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## Retrospective evaluation of microbial presence in existing saliva repository: A PCR based molecular survey of oral microbial populations from existing saliva samples

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RETROSPECTIVE EVALUATION OF MICROBIAL PRESENCE IN EXISTING  
SALIVA REPOSITORY: A PCR BASED MOLECULAR SURVEY OF ORAL  
MICROBIAL POPULATIONS FROM EXISTING SALIVA SAMPLES

by

Jay Ericksen Davis

Doctor of Dental Medicine

University of Nevada, Las Vegas

2008

A thesis submitted in partial fulfillment  
of the requirements for the

**Master of Science in Oral Biology  
Department of Orthodontics  
School of Dental Medicine**

**Graduate College  
University of Nevada, Las Vegas  
December 2011**

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**THE GRADUATE COLLEGE**

We recommend the thesis prepared under our supervision by

**Jay Davis**

entitled

**Retrospective Evaluation of Microbial Presence in Existing Saliva  
Repository: A PCR Based Molecular Survey of Oral Microbial  
Populations from Existing Saliva Samples**

be accepted in partial fulfillment of the requirements for the degree of

Master of Science in Oral Biology  
Department of Orthodontics

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**December 2011**

## ABSTRACT

### Retrospective Evaluation of Microbial Presence in Existing Saliva Repository: A PCR Based Molecular Survey of Oral Microbial Populations from Existing Saliva Samples

The overall purpose of this research project is to explore the link between oral health and disease in the University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM) clinic patient population. More specifically, the population of interest is the UNLV-SDM orthodontic clinic patient population, which is mostly composed of adults, females, and minorities. The University of Nevada, Las Vegas, School of Dental Medicine, with more than 70,000 active patients and a post-graduate specialty program in Orthodontics is uniquely positioned to perform this type of oral health epidemiology survey, as well as targeted oral microbial testing among populations within the clinic and the local community.

A UNLV Office of Research Integrity - Human Subjects Exemption (OPRS#1104-3801M - *Retrospective Evaluation of Microbial Presence in Existing Saliva Repository: A PCR-Based Molecular Survey of Oral Microbial Populations from Existing Saliva Samples*) was filed and approved May 10, 2011 to facilitate the use of an existing saliva database of more than one hundred clinical samples that have already been collected from adult patients in the UNLV-SDM clinic, including patients from the orthodontic clinic. These saliva samples contain DNA representative of the host (human), as well as any endogenous microbial flora (bacteria, viruses). Analysis of these samples to test for microbial presence, as well as quantitative analysis of microbial burden will provide

UNLV-SDM clinicians with information about the types of public health interventions that may be needed to serve the local population.

This project will attempt to determine if there are oral microbial burden differences within the patient population that could positively or negatively influence their orthodontic needs. Variables to be assessed will include: gender, age, and ethnicity. No previous survey of this population has been attempted; therefore, this study will be among the first to report on the oral microbial burden within this patient pool.

## ACKNOWLEDGEMENTS

To my wife, Keri, without her support, none of this would have been possible.

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## CHAPTER 1

### INTRODUCTION

#### **Background**

Malocclusions are the third most common oral health problem, and are associated with a number of complications (Huser et al., 1990). Orthodontic treatment often can correct these complications or at least prevent them from progressing, but may also represent some potential for harm to teeth and periodontal tissues (Bollen et al.,k 2008). For example oral hygiene may be difficult to maintain during treatment, which may lead to plaque accumulation and gingival inflammation (Balenseifen and Madonia, 1970). Evidence now demonstrates that orthodontic treatment induces changes in the oral environment, with an increase in oral bacterial concentrations, and significant alterations in salivary buffer capacity, pH acidity and salivary flow rates (Chang et al., 1999).

Fixed or removable orthodontic appliances impede the maintenance of oral hygiene and result in plaque accumulation (Balenseifen and Madonia, 1970; Huser et al., 1990). Plaque retention surrounding orthodontic appliances leads to enamel demineralization caused by organic acids produced by bacteria in the dental plaque (Sakamaki and Bahn, 1968). The literature clearly demonstrates that fixed orthodontic appliances increase plaque accumulation, bacterial colonization, and resultant enamel decalcification (Glans et al., 2003).

Generally, adult periodontitis is supposed to be caused by periodontopathic bacteria, such as the clinically important organism *Porphyromonas gingivalis* (*P. gingivalis*), as well as others, including *Aggregatibacter actinomycetemcomitans*, and *Tannerella forsythensis* (Sinclair et al., 1987). It is important to identify the changes in the oral environment in patients undergoing orthodontic treatment and other variables that may influence the development or progression of periodontal disease, including levels of *P. gingivalis*, and the development or progression of cariogenic disease (Kimmel and Tinanoff, 1991). In some cases involving extended treatment duration, it is imperative to preserve the oral health of the patient and it is the clinician's goal to reduce plaque accumulation during orthodontic treatment in order to prevent the onset of periodontal disease (Balenseifen and Madonia, 1970; Bollen et al., 2008).

Previous studies have documented that individuals with high levels of *Streptococcus mutans* are "at risk" for dental caries (Corbett et al., 1981; Mattingly et al., 1983; Scheie et al., 1984; Rosenbloom and Tinanoff, 1991). The research indicates that females in this age group are potentially at greater risk for dental caries than their male counterparts due to the observed higher levels of endogenous *S. mutans* (Rosenbloom and Tinanoff, 1991). Interestingly, another recent research study found that minority patients were not at greater risk for dental caries, but rather were at much greater risk for periodontal disease due to a lack of oral health knowledge and skills related to flossing (Macek et al., 2011). These preliminary studies highlight clinically relevant findings that may apply to the orthodontic clinic population at UNLV-SDM.

Population in the United States is projected by the US Bureau of the Census to grow from 310.2 million in 2010 to 439.0 million by 2050. This represents a population increase of over 41% over the next 40 years. Recent analysis of these data now suggest the most relevant age groups for orthodontics consist of two (2) main subgroups of the entire population; teenagers and young adults (Brown and Nash, 2009). However, recent reports also suggest that the percentage of patients currently seeking orthodontic care over the age of 18 is expected to increase, as are the numbers and percentage of minorities (Brown and Nash, 2009).

