RNA-oligonucleotide quantification technique (ROQT) for the enumeration of uncultivated bacterial species in subgingival biofilms

F.R.F. Teles[1,3], **R.P. Teles**[1,3], **Y. Siegelin**[1], **B. Paster**[2,3], **A.D. Haffajee**[1,3], and **S.S. Socransky**[1,3]

[1] Department of Periodontology, The Forsyth Institute, Boston, MA, USA

[2] Department of Molecular Genetics, The Forsyth Institute, Boston, MA, USA

[3] Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, Boston, MA, USA

SUMMARY

Approximately 35% of the species present in subgingival biofilms are as yet uncultivated, so their role in periodontal pathogenesis is unknown. The aim of the present study was to develop a high throughput method to quantify a wide range of cultivated and uncultivated taxa in subgingival biofilm samples associated with periodontal disease or health. Oligonucleotides targeting the 16S ribosomal DNA gene were designed, synthesized and labeled with digoxigenin. These probes were hybridized with the total nucleic acids of pure cultures or subgingival biofilm samples. Target species included cultivated taxa associated with periodontal health and disease, as well as uncultivated species, such as TM7 *sp* OT 346, *Mitsuokella* sp. OT 131 and *Desulfobulbus* sp. OT 041. Sensitivity and specificity of the probes were determined. A Universal probe was used to assess total bacterial load. Sequences complementary to the probes were used as standards for quantification. Chemiluminescent signals were visualized after film exposure or using a CCD camera. In a pilot clinical study, 266 subgingival plaque samples from eight periodontally healthy people and 11 patients with periodontitis were examined. Probes were specific and sensitivity reached 10^4 cells. *Fusobacterium nucleatum* ss *polymorphum* and *Actinomyces gerencseriae* were the most abundant cultivated taxa in clinical samples. Among uncultivated/unrecognized species, *Mitsuokella* sp. OT 131 and *Prevotella* sp. OT 306 were the most numerous. *Porphyromonas gingivalis* and *Desulfobulbus* sp. OT 041 were only detected in patients with periodontitis. Direct hybridization of total nucleic acids using oligonucleotide probes permitted the quantification of multiple cultivated and uncultivated taxa in mixed species biofilm samples.

Keywords

bacteria; biofilms; oral; periodontal; uncultivated

INTRODUCTION

Periodontal diseases are polymicrobial infections that can lead to periodontal inflammation and alveolar bone and tooth loss. One key objective in studying these diseases is the discrimination of host-compatible and pathogenic species. Cultivated species including

Correspondence: Flavia R. F. Teles, Department of Periodontology, The Forsyth Institute, 245 First Street, Cambridge, MA, 02142, USA Tel.: + 1 617 892 8562; fax: + 1 617 262 4021 ; fteles@forsyth.org.

Aggregatibacter actinomycetemcomitans, *Porphyromonas gingivalis* and *Tannerella forsythia* were determined to be periodontal pathogens (American Academy of Periodontology, 1996), in part because increased levels of these species were associated with periodontitis and their levels decreased after successful periodontal therapy. Using similar criteria, other species were associated with periodontal health or stability, including members of the genera *Actinomyces* and *Streptococcus*.

The oral cavity harbors more than 700 taxa, 35% of which have not yet been cultivated (Chen *et al*., 2010; Dewhirst *et al*., 2010). It is likely that the uncultivated segment of the microbiota harbors pathogenic as well as beneficial species. Oral uncultivated taxa have been 'discovered' by cloning (Paster *et al*., 2001b) and further investigated using polymerase chain reaction-based techniques (Brinig *et al*., 2003), *in situ* hybridization (Zuger *et al*., 2007) and more recently microarrays (Colombo *et al*., 2009) and next generation sequencing (Keijser *et al*., 2008; Zaura *et al*., 2009). However, none of these techniques has the ability to quantify the levels of multiple uncultivated species in large numbers of individual biofilm samples simultaneously. Absolute numbers and proportions of organisms in multiple individual biofilm samples are important in distinguishing species associated with periodontal health or disease and to evaluate the effects of periodontal therapy (Haffajee *et al*., 1998, 2006; Socransky *et al*., 1998; Socransky and Haffajee, 2005). When attempting to distinguish possible periodontal pathogens from non-pathogenic 'uncultivable' species, it is likely that the investigator will have to examine a wide range of candidate taxa in a large number of subgingival biofilm samples from various states of periodontal health or disease. This is because of the rather large variability encountered in the microbial composition of subgingival biofilm samples. Hence, the purpose of the present investigation was to develop a high throughput method to quantify a wide range of uncultivable and cultivable taxa in large numbers of subgingival biofilm samples.

METHODS

Sample preparation

Two types of samples were employed: (i) total nucleic acids extracted from bacterial cells and (ii) total nucleic acids (TNA) extracted from subgingival biofilm samples.

Bacterial cells—As a result of the unavailability of cells from uncultivated/unrecognized bacterial species, cells from cultivated taxa were used as test species for the development of the method and validation purposes in this study.

The majority of strains (Table 1) were grown on trypticase soy agar supplemented with 5% defibrinated sheep blood (Baltimore Biological Laboratories, Cockeysville, MD). *Tannerella forsythia* was grown on trypticase soy agar supplemented with 5% sheep blood and 10 µg ml^{-1} *N*-acetylmuramic acid (Sigma Chemical Co., St Louis, MO). *Porphyromonas gingivalis* was grown on trypticase soy agar supplemented with 5% sheep blood, 0.3 µg ml^{-1} menadione (Sigma) and 5 µg ml^{-1} hemin (Sigma). *Eubacterium* and *Neisseria* species were grown on fastidious anaerobic agar (Baltimore Biological Laboratories) with 5% defibrinated sheep blood. *Treponema denticola* and *Treponema socranskii* were grown in *Mycoplasma* broth (Difco Laboratories, Detroit, MI) supplemented with 1 mg ml^{-1} glucose, 400 µg ml^{-1} niacinamide, 150 µg ml^{-1} spermine tetrahydrochloride, 20 µg ml^{-1} sodium isobutyrate, 1 mg ml^{-1} L-cysteine, 5 µg ml^{-1} thiamine pyrophosphate and 0.5% bovine serum. All strains were grown at 35°C under anaerobic conditions (80% N$_2$, 10% CO$_2$, 10% H$_2$).

