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DEFINING THE BACTERIAL FLORA OF PERIODONTAL POCKETS IN CHRONIC PERIODONTITIS PATIENTS

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DEFINING THE BACTERIAL FLORA OF PERIODONTAL POCKETS IN CHRONIC PERIODONTITIS PATIENTS

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Dentistry at Virginia Commonwealth University.

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Abstract

DEFINING THE BACTERIAL FLORA OF PERIODONTAL POCKETS IN CHRONIC PERIODONTITIS PATIENTS

By Rafael Karlo Rodriguez, D.M.D.

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Dentistry at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

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PURPOSE: The purpose of this study is to describe the subgingival bacterial biodiversity in untreated chronic periodontitis patients through the use of next generation 16S rRNA molecular analysis, and to determine similarities or differences between deep and shallow pockets within the same patients.

METHODS: The analysis involved paired subgingival plaque samples from 24 subjects diagnosed with Generalized Moderate to Severe Chronic Periodontitis. One sample was selected from a single site having a probing depth $\geq$5 mm (i.e. Deep Site), and the other from a site with a probing depth $\leq$3mm (i.e. Shallow Site) within each subject. Bacterial DNA amplification of the V4-V6 region of the 16S rRNA was performed. The amplicons were sequenced via 454 Roche Genome Sequencer FLX System. The identified sequences were evaluated, and then compared to calculated false discovery rates.

RESULTS: A total of 119 independent microbial genera were identified within the samples analyzed. Seven genera were identified to be statistically significant ($p<0.05$) in their association to deep or shallow sites following t-test and boot strap randomization: Actinomyces ($p=0.004$), Methylobacterium ($p=0.028$), Veillonella ($p=0.028$), and Rothia ($p=0.038$), and Streptococcus ($p=0.033$) in Shallow sites; while Mycoplasma ($p=0.007$) and Fusobacterium ($p=0.016$) were associated with deep sites. However, taking into account the calculated false discovery rates, it is suggested that none of the 119 microbial genera identified in this study were significantly associated with either deep nor shallow sites.

CONCLUSION: The microbial genera identified within this study to be associated with deep and shallow sites follows the traditional pattern anticipated from the literature. However, the calculated false discovery rates suggest that these results may have occurred by chance and not due to a true difference.

CHAPTER 1

INTRODUCTION

Early research of the oral micro-flora can be traced to Antoni van Leeuwenhoek (1632-1723), who illustrated findings observed from his own dental plaque.[1] In his notebook, he wrote "I didn't clean my teeth for three days and then took the material that had lodged in small amounts on the gums above my front teeth. . . I then most always saw, with great wonder, that in the said matter there were many very little living animalcules."[2,3] Substantial advancements in research methods over the last century have significantly improved microbial findings since this report. Despite such progress, only associations between specific pathogens and periodontitis have been noted. In fact, a precise spectrum of the microbial flora within the gingival biofilm that is responsible for eliciting periodontitis has not been established.[4] The main impediments to this goal have stemmed from technical research limitations, and the uniqueness of the pathogenesis of periodontal diseases.

It is generally accepted that the primary etiology for periodontal disease is the dental plaque including the bacteria, bacterial products, and the resulting inflammatory cascade. However, the human oral cavity presents a unique microbiological environment from other surfaces of the body. Teeth provide a solid and non-shedding surface that remains in close proximity to epithelial cells and tissues of the periodontium.[5] This environment allows for the extended colonization and development of microbial communities.

Biofilms are natural communal aggregations of microorganisms that form on liquid-air and liquid-solid interfaces.[6,7] The establishment of these systems involves a sequential process by which early colonizing microbes such as *Streptococci gordonii* adhere to, and begin to condition, the tooth surface and gingival sulcus. Other cells attach and organize by means of autoaggregation and coaggregation. The local environment begins to change (eg. from aerobic to facultative anaerobic) as extracellular matrix products are produced by the various flora at each stage.[8,9] These ubiquitous aggregations occur in health but can also alter their environments to promote pathology, as is the case in periodontal diseases. In fact, it is estimated that 65-80% of all physiologic infections are biofilm related.[10,11]

Supragingival and subgingival dental plaques are classic examples of liquid-solid surface biofilms. Highlighting the diversity of these biofilms, studies have identified more than 700 species in the oral cavity[48,47] and over 400 bacterial species in subgingival sites.[12] Additionally, recent studies have illustrated the complexity of such biofilm communities by identifying the process of quorum sensing.[13] 'Quorum Sensing' bacteria produce and release chemical signal molecules that enable them to communicate with one another to coordinate gene expression, metabolic functions, and behavior of the entire community. These behaviors include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation. Research suggests that this process can also be used by biofilms to elicit specific responses from their corresponding host, thereby altering or controlling their local environment.[14] The capacity of biofilms to coordinate these behaviors is thought to be a significant reason for

the failure of antimicrobial therapies to infections.[11] These complex interactions also present a challenge in illustrating a complete description of the subgingival environment.

In light of these discoveries, it is essential to gain a complete understanding of all the microbes within the oral flora in order to better define the role of plaque as the primary etiology of periodontitis. This will better inform researchers to find more effective methods to evaluate the etiology and pathogenesis of periodontal diseases. The following information provides a brief summation of the primary research techniques that have been employed in the effort to describe the natural and pathologic flora of human gingival sites. Discovery and refinement of these methods, have led to the development of our current research methods.

