Etiology of Oral Disease in View of Microbial Complexity

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Summary: The number of different microorganisms recognized in the oral cavity using molecular methods has more than doubled compared with the number isolated using cultural techniques. This finding necessitates a reevaluation of which species may be pathogens in dental infections. Molecular methods used to determine microbial diversity include broad range target Polymerase Chain Reaction with 'universal primers', and cloning amplicons or denaturing gradient gel electrophoresis to separate DNA fragments before sequencing. These molecular methods have clarified and expanded the taxonomy of oral microbial species. Discrepancies between comprehensive molecular and cultural methods suggest that neither method alone can adequately evaluate associations of specific microorganisms with disease. Oligonucleotide DNA probes, direct PCR, and DNA arrays of the future, can detect cultured and uncultivated phylotypes, so these methods have the potential for use in reevaluating microbial associations with oral diseases. Assigning pathogenicity to newly described and uncultivated species will require new approaches. These include linking the presence of species in biofilms or intracellularly as well as assessing host reactions to stimulation of pathogens within the microbiome.

Conclusions: A wide range of microorganisms has been identified in the oral cavity, and rapid methods have been developed to evaluate their associations with oral infections. New methods are needed to asses the role of phylotypes and fasitidious new species, or specific groups of microorganisms, as etiologic pathogens for oral infections.

Key words: oral bacteria, caries, periodontal disease

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INTRODUCTION

Most infectious diseases, including those of the oral cavity, have been associated with a dominant pathogen, with a limited number of alternative likely pathogens. For example, the dominant pathogen for dental caries is *Streptococcus mutans (S. mutans)*, for chronic and aggressive periodontal diseases *Porphyromonas gingivalis (P. gingivalis)* and *Actinobacillus actinomycetemcomitans* respectively, and for endodontic infections, *Enterococcus faecalis*. These key pathogens, however, were not the only species isolated from sites of infection. Pathogens from culture studies were selected for by having mediators of pathogenicity for the associated infection. The advent of molecular methods that can detect species that are either difficult to culture or, so far, have not been cultivated, has expanded the diversity of phylogenic types, and also increased the number of different species or phylotypes detected in oral sites from 200-300 to over 600 (Paster et al, 2001). This review will summarize the microbial diversity in oral infections, and visit steps needed to evaluate the expanded set of species as etiologic agents in oral infections.

DIVERSITY OF MICROORGANISMS IN ORAL SAMPLES

In addition to classical cultural techniques, microbial diversity has been characterized from PCR/cloning/sequencing studies, adapted from methods devised by Norman Pace to examine the diversity of soil microorganisms. Using this approach, samples underwent PCR amplification using broad-range primers designed to amplify 16S rRNA in bacteria (Paster et al, 2001; Munson et al, 2002), or archea (Lepp et al, 2004) in oral

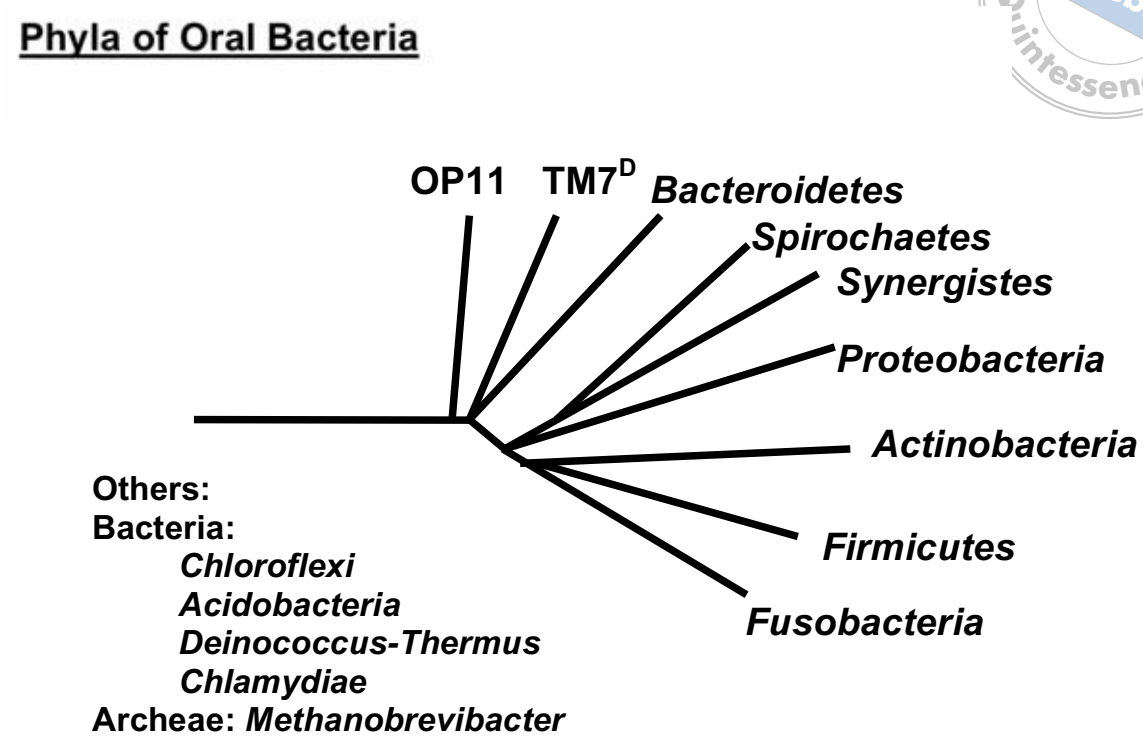

Fig 1 illustrates a phylogenetic tree for the dominant bacterial Phyla and divisions (D) with microorganisms, other bacterial genera with oral species, and a genus within Archeae, *Methanobrevibacter*.