Bacterial cells were harvested from agar plates, placed into 100 µl of RNAse-free TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.6) and kept at −80°C until extraction of TNA.

Subgingival plaque samples—Subgingival plaque samples were collected from healthy subjects and from patients with periodontitis. Samples were taken from mesio-buccal sites using 11/12 sterile Gracey curettes (HuFriedy, Chicago, IL) and placed in individual microcentrifuge tubes containing 100 μl RNAse-free TE buffer. Samples were kept at −80°C until TNA extraction.

Extraction of total nucleic acids

Extraction of TNA from all samples was performed using a Masterpure RNA purification kit (Epicentre, Madison, WI). Cells were pelleted by centrifugation at 3500 r.p.m. for 10 min. After the supernatant was discarded, the pellet contained in 25 μl TE was resuspended by vortex mixing. One microliter of proteinase K (50 μg μl^{-1}) and 300 μl of tissue and cell lysis buffer (provided by the manufacturer) were added and the solution was incubated in a 65°C waterbath for 15 min. After 5 min in ice, 175 μl MCP protein precipitation reagent (provided by the manufacturer) was added to each sample. The debris was pelleted by centrifugation at 12,000 r.p.m. for 10 min. The supernatant was transferred to a new tube and 500 μl isopropanol was added. Tubes were mixed by inversion for 2 min and TNA were pelleted by centrifugation at 12,000 r.p.m. for 10 min at 4°C. After carefully pouring off the isopropanol, pellets were rinsed twice with 70% ethanol. Pellets were air dried for 10 min and resuspended in 35 μl TE buffer at 37°C for 10 min. One microliter of ScriptGuard (Epicentre) was added to each sample and samples were kept at −80°C until analysis. The TNA from individual bacterial species were measured with a spectrophotometer (Nanodrop, Wilmington, DE) at 260 nm wavelength. The TNA from bacterial mixtures and from clinical samples were not measured, rather, the entire sample was laid onto a positively charged nylon membrane.

Ninety microliters of 2% glutaraldehyde and 910 μl 6 × SSC (where 1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) were added to each sample. The final solutions were deposited in individual lanes of a Minislot (Immunetics, Cambridge, MA), concentrated onto a nylon membrane (Boehringer Mannheim) by vacuum and fixed onto the membrane by cross-linking using ultraviolet light (Stratalinker 1800, La Jolla, CA) and dried at room temperature. The Minislot device permitted the deposition of 28 different plaque samples in individual 'lanes' on a single 15 × 15-cm nylon membrane as well as two control lanes containing standards for quantification of each test species.

Probe preparation

Oligonucleotide probes were prepared for 23 cultivated and 19 uncultivated bacterial taxa. The sequences were 18–22 nucleotides in length and had minimal secondary structure. Each sequence included in this group of probes targeted the 16S ribosomal DNA (rDNA) gene of the species or phylotypes listed in Table 2. The probe panel also included one universal (eubacterial) probe. This sequence was based on a conserved region of the bacterial 16S rDNA gene. All probes used in this study were based on sequences routinely employed in the Human Oral Microbial Identification Microarray (HOMIM). The full list of probe sequences has been published elsewhere (Colombo *et al*., 2009). One hundre picamoles of each sequence were labeled using a digoxigenin 3' end labeling kit (Roche, Indianapolis, IN).

Hybridization using oligonucleotide probes

Before hybridization, the membranes were pre-wetted in 2 × SSC. The membranes were pre-hybridized in 35 ml of a solution containing 50% formamide, 5 × SSC, 1% casein (Sigma, St Louis MO), 5 × Denhardt's reagent (Maniatis *et al*., 1982), 25 mM sodium phosphate (pH 6.5) and 0.5 mg ml^{-1} yeast RNA (Boehringer Mannheim). The solution was placed into a plastic hybridization bag containing the membrane. The sealed bag was incubated at 42°C

for at least 90 min. Each membrane with fixed TNA was placed in a Miniblotter 45 (Immunetics, Cambridge MA), with the "lanes" of TNA at 90° to the channels of the device. A 30 × 45 'checkerboard' pattern was produced

Probes were diluted in a proprietary hybridization buffer (UltraHyb Oligo buffer; Ambion, Austin, TX). The final concentration of the different probes in each lane in the Miniblotter 45 varied from 2 to 60 pM. The digoxigenin-labeled oligonucleotide probes, diluted in UltraHyb Oligo buffer, were placed in individual lanes of the Miniblotter. Empty lanes were filled with hybridization solution. The apparatus was placed in a plastic bag and the membranes were hybridized at 42°C for 80 min. Membranes were washed on a rotator with 250 ml sterile 2 × SSC, 0.5% sodium dodecyl sulfate at 37°C for 1 h. To detect hybrids, membranes were blocked in a maleic acid buffer containing 10% casein and then incubated with a 1 : 2500 dilution of anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche, Indianapolis, IN). The membrane and the solution were placed in a sealed hybridization bag and kept on a rotator for 30 min. The membranes were rinsed with maleic acid buffer for 5 min to remove excess antibody and then washed three times with that buffer for 15 min each time. The membranes were washed with 200 ml 'buffer 3' [0.04% $MgCl_2$ and 2.1% diethanolamine (pH 9.5), equal volumes] for 5 min. Finally, 1 ml of a chemiluminescent substrate (CDP Star; Tropix, Bedford, MA) was diluted in 4 ml of 'buffer 3'. The final solution was deposited onto the membrane surface. The membrane was exposed to an X-ray film in a radiograph cassette for 60 min. The films were scanned and signal intensities of the samples and the standards were measured using TOTAL LAB software (NonLinear USA, Durham, NC). Signals were converted to absolute counts by comparison with standards on the membrane. Failure to detect a signal was recorded as zero.