These changes in population demographics suggest the need for research to address the needs of these new patients, including many under-represented minority subgroups. The University of Nevada, Las Vegas (UNLV) opened a new School of Dental Medicine (SDM) in 2002 to address the needs of the racially and ethnically diverse population of Southern Nevada, primarily focusing on the treatment and care of low-income and minority patients. The recent addition of an orthodontic residency program and clinic has functioned to serve the needs of this specific population. There is a paucity of research to describe the needs and oral health status of minority adults in the US, particularly those seeking or in need of orthodontic treatment (Brown and Nash, 2009). Based upon this information, the primary goal of this study was to describe the microbial burden of the cariogenic and periodontal pathogens, *P. gingivalis* and *S. mutans* within the UNLV-SDM patient population.

## **Research Questions and Hypotheses**

This study will assess what the local community (Clark County, Nevada) demographics are and how the patient population at UNLV-SDM reflects these demographics. Given the UNLV-SDM mandate to serve the needs of the racially and ethnically diverse population of Southern Nevada, primarily focusing on the treatment and care of low-income and minority patients – the first steps must be to establish the composition of these populations.

Research Question 1. How does the UNLV-SDM patient population (~ 70,000) compare with the local population of Southern Nevada (Clark County ~ 1,400,000)?

Null hypothesis  $H_0$ :

There will be no differences between the patient and local populations of UNLV-SDM and Clark County, respectively.

Alternative hypothesis  $H_A$ :

There will be differences between the patient and local populations of UNLV-SDM and Clark County, respectively.

Research Question 2. How does the Orthodontics patient population (~ 800) compare with the UNLV-SDM patient population (~ 70,000)?

Null hypothesis  $H_0$ :

There will be no differences between the Orthodontic and main UNLV-SDM clinic populations.

Alternative hypothesis  $H_A$ :

There will be differences between the Orthodontic and main UNLV-SDM clinic populations.

Research Question 3. Are there any differences in the prevalence of specific pathogens, such as *P. gingivalis* and *S. mutans* between demographic groups within the sample population?

*Porphyromonas gingivalis*

*Streptococcus mutans*

Null hypothesis  $H_0$ :

There will be no differences in microbial pathogens among different subgroups.

Alternative hypothesis  $H_A$ :

There will be differences in microbial pathogens among different subgroups.

### **Experimental Design**

This study utilizes a retrospective, cluster design, and involves a convenience sample or cluster, derived from patients who seek treatment at UNLV-SDM. The investigators randomly selected healthy, adult patients for participation. The inclusion criteria required participants to: be a current patient at UNLV-SDM; be over eighteen years of age; and agree to participate and provide Informed Consent. The exclusion criteria excluded participants under the age of 18 and those who did not wish to participate.

### **Preliminary Results and Analysis**

To more accurately assess the demographic profile of the local community, data were obtained from the US Census Bureau for analysis of specific subgroups. Using the available data from UNLV-SDM, a comparison was made using Chi-square analysis to determine if any statistically significant differences between subgroups were apparent. These results demonstrate that the proportion of males and females in Clark County, NV is roughly equal, as are the percentages at UNLV-SDM (Table 1). More specifically, these percentages are nearly equal, although there are slightly more females at UNLV-SDM (50.6%) than the local community (49.1%), which was not statistically significant ( $p > 0.05$ ).

Further analysis of these data also revealed that nearly one third (28.5%) of the local community were minorities, a far larger percentage of patients at UNLV-SDM were minorities (59.2%), which was statistically significant ( $p < 0.05$ ). No significant differences in the age distribution of patients were found.



Table 1. Analysis of demographic profile to assess specific subgroups within the local community and UNLV-SDM clinic population.

<b>Variables</b>	<b>UNLV-SDM</b>	<b>Clark County</b>	<b>Statistical analysis</b>
<i>Gender</i>			
Female	n = 35,952 (50.6%)	n = 676,037 (49.1%)	$\chi^2 = 0.064$ , d.f.=1
Male	n = 35,099 (49.4%)	n = 699,728 (50.9%)	<b><math>p = 0.8001</math></b>
<i>Race</i>			
White	n = 28,989 (40.8%)	n = 984,796 (71.6%)	$\chi^2 = 401.33$ ,d.f.=1
Non-White	n = 42,062 (59.2%)	n = 390,969 (28.4%)	$p = 0.0000001$
<i>Age</i>			
18 - 64 years	n = 60,598 (85.3%)	n = 1,019,442(74.1%)	$\chi^2 = 1.997$ , d.f. = 1
<18	n = 10,453 (14.7%)	n = 356,323 (25.9%)	<b><math>p = 0.1576</math></b>

These data provide some evidence to address Research Question 1, which sought to analyze any differences between the UNLV-SDM patient population (~ 70,000) with the local population of Southern Nevada (Clark County ~ 1,400,000). These data suggest that there are differences between UNLV-SDM and Clark County (higher percentage of minorities at UNLV-SDM), and therefore the alternative hypothesis  $H_A$  must be accepted.

To determine if the orthodontic clinic services a similar population profile as UNLV-SDM, the demographic profiles of the main UNLV-SDM patient clinic and Orthodontic clinic were analyzed. These data demonstrate that the Orthodontic clinic services a

significantly higher proportion of females (61.3%) than males (38.7%), which is statistically different ( $p < 0.05$ ) from the main UNLV-SDM clinic (50.6%, 49.4%) (Table 2). Further analysis of these data also revealed that the percentage of minority patients was higher in the Orthodontic clinic (64.9%) than in the main UNLV-SDM patient clinic (59.2%), which was statistically significant ( $p < 0.05$ ). Finally, the age distribution in the orthodontic clinic was different, due mainly to the fact that UNLV-SDM maintains a separate pediatric clinic – thereby separating those patients under the age of eighteen (18) from the adult patient population.

Table 2. Analysis of demographic profile to assess specific subgroups within the main UNLV-SDM and Orthodontic clinic populations.