Microscopy

Early investigations of the periodontal flora began during the "golden age of microbiology" (~1857-1914) when the understanding of the association between microbes and diseases led to many medical discoveries of etiologic pathogens. These studies were primarily based on observations from wet mount or stained smear microscopy and limited bacterial culturing. Investigators from this period identified amoebae, spirochetes, fusiforms, and streptococci as the four possible etiologic microbes of periodontal lesions.[5,15]

It is now abundantly clear that these observations were heavily influenced by the methods employed in each investigation. Those suggesting amoebae and spirochetes as the etiology were using wet mounts or specific stains that selectively identified these

microbes within samples.[16,17,18] The implication of fusiforms was based on the frequency of observation noted in microscopic analyses of subgingival plaque, and their association with Vincent's infection.[5,19,20]

Successive observations progressed with the development of the microscope. At a time when culture studies still experienced limitations, Listgarten[21] (1976) was able to report a clear differential composition between the microflora of the periodontium in health and disease based on observations from light and electron microscopes. This report indicated more spirochetes, gram-negative, and flagellated species in disease. Another development was the use of dark-field microscopy. Many of the studies involving this method were able to reveal more dramatic differences than were previously reported from culture data.[22,23]

Bacterial Culturing

Streptococci were initially identified as prevalent periodontal pathogens based on methods of microbial culturing. The ease of growing these microbes in artificial laboratory conditions led to their frequent observation. Unfortunately, culturing techniques inherently limit observational findings to those microbes that can be cultivated by the in-vitro methods employed. These limitations result from the variable growth or inhibited growth among the sampled species on the selected media.[24] In fact, it was estimated that only ~0.5% of microbes could be counted based on the techniques available during the early 20th Century.[25]

Limited clinical applications from such findings led to a decrease in the enthusiasm to search for etiologic microbes. By the 1930's, research in this area virtually ceased.[5,26] Pathogenesis of the disease was attributed to several factors including a constitutional defect of the patient or trauma from occlusion.

A resurgence of interest in identifying a specific microbial etiology for periodontal disease was renewed by the studies of Keyes & Jordan in the 1960's.[5] These researchers demonstrated the transmisability of periodontal disease to healthy/non-diseased hamsters by housing the animals in single cages, as well as by swabs from plaque and feces.[27] Studies illustrating the invasive potential of spirochetes into the connective tissue and epithelium of ANUG lesions also emphasized the possibility of a specific microbial etiology.[28]

The subsequent cultural studies undertaken during the 1960's, like that from Socranski, et al.[29] attempted to analyze the microbiota of both healthy and diseased sites. Unfortunately, these studies were still affected by many of the limitations from earlier reports and, therefore, were not able to identify significant differences between sites. These studies continued to be limited by growth media selection, challenges in re-creating the subgingival atmosphere (anaerobic, etc), and difficulty in maintaining this atmosphere following sampling.[24] Studies have also illustrated that plaque dispersion techniques employed during this time preferentially killed gram-negative anaerobic organisms.[30]

Necessary advances in culturing techniques were made following this period including the development of balanced anaerobic transport mediums such as RTF[31], more effective growth media such as TSBV[32] for *A. actinomycetemcomitans*, and refinements

of anaerobic incubators. Due to these advances, a report in 1977 estimated that up to 70% of the enumerated species identified microscopically could be cultivated.[33] However, this estimate did not approximate the number of species that had yet to be identified. One recent study confirms that approximately 50% of oral microbes do not grow on conventional in-vitro culture media/environments.[34]

During the 1980's sufficient studies were available for comparison whereby researchers noted associations of microbes with inflammatory periodontal diseases; the so called 'Perio-pathogens'. By 1994, Haffaje A. & Socranski S.[35] proposed a list of microbes ranked according to their likely involvement in the etiology and progression of periodontal diseases. In reviewing the literature, evidence for each microbe was organized based on a modified version of the classic postulates of Dr. Robert Koch. The following periodontal pathogens were listed as having a 'Very Strong' or 'Strong' relationship to periodontitis: *A. actinomycetemcomitans*, spirochetes (in ANUG), *P. gingivalis, B. forsythus, P. intermedia, C. rectus, E. nodatum*, and *Treponema* species.[35] This list provided direction as to which microbes would be selected for analysis by future culture-independent techniques such as immunological assays, bacterial enzyme assays, DNA probes, and PCR.

Immunological Assays

Based on these findings, techniques were developed to improve the sensitivity in the identification of the "Periodontal Pathogens" from subgingival plaque samples. Immunofluorescence is a method based on the development of rabbit antisera against

whole cells and/or monoclonal antibodies against a specific antigen.[36] In 1989, Seida, et al. confirmed immunofluorescence as comparable to culture methods for microscopic counting.[37] In 1997, Ellwood R, et al. found *P. gingivalis* to be associated with sites having a deep probing depth of >3mm, BOP, and calculus using enzyme-linked immunosorbant assay (ELISA). However, these techniques require a thorough knowledge of the serology behind the periodontal pathogen(s) in question. Furthermore, antigenic variability of cell surface markers can lead to cross-reactivity of polyclonal antibodies.[38] This type of error produces false-positive results, thereby affecting the accuracy of the test.

