$4{\cdot}2 \times 10^9$
Staphylococcus aureus	$1{\cdot}3 \times 10^9$	$2{\cdot}5 \times 10^9$
Mixed culture‡	$1{\cdot}4 \times 10^9$§	$\underline{1{\cdot}3 \times 10^9}$

* The data are the means of duplicate determinations. Variation between duplicates was $\leqslant 5{\cdot}2\,\%$.

† Based on a standard graph generated by *Escherichia coli* DNA within the range 238 fg–2·38 ng. The mean of duplicate determinations for each of two dilutions of DNA are shown. Variation between duplicates did not exceed 3·0 %, except for one dilution where the variation was 8·8 % (underlined).

‡ The mixed culture consisted of equal volumes of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* cultures.

§ Estimated from the viable cell numbers measured in each of the three cultures.

Table 4. Real-time PCR estimation of anaerobic bacteria in carious dentine compared with the total viable anaerobic load

The method of DNA extraction lyses anaerobic Gram-negative and Gram-positive bacteria, but not facultative Gram-positive bacteria.

Sample	Estimation of Gram-negative bacteria by real-time PCR [cells (mg dentine)$^{-1}$]*	Viable c.f.u. [c.f.u. (mg dentine)$^{-1}$]†	Ratio [cells (c.f.u.)$^{-1}$]‡
1	$3{\cdot}4 \times 10^8$	$9{\cdot}0 \times 10^6$	38
2	$\underline{4{\cdot}5 \times 10^8}$	$5{\cdot}5 \times 10^6$	82
3	$4{\cdot}8 \times 10^8$	$9{\cdot}8 \times 10^6$	49
4	$1{\cdot}3 \times 10^8$	$4{\cdot}8 \times 10^6$	27
5	$3{\cdot}8 \times 10^8$	$1{\cdot}2 \times 10^7$	32
6	$\underline{5{\cdot}5 \times 10^8}$	$1{\cdot}2 \times 10^7$	46
7	$1{\cdot}4 \times 10^8$	$6{\cdot}9 \times 10^6$	21
8	$1{\cdot}1 \times 10^8$	$2{\cdot}0 \times 10^6$	55
9	$1{\cdot}9 \times 10^8$	$1{\cdot}5 \times 10^7$	13
10	$3{\cdot}7 \times 10^8$	$2{\cdot}2 \times 10^7$	17
11	$\underline{1{\cdot}4 \times 10^8}$	$3{\cdot}1 \times 10^6$	45
12	$3{\cdot}6 \times 10^8$	$5{\cdot}9 \times 10^6$	61
13	$1{\cdot}5 \times 10^8$	$2{\cdot}2 \times 10^6$	68
14	$1{\cdot}1 \times 10^9$	$1{\cdot}2 \times 10^7$	92
15	$\underline{2{\cdot}6 \times 10^8}$	$1{\cdot}4 \times 10^7$	19
16	$\underline{2{\cdot}5 \times 10^8}$	$1{\cdot}5 \times 10^7$	17
17	$\underline{2{\cdot}8 \times 10^8}$	$8{\cdot}2 \times 10^6$	34
18	$6{\cdot}5 \times 10^8$	$1{\cdot}6 \times 10^7$	41
19	$2{\cdot}5 \times 10^8$	$5{\cdot}6 \times 10^6$	45
20	$6{\cdot}7 \times 10^8$	$3{\cdot}7 \times 10^7$	18

* Based on a standard graph generated by *Prevotella melaninogenica* DNA within the range 82·9 fg–8·29 ng, where 2·36 fg *Prevotella melaninogenica* DNA represents one cell. The data are the means of triplicate determinations. The standard deviation of the means varied by $\leqslant 1{\cdot}0\,\%$, except where underlined where the variation was in the range 1·7–4·4 %.

† The data are the means of duplicate determinations. Variation between duplicates was $\leqslant 10{\cdot}0\,\%$.