<b>Variables</b>	<b>UNLV-SDM</b>	<b>Orthodontic clinic</b>	<b>Statistical analysis</b>
<i>Gender</i>			
Female	n = 35,952 (50.6%)	n = 376 (61.3%)	$\chi^2 = 28.033$ , d.f. = 1
Male	n = 35,099 (49.4%)	n = 237 (38.7%)	$p = 0.0001$
<i>Race</i>			
White	n = 28,989 (40.8%)	n = 215 (35.1%)	$\chi^2 = 8.255$ , d.f. = 1
Non-White	n = 42,062 (59.2%)	n = 398 (64.9%)	$p = 0.0041$
<i>Age</i>			
18 - 64 years	n = 60,598 (85.3%)	n = 800 (65.3%)	$\chi^2 = 378.359$ , d.f. = 1
<18	n = 10,453 (14.7%)	n = 426 (34.7%)	$p = 0.000001$

These data provide some evidence to address Research Question 2, which sought to analyze any differences between the UNLV-SDM patient population (~ 70,000) and the Orthodontics patient population (~ 800). These data suggest that there are differences between UNLV-SDM and the Orthodontic Clinic. More specifically, the UNLV-SDM Orthodontic clinic is different from the main patient clinic in that more females than males are seeking and receiving treatment. Furthermore, although UNLV-SDM treats a large percentage of minority patients, the Orthodontic clinic has an even greater proportion of minority patients seeking and receiving treatment. Based upon these differences, the alternative hypothesis  $H_A$  must be accepted.

Very little evidence exists to provide information about the oral health needs of minority patients seeking orthodontic treatment, which provides the rationale for addressing the third Research Question, which seeks to understand if there are any differences in the prevalence of specific pathogenic microbial populations between different demographic groups within the sample population. This is addressed in Chapter 2.

## CHAPTER 2

This chapter has been prepared for submission to the peer-reviewed scientific journal *Journal of Orthodontics* and is presented in the style of that journal.

The complete citation is:

Jay Ericksen Davis<sup>1</sup>, Nicholas Freel<sup>2</sup>, Allison Findley<sup>3</sup>, Keaton Tomlin<sup>3</sup>, Katherine Howard<sup>3</sup>, Clifford C. Seran<sup>1</sup>, Patricia Cruz<sup>4</sup>, Karl Kingsley<sup>3</sup>

A molecular survey of *S. mutans* and *P. gingivalis* oral microbial burden in human saliva using RE-PCR within the minority population of a Nevada dental school.

# **A molecular survey of *S. mutans* and *P. gingivalis* oral microbial burden in human saliva using RE-PCR within the minority population of a Nevada dental school.**

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## **Abstract**

Background: The University of Nevada, Las Vegas School of Dental Medicine recently opened an orthodontic treatment clinic to address the needs of the racially and ethnically diverse population of Southern Nevada, primarily focusing on the treatment and care of low-income and minority patients. Although orthodontic treatment and therapy has been shown to induce changes in the oral cavity, much of this evidence was collected from

traditional White, teenage orthodontic clinic populations. The primary goal of this study was to describe the microbial burden of the cariogenic and periodontal pathogens, *Streptococcus mutans* and *Porphyromonas gingivalis* within the UNLV-SDM patient population.

Methods: Representative saliva samples were collected from healthy adult patients for DNA isolation. Relative endpoint polymerase chain reaction (RE-PCR) was performed to ascertain the presence and relative microbial burden of these oral pathogens.

Results: Nearly one quarter (13/52) or 25% of these patients had elevated levels of *S. mutans*. Only (10/52) or 19.2% of these samples were found to have elevated levels of *P. gingivalis*, however, a significant percentage (90%) were from minority patients ( $X^2=17.921$ , d.f. =1;  $p<0.0001$ ).

Conclusions: These findings of elevated *P. gingivalis* levels, primarily among minority patients, may suggest underlying oral health practices contributing to adverse oral health conditions within this population. Oral health knowledge and practices among minority patients may be strongly influenced by other factors, including education and socioeconomic status, suggesting additional research may be needed to accurately determine the most appropriate standards for care and oral health education within this patient population.

## Background

Orthodontic treatment and therapy has been associated with changes to the oral mucosa, gingiva and the oral microflora.<sup>1</sup> Alterations to oral hygiene and the addition of new surfaces and microenvironments during orthodontic treatment often precipitate increases among cariogenic bacteria, including *Streptococcus mutans* and *Lactobacillus acidophilus*.<sup>2,3</sup> In addition, these alterations also affect the periodontal status of patients during orthodontic therapy, increasing the burden of anaerobic and facultative subgingival bacteria such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*.<sup>4,5</sup>

Much of the evidence regarding changes to the oral flora and cariogenic risk has been collected from traditional orthodontic clinic populations, which have been mainly White adolescents from middle- or upper-income families.<sup>6-9</sup> Similarly, clinical studies evaluating periodontal status and subgingival flora have mostly involved White affluent teenage populations.<sup>10,11</sup> However, the increase in the percentage of minorities in the United States, as well as other industrialized countries, may be leading to changes in the demographic profile of orthodontic patients seeking treatment.<sup>12</sup>

Recent evidence suggests that orthodontic treatment needs are similar in all population subgroups in the U.S., although the percentage of White patients receiving treatment far exceeds that of minorities, including Hispanics or Blacks.<sup>12-14</sup> Other studies have suggested that orthodontic needs among minority patients in the U.S. are often unmet, with many clinics reporting vanishingly small numbers of Medicaid or low-income

patients receiving treatment.<sup>15,16</sup> Although scant evidence is available to evaluate the oral health status of minorities seeking orthodontic treatment in the U.S., studies of Hispanic and Latino orthodontic populations from Latin and South America have recently become available.<sup>17-19</sup>

The University of Nevada, Las Vegas opened a new School of Dental Medicine (UNLV-SDM) in 2002 to address the needs of the racially and ethnically diverse population of Southern Nevada, primarily focusing on the treatment and care of low-income and minority patients. The more recent addition of an orthodontic residency program and clinic has functioned to serve the needs of this specific population. In fact, unlike the demographic profiles of local and regional orthodontic clinics, UNLV-SDM patients are primarily adults, low-income, and minority. Although a preliminary study of White adult orthodontic patients has described periodontal changes among these patients, there is a paucity of research to describe the needs and oral health status of minority adults in the U.S., particularly those seeking or in need of orthodontic treatment. Based upon this information, the primary goal of this study was to describe the microbial burden of the cariogenic and periodontal pathogens, *S. mutans* and *P. gingivalis* within the UNLV-SDM patient population.