Standards for the oligonucleotide probes

The standards for detection using oligonucleotide probes were a mixture of sequences complementary to the oligonucleotide probes designed to detect both the cultivable and as yet uncultivated taxa. The 'complementary' sequences were synthesized by SigmaGenosys (Woodlands, TX). The final mixtures of standards had 0.004 or 0.04 pM of each sequence. Ninety microlitres 2% glutaraldehyde and 910 μl 6 × SSC were added to the standards. The final solutions of the standards were deposited as the last two lanes of each membrane.

Relation of complementary sequence concentration to bacterial counts

It was estimated that 0.004 and 0.04 pM complementary probe sequence would approximate 10^5 and 10^6 bacterial cells, respectively. As bacterial cells were not available from uncultivated taxa, cultivated bacterial species were employed to test this estimate. Signals were compared from samples containing 10 ng TNA, 10^6 bacterial cells and 0.04 pM oligonucleotide sequences. These targets were hybridized with probes to the species and the intensities of the signals were compared.

Determination of probe sensitivity

Bacterial suspensions were prepared from pure cultures. Upon harvesting, the cell density of each species was adjusted to an optical density at 600 nm of 1 and 10^8 cells of each species were pipetted into a microcentrifuge tube. The resulting suspension was serially 10-fold diluted and TNA from each dilution was individually extracted using the Masterpure RNA purification kit. The samples were quantified as described above.

Determination of probe specificity

To determine the specificity of the probes, 10 ng TNA from 96 different bacterial species commonly found in the oral cavity, as well as 20 'complementary' sequences for the

uncultivated phylotypes, were laid on the nylon membranes using a Minislot. The membranes were then 'probed' in a checkerboard format using all the oligonucleotide probes.

Effect of multiple species in different levels on signal detection

To determine the influence of the presence of different numbers of cells from multiple species on quantification of target species, a series of bacterial mixtures were prepared. Suspensions containing 10^3 to 10^6 cells from a range of bacterial species were prepared. The TNAs were extracted from these mixtures and laid on a nylon membrane and quantified as described above.

Pilot clinical study

To assess the feasibility of the proposed method, a small pilot study was conducted. Eight periodontally healthy individuals and 11 patients with periodontitis were selected for study. Periodontally healthy individuals had more than 24 teeth and presented no sites with pocket depth and/or attachment level greater than 3 mm. Patients with periodontitis had at least 20 teeth, at least 5% of sites (measured at up to 168 sites) with pocket depth of 5 mm or greater and attachment level greater than 3 mm. Subjects included in the study had no systemic condition that might influence periodontal disease or dental treatment. Upon enrollment in the study, all subjects signed an informed consent. The informed consent and study protocol were approved by the Institutional Review Board at The Forsyth Institute, where all of the subjects were monitored and sampled. Samples were taken from two randomly selected contralateral quadrants in each subject, providing a total of 14 samples per subject. After removal of supragingival plaque, subgingival plaque samples were individually taken from each mesio-buccal site in the selected quadrants using sterile 11/12 Gracey curettes. Each sample was placed in a microcentrifuge tube containing 100 μl RNAse-free TE buffer. Samples were kept at −80°C until TNA extraction.

Data analysis

Signals were converted to approximate 'counts' by comparison with the standards on each membrane. The 'counts' were computed by estimating that 0.04 pM of target sequences in the standard was equivalent to approximately 10^6 cells and that 0.004 pM target sequences approximated 10^5 cells. Absence of signal detection was recorded as zero. In clinical samples, 'counts' for each taxon were averaged within a subject and then across subjects in the periodontitis and periodontally healthy groups, separately. Significance of differences between subject groups was determined using the Mann–Whitney U-test. In this pilot investigation, no effort was made to correct for multiple comparisons.

RESULTS

Sensitivity of detection

Figure 1 demonstrates the checkerboard format of the assay as well as the sensitivity of detection of the oligonucleotide probes. For all the species tested, signals could be detected on film at the level of 10^5 bacterial cells and for most species at 10^4 cells, using a newly acquired CCD camera.

Specificity of detection

Probes for cultivated species were tested for specificity against cultivated species, using 10 ng of TNA as target (Fig. 2). Because of the unavailability of cells from uncultivated taxa, probes from those species were tested against their complementary oligonucleotide sequences. These probes were also tested against TNA extracted from 96 cultivated bacterial

species (Table 1). When a signal provided by the hybridization of a test probe with the TNA from any of those species was greater than 10% of the signal provided by the Universal probe for the same TNA, the probe was eliminated from the panel. This was the case for probes for *Haemophilus* sp. oral taxon (OT) 035, *Selenomonas* sp. OT 134 and *Synergistetes* sp. OT 363 [throughout this manuscript, OT or oral taxon designations for uncultivated/unrecognized taxa are provided in accord with the Human Oral Microbiome Database–HOMD (Human Oral Microbiome Database, 2009), when available; GenBank assession numbers can also be found in the HOMD website]. None of the probes for the cultivated taxa showed cross-reactions.

