

A Comprehensive Metagenomic Approach to Determine the Relationship Between Periodontal Disease and Cardiovascular Disease in Young Adults

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ABSTRACT:

Periodontal Disease (PD) is shown to be associated with an increased risk of a number of systemic diseases - including atherosclerosis. We are investigating a possible causal relationship in a young population of individuals with PD (ages 20-30) to determine if they show early signs of atherosclerosis. Using powerful new culture-independent molecular approaches based on pyrosequencing technology, we are conducting a comprehensive metagenomic analysis to identify, classify, and quantify bacterial species associated PD and atherosclerosis (both pre- and post-treatment for PD). This survey of microbial diversity is expected to cover between 300,000-500,000 16S sequences for 300 samples from 40 patients. The bioinformatics pipeline will include 1) databasing data sets of unique 100-250-base 16SrRNA sequences per species, 2) sequence alignment and phylogenetic analysis, and 3) computing distance metrics for differences between microbial communities (samples) at different stages of PD and with or without changes in brachial artery flow dynamics. Multivariate analyses, such as Principle Coordinates Analysis (PCoA), will be used to show the distribution of 16S community samples. Subsequently, functional annotation of the genes combined with relative abundance data for each species will allow for the prediction of important functional relationships among potential subgroups of species that support the persistence of PD and that could be involved in the mechanism of action leading to atherosclerosis. Ultimately, this knowledge could be used to develop an effective probiotic treatment for PD.

BACKGROUND AND SIGNIFICANCE:

Periodontal disease (PD) is a risk factor for atherosclerosis. Endothelial function is a sensitive and reliable indicator of vascular health. PD is accompanied by a local inflammatory response. The magnitude of inflammatory burden is related to the progression of atherosclerotic calcifications in patients with advanced disease (Li et al. 2007). Two studies implicated *P. gingivalis* as a particular risk factor for atherosclerosis (Chou et al. 2005; Yamazaki et al. 2007). Some bacteria secrete factors that limit growth of other species, likely substantially altering the entire ecosystem. Comprehensive characterization of the oral microbial ecosystem including many anaerobic species and those never before cultured (Lepp et al. 2004) will prove valuable. Identifying those strongly associated with PD, as well as oral flora specifically associated with atherosclerosis (as reflected by endothelial dysfunction) is highly probable with new high-throughput molecular methods.

Culture-independent molecular methods: Culture-independent molecular techniques, based on PCR and molecular cloning of small subunit ribosomal (16S rRNA) gene sequences, revolutionized our ability to study previously uncultured microbial organisms. The 16S rRNA gene is the "gold-standard" of markers for culture-independent microbial studies because: (1) It is found in all forms of cellular life: bacterial, archaeal and eukaryal; (2) Enormous databases of 16S sequences from cultured and uncultured microbes, allow for easy identification and phylogenetic analyses; (3) This molecule contains both highly conserved regions necessary for universal primer design, and highly variable regions necessary for identifying species and phylogenetic relationships (Pace 1997; Hugenholz et al. 1998). Our lab has studied microbial communities in sheep respiratory tracts (Safaei et al. 2006), acidic hot springs (Mathur et al. 2007), contaminated human environments (McManus and Kelley 2005; Lee et al. 2007) and oceanic waters (Breitbart et al. 2004; Angly et al. 2006). Although we cannot completely rule out bias, particularly PCR bias, culture-independent diversity estimates, are considerably less biased than culturing.

RESEARCH DESIGN AND METHODS:

Preliminary Results:

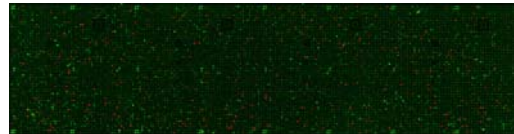
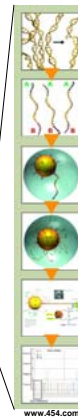
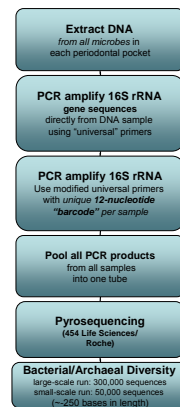


Figure 1. Microarray Analysis (left)

This CustomArray chip (Combinatrix) demonstrates the ability to rapidly detect the relative presence or absence of 16S rRNA transcripts uniquely representing known bacterial species as well as specific toxin and antibiotic-resistance genes in two environmental samples (communities). Due to the diversity of bacterial cells, it is much more challenging to quantify their relative amounts in this way. However, PCR combined with new pyrosequencing technology (Diagram Below) allows us to identify and quantify the relative abundance of 1000's of species per sample. (courtesy of Lesley Lee, Kelley Lab, Dept of Biology) The pyrosequencing approach is an especially tractable method that increases data generation by several orders of magnitude, while eliminating the tedious and error-prone step of cloning each PCR product and picking colonies for sequencing.

