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Molecular Microbial Diagnosis

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Based on culture-independent molecular methods using sequence analysis of 16S rRNA genes, most of the predominant bacterial species in the oral cavity have been identified (1,2,3,6,[14],[17],[20],[26],[28],[30],[36],39,40,46,48). Collectively speaking, there are about 620 predominant oral bacterial species, of which about 35% have not yet been cultivated in vitro. Whereas there is considerable debate as to what defines a bacterial species (53,54), the 16S rRNA approach defines, in general, a species (or more precisely a phylotype) as 16S rRNA gene sequences of strains or cloned 16S rRNA inserts with >98.5% similarity. Consequently, strain or clone sequences with <98.5% similarity to previously defined phylotypes are considered representatives of new phylotypes (for details, see the Human Oral Microbiome Database [HOMD, http://www.homd.org/]). More recently, based on evaluations of 35,000 16S rRNA gene sequences from about 400 patients, it is estimated that there are approximately 1,200 predominant species (unpublished data, HOMD). In contrast, investigators in a recent study utilizing pyrosequencing analysis of 197,600 sequences derived from the oral cavity have suggested that the microbial diversity of the human oral microbiome is much greater with approximately 19,000 phylotypes (27). However, most of these phylotypes are those found at very low densities.

Bacterial species colonizing the surfaces of the human oral cavity are known to play an important role in oral health and disease and thus a rapid and accurate means of identification is crucial. Traditionally, identification has been based on phenotypic and biochemical criteria, including microscopy, biochemical reactivity, growth conditions, dye and immunofluorescence staining, bacterial end product analysis, cell membrane composition, and antibiotic sensitivity. However, these tests are labor-intensive and costly, providing sometimes inconsistent results that make identification rather tentative. This can be due to strain variation within a species. More recently, molecular DNA-based techniques have been used to identify bacteria directly from clinical samples circumventing the need for in vitro cultivation. Results from these studies implicate specific bacterial species or complexes of species that are associated with oral health and disease. In general, there are 3 main categories of molecular microbial analyses to consider, namely 1) PCR-based methods, including single target PCR, multiplex PCR and quantitative PCR; 2) DNA-DNA hybridization methods such as in situ hybridization, checkerboard hybridization, and 16S rRNA-based microarrays; and 3) sequencing methods including the latest, next-generation sequencing (NGS) techniques, such as pyrosequencing, real-time single-molecule DNA sequencing, and nanopore-based sequencing. The focus of this review is to describe the current status of these DNA-based, culture-independent methods for use or potential use in molecular microbial diagnosis.

PCR-based methods

Single target PCR applications

Many studies have utilized PCR-based methods to detect specific species directly from oral clinical samples. These studies focused on the detection of a few species typically associated with the putative periodontal and caries pathogens, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Streptococcus mutans*, and *Aggregatibacter actinomycetemcomitans* (13,31,34,47,55). In previous studies of sequence analysis of 16S

rRNA genes from the oral cavity, a number of bacterial species were implicated as candidates as putative pathogens for periodontitis, including the more traditional pathogenic species, such as *P. gingivalis*, *T. denticola* and *T. forsythia* (36). Species or phylotype-specific PCR primers were designed and were subsequently used in highly stringent, individual PCR reactions to detect the prevalence of target species in plaque samples of healthy subjects and diseased subjects (29). These investigators confirmed that several additional species, including those that have not yet been grown in vitro, were associated with oral health or periodontitis.

Multiplex PCR

This technique is an expansion of single target PCR methodology in which more than one pair of species-specific primers are used in a single PCR assay that allows for the simultaneous detection of multiple species. Such assays have been used to simultaneously detect *A. actinomycetemcomitans*, *T. forsythia*, and *P. gingivalis* (21,57,62). Optimization of multiplex PCR can be laborious to establish, but ultimately these assays are quite sensitive with detection limits of 10 to 100 cells per PCR reaction (57).

MicroDent® Test is a commercially available method using multiplex PCR that tests for 5 oral species and has been used to compare the microbial profiles of subgingival plaque samples in oral health and periodontitis (19,52).

Real-time PCR

Real-time PCR, also referred to as qPCR, qRT-PCR, RT-qPCR and kinetic PCR, is a method to quantify the copy numbers of DNA in clinical samples. There are two types of real time PCR, namely an intercalator-based method and a probe-based method. The intercalator-based method, also known as SYBR Green method, intercalates SYBR green which binds to newly synthesized double-stranded DNA producing a fluorescently-labeled PCR amplicon. The probe-based method, or TaqMan PCR, is more specific in that it utilizes a fluorogenic-labeled probe that binds only to its complementary sequence in the internal portion of the generated PCR amplicon. Real-time PCR has been used to detect and quantify several periodontal pathogens including *A. actinomycetemcomitans*, *P. gingivalis*, *Prevotella intermedia*, the tetQ gene, and total bacteria, in clinical samples (5,7,32,33).