Relation of complementary sequence concentration to bacterial counts

The levels of bacterial taxa in each sample were determined by comparison with standards containing known picomolar levels of sequences complementary to the detection probe sequences. The equivalency between cell numbers and picomolar levels of complementary sequences was individually assessed for 20 cultivated bacterial species. These samples were laid on a nylon membrane and probed using the corresponding specific oligonucleotide probes. It was observed that, on average, 10^6 bacterial cells were equivalent to 0.068 ± 0.048 pM (mean ± SD).

Effect of multiple species at different levels on signal detection

The presence of one or more species in a sample at high levels might affect the detection of other taxa present at lower levels. As there is marked variation in species concentrations in single samples of subgingival biofilms, it was crucial to determine whether the presence of high numbers of cells from a given species would overshadow the presence of less abundant taxa. To evaluate this possibility, mixtures of bacterial species at different levels were prepared. Figure 3 demonstrates that TNA from individual bacterial species could be detected in the range of 10^4 to 10^6 cells when in the presence of different levels of cells of other species. For instance, even in the presence of 10^6 cells of *Actinomyces odontolyticus* and *Streptococcus parasanguinis*, 10^4 cells of *Porphyromonas endodontalis* and 10^3 cells of *Aggregatibacter actinomycetemcomitans* and *Parvimonas micra* could be detected.

Enumeration of taxa in clinical samples

In a pilot clinical study to assess the feasibility of the method, cultivated and uncultivated bacterial taxa could be detected and quantified. Figure 4 shows an example of a typical membrane from this study. The use of a universal (eubacterial) probe gave an estimate of the total bacterial load in each sample. Samples from patients with periodontitis usually showed higher levels of subgingival bacteria based on the universal probe signals than samples from periodontally healthy subjects. Overall, the most commonly detected uncultivated/unrecognized species in the subject population were TM7 sp. OT 346, *Prevotella* sp. OT 306, *Mitsuokella* sp. OT 131 (Fig. 5A). In the cultivated segment of the microbiota, the most abundant taxa were *Fusobacterium nucleatum* ss. *polymorphum*, *Actinomyces gerencseriae* and *Parvimonas micra*. When the data from periodontal health and disease were compared, different mean microbial profiles were observed (Fig. 5B). Patients with periodontitis showed higher mean counts of *Prevotella* sp. OT 306. *Desulfobulbus* sp. OT 041 was detected only in the periodontitis group. Mean counts of *Prevotella intermedia*, a member of the 'orange complex' (Socransky *et al.*, 1998), was also elevated in this group. *Porphyromonas gingivalis*, a periodontal pathogen of the 'red complex' could only be detected in the periodontitis group.

DISCUSSION

It has long been recognized that many taxa in subgingival plaque were not being cultivated, as there were marked differences between total viable counts (representing cultivated species) and total microscopic counts representing all organisms (Socransky *et al*., 1963; Newman and Socransky, 1977). This phenomenon has been described as the 'great plate count anomaly' (Staley and Konopka, 1985) and it seems to be a constant in bacterial samples originating from different environments, including the oral cavity.

It seems likely that periodontal pathogens are among the uncultivated segment of the microbiota. A number of studies have used cloning to seek novel and uncultivated bacterial species (Paster *et al*., 2001a; Becker *et al*., 2002; Dewhirst *et al*., 2000; Kazor *et al*., 2003). Cloning, however, is time-consuming, limiting the number of samples and clones that can be analysed at a time, and it does not allow direct quantification. Recently, next generation sequencing (NGS) methods have surpassed the capabilities of cloning and enabled deeper coverage and less labor-intensive sequencing of microbial communities. Even though NGS represents a major advance in the study of oral microbiology, only small numbers of subjects and samples have been reported (Zaura *et al*., 2009; Li *et al*., 2010). Furthermore, those studies report data at the genus level, preventing the identification of pathogenic or health compatible species.

Because the differences between periodontal health and disease and before and after therapy are quantitative (Haffajee *et al*., 1998, 2006; Socransky *et al*., 1998; Socransky and Haffajee, 2005) and because of the site-specific nature of periodontal diseases, quantification of individual biofilm samples is necessary. Species quantification in samples from periodontal sites with different clinical status in the same or different oral cavities is a powerful first step in discriminating pathogens from host-compatible species. High throughput is another prerequisite. Species counts are highly variable in biofilm samples, requiring the analysis of large numbers of samples from many subjects to detect meaningful differences in species counts. In the present study, the standards for quantification of individual taxa contained 0.004 and 0.04 pM of the sequences complementary to the probes. These levels were estimated based on the molecular weight of the 16S rRNA molecule and the number of copies thought to be present in bacterial cells. Subsequent experiments indicated that 0.04 pM yielded signals equivalent to about 0.44×10^5 bacterial cells.

Ribosomal RNA was selected as the target molecule because it is more abundant in bacterial cells than DNA, and so could enhance the sensitivity of oligonucleotide probes. Additionally, because an actively growing cell has 10^3 to 10^4 rRNA molecules (Scheu *et al*., 1998), rRNA is more associated with cell viability than is DNA (McKillip *et al*., 1998). Hence, the use of rRNA can provide insights on the relevance of the test species in the ecosystem of interest and avoid biases in the results due to the presence of dead cells (Nocker and Camper, 2006).

Levels of periodontal bacteria in single curette stroke samples of subgingival plaque commonly range from 10^4 to 10^7 cells (Socransky and Haffajee, 2005). RNA-oligonucleotide quantification technique (ROQT) is able to consistently detect 10^5 bacterial cells, and 10^4 cells of most species (Fig. 1). This is in line with the level of sensitivity obtained using whole genomic DNA probes.