Bacterial Enzyme Assays

Bacterial enzyme assays provide another method for testing the presence of periodontal pathogens within gingival sites. These tests (ex. BANA and BAPNA) are based on the ability of *T. denticola, P. gingivalis, B. forsythus*, and unspeciated *Capnocytophaga* to hydrolyze β-napthylamide derivatives. Evidence shows a good correlation between the detection of the 3 BANA periodontal pathogens and the results from ELISA exams.[39] A common drawback to both the immunological assays and these enzymatic assays is their requirement for a detection level of at least 10^4 cells. Another limitation from this system is that the BANA test does not provide any qualitative or quantitative information on which of the three test species is present in a given site. Additionally, false-positive reactions may occur by other enzymatic activity produced by the host.[40]

PCR

The development of polymerase chain reaction (PCR) methods to amplify genetic material has created an especially powerful molecular research tool. These techniques have illustrated such extreme sensitivity as detecting a single *Treponema pallidum* cell, and as few as 50 *A. actinomycetemcomitans* and *P. gingivalis* cells in clinical samples.[39] This technology is the basis for culture-independent research methods. Single target PCR, Multiplex PCR, and quantitative or 'real-time' PCR are the three predominant applications of this method in microbial analyses. PCR has been coupled with DNA probe research but, in recent years, it has also been applied to studies involving sequencing of 16S rRNA.

Nucleic acid probes

With the advancements in the understanding and manipulation of genomic material, DNA probe methods became useful for identifying pathogens that are difficult to grow, present in low numbers, and exist in mixed samples.[41] This method is based on DNA hybridization, or the ability of a portion of DNA to bind to complementary strands of DNA. This allows for more specific analysis with subspecies differentiation, and the ability to reveal associations of microbes within plaque samples. For example, such studies have been able to identify that patients, as well as individual sites, are more likely to harbor single clonal types of *P. gingivalis* and *A. actinomycetemcomitans*.[42]

Additionally, Socransky et al. (1998) analyzed 13,261 plaque samples using whole genomic DNA probes to 40 culturable bacterial species using checkerboard hybridization assays to define bacterial complexes, rather than individual species, that were associated with periodontal disease and health.[43]

Although all of these highly sensitive methods have been useful in research, they are not ideal for completely describing the microbiology of an environment because their scope is limited to those known microbes whose genomic information is already cataloged. Traditionally, these studies focused on the search for the species that have been identified from culture based studies. It is possible to detect uncultured species only when the genome for these microbes, or their near relatives, have been characterized. This allows for the preparation of specific primers that will selectively detect them. It is for these reasons why PCR, DNA hybridization, and microarray assays are considered 'closed-ended' culture-independent approaches.

16S rRNA

A tremendous advancement in the development of an 'open-ended', culture-independent research technique resulted from the analysis of the nucleotide sequence of ribosomal RNA (rRNA). This approach allows for the identification of nearly all the bacteria in a sample population including uncultivated or previously unknown species. Microbiologist George E. Fox, et al. described this innovation as an impending "revolution" in bacterial taxonomy promising to change the existing "uncertain discipline".[44] Evaluation of the rRNA sequence was quickly applied in research to

estimate the evolutionary relationships among species because it is one of the most conserved units of genetic material, and it is present in all free-living organisms.[45] It is now possible to analyze this genetic sequence and identify unknown bacterium to a given genus or species by comparing the results to large databases of known sequences such as GenBank[46]. This method has led to the discovery of many previously unrecognized species.

This culture-independent, 16S rRNA technique has recently been employed in intraoral microbiology studies. In 2001, Paster, et al. performed a comprehensive study of 31 subjects with a variety of periodontal diseases. The researchers reported 347 phylotypes within the subgingival plaque samples, 40% of which were novel.[47] Later, Jorn A. Aas, et al. sampled nine intraoral sites of five clinically healthy patients with this new technique. Over 700 bacterial species or phylotypes were detected and more than 50% of the bacterial flora from the samples taken represented phylotypes which had not yet been cultivated.[48] Faveri, M. et al examined subgingival samples from 10 generalized aggressive periodontitis subjects and found that 57% of the phylotypes were previously uncultivated species and that the species *Selenomonas* may be more associated with this form of periodontitis than previously expected.[49]

Technological advances in this high-thoroughput sequencing technique has continued to improve the insight into microbial communities. Previous studies were based on methods whereby the 16S ribosomal sequences were isolated, amplified by PCR, cloned into *Escherichia coli*, and then sequenced.[50] Next-generation sequence analysis involves partial sequencing of variable 16S rRNA gene regions. There are nine different variable gene regions surrounded by conserved stretches that can be targeted by

selected PCR primers.[51] At this time, there is no consensus on a single best region, though V2 and V4 have been reported to be suitable for community analysis given their low error rates when assigning taxonomy.[52] Researchers also combine analysis to include these moderately conserved regions with analysis of variable regions such as V6.[53] These selected amplicons are typically quantified by pyrosequencing. The shorter sequence reads may be less discriminatory than full-length 16SrRNA genes. However, pyrosequencing offers the significant advantages of higher coverage per sample, much greater resolution of the community composition, cheaper, faster, and eliminating the need of preparing clone libraries.[54]

These results provide encouragement for the discovery of additional novel species, as well as gaining a further understanding of the subgingival microflora. It is evident from the history of research studies that previous findings have been influenced by the research design and methods employed. The heterogeneous nature of periodontal infections requires a comprehensive understanding of the complete gingival microflora associated with health and disease. The recent findings illustrate the complexities and host modifying ability of biofilms, and emphasize the importance of attaining this information. The new open-ended culture independent techniques offer a method to explore and identify the phylotypes of the oral biofilm completely. The purpose of this study is to describe the subgingival bacterial biodiversity in untreated chronic periodontitis patients through the use of 16S rRNA molecular analysis, and to determine similarities or differences between deep and shallow pockets within the same patients.