MYPERIOPATH™ from OralDNAlabs™ is a commercially available service that utilizes TaqMan PCR which determines the microbial profiles of 13 putative periodontal pathogens from oral specimens provided by clinicians. Treatment considerations and follow up recommendations are given with the final report.

DNA-DNA hybridization methods

Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH), or more specifically whole cell hybridization, can be used to quantify, determine the spatial configuration, and demonstrate the morphology of individual bacterial cells in complex natural communities, such as dental plaque (4). Basically, fluorescently labeled, rRNA-targeted oligonucleotides are hybridized to partially fixed, whole cells on microscope slides and are visualized using fluorescence or confocal fluorescence microscopy. Known oral bacterial species including *A. actinomycetemcomitans*, *P. gingivalis*, *Actinomyces* spp. and *Streptococcus* spp. (15,16,44,45) and phylotypes known only from 16S rRNA sequence analysis (35,58) have been detected using FISH. Furthermore, whole-cell hybridizations in solution can be combined with flow cytometry for analysis of mixed microbial populations (63).

Checkerboard hybridization

In order to make definitive bacterial associations with oral health and disease states, the microbial profiles of large numbers of clinical samples must be determined. Consequently in 1994, a method was introduced that enabled the hybridization of 45 DNA samples against 30 DNA probes (i.e., up to 1350 simultaneous hybridizations) on a single support membrane (51). There are two types of checkerboard hybridization, one utilizing whole genomic DNA probes that are hybridized to sample DNA on the membrane (51), and the other utilizing labeled 16S rRNA amplicons that are hybridized to 16S rRNA-based probes that are on the membrane (36). This latter method has been referred to as reverse-capture, 16S rRNA-based oligonucleotide checkerboard hybridization. In both methods, hybridization signals are typically detected using chemifluorescence procedures.

Although there is potential use for checkerboard hybridization as a diagnostic tool, the checkerboard hybridization methodology is used more routinely for research purposes. Most of the publications have used the whole genomic DNA probes checkerboard hybridization to study the roles of bacteria in oral health and disease. In a landmark paper, Socransky et al (49) analyzed 185 subjects representing about 13,000 plaque samples using whole genomic DNA probes to 40 bacterial cultivable species in checkerboard hybridization assays to define bacterial complexes, rather than individual species, that were involved in oral health and periodontal disease. Many publications since then have utilized whole genomic checkerboard hybridization to answer many biologic, research-related questions in oral ecology (23,24,50). Recently, checkerboard hybridization has also been used for the quantification of multiple inflammatory mediators in gingival crevicular fluid (GCF) samples (56)

The reverse-capture checkerboard hybridization has also been used to determine the role of bacteria in oral health and disease, including caries of the primary dentition (6,11), caries of the secondary dentition (3), necrotizing ulcerative periodontitis (39), and periodontal diseases associated with HIV infected individuals (2). The advantage of the reverse capture method is that species that have only been identified as 16S rRNA phylotypes, e.g., the “uncultivables”, can be monitored just as easily as known cultivable species (38).

Oligonucleotide microarray technology

As an extension of the 16S rRNA-based, reverse-capture DNA-DNA checkerboard hybridization, the Human Oral Microbe Identification Microarray, or HOMIM, was developed in order to examine the complex oral microbial diversity in a single hybridization on glass slides (38,41,42). This high sample-throughput, 16S rRNA-based technology allows the simultaneous detection about 300 key and predominant bacterial species, including species that have not yet been cultivated (for details, see http://mim.forsyth.org).

Briefly, 16S rRNA-based, reverse-capture oligonucleotide probes (typically 18 to 20 bases) are synthesized with a 5’ - (C6)-amine modified base and eight spacer thymidines. Probes are printed on aldehyde-coated glass slides. 16S rRNA genes are PCR-amplified from DNA isolated from clinical samples using 16S rRNA universal forward and reverse primers and labeled via incorporation of Cy3-dCTP in a second nested PCR (41). Labeled amplicons are hybridized to the probes on the slides. Details of the protocol have been described (41). Typical arrays are shown in Figure 1.