A number of previous studies have investigated uncultivated species (Paster *et al*., 2001a; Kumar *et al*., 2005; Li *et al*., 2006). Kumar *et al*. (2005) evaluated the subgingival microbiota of periodontal health and disease. The most abundant species belonged to the genera *Selenomonas*, *Streptococcus*, *Veillonella*, *Campylobacter* and *Peptostreptococcus*. *Fusobacterium* species and *Actinomyces* species, both routinely found by culture and DNA

probes (Slots, 1977; Ximenez-Fyvie *et al.*, 2000; Teles *et al.*, 2008) were rarely detected. In contrast, in our study, *Fusobacterium nucleatum* ss *polymorphum* and *Actinomyces gerencseriae* were the most abundant taxa detected. The authors found a robust association of peptostreptococci with periodontitis: *Peptostreptococcus* strains BS044 (not in HOMD) and CK035 (HOMD: *Peptostreptococcus stomatis)* were numerous. Targeted DNA approaches have found *Parvimonas micra* (formerly *Peptostreptococcus micros*) to be elevated in patients with periodontitis (Nonnenmacher *et al.*, 2004). In the present study, *Parvimonas micra* was also more prevalent in periodontitis samples. The authors also reported that *Desulfobulbus* sp. CH031 (not in HOMD) and OT 041 were significantly associated with deep periodontal sites. In our study, although in low numbers, *Desulfobulbus* sp. OT 041 was only detected in periodontitis samples. *Selenomonas* clones were not associated with periodontal disease in the Kumar *et al.* study. In the present study, *Selenomonas noxia* was present in low numbers in healthy subjects and *Selenomonas* sp. CS002 (HOMD: *Mitsuokella* sp. OT 131) was increased in samples from healthy individuals. *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* were rarely detected in periodontitis samples by cloning, which contrasts with investigations using culture, polymerase chain reactions, real-time polymerase chain reactions, immunofluorescence, *in situ* hybridization, immunocytochemistry, DNA probes and oligonucleotide probes (Tanner *et al.*, 1979; Kigure *et al.*, 1995; Klein and Goncalves, 2003; Nonnenmacher *et al.*, 2004; Yang *et al.*, 2004). In our study, *Porphyromonas gingivalis* was solely detected in patients with periodontitis and accounted for a significant portion of the total probe count (Fig. 5B).

In our study, *Tannerella* sp. OT 286 was the least abundant uncultivated taxon present in patients with periodontitis, corroborating findings by others (Zuger *et al.*, 2007). TM7 sp. OT 346 was the most prevalent uncultivated phylotype overall and in the periodontitis group, which is in accord with previous reports (Brinig *et al.*, 2003). The Human Oral Microbe Identification Microrray (HOMIM) has been employed to study the subgingival microbiota of periodontal health and disease (Colombo *et al.*, 2009). The frequency of detection of *Porphyromonas gingivalis*, *Selenomonas noxia*, *Streptococcus anginosus*/ *gordonii*, *Actinomyces gerencseriae* and TM7 OT 346 was significantly higher in diseased sites and the frequency of *Capnocytophaga sputigena* was elevated in healthy sites. These results were in line with the levels of taxa found in the present study.

The aim of the present study was to devise a method to detect and quantify uncultivated bacterial species in subgingival biofilm samples in periodontal health and disease. The specificity of the probes used was confirmed by the absence of cross-reactions with any of the 96 bacterial taxa tested, representing the most prominent cultivable oral bacterial taxa. The small clinical study demonstrated the feasibility of the method for its use in clinical trials. The strengths of the proposed method include the absence of pooling, amplification or dilution bias, because an entire individual sample is laid onto the membrane. It allows the quantification of both cultivable and uncultivable bacterial taxa. It is high throughput, in that multiple samples can be analysed for the levels of multiple species at the same time on a single membrane and it is relatively inexpensive. The method also has limitations. The standard curve presented has three data points, which enables quantification of taxa in the 10^4 to 10^7 cells range in a given sample. However, additional levels of standards can be added to provide a tighter or more comprehensive standard curve. The data presented above were obtained using film exposure, which has a dynamic range of about two orders of magnitude. We have recently switched to image capture by a CCD camera, reaching a dynamic range of 4.8 orders of magnitude and enhancing the accuracy of the method. Although the format of the ROQT may resemble that of the checkerboard DNA–DNA hybridization technique (Socransky *et al.*, 1994, 2004), it is not meant to represent a 'more

sensitive checkerboard'. The techniques differ in the nature of the probes, their target molecules, their hybridization protocols and the nature of the species sought.

It seems that an ever growing number of taxa can be detected in the oral cavity and more than 35% of them cannot be cultivated. It is likely that only a subset of this segment is relevant to disease development. ROQT provides the ability to examine large numbers of biofilm samples from large numbers of subjects for the levels of uncultivated taxa. This approach can indicate the most numerous, and thereby possibly the most relevant, taxa associated with periodontal diseases, clarifying their potential role in initiation and progression of periodontal infections. By identifying the more relevant uncultivated/ unrecognized taxa, this technique will guide the isolation and cultivation of disease-associated uncultivated and unrecognized taxa. Such taxa merit further pursuit by cultivation, to evaluate the pathogenic mechanisms of the selected taxa and to develop more targeted treatment and prevention strategies.