CHAPTER 2

METHODS

Subject Population

The protocol for this cross-sectional study was evaluated and approved by the Institutional Review Board of Virginia Commonwealth University. Subjects were recruited from two locations: the dental clinics of Virginia Commonwealth University's School of Dentistry in Richmond, Virginia and from a community health fair in Wise, Virginia. One examiner performed the clinical intra-oral exam as well as the review of medical and dental history information. A total of 92 subjects (age range 32-67 years) diagnosed with Generalized Moderate to Severe Chronic Periodontitis were identified and signed the committee-approved informed consent. This pilot study report is based on a subset of 24 from the 92 total subjects.

Inclusion and exclusion criteria

The inclusion criteria were as follows: subjects with clinical and radiographic evidence of having generalized moderate to severe chronic periodontitis, within ages 30 to 65, with a minimum of 16 teeth excluding 3rd molars and implants, five sites with probing depths of $\geq$5 mm, and at least one proximal area with probing depth $\leq$3mm.

The exclusion criteria were as follows: pregnant females, any periodontal therapy within the previous 3 months, systemic or local antibiotic therapy within three months of

enrollment, subjects with characteristics of aggressive periodontitis, and individuals requiring prophylactic antibiotics.

Clinical Examination

The following information was recorded for each of the selected sites: tooth number, surface, probing depth, clinical attachment loss, bleeding on probing, Miller tooth mobility, plaque index[55], and gingival index[56]. Pocket depth and clinical attachment levels were recorded to the nearest millimeter using a North Carolina probe (Hu-Friedy, Chicago, IL). Additional subject-level information was documented including the overall periodontal diagnosis, diabetes status (via blood sugar and/or HbA1C when available), smoking habit/history, and caries risk.

Caries risk was categorized into three levels. The first level included low risk subjects exhibiting no clinical or radiographically detectable areas of decay. The second level involved subjects with (1-6 surfaces) of decay. A third level documented included subjects with rampant or severe decay in which the teeth surfaces sampled were also near carious lesions.

Subjects using tobacco were included in the study. The number of years that subjects had smoked and the number of packs were recorded, so 'pack years' could be calculated. Three subjects reported use of chewing tobacco.

Sample Collection

Sample collection was performed after clinical parameters were recorded. Two independent subgingival plaque samples were collected from each subject based on their

clinical probing depth. One sample was selected from a single site having a probing depth ≥5 mm (ie. Deep Site), and the other from a site with a probing depth ≤3mm (ie. Shallow Site). The selected sites were isolated from supragingival plaque with sterile gauze. Samples were collected with individual, detachable, sterile Gracey curettes (Hu-Friedy, Implacare) inserted to the depth of the crevice. The curette-end containing the retrieved subgingival plaque sample was detached from the curette handle and immediately placed into separate polypropylene tubes containing 1mL of sterile Phosphate buffered saline solution (7.4 pH).

Isolation and PCR Amplification of 16S rRNA

Bacterial lysis and DNA isolation was achieved with a MO BIO PowerLyzer genomic DNA isolation kit. Eubacterial primers were selected to amplify the 16S ribosomal gene from the community DNA of each sample. These primers were identified based on comparisons to a database of known genetic sequences of 700 oral bacteria.[57] The primers were selected to recognize the conserved genetic regions flanking the variable regions V4-V6 (~485 bp) of the 16S rRNA. Degenerate sequences were further used in designing PCR primers to increase the number of included oral strains. High fidelity Taq DNA polymerase was employed for the real-time PCR amplification.

During PCR amplification, a barcode sequence tag of 6 bases was attached to the amplified sequences. Barcodes were employed during amplification in order to pool multiple samples, up to 96, for the sequencing reaction. This allowed for the identification of the amplified strains to match each individual sample number following the subsequent pyrosequencing. The amplicons (~500bp) were purified and sequenced

using the next generation sequencer, 454 Roche Genome Sequencer FLX System as described by the manufacturer. The amplicon squencing was performed off-site in a DNA core facility (VCU Center for the Study of Biological Complexity; Dr. Gregory Buck).

Sequence Analysis

Following sequencing, the barcodes were used to identify and assign individual sequence amplicons to their originating sample number while dropping low quality reads such as those from shorter or longer sequences (<300 bp or >500 bp). Barcodes were then trimmed and the read amplicons were aligned into multiple 16S rRNA sequence contigs based on sequence overlap. These contigs also represent related sequences creating OTUs (operational taxonomic units) for downstream analysis. The contigs were further classified taxonomically in Ribosomal Database Project (RPD) database using Classifier[58] and GenBank microbial DNA database to identify the microbial genus.

Characterizing sequences to the species level will be performed at a later date with methods of comparative genomic analysis previously described.[59,60] The characterized V4-V6 sequences will be collected and stored in a local database. Specific base changes of the sampled oral bacterial species will also be identified and documented. The described phyla will also be evaluated for association to the clinical data recorded for each subject and sample.