## **Methods**

### *Human Subjects*

The protocol for this study was approved by the UNLV Office of Research Integrity – Human Subjects (OPRS#1104-3801M) on April 25, 2011. Saliva samples were



originally collected under a separate protocol titled “The Prevalence of Oral Human Papilloma Virus (HPV) in the University of Nevada, Las Vegas - School of Dental Medicine (UNLV-SDM) Clinic Population”, approved by the UNLV Office of Research Integrity – Human Subjects (OPRS#1002-3361) on April 9, 2010. Briefly, subjects in this convenience sample were recruited by members of the UNLV-SDM Clinic during their dental visit on one of 15 clinic dates. Informed consent was required and was conducted onsite. Inclusion criteria: subjects had to be 18 years old or older and had to agree to participate. Subjects younger than 18 years of age, subjects that declined to participate, and subjects with prior diagnosis of oral cancer were excluded.

#### *Sample size*

To determine an appropriate sample size for this study, the standard recovery rate from the sample-limiting step of DNA extraction (90-95%) was used to determine the minimum expected difference of 10% or 0.10.<sup>20</sup> Using chi-square ( $\chi^2$ ) analysis, a significance level of  $\alpha=0.05$ , and power  $p=0.80$ , a minimum required sample size of  $N=51$  was obtained.<sup>21</sup> At a minimum, twenty two (22) individuals from each category (Females, Males and Whites, Minorities) would be required to meet the standard assumptions of a two-tailed  $t$ -test.<sup>21</sup> Based upon this combined information, the minimum sample size was estimated to be 50, with a minimum of 22 within each demographic comparison sub-group.

#### *Saliva Collection Protocol*

In brief, healthy adults who agreed to participate were given a sterile 50 mL sterile

polypropylene tube obtained from Fisher Scientific (Fair Lawn, NJ). Participants were then asked to chew on a small piece of paraffin wax for one minute and then to expectorate. Samples were stored on ice until transported to the laboratory for analysis. Each saliva sample was assigned a unique, randomly-generated number to prevent research bias. Demographic information regarding the sample was concurrently collected, which consisted of age, gender, and ethnicity only. Based upon the demographics of the clinic, freely available from the UNLV website<sup>22</sup>, fifty six (56) samples were randomly selected that represented the approximate distribution of males (40%, n=22) and females (60%, n=32), as well as Whites (37%, n=20) and Minorities (63%, n=34).

#### *Cell enumeration and DNA isolation*

Cell number was determined from a small aliquot (100  $\mu$ L) of saliva using Trypan Blue (Fisher Scientific), a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany), and a hemacytometer (Fisher Scientific).

DNA was isolated from each saliva sample using a standard volume, containing a minimum of  $3.5 \times 10^5$  cells, using the GenomicPrep DNA isolation kit (Amersham Biosciences: Buckinghamshire, UK), following the procedure recommended by the manufacturer as previously described.<sup>23-25</sup> DNA purity was calculated using ratio measurements of absorbance at 260 and 280 nm. DNA purity has been established as the A260/A280 ratio between 1.7 and 2.0.<sup>26</sup>

#### *Polymerase chain reaction (PCR)*

DNA from each sample was then used to perform PCR with the exACTGene complete PCR kit (Fisher Scientific) and a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) using the following primers for glyceraldehyde- 3- phosphate dehydrogenase (GAPDH)<sup>27</sup>, *S. mutans*<sup>28</sup>, *P. gingivalis*<sup>29,30</sup>, synthesized by SeqWright (Houston, TX):

GAPDH forward primer, ATCTTCCAGGAGCGAGATCC;

GAPDH reverse primer, ACCACTGACACGTTGGCAGT;

*Streptococcus mutans* forward primer, GCCTACAGC TCAGAGATGCTATTCT;

*Streptococcus mutans* reverse primer, GCC ATACACCACTCATGAATTGA;

*Porphyromonas gingivalis* forward primer, TACCCATCGTCGCCTTGGT;

*Porphyromonas gingivalis* reverse primer, CGGACTAAAACCGCATACTTG;

Cycle number for relative endpoint (RE) PCR was established using a pure standard of DNA extracted from Human Gingival Fibroblast cells (American Tissue Culture Collection, HGF-1, CRL-2014) at a concentration of approximately one (1) µg/µL to establish the detection limit (floor), saturation limit (ceiling), and the exponential phase (EP). One µg of template DNA was used for each reaction. The initial denaturation step ran for three minutes at 94°C. A total of 30 amplification cycles were run, consisting of 30 second denaturation at 94°C, 60 seconds of annealing at 58°C, and 30 seconds of extension at 72°C. Final extension was run for five minutes at 72°C. The PCR reaction products were separated by gel electrophoresis using Reliant 4% NuSieve® 3:1 Plus Agarose gels (Lonza, Rockland, ME). Bands were visualized by UV illumination of

ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

### *Statistical evaluation*

Data were analyzed and basic descriptive statistics, which included concentration averages, Pearson's correlation (R) and coefficient of determination ( $R^2$ ), were graphed using Microsoft Excel (Redmond, WA). The demographic comparisons, as well as the differences between the population sub-groups (Males, Females, Whites, Minorities) were measured using chi-square ( $\chi^2$ ) test. A probability level of alpha ( $\alpha$ ) = 0.05 and two-tailed *p*-values were used to determine statistical significance.<sup>21</sup>

## **Results**

Fifty six (56) saliva samples, collected from UNLV-SDM patients between June and October 2010, were selected at random for this study. Demographic analysis revealed this sample was not statistically different from the demographic composition of the orthodontic clinic patient population with respect to gender or race (Table 1). More specifically, the percentage of females (n=32, 59.3%) and males (n=22, 40.7%) in the sample was not significantly different than the percentage of females (n=376, 61.3%) and males (n=237, n=38.7%) in the Orthodontic clinic ( $\chi^2= 0.0902$ , d.f.=1, p=0.7639). Similarly, there was approximately the same percentage of White (n=20, 37%) and Minority (n=34, 63%) patients in the study sample compared with Whites (n=215,35.1%) and Minorities (n=398, 64.9%) in the overall Orthodontic clinic population ( $\chi^2= 0.838$ , d.f.=1, p=0.7722). However, because only saliva from adult patients was collected, there were no samples from patients under 18 years old in the study sample (n=0, 0%), which

was statistically different from the ratio within the overall clinic (n=426, 34.7%) ( $p < 0.0001$ ).