Acknowledgments

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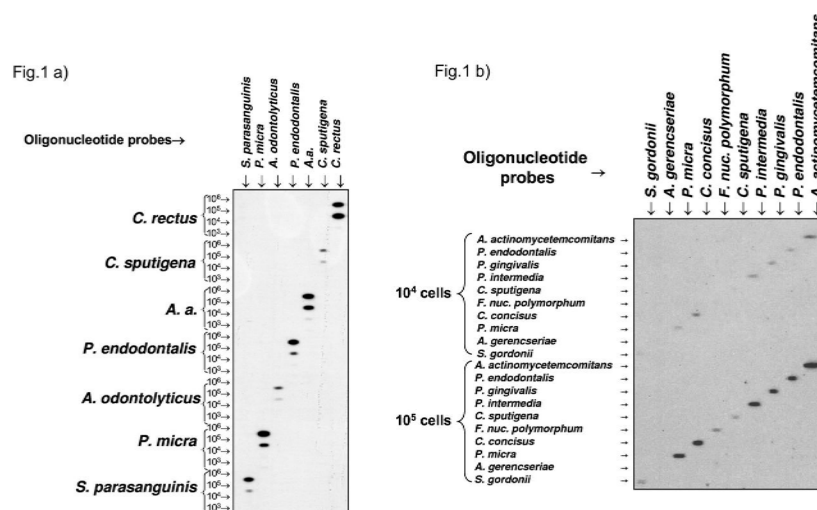
Figure 1.
RNA oligonucleotide quantification technique (ROQT) membrane used to assess probe sensitivity. Total nucleic acids extracted from selected bacterial species were used as targets in the horizontal lanes. Oligonucleotide probes in the vertical lanes were hybridized against these targets. (A) Targets at 10^6 to 10^3 bacterial cells. because of higher than ideal concentration of the *Campylobacter rectus* probe, signals became saturated for both cell concentrations. (B) Targets at 10^5 to 10^4 bacterial cells.
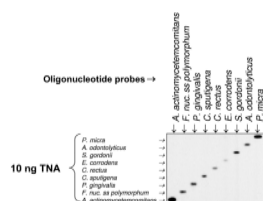
Figure 2.
Assessment of probe specificity. Total nucleic acids (TNA) from selected bacterial species were used as targets in the horizontal lanes. Probes to the same species were hybridized against these targets in the vertical lanes. This figure shows nine of the 23 target taxa tested.