Statistical Analysis

Power analysis was based on a previous study involving 15 subjects with moderate to severe chronic periodontitis and 15 healthy control subjects.[61] Of the 274 phylotypes noted in that study, 38 were found to be significantly associated with diseased and healthy patients. The intended comparative analysis of this study involved t-tests, boot-strap randomized t-tests, and false discovery rates (q-value). The results indicated a sufficient power existed within the present study design based on the results from the previous analysis.

Data from the sequencing analysis allowed for the identification of the microbial population and genus distribution within each sample. The number of sequences in each 16S rRNA contig were evaluated to report the bacterial distribution. In addition, the percentage of 16S rRNAs of the individual bacterial genus from each sample was calculated.

The goal of the analyses was to determine the relationship, if any, of the microbial diversity between deep and shallow pockets. Paired t-tests were used to test for differences in percentage of individual organisms. The significance levels of the t-tests were verified by boot strap randomization (1000 samples were used). Q values were then estimated to estimate the false discovery rates (http://genomics.princeton.edu/storeylab/qvalue/).[62]

CHAPTER 3

RESULTS

The analysis within this preliminary report involves paired samples from 24 of the 92 total subjects. Samples from two subjects were insufficient for analysis rendering a total of 44 samples from 22 subjects for examination. The subgroup examined in this study was part of the population recruited from the dental clinics of Virginia Commonwealth University School of Dentistry.

The demographic and clinical parameters are described in Table 1. The mean age of the participants was 50.6 years, ranging from 35 to 71 years of age. Ten of the subjects were current smokers with a mean pack year history of 20.9. The mean probing depth and clinical attachment level for the deep sites sampled were 6.79 $\pm$ 1.7mm and 5.9 $\pm$ 2.25mm, respectively. The corresponding clinical values for the shallow sites were 2.9 $\pm$ 0.3mm and 2.0 $\pm$ 1.1mm respectively. Bleeding on probing was noted for 87.5% of the deep, and 20.1% of the shallow sites sampled.

A total of 119 independent microbial genera were identified within the samples analyzed. For every deep and shallow site sampled, the percentage of each individual genus from the overall microbial population was calculated. Figure 1 illustrates the 12 microbial genera that were present by the highest mean percentage in deep and shallow sites. Genera such as *Streptococcus, Actinomyces, and Veillonella* were found in higher percentages in shallow sites while *Fusobacterium, Porphyromonas*, and *Prevotella* were

associated with deep sites. To calculate the significance of these findings and identify other microbes associated with deep or shallow sites, paired t-tests were performed.

Figure 2 illustrates the mean difference and standard deviation of all 119 genera noted in this analysis for the deep and shallow sites. Those microbes exhibiting distinct positive values were associated with deep sites, where as the microbes skewed toward the negative values were associated with shallow sites. The majority of microbes identified did not exhibit a significant difference in their prevalence within sites of deep or shallow probing depths.

Among the 119, seven genera were identified to be statistically significant ($p<0.05$) in their association to deep or shallow sites following t-test and boot strap randomization. These organisms are outlined in Figure 3. The most significant differences were found with *Actinomyces* ($p=0.004$) in Shallow sites, and Mycoplasma ($p=0.007$) found to be mildly elevated in Deep sites. *Fusobacterium* ($p=0.016$) was associated with deep sites, while *Streptococcus* ($p=0.033$), *Methylobacterium* ($p=0.028$), *Veillonella* ($p=0.028$), and *Rothia* ($p=0.038$) were found to be associated with shallow sites.

To verify the significance of the findings from the t-test, the false discovery rate (q-value) for these microbes was calculated, as previously described. Table 3 presents these p-value ranges (ie. the Q-value or false discovery rate) for the microbes noted as significant in Figure 3. Taking into account the false discovery rate, it is suggested that none of the 119 microbial genera identified in this study were significantly associated with either deep nor shallow sites including those presented in Figure 3.

CHAPTER 4

DISCUSSION

The present study presents a preliminary report of 24 untreated subjects with Generalized Moderate to Severe Chronic Periodontitis from a larger sample population set (n=92). The main objectives of the study were to characterize the subgingival microbiota, and to determine similarities or differences between deep and shallow periodontal pockets within the same patients.

Previous periodontal studies utilizing 16S rRNA microbial analysis involved 16S cloning prior to sequencing.[12,47,49,61] The next generation technique employed involved barcoded pyrosequencing with the 454 Roche Genome Sequencer FLX machine following PCR amplification. This system demonstrated to be a highly sensitive approach to the evaluation of the oral microecology. A total of 119 genera were identified from the samples reviewed. The present report was also able to identify genera such as *Actinomyces* and *Fusobacterium* occupying significant proportions of the microbiota. These genera have traditionally been identified clearly from cultural studies, but molecular analysis techniques utilizing 16S clonal techniques have repeatedly underreported their prevelance.[61,63,64] The significance of these differences have been speculated to be altered by several possible mechanisms including: over-representation from cultivation techniques, primer bias during PCR amplification, or the detection of non-viable cells as present by their residual genetic material.