To analyze data from HOMIM arrays, individual signals are translated to a “bar code” format and are normalized by comparing individual signal intensities to the average of signals from universal probes. The bands correspond to presence or absence and band intensities correspond to 1+, 2+, 3+4+, or 5+—thus more intense bands reflect higher proportions. Figure 2 illustrates the bar code format of HOMIMs comparing the microbial

profiles of approximately 300 bacterial species from subjects with periodontal health and periodontitis. HOMIMs have been used to define the microflora of root surface caries in the elderly (41) and to compare and define the normal flora in 5 sites in the oral cavity of the elderly (42). Most recently, HOMIM has been used to compare the subgingival microbiota of refractory periodontitis, successfully treated periodontitis, and periodontally healthy (10)

One reverse-capture, 16S rRNA-based microarray is currently commercially available for diagnostic use. ParoCheck® DNA chip targets 20 oral bacterial species and has been used to determine the microbial profiles in clinical samples including endodontic lesions (59) and normal microflora of gingival biopsies (18).

Recently, high density 16S rRNA-based microarrays have also been developed. The Phylochip as developed by the Affymetrix Corporation ® and Lawrence Berkeley Labs can detect up to 32,000 16S rRNA phylotypes (9). Huyghe et al. (25) reported on a similar microarray design to study complex bacterial communities that targets 9,500 16S rRNA phylotypes. Most of the studies using such microarrays have focused on analysis of environmental samples. Probes on these high-density microarrays target mainly family and genus rather than species level distinctions.

Sequencing methods

Kumar et al (30) used culture-independent, quantitative analysis of subgingival plaque samples to identify putative pathogenic and health associated species. In this study, DNA from samples was analyzed using ribosomal 16S cloning and subsequent sequencing, but the results were subjected to quantitative analysis. Most studies of this type are descriptive in nature. This technique would be far too laborious to be used as a diagnostic tool.

Next generation sequencing, or NGS, is the newest technology for high-throughput genomic analysis using a pyrosequencing platform (43). Most NGS technologies eliminate the need for cloning and sequencing by amplifying a single DNA molecule (60,61). The 3 main technologies for NGS are as follows: 1) 454 pyrosequencing in which DNA is fragmented and amplified with special adaptors in an emulsion PCR which bind to an agarose bead. This amplification produces up to 1 million copies around one bead. This methodology allows reads 400,000 DNAs that are each about 250 bases in length although newer technology allows for longer reads of up to 500 bp; 2) SOLiD which is similar to 454 pyrosequencing in that fragmented DNA is amplified on an agarose bead. However, this technique utilizes the incorporation of a ligase and universal oligonucleotides. 3) Illumina/Solexa methodology also utilizes fragmented DNA and specialized adaptors, but attaches to a slide rather than a bead. SOLid and Illumina allow for millions of reads, albeit only 35 to 50 bp.

Even newer technologies such as real-time, single-molecule DNA sequencing, which uses RNA polymerase (22), and nanopore sequencing (8,12), which measures the change in current as a single DNA molecule is driven through a tiny pore, allows reading over a thousand bases per second. Such technology greatly reduces the cost per sample.

Concluding remarks

Most of the DNA-based, culture-independent methods described in this review are used more for research purposes in order to answer specific scientific questions rather than for use in molecular microbial diagnosis. For example, 16S rRNA-based and DNA hybridization-based methods are being used to identify the role of particular bacterial species or bacterial complexes in oral infectious diseases, oral cancer, and systemic diseases associated with oral bacteria. Consequently, specific bacterial profiles may be useful to determine those people at risk for disease, e.g., a “unhealthy” profile may be an early

indicator of disease. As the scientific questions are answered and the technology improves, then many of these techniques will likely be utilized as diagnostic tools.

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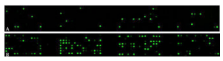

Figure 1.
Microbial profiles of clinical samples. HOMIMs of DNA isolated from subgingival plaque from a healthy site of a healthy subject (A) and from a 5 mm pocket of a subject with periodontitis (B) are compared using 461 oligonucleotide probes that were organized phylogenetically. Note that the diseased profile is considerably more diverse than the healthy profile.

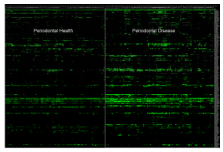

Figure 2.
Bacterial profiles of 461 bacterial taxa (representing about 300 species) comparing subgingival plaque from 105 healthy sites in periodontally healthy subjects (n=20) to 154 diseased sites from periodontally diseased subjects (n=47). Differences in profiles can be seen at a glance. (Courtesy of A.P. Colombo)