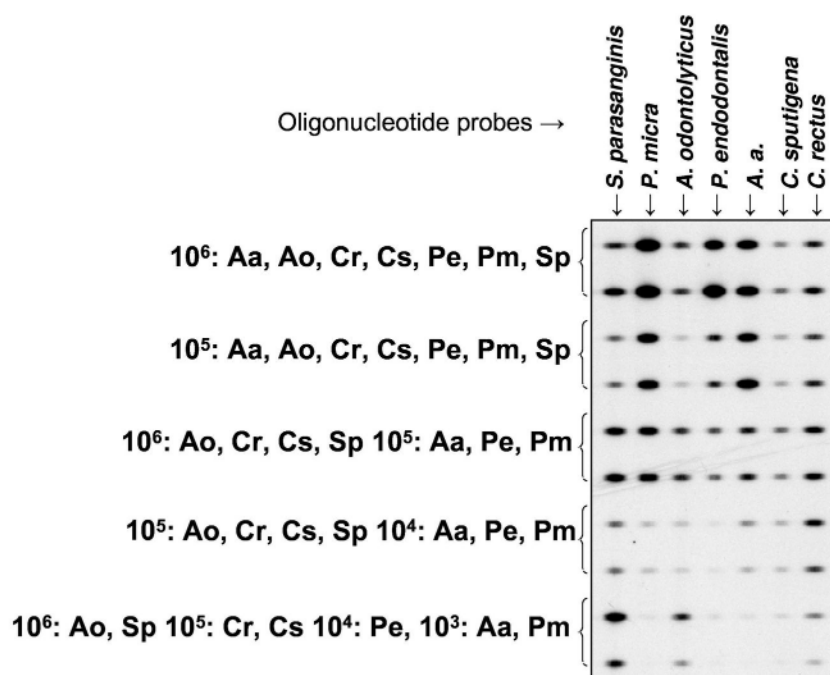
Figure 3.
Effects of different levels of species in mixtures on signal intensity. Aa, *Aggregatibacter actinomycetemcomitans;* Ao, *Actinomyces odontolyticus*; Cr, *Campylobacter rectus*; Cs, *Capnocytophaga sputigena*; Pe, *Porphyromonas endodontalis*; Pm, *Parvimonas micra*; Sp, *Streptococcus parasanguinis*.
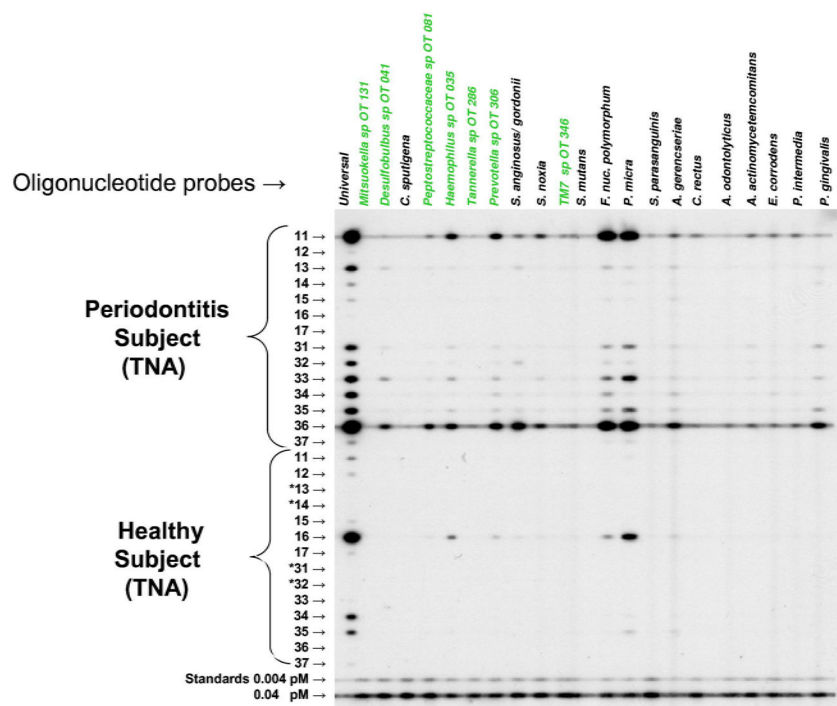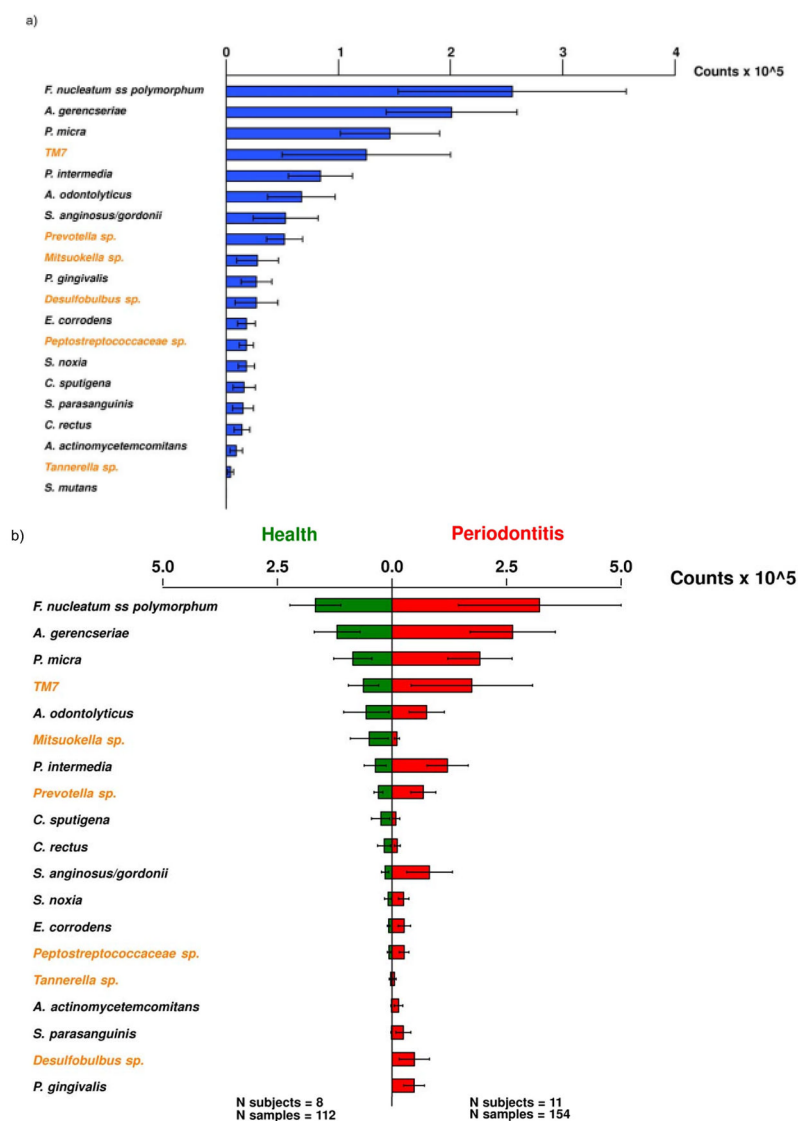
Figure 4.
A checkerboard membrane showing hybridization of clinical samples with oligonucleotide probes. Probes for cultivated species (black) and as yet uncultivated species (green) are listed across the top. Each horizontal lane represents the total nucleic acids (TNA) from a sample from the indicated numbered tooth. Standards comprised a mixture of 'complementary' sequences from all the test taxa at 0.004 and 0.04 pM, respectively. Teeth marked with an asterisk (*) were absent.