Culture-Independent Molecular Microbiological Methods:



Sample Input and Fragmentation

The Genome Sequencer FLX System sequences samples from PCR products, fractionates them into small, 300- to 800-basepair fragments.

Library Preparation

Single-stranded fragments with added short adaptor sequences (A and B) - specific for both the 3' and 5' ends - compose the sample library used for subsequent workflow steps.

One Fragment = One Bead

The single-stranded DNA library is immobilized onto DNA Capture Beads. Each bead carries a unique single-stranded DNA library fragment. The bead-bound library is emulsified with amplification reagents in a water-in-oil mixture resulting in microdroplets containing just one bead with one unique sample-library fragment.

emPCR (Emulsion PCR) Amplification

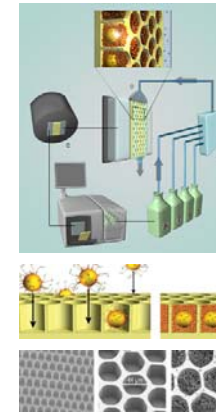
Each unique sample library fragment is amplified within its own microreactor, excluding competing or contaminating sequences. Amplification is done in parallel; for each fragment, resulting in a copy number of several million per bead. Subsequently, the emulsion PCR is broken while the amplified fragments remain bound to their specific beads.

One Bead = One Read

Clonally amplified fragments are enriched, loaded onto a PicoTiterPlate device for sequencing. Its small well diameter allows for a single bead carrying amplified DNA per well. After addition of sequencing enzymes, the fluidics subsystem of the instrument flows individual nucleotides in fixed order across hundreds of thousands of wells each containing one bead. Addition of one (or more) nucleotides complementary to the template strand results in a chemiluminescent signal recorded by CCD camera.

Sequencing Data Analysis

Signal intensity and positional information generated across the PicoTiterPlate device allows the software to determine the sequence of more than 400,000 individual reads per 7.5-hour instrument run simultaneously. For sequencing data analysis, amplicon variant detection by comparison with a known reference sequence.



Bioinformatics and Data Analysis Pipeline:



Figure 2: Flow of Analysis of Sequence Data

Phylogeny: Due to the size of the trees, the simple and rapid Neighbor-joining (NJ) algorithm will be used to determine relationships among individual sequences. **Alignment:** Python programming scripts will be used to sort sequences by barcode and determine basic similarity (e.g. BLAST) to known sequences in the database. The scripts will also be used to de-replicate the sequences (1% divergence) to quantify how often particular microbes are present in the samples. Conditions for alignment (e.g. % identity and coverage) are yet to be determined. **UniFrac software** measures the fraction of total branch length in a phylogenetic tree that leads to sequences from one community or the other but not both in each pair-wise comparison. A distance matrix of values determined for all pairs of environments is used to either hierarchically cluster using an Unweighted Pair-Group Method with Arithmetic mean (UPGMA) or to perform dimensionality reduction using Principle Components Analysis (PCA). Samples will be clustered by microbial community, by disease state, treatment status, and by other clinical parameters, in terms of phylogeny.

Patient Parameters for clustering analysis:

Brachial artery ultrasound reactivity: (Corretti et al. 2002). Right brachial artery diameter and reactive hyperemia vs baseline blood flow;

Carotid artery intima-media wall thickness: B-mode ultrasound images to measure carotid artery intima-media thickness (Selzer et al. 1994).

Bloodwork: Fasting blood glucose, BUN, Cr, lipid profile, Urinalysis, Cr, albumin. Serum will be stored for subsequent analysis of IL-6, TNF α , CRP, and antibodies to LPS* for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*.

EXPECTED OUTCOMES:

This will be the first study to evaluate endothelial function in relation to periodontal disease in young adults aged 20-30. If periodontal disease contributes to vascular dysfunction and the development of atherosclerosis, the follow-up study will reveal whether vascular dysfunction is reversible in this age group and may provide insight into the role of poor oral hygiene as a contributing factor to health disparity.

The most important information derived from this work will be the evaluation of the complex oral microbial flora in this patient population. Previously uncharacterized species of bacteria may be identified, some of which are expected to correlate with periodontal disease. We hypothesize that some organisms may correlate strongly with endothelial dysfunction. We also hypothesize that some organisms will correlate with good oral (and/or cardiovascular) health. Such organisms may secrete factors that potentially suppress the overgrowth of oral pathogens and could be beneficial.

This study, which will demonstrate whether treating periodontal disease in young adults can normalize endothelial function, represents an important step towards demonstrating causality and will furthermore establish the characteristics of a microbial ecosystem that may correlate more strongly with vascular dysfunction than the presence or absence of a single species.

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