Within the results of this study, the mean percentage of the microbial genera *Streptococcus, Actinomyces, Veillonella, Rothia, Granulicatella,* and *Methylobacterium* were elevated within Shallow sites. Meanwhile *Fusobacetrium, Porphyromonas, Prevotella*, *Mycoplasma*, and *Aggregatibacter* were noted to occupy higher percentages in Deep Sites. *Treponema* were found to reside in a nearly equal percentage of the microflora within deep and shallow sites. These findings confirm the traditional pattern of oral microflora associated with chronic periodontitis anticipated from the literature.[21,28,35,43,47,61]

Among these genera, only seven were found to be significantly associated with deep or shallow sites as illustrated in Figure 3. The p-values were determined by way of paired t-test, with and without boot-strap randomization. Shallow sites were associated with *Actinomyces* (p=0.004), *Streptococcus* (p=0.033), *Methylobacterium* (p=0.028), *Veillonella* (p=0.028), and *Rothia* (p=0.038). Conversely, *Mycoplasma* (p=0.007) and *Fusobacterium* (p=0.016) were found to be significantly elevated in deep sites.

Although commonly attributed to periodontal disease, *Porphyromonas* and *Prevotella* were not significantly associated with deep sites in this study. This finding is in agreement with another recent report that found greater differences among healthy and diseased patients, but fewer differences between the microbiota of deep and shallow sites within the mouth of diseased patients.[61] This suggests that patients with periodontitis may have an overall shift in their microecology for all sites. Another factor leading to this finding may be the small sample population reported in this preliminary analysis, or that the sequences have not been categorized to the level of species.

False Discovery rate (FDR) is a relatively new method for controlling the amount of anticipated false positive rates in large multiple comparison studies such as those of proteomic or metagenomic evaluations. FDR is more sensitive than traditional methods, less conservative than the Bonferroni approach, and has greater power because it is adaptive to the amount of signal within the data.[6262,65] The p-value ranges representing the FDR in Table 3 suggest that the results presented in this study may have occurred by chance alone and not due to a true difference.

This suggests prudence to the interpretation of results from the analysis of high through-put 16S RNA molecular studies. Given that the genera *Streptococcus, Actinomyces, Veillonella,* and *Fusobacetrium* follow the paradigm of results anticipated from the body of literature,[21,29,35,43,47,61] it is doubtful that those findings occurred by chance alone. However, the FDR is noteworthy for the interpretation of results suggesting the significant prevalence of novel species. To our knowledge, this statistical instrument has not been applied to the previous study designs involving periodontal microbial evaluations. Indeed, the novel phlyotypes associated with disease reported by these studies may have also been identified by chance alone.

This report is based on a preliminary analysis from a larger study population. One of the factors affecting the lack of significance of the findings in the present preliminary report is the small sample population (24 subjects). This subject population size is similar to, if not larger than many of the previous reports utilizing 16S rRNA analysis. Most studies in this field have presented subject populations ranging from 5-30 participants likely due to the high cost of the sequencing and the statistical challenge of data analysis.[47,48,49,61] For instance, one of the most recent publications involved the

analysis from a total of 30 patients including 15 patients with chronic periodontitis and 15 age matched, healthy controls.[61] The largest study noted by the author involved 66 periodontal subjects and 66 healthy controls based on a database of previously acquired samples.[66] Neither of these previous studies included the statistical method of False Dicovery rate to adjust for the chance false positive findings from their multiple comparitive analysis. The power analysis for this study suggested a sufficient sample size of 92 subjects. The subsequent study will complete the analysis of 92 subjects, characterize data to the species-level, compare clinical/demographic parameters, and catalog the identified V4-V6 regions. The following report may be able to verify whether further analysis of the remaining samples will result in the identification of significant differences among the reported microbes, or of significant novel microbes. This additional analysis will also provide further insight into the most effective statistical methods for analyzing data from these studies.

The progression of technology has provided innovative measures by which researchers unravel the complexity of diseases. Heterogeneous infections such as periodontitis continue to challenge researches and clinicians alike. Each study method has provided further insight by complementing the strengths and weaknesses of those previously employed. Further studies are necessary to identify the significant microbiota of the subgingival environment associated with health, and the initiation and progression of periodontal diseases.

CHAPTER 5

CONCLUSION

In conclusion, this preliminary report suggests that barcoded pyrosequencing of the V4-V6 region of 16S rRNA sequences is a viable and sensitive method for the analysis of the oral microbiota. The mean percentages of microbial genera noted to be elevated in this study within the deep and shallow sites follow the traditional pattern anticipated from the literature. In this study, the calculated false discovery rates (FDR) suggest that the results may have occurred by chance and not due to a true difference. This finding suggests that future studies design, include power analysis, and interpret their data in perspective with FDR or other statistical methods that adjust the p-values appropriately, particularly in the identification of significant novel species.

Table 1. Demographic and Clinical Parameters

Variables	Mean values ± SD
Age	50.6 ± 11.4
Race (African American/Caucasian)	10/14
Gender (female/male)	15/9
Smokers (Y/N)	10/14
Mean Pack Years	20.9
Caries Risk (Lo/Moderate/High)	5/17/2
Mean Probing Depth (mm; Shallow sites)	2.9 ± 0.3
Mean Probing Depth (mm; Deep sites)	6.79 ± 1.7
Mean Clinical Attachment Level (mm; Shallow sites)	2.0 ± 1.1
Mean Clinical Attachment Level (mm; Deep sites)	5.9 ± 2.25
Bleeding on probing (%; Shallow sites)	20.1
Bleeding on probing (%; Deep sites)	87.5

Table 2. P-values of Significant Genera

Genus	p-value (t-test)	p-value (t-test, boot strap)
Actinomyces	0.007	0.004
Mycoplasma	0.016	0.007
Fusobacterium	0.018	0.016
Methylobacterium	0.066	0.028
Veillonella	0.043	0.028
Streptococcus	0.031	0.033
Rothia	0.060	0.038