Table 1. Demographic analysis of salivary samples

<b>Variables</b>	<b>Orthodontic clinic</b>	<b>Saliva samples</b>	<b>Statistical analysis</b>
<i>Gender</i>			
Female	n = 376 (61.3%)	n = 32 (59.3%)	$\chi^2 = 0.0902$ , d.f. = 1
Male	n = 237 (38.7%)	n = 22 (40.7%)	<b><math>p = 0.7639</math></b>
<i>Race</i>			
White	n = 215 (35.1%)	n = 20 (37.0%)	$\chi^2 = 0.0838$ , d.f. = 1
Non-White	n = 398 (64.9%)	n = 34 (63.0%)	<b><math>p = 0.7722</math></b>
<i>Age</i>			
18 - 64 years	n = 800 (65.3%)	n = 54 (100.0%)	$\chi^2 =$ , d.f. = 1
<18	n = 426 (34.7%)	n = 0 (0.0%)	<b><math>p &lt; 0.0001</math></b>

DNA was then successfully isolated from all 56 patient samples using equal volumes of saliva (Figure 1A). More specifically, the average DNA concentration yield was 852.25 ng/ $\mu$ L, +/- 150.6. The DNA concentrations ranged between 445 ng/ $\mu$ L and 1095 ng/ $\mu$ L.

Absorbance measurements and subsequent A260/A280 ratio analysis confirmed the purity of the DNA isolates, which averaged 1.78 +/- 0.18.

To establish the conditions and cutoff cycle for relative endpoint polymerase chain reactions (RE-PCR), DNA from human gingival fibroblast (HGF-1) cells was extracted and diluted (1196 ng/ $\mu$ L) to approximate the highest DNA concentration from the saliva sample isolates (1095 ng/ $\mu$ L). PCR reactions, 0 – 50 cycles (C), were performed using the internal control primer, GAPDH, and amplification products were separated on agarose gels to quantify ethidium bromide (EtBr) band intensity (Figure 1B). Analysis of these results indicated the limit of detection (floor) was evident at 10 cycles (C10) and saturation (ceiling) was observed at all cycles exceeding 35 (C35), which established the exponential phase between C10 and C30.

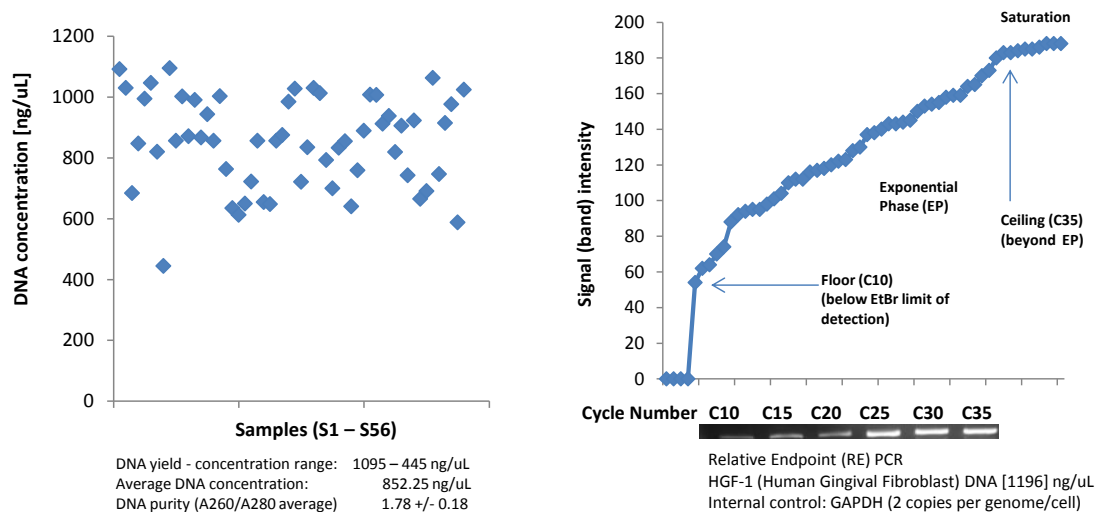


Figure 1. DNA isolation and RE-PCR parameters. (A) Average DNA concentration from 56 samples (S1-S56) was 852.25 +/- 150.6 ng/ $\mu$ L. (B) RE-PCR floor and ceiling at cycles 10 and 35, respectively (C10, C35) described exponential phase (EP).

To determine the number of exfoliated human oral cells, which varied between saliva samples, aliquots from each sample were examined to determine cell number and relative concentrations (Table 2). More specifically, these data revealed cell counts varying between  $0.8 - 2.4 \times 10^6$  cells/mL, which could then be grouped into three broad categories:  $0.8 - 1.2$  (low),  $1.6 - 1.9$  (mid), and  $2.1 - 2.4$  (high) cells/mL. The average DNA concentration in the group with the lowest cell number ( $0.8 - 1.2 \times 10^6$  cells/mL) was higher than the average DNA concentration in the group with the middle level ( $1.6 - 1.9 \times 10^6$  cells/mL), which suggests the presence of additional bacterial, viral or fungal DNA. Four (4) samples were not found to contain any identifiable human cells.

Table 2. Cell enumeration and DNA concentrations

<b>Cell count (cells/mL)</b>	<b>Average DNA concentration (ng/<math>\mu</math>L)</b>	<b>Samples (n)</b>
$2.1 - 2.4 \times 10^6$	886.47 +/- 167.9	20
$1.6 - 1.9 \times 10^6$	814.89 +/- 137.6	18
$0.8 - 1.2 \times 10^6$	843.94 +/- 138.2	14

To verify the internal control primer on saliva samples, RE-PCR at C30 was performed on DNA isolates from samples representing the high and low for each cell concentration range ( $0.8, 1.2, 1.6, 1.9, 2.1,$  and  $2.4 \times 10^6$  cells/mL) in triplicate (Figure 2A). Analysis of these results revealed consistent, band intensities with relatively low variability, ranging from 3.3 to 12.5%.