samples. Amplicons were cloned via a vector into *Escherichia coli*, and then individual clones were reisolated and sequenced. An alternative method used denaturing gradient gel electrophoresis (DGGE), and sequencing of bands rather than cloning (Li et al, 2005). Sequences were compared with those of known species, phylotypes or clones, to either identify the sequence as a known species, a previously sequenced phylotype, or to novel previously unrecognized microbial type.

Most studies of the microbial flora of the oral cavity have focused on periodontal samples. The genera in Fig 1 were recognized from subgingival samples (Paster et al, 2001; Lepp et al, 2004). Molecular methods have also examined the diversity of the microbiota of dental caries (Munson et al, 2004; Li et al, 2005) and endodontic infections (Munson et al, 2002; Siqueira and Rocas, 2005). Molecular methods that involved sequencing have proved invaluable in taxonomy to determine phylogenetic relationships between known and newly recognized microorganisms. This has led to taxonomic rearrangements and the creation of new names, further increasing microbial diversity. In many cases, however, these names represent regrouping of previously recognized species, rather than the creation of entirely new phylogenic groups. *Firmicutes* and *Synergistes* represent relatively new Phylum names. The phylum *Synergistes* does include perhaps the unfamiliar genera *Synergistes* and *Desulfothiovibrio*, both genera comprise anaerobic sulfate reducing species. In contrast, the phylum *Firmicutes* includes the familiar 'Gram positive' genera *Streptococcus*, *Gemella*, *Lactobacillus*, *Peptostreptococcus*, *Eubacterium*, *Propionibacterium*, and *Bacillus*, and the 'Gram negative' genera *Selenomonas* and *Veillonella*. The increased complexity of the oral microbiota has come mainly from recognition of "Gram positive" species in *Actinobacteria* and *Firmicutes* including *Slackia, Eggerthella, Cryptobacterium, Olsenella, Scardovia, Parascardovia, Catonella, Filifactor, Mogibacterium, Pseudoramibacter, Shuttleworthia* and *Bulleidia*. Many other new or reclassified species are in recognized genera, including *Treponema, Porphyromonas, Prevotella* and *Leptotrichia*. Of equal, or likely of greater importance to the complexity of the oral microbiota, has been the identification of clones representing uncultivated phylotypes in all the Phyla of Fig 1. These phylotypes include those of genera with no cultivable representatives, for example, the TM7 division originally detected in soil samples and Obsidian pool microorganisms (Paster et al, 2001; Brinig et al, 2003).

While molecular methods have revealed tremendous diversity, they can selectively and preferentially enrich for different segments of the resident microbiota, due to the efficiency and selectivity of 'universal primers'. In

the past, detecting different segments of the microbiota using cultural methods has been instrumental in initiating improvement of sample analysis techniques. Observing a greater number of bacterial morphologic types in dark-field or plaque sections than cultured from samples, led microbiologists to expand and improve their tools. To elucidate microbial diversity in oral samples comparisons were made between microorganisms detected using anaerobic culture on enriched media and PCR using universal primers, cloning and sequencing (Munson et al, 2002, 2004). Both relatively 'non-selective' methods were designed to allow detection of the widest range of taxa. While only a few samples were compared, consistent differences were noted in microbial diversity by the two methods. Increased numbers of *Firmicutes* were detected in endodontic samples by molecular methods compared to culture, but more *Bacteroidetes* were detected by culture (Munson et al, 2002). Further differences were noted in the microbiota detected between broad-range primer sets. In a similar cultural and molecular analysis of samples from deep carious lesions, both microbiological methods detected *S. mutans (Phylum Firmicutes)* but higher proportions of *Actinomyces (Phylum Actinobacteria)* were detected by culture compared with molecular methods (Munson et al, 2004). These findings suggest that neither cultural nor molecular methods alone can reliably evaluate the microbiota of oral diseases and that either both are needed concurrently, or different approaches are needed to reduce bias. Furthermore, both these comprehensive methods are labor-intensive and technique sensitive.