Figure 5.
(A) Mean estimated 'counts' ($\times 10^5 \pm$ SEM) of 20 bacterial taxa in 266 subgingival plaque samples obtained from eight periodontally healthy people and 11 patients with periodontitis. The species were ordered according to mean counts. Taxa in black represent cultivated species, whereas those in orange represent uncultivated taxa. (B) Mean estimated 'counts' ($\times 10^5 \pm$ SEM) of 20 bacterial taxa in 266 samples obtained from eight periodontally healthy people (green) and 11 patients with periodontitis (red). The species were ordered according to mean counts in health. Taxa in black represent cultivated species, while those in orange represent uncultivated taxa.

Table 1

Reference bacterial strains used to test specificity of oligonucleotide probes and their American Type Culture Collection numbers

Aggregatibacter actinomycetemcomitans [1]	*Lactobacillus salivarius* (11741)
Actinomyces georgiae (49285)	*Lactobacillus oris* (49062)
Actinomyces gerencseriae (23860)	*Legionella pneumophila* (33153)
Actinomyces israelii (12102)	*Leptotrichia buccalis* (14201)
Actinomyces meyeri (35568)	*Neisseria mucosa* (19696)
Actinomyces naeslundii I (12104)	*Olsenella uli* (49627)
Actinomyces naeslundii I (43146)	*Parvimonas micra* (33270)
Actinomyces odontolyticus (17929)	*Peptostreptococcus anaerobius* (27337)
Atopobium rimae (49626)	*Porphyromonas endodontalis* (35406)
Bacteroides fragilis (25285)	*Porphyromonas gingivalis* (33277)
Campylobacter concisus (33237)	*Prevotella denticola* (35308)
Campylobacter curvus (35224)	*Prevotella intermedia* (25611)
Campylobacter gracilis (33236)	*Prevotella loescheii* (15930)
Campylobacter rectus (33238)	*Prevotella melaninogenica* (25845)
Campylobacter showae (51146)	*Prevotella nigrescens* (33563)
Campylobacter sputorum ss *sputorum* (35980)	*Prevotella oris* (33573)
Campylobacter ureolyticus (33387)	*Prevotella tannerae* (51259)
Capnocytophaga gingivalis (33624)	*Propionibacterium acnes I* [2]
Capnocytophaga ochracea (33596)	*Propionibacterium acnes II* [2]
Capnocytophaga sputigena (33612)	*Propionibacterium propionicum* (14157)
Dialister invisus (GBA27)	*Rothia dentocariosa* (17931)
Eikenella corrodens (23834)	*Selenomonas artemidis* (43528)
Enterococcus aerogenes (13048)	*Selenomonas infelix* (43523)
Enterobacter sakazaki (12868)	*Selenomonas noxia* (43541)
Enterococcus faecalis (10100 & 29212)	*Selenomonas sputigena* (35185)
Escherichia coli (10799)	*Slackia exigua* (700122)
Eubacterium brachy (33089)	*Staphylococcus aureus* (33591)
Eubacterium limosum (8486)	*Staphylococcus epidermidis* (14990)
Eubacterium nodatum (33099)	*Staphylococcus warneri* (27836)
Eubacterium saburreum (33271)	*Streptococcus anginosus* (33397)
Eubacterium saphenum (49989)	*Streptococcus constellatus* (27823)
Filifactor alocis (35896)	*Streptococcus gordonii* (10558)
Fusobacterium necrophorum (25286)	*Streptococcus intermedius* (27335)
Fusobacterium nucleatum ss *nucleatum* (25586)	*Streptococcus mitis* (49456)
Fusobacterium nucleatum ss *polymorphum* (10953)	*Streptococcus mutans* (25175)
Fusobacterium nucleatum ss *vincentii* (49256)	*Streptococcus oralis* (35037)
Fusobacterium periodonticum (33693)	*Streptococcus parasanguinis* (15912)
Fusobacterium naviforme (25832)	*Streptococcus pneumoniae* (49619)
Gemella haemolysans (10379)	*Streptococcus salivarius* (27945)

Gemella morbillorum (27824)	*Streptococcus sanguinis* (10556)
Granulicatella adjacens (49175)	*Streptococcus vestibularis* (49124)
Haemophilus aphrophilus (33389)	*Tannerella forsythia* (43037)
Haemophilus influenza (33533)	*Veillonella dispar* (17748)
Haemophilus paraaphrophilus (29242)	*Veillonella parvula* (10790)
Haemophilus segnis (33393)	*Treponema denticola* (B1)
Lactobacillus acidophilus (4356)	*Veilonella atypica* (17744)
Lactobacillus brevis (14869)	*Bifidobacterium denticum* (27534)
Lactobacillus fermentum (14931)	*Moraxella catarrhalis* (24240)

All cultivated strains were obtained from the American Type Culture Collection (ATCC number in parenthesis), except for *Treponema denticola* (B1), which was obtained from The Forsyth Institute.

[1] ATCC strains 43718 and 29523.

[2] ATCC strains 11827 and 11828.

Table 2

Taxa for which oligonucleotide probes have been validated

Cultivated	Uncultivated/Unrecognized
Aggregatibacter actinomycetemcomitans [1,2]	*Acidaminococcus* [G-1] oral taxon 135
Actinomyces gerencseriae [1,2]	*Acidaminococcus* [G-1] oral taxon 148
Actinomyces odontolyticus [1,2]	*Bacteroidetes* [G-2] sp. oral taxon 274
Campylobacter concisus [2]	*Capnocytophaga* sp. oral taxon 326
Campylobacter rectus [1,2]	*Capnocytophaga* sp. oral taxon 329
Capnocytophaga sputigena [1,2]	*Capnocytophaga* sp. oral taxon 336
Eikenella corrodens [1,2]	*Desulfobulbus* sp. oral taxon 041 [1]
Eubacterium brachy [2]	*Mitsuokella* sp. oral taxon 131 [1]
Fusobacterium nucleatum ss. *polymorphum* [1,2]	*Neisseria* sp. oral taxon 020
Gemella haemolysans [2]	Peptostreptococcaceae [11][G-7] sp. oral taxon 081 [1]
Haemophilus parainfluenza	Peptostreptococcaceae [13][G-1] sp. oral taxon 113
Parvimonas micra [1,2]	*Porphyromonas* sp. oral taxon 279
Porphyromonas endodontalis [2]	*Prevotella* sp. oral taxon 292
Porphyromonas gingivalis [1,2]	*Prevotella* sp. oral taxon 306 [1]
Prevotella denticola [2]	*Streptococcus* sp. oral taxon 055
Prevotella intermedia [2]	*Streptococcus* sp. oral taxon 057
Selenomonas noxia [1,2]	*Streptococcus* sp. oral taxon 066
Shuttleworthia satelles	*Tannerella* sp. oral taxon 286 [1]
Streptococcus anginosus [1]	TM7 [G-1] sp. oral taxon 346 [1]
Streptococcus gordonii [1,2]	
Streptococcus mutans [1,2]	
Streptococcus parasanguinis [1,2]	
Tannerella forsythia [2]	
Treponema denticola	

A subset of the probes employed were 'combination probes', in that they could not distinguish species/phylotypes. This was the case for *S. anginosus/gordonii; C. rectus/concisus* and *Streptococcus* sp. OT055/057/066.

[1] Taxa for which probes were used in the pilot clinical study.

[2] Taxa for which probes were used in the experiments to correlate bacterial cell counts and picomolar levels of complementary sequences.