‡ The ratio represents the *n*-fold increase in anaerobic bacteria detected by real-time PCR over the total colony count which includes facultative Gram-positive bacteria.

that in *Pseudomonas aeruginosa* is four (Farelly *et al.*, 1995) and *Staphylococcus aureus* is nine (Gurtler & Stanisich, 1996), and the further expectation that *Pseudomonas aeruginosa* would be underestimated (as was apparently the case) and *Staphylococcus aureus* overestimated against the *Escherichia coli* standard DNA.

Comparison of the number of anaerobic bacteria in carious dentine by real-time PCR with the total anaerobic colony count

Determination of bacterial numbers in carious dentine has routinely utilized culture-based techniques. The presence of bacteria adhering to the dentinal matrix or internalized as co-adhering colonies within the dentinal tubules precludes enumeration by microscopic or fluorescence techniques. While colony counting has been the method of choice, this is clearly limiting due to the complex nature of the bacterial flora and their fastidious nutritional requirements. Furthermore, a colony on a plate can be formed by more than one proximal cell, as is the case, for instance, with chains of streptococcal cells. The value of using the universal probe and primers set in estimating the anaerobic bacterial load in carious dentine was therefore determined in 20 clinical samples using *Prevotella melaninogenica* ATCC 25845 DNA from anaerobically grown cells as the standard. Comparison was made with the total anaerobic colony count for each of the samples. The mean number of anaerobic bacteria determined by real-time PCR was $3{\cdot}6 \times 10^8$ (mg dentine)$^{-1}$ [range $1{\cdot}1 \times 10^8$–$1{\cdot}1 \times 10^9$ (mg dentine)$^{-1}$], while that for the total viable cell count was $1{\cdot}1 \times 10^7$ (mg dentine)$^{-1}$ [range $2{\cdot}0 \times 10^6$–$3{\cdot}7 \times 10^7$ (mg dentine)$^{-1}$] (Table 4).

It should be noted that the ATL buffer/proteinase K procedure used in this study not only extracted DNA from Gram-negative anaerobic bacteria but also partially from Gram-positive anaerobic bacteria (data not shown). This is in line with the finding that the cell-wall integrity of Gram-positive anaerobes is compromised when the bacteria are exposed to oxygen (Johnson *et al*., 1995). Other microaerophilic or facultative Gram-positive bacteria, including streptococci, lactobacilli and *Actinomyces*, were not lysed by this procedure (data not shown). Consequently, the finding that the anaerobic bacterial load in samples of carious dentine was 40-fold greater on average than the total anaerobic colony count, which also includes 5–10 % Gram-positive facultative bacteria (Massey *et al*., 1993) that are not lysed by the technique employed, clearly demonstrates the value of our universal probe and primers set in enumerating bacterial numbers by real-time PCR in the presence of a contaminating matrix. Furthermore, the real-time PCR results could be obtained in 6–8 h from patient sampling compared with 14 d for the colony counts. By using species-specific probes with real-time PCR, it will also be possible to rapidly enumerate the numbers of different types of bacteria within these samples and compare them to the cultivable bacteria grown on selective media.

In conclusion, the universal probe and primers set that we have developed for the TaqMan system enables the sensitive detection of numerous bacterial species and strains belonging to the major groups of bacteria defined in *Bergey's Manual of Determinative Bacteriology* (Holt *et al*., 1994), without cross-detection of DNA from *Eucarya* or *Archaea*. Detection was achieved in minimum time and with no additional handling of the PCR product, thereby reducing the chances of contamination. We therefore believe that our designed universal probe and primers set should universally estimate total bacteria by real-time PCR in the shortest possible time. The greatest potential of our probe and primers set lies in its ability to detect bacteria from environmental samples which are difficult to cultivate and that would in all practicality remain undetected or underestimated by viable culture count methods or, alternatively, bacteria that are in an aggregated or coaggregated state or contained within matrix material, such as the carious dentine samples examined in this study, where fluorescent detection and/or microscopic enumeration are also impractical. In addition, the application of this universal probe and primers set could enable rapid differentiation of bacterial from viral infections within the limited time constraints sometimes experienced in life-threatening clinical situations.

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