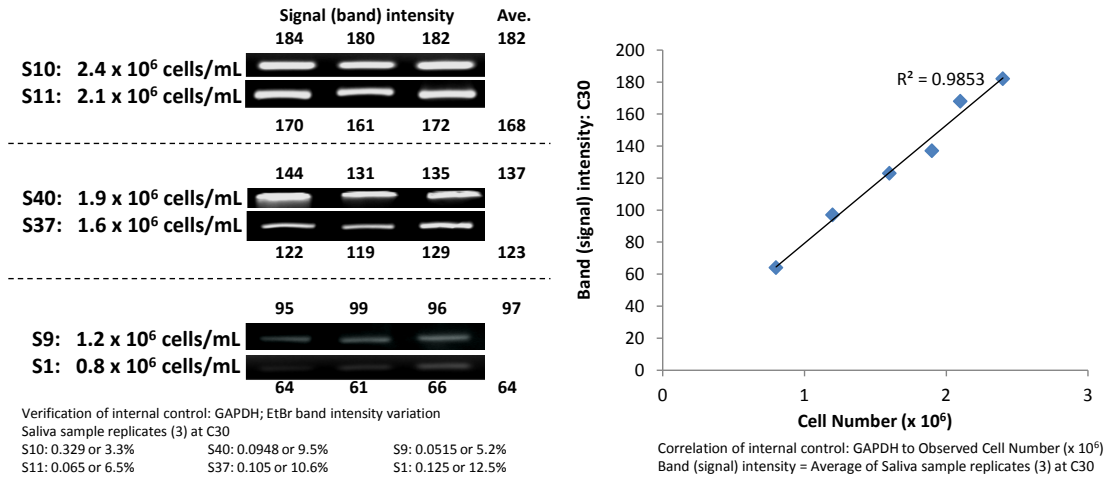


Figure 2. Association between cell number and RE-PCR band intensity. (A) Cell enumeration revealed RE-PCR band intensity at C30 varies according to cells/mL. (B) RE-PCR band intensity correlates significantly with cell number (cells/mL).

The average band intensities were then plotted against cell number to determine the correlation between observed human cell number and GAPDH band intensity at C30 (Figure 2B). These results demonstrated a strong positive correlation between GAPDH band intensity at C30 and observed cell number. Analysis of these data using Pearson's correlation revealed a near perfect linear association ( $R=0.9926$ ) and robust coefficient of determination ( $R^2=0.985$ ).

All samples ( $n=56$ ) were subsequently screened using the internal control, GAPDH (Figure 3A). Analysis of these results revealed positive results for 52/56 or 92.8% of the DNA isolates from the original saliva samples. The four (4) samples previously identified without any observable human cells did not have detectable amplification products, although each were found to contain some measurable quantity of DNA.



The band intensities from the 52 GAPDH-positive samples were then plotted against cell number to determine the correlation between observed human cell number and GAPDH band intensity at C30 (Figure 3B). These results confirm the strong positive correlation between GAPDH band intensity at C30 and observed cell number in all 52 samples. Analysis of these data using Pearson's correlation revealed a strong, positive correlation ( $R=0.9642$ ) and coefficient of determination ( $R^2=0.92968$ ).

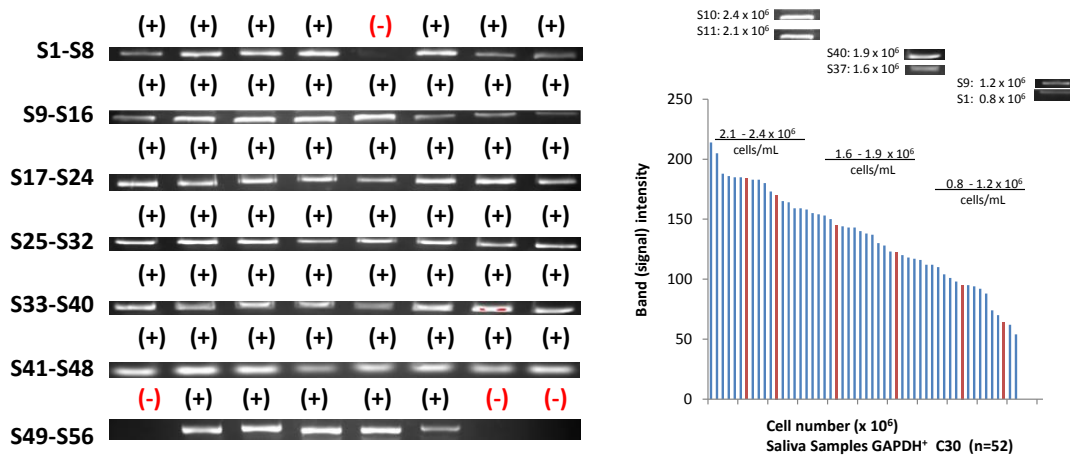


Figure 3. Saliva sample screening using internal control primer, GAPDH. (A) RE-PCR using the control primer resulted in fifty two (52) samples with detectable bands (GAPDH<sup>+</sup>). Four (4) samples had no detectable bands. (B) Strong, positive association was observed between RE-PCR band intensity and cell number (cells/mL).

Based upon these results, all samples (n=56) were subsequently screened using primers to detect the cariogenic oral pathogen, *S. mutans* (Figure 4A). Analysis of these data revealed 13/56 or 23.3% of these samples had detectable levels of *S. mutans*. Demographic analysis using chi-square revealed that the percentage of *S. mutans*-positive

samples from females (n=7, 53.8%) and males (n=6, 46.2%) was not significantly different ( $X^2=0.126$ , d.f. =1;  $p=0.7224$ ) than their respective percentages in the overall sample (59.6, 40.4%, respectively). Similarly, the percentages of *S. mutans*-positive samples from Whites (n=3, 23.1%) and Minorities (n=10, 79.6%) was also not significantly different ( $X^2=0.906$ , d.f. =1;  $p=0.3412$ ) from the overall sample (35.1%, 64.9%, respectively). Further analysis of band intensity revealed significant variation between samples, which could be categorized similar to GAPDH (low, mid, high). Three (3) samples had relatively high *S. mutans* band intensities, while five (5) had mid- and low-intensities (Figure 4B). Of the GAPDH-negative samples, none tested positive for *S. mutans*.

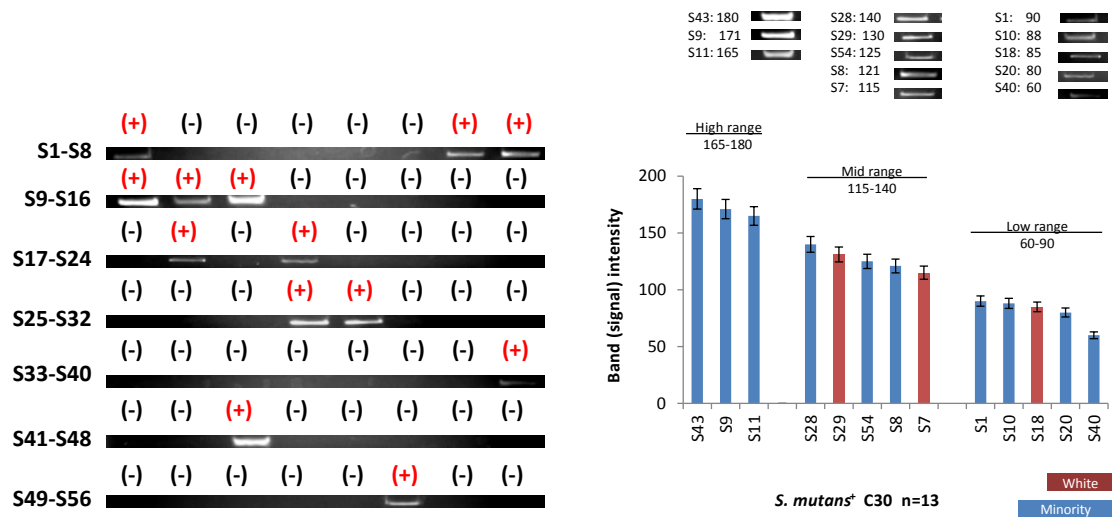


Figure 4. RE-PCR screening for cariogenic pathogen, *S. mutans*. (A) RE-PCR using a specific *S. mutans* primer revealed thirteen (13) samples with detectable bands (*S. mutans*<sup>+</sup>). (B) Three (3) samples had strongest band intensity, while five (5) had mid- or low-level band intensity, respectively. No significant differences among subgroups were identified.

Finally, all samples (n=56) were subsequently screened using primers to detect the periodontal disease-related oral pathogen, *P. gingivalis* (Figure 5A). Analysis of these data revealed 10/56 or 17.8% of these samples had detectable levels of *P. gingivalis*. Demographic analysis using chi-square revealed that the percentage of *P. gingivalis*-positive samples from females (n=6, 60%) and males (n=4, 40%) was not significantly different ( $X^2=0.021$ , d.f. =1;  $p=0.885$ ) than in the overall sample (59.6, 40.4%, respectively). However, the percentages of *P. gingivalis*-positive samples from Whites (n=1, 10%) and Minorities (n=9, 90%) was significantly different ( $X^2=17.921$ , d.f. =1;  $p<0.0001$ ) than the overall sample (35.1%, 64.9%, respectively). Analysis of band intensity revealed significant variation between samples, with four (4) samples exhibiting relatively high- or mid- *P. gingivalis* band intensities, and two (2) exhibiting low-intensities (Figure 5B). None of the GAPDH-negative samples tested positive for *P. gingivalis*.

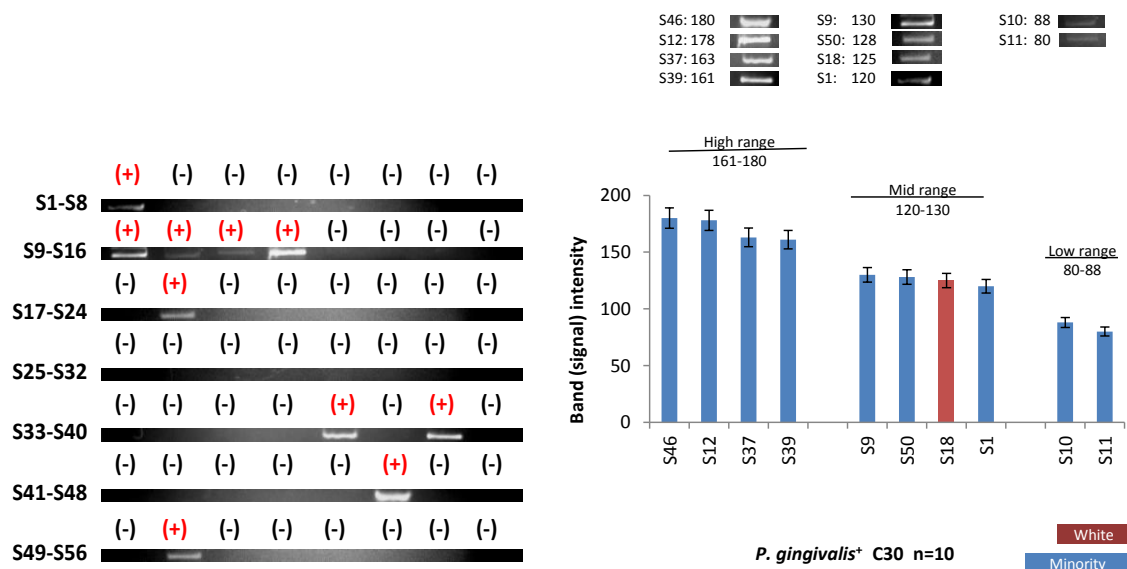


Figure 5. RE-PCR screening for periodontal pathogen, *P. gingivalis*. (A) RE-PCR using a specific *P. gingivalis* primer revealed ten (10) samples with detectable bands (*P. gingivalis*<sup>+</sup>). (B) Four (4) samples had strongest band intensity, while four (2) had mid- and two (2) had low-level band intensity, respectively. Although no differences in gender were identified, the percentage of Minorities (9/10=90%) was significantly higher than expected ( $X^2=17.921$ , d.f. =1;  $p<0.0001$ ).

## Discussion

Although patient populations vary from clinic-to-clinic, and from state-to-state, some unique features distinguish the UNLV-SDM patient profile from many other clinics. For example, the gender ratio is much different than the statistical averages in many other local and regional orthodontic clinics<sup>10-16</sup> – with females accounting for nearly two-thirds (61%) of all clinic patients. Moreover, the percentage of adult patients is much higher (65%) than might otherwise be expected, as is the proportion of minority patients (64%) currently seeking or undergoing orthodontic therapy.<sup>12,15</sup> These demographic differences in the composition of the patient population suggest additional research may be warranted in order to provide the most appropriate level of care for the many adult female and minority patients seeking treatment.

Although some evidence has suggested that adult patients are more likely to have acquired sufficient oral health literacy prior to seeking orthodontic treatment than juveniles or adolescents<sup>1</sup>, much less evidence exists to assess the oral health status of adult minorities or any specific differences based upon gender. This study sought to address this paucity of information by randomly screening saliva samples collected from

the patient pool at UNLV-SDM to assess the oral microbial burden of two specific oral microbes related to caries formation (*S. mutans*) and periodontal disease (*P. gingivalis*), the major complications and sequelae that result from orthodontic treatment.<sup>1-5</sup> Using this sensitive and specific method for comparing oral microbial burden for these organisms, the results clearly indicate that a significant subset of this sample had detectable levels of one or both oral pathogens.

More specifically, one quarter (13/52=25%) of patients had detectable *S. mutans* levels. These patients could further be subdivided into categories that more accurately assess their cariogenic risk. For example, three patients had the highest levels of *S. mutans* (3/13), suggesting that overall only 5.8% (3/52) had elevated risk that might necessitate ancillary treatments, interventions, or additional oral health education. These data are consistent with other research suggesting that only a small percentage of orthodontic treatments are interrupted by complications involving oral infections or other complications<sup>5-9</sup>, with detectable *S. mutans* levels ranging from 14 – 40%.

Although these data also revealed that even fewer patients (10/52=19.2%) had detectable, elevated levels of *P. gingivalis* – the results revealed by this study are more disconcerting for two specific reasons. First, nearly all of these patients were minorities (90%), which may suggest additional underlying factors influenced the outcome of these results that may be specific to these minority groups in Southern Nevada. Second, half of the *P. gingivalis*-positive samples (5/10) were found to be in the highest risk category, suggesting that many more of these patients had underlying conditions related to periodontal disease risk that may be more readily exacerbated by orthodontic treatment

and therapy. Other research studies have demonstrated detectable *P. gingivalis* levels ranging from 5 – 19%, which may suggest the results of this study are among the highest reported.<sup>3,11</sup>

These results are consistent with the most recent study of oral health literacy among minority populations, which found that although 82% of minority patients knew how to brush, thereby reducing *S. mutans* populations and overall caries lesions, only 15% of patients knew how to floss, and flossed regularly<sup>31</sup>. The fact that nearly all of the patients testing positive for *P. gingivalis* were minority, and half of those were in the highest cell number category, may point to a larger issue affecting many populations that have lower health literacy, in general, and more specifically, much lower oral health literacy. The lack of statistically significant differences between males and females further suggests that these phenomena are not specific to gender, but are more pervasive among minority populations in this area who may benefit from additional oral health information, training, and targeted education initiatives.

While these results provide new information regarding oral health in minorities, there are several limitations of this study which should also be considered. The most obvious of these issues involves the size and composition of the sample. An analysis of previous orthodontic studies that performed similar saliva screenings for oral pathogens uncovered a range of sample sizes, which varied greatly from a low of only 14 to 70/<sup>3,4,6,8,10,11,17,32-34</sup> Previous research at UNLV and the UNLV-SDM clinic demonstrated low participation rates for invasive, blood-based screenings<sup>35</sup>, but higher rates of participation using non-invasive biomonitoring and screening methods, including saliva collection (unpublished

data). These studies had sample sizes ranging from 16 – 151, which suggest that the final sample size of the present study (N=52) is comparable and well within the range of similar studies. In addition, the sample population consisted solely of adult patients, which does not provide any information regarding the adolescent orthodontic population (<18). These younger populations have been the focus of intense study in previous research efforts because they have been the more traditional orthodontic patients until very recently.<sup>34</sup> In addition, no other demographic information about oral disease risk was collected, which may provide more information and additional insights in future studies of this population.

## **Conclusions**

In summary, although the overall implications of this study suggest that the screening of these samples did not reveal underlying differences between males and females, significant differences in risk for specific oral pathogens were evident among a subgroup of mainly minority patients. More specifically, elevated levels of *P. gingivalis* among minority patients may suggest that underlying oral health practices may contribute to this condition among both males and females. Moreover, because oral health knowledge and oral health practices among minority patients may be strongly influenced by other factors, including education and socioeconomic status, additional research may be needed to more accurately determine the most appropriate methods for addressing this newly identified health disparity within this patient population.

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## CHAPTER 3

### DISCUSSION & CONCLUSION

This study sought to determine the demographic patterns associated with oral microbial burden within the UNLV-SDM clinic population. Using well-established methods for oral health research, relative levels of cariogenic (*S. mutans*) and periodontal (*P. gingivalis*) disease associated bacteria were determined from saliva samples collected in the UNLV-SDM clinic. These results clearly demonstrate that levels of oral bacteria in the overall sample population are not significantly different from other published studies. However, when these data are analyzed by subgroup (gender, race), minorities within this sample are more likely to exhibit elevated levels of the periodontal pathogen, *P. gingivalis*.

These results are significant in two important ways. To date, this study is the first to evaluate oral microbial burden from the UNLV-SDM population in order to compare endogenous levels of these bacteria in saliva samples. This represents an important first step in a broader attempt to further define the patient population and assess their basic oral health needs. These needs may be different based upon cultural, ethnic, racial and health-literacy background. Second, this study is one of the largest to evaluate oral microbial burden using PCR analysis from saliva samples. A recent review of the literature revealed that although saliva, a non-invasively collected biomonitoring matrix, is more readily accepted by patients for research study participation, more easily collected, and facilitates both qualitative and quantitative analysis, many orthodontic

studies do not have sufficient sample sizes to make broader inferences. The average sample size of these published studies is 37, with the current study evaluating a larger sample than seven of the nine previous saliva-based PCR studies of oral microbial burden relating to orthodontic treatment (7/9 = 78%). Moreover, these samples were composed almost entirely of juveniles and adolescent patients (Table 3). Only two of these studies evaluated saliva samples from adults undergoing orthodontic therapy (Fischer et al., 2008; Sanpei et al., 2010).

Table 3. Survey of PCR-based orthodontic saliva studies

<b>Authors</b>	<b>Year of Publication</b>	<b>Organism</b>	<b>Sample size</b>
Jordan, LeBlanc	2002 / US	<i>S. mutans</i>	n = 27
Turkkahraman et al.	2005 / Turkey	<i>S. mutans</i> <i>P. gingivalis</i>	n = 21
Motisuki et al.	2005 / Brazil	<i>S. mutans</i> <i>L. acidophilus</i>	n = 30
Kupietzky et al.	2005 / Israel	<i>S. mutans</i> <i>L. acidophilus</i>	n = 64
Ristic et al.	2008 / Serbia	<i>P. intermedia</i> <i>P. gingivalis</i>