ETIOLOGY OF DISEASE IN VIEW OF COMPLEXITY

The recognition of an increased complexity of microorganisms in the oral cavity indicates a need for an evaluation of the new species and phylotypes as pathogens. New approaches will be required for microorganisms recognized only from sequence-based data. Several stages have been proposed to assign etiology of species in infections based on 'Koch's postulates'. Criteria to include sequence-based microbial identifications have been proposed (Fredericks and Relman, 1996), and revised recently (Relman, 2002) to include use of 'molecular signatures' of infection. These molecular signatures include features of microorganisms, and features of host response (Table 1). The proposed first step was to detect the 'association with disease' particularly disease prediction, and lack of detection with successful therapy and health (Relman, 2002). To do this, one needs tools that avoid the limitations of the comprehensive cultural and broad-range target PCR because of limitations described above. Currently available tools to detect cultivable species include DNA probes and direct PCR technologies (Fredericks and Relman, 1996; Siqueira and Rocas, 2005) in addition to immunoassays. Fastidious and difficult to grow species and uncultivated phylotypes, can be detected using PCR-based methods, for example, oligonucleotide DNA probes and PCR. Multiplex PCR allows detection of two to about five species simultaneously and macroarray, Checkerboard DNA probe assays allow for simultaneous assay of 30-40 species on a single membrane.

Newly recognized species and uncultivated phylotypes have been sought in oral samples using methods to evaluate associations with disease. For periodontal samples, direct PCR to species selected from broad-range polymerase chain reaction and cloning analysis was used to compare the microbiota of periodontitis and periodontal health (Kumar et al, 2003). Several new phylotypes and species in TM7, *Synergistes*, and *Treponema* were associated with periodontitis. Our recent clinical study used a combination of multiplex PCR and oligonucleotide probes in a checkerboard assay to screen over 100 subjects for the presence of over 50 species or uncultivated phylotypes (Tanner et al, submitted). Several periodontally associated phylotypes, and newly recognized species, were detected in the population of healthy and initial periodontitis subjects using the DNA probe assay including *Filifactor alocis*, *Dialister invisus* and clone TM7 1025. Multiplex PCR was more sensitive for *P. gingivalis* than the DNA probe assay and, with *Tanerella forsythensis*, was associated with initial periodontitis using the direct PCR assay.

An associated challenge is to quantitate microorganisms in samples to ascertain their role, by species detection and proportions of microbiota, during disease progression and remission. In addition, studies that can localize microorganisms in the plaque biofilm, particularly whether species are adjacent to diseased host tissues, using fluorescent labeled gene probes (FISH) will likely be critical in assessing association with disease. Future studies will likely use high-density microarrays of oligonucleotide or amplified DNA products to rapidly screen for specific phylotypes (Relman, 2002). To determine pathogenic potential, evaluating individual bacterial cell, and biofilm, biology will be indicated (Table 1).

Detecting disease-associated mediators in host tissues in association with detecting selected species would provide additional evidence to assess pathogenicity (Table 1). In periodontal infections, this could be from changes in gene expression in local tissues in response to microbes. In caries, for enamel lesions, this might entail measuring tissue response to pH change by physical methods. Furthermore, methods that investigate cross-talk between host and microbiome, while challenging, will likely be instrumental is determining microbial pathogenicity. Thus, assessing microbial etiology in

Table 1 Approaches to assess etiology for new species, uncultivated phylotypes, and bacterial complexes in oral infections

Approach	Data to be acquired	Examples of methods
Microorganism detection and quantitation	Strength of association between microorganism(s) and disease Risk factor analysis of organisms associated with disease, disease progression and healing	16S rRNA libraries Macroarrays (Checkerboards) DNA microarrays Quantitative PCR, FISH Culture
Bacterial cell biology	Genome-wide transcript profile for species at the disease site (or disease model) Genetics of cell physiology Cell proliferation Conditional expression of pathogenic factors and their effects	Expression microarrays Quantitative RT-PCR Full, partial genome sequencing Protein chemistry Induction regulation studies Biochemistry of interactions
Biofilm biology	Survey of genes in microbiota (Metagenome analysis) for pathway shifts or factor representation Protein profiling at disease site (or model) Small molecule and protein detection at the disease site (or disease model) Biofilm formation in host	Shotgun sequencing Fosmid library sequencing Mass spectrometry Microscopy and cell biology
Host response	Host genome-wide transcript profile Host protein profiling Small molecule and protein detection at the disease site (or disease model), including response to inflammation, and bacterial stimuli Genetic background of the host for disease susceptibility to specific pathogens	Expression microarrays Mass spectrometry Epidemiology Biochemistry of cell interaction Biochemistry Co-culture
Cross-talk host-microbiome	Differential display or representational difference for genes, transcripts, proteins and small molecules between health and disease	Microarrays Mass spectrometry Microscopy Cell biology Biochemistry

the presence of a diverse and complex microbiota will require multi- and interdisciplinary research, combined with "shrewd clinical insight" (Relman, 2002). Finally, a comparison of disease associations and molecular signatures of pathogenicity of newly defined microorganisms with currently recognized pathogens to oral infections will be important to assess the relative importance of new etiologic agents.

CONCLUSIONS

Molecular tools have revealed an extraordinary diversity in the oral microbiota. While a challenging task, currently available molecular tools and those devised in the future will provide methods to tease out which of these newly recognized microbes might also be of great influence on disease outcome.

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