Table 3. False Discovery Rates

Genus	Q-value (p-value range)
Actinomyces	0.004 - 0.226
Mycoplasma	0.007 - 0.226
Fusobacterium	0.016 - 0.345
Methylobacterium	0.028 - 0.351
Veillonella	0.028 - 0.351
Streptococcus	0.033 - 0.351
Rothia	0.038 - 0.351

Figure 1. – Genus Distribution – Deep vs. Shallow

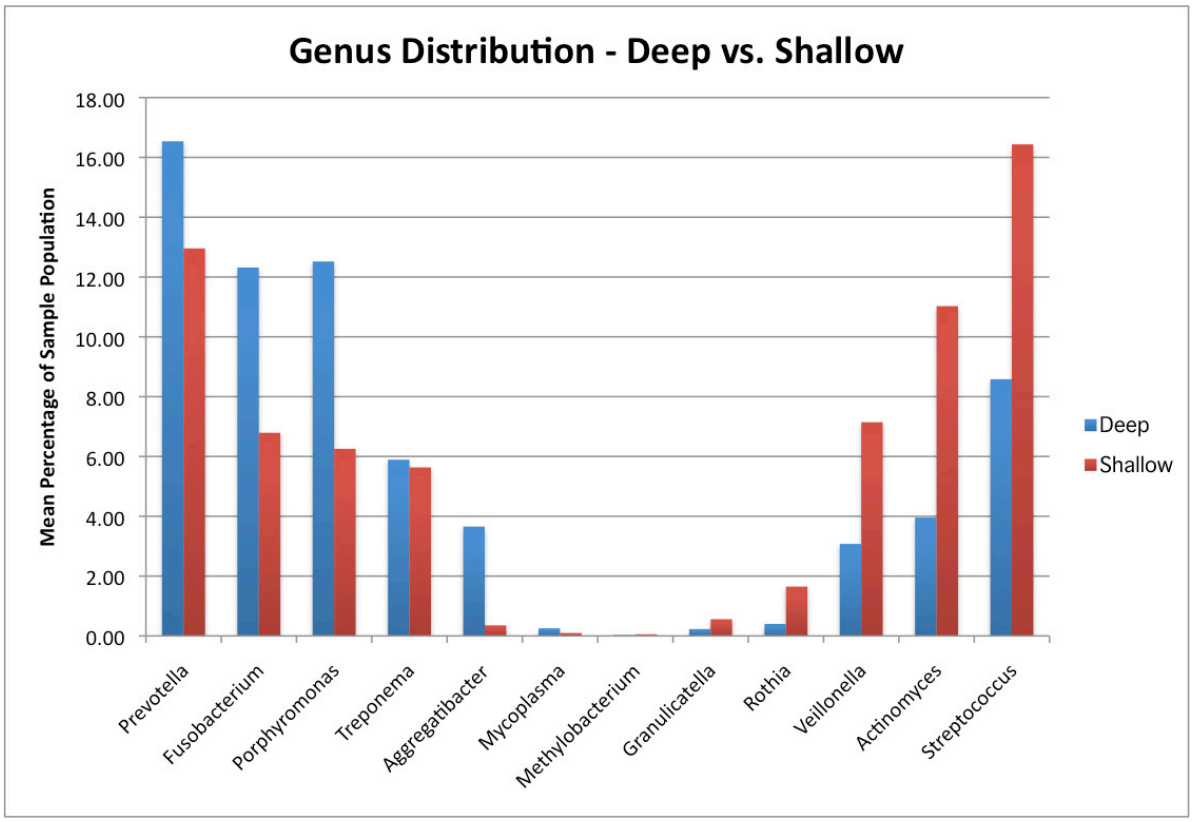


Figure 2. – Difference of Microbial Distribution

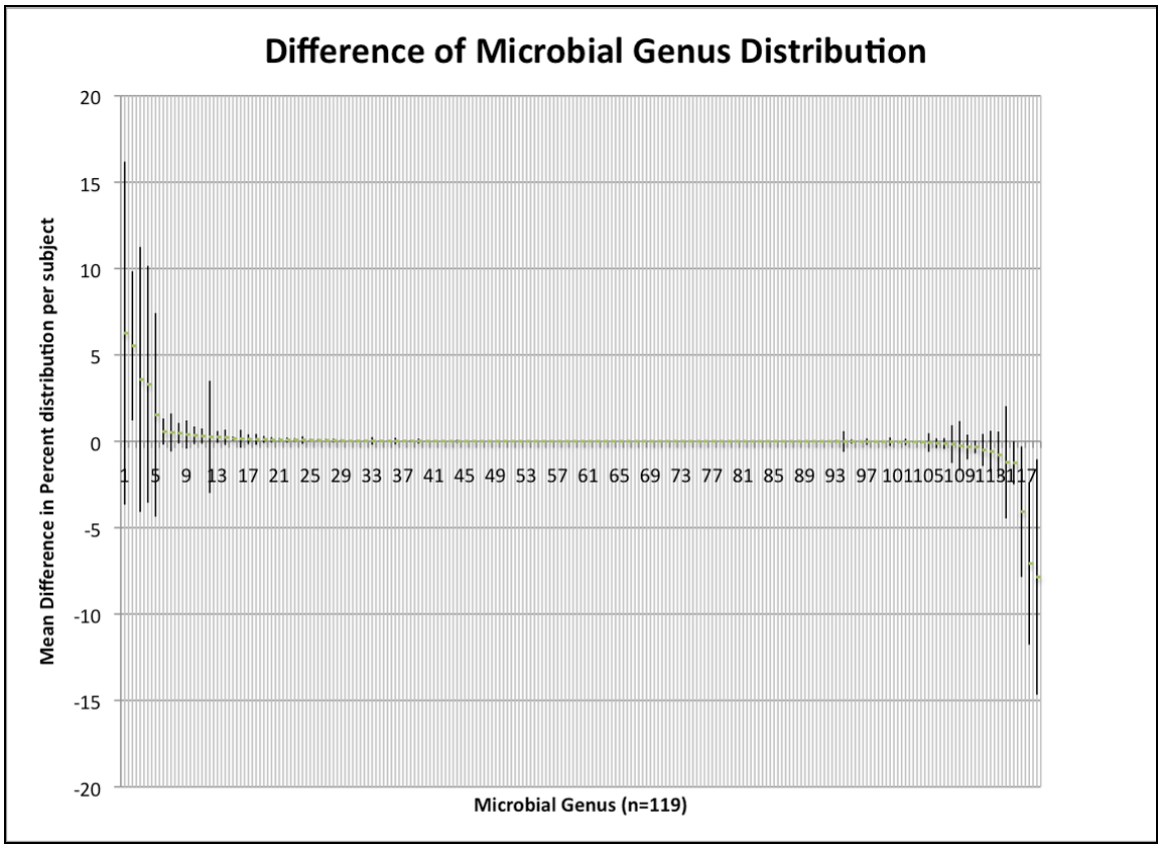
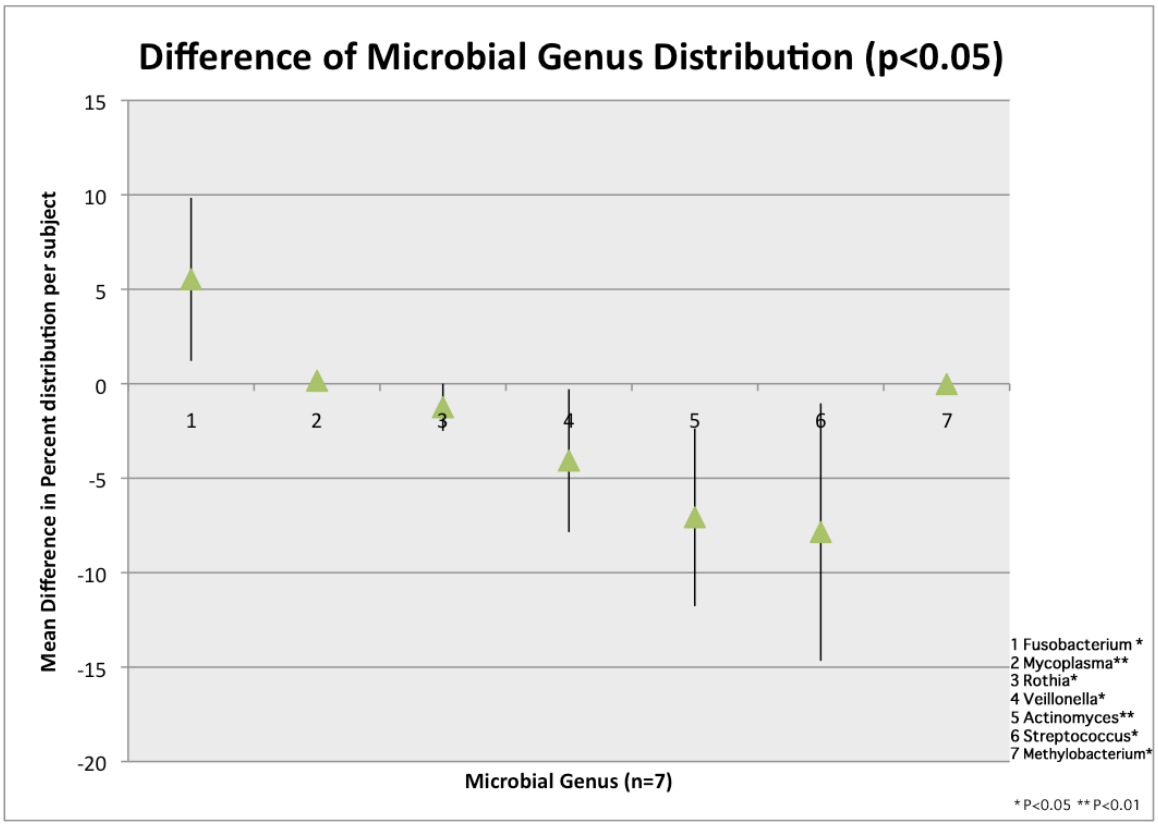


Figure 3. – Difference of Microbial Genus Distribution (p<0.05)



VITA

Rafael K Rodriguez was born on March 15th, 1982 in Miami, Florida. He graduated from Belen Jesuit Preparatory High School, Miami, FL in 2000. Dr. Rodriguez attended the University of Florida in the City of Gainesville, FL on an academic scholarship. He went on to earn a Bachelor of Science degree in zoology. Upon graduation, he entered the University of Florida College of Dentistry and received his Doctor of Dental Medicine Degree in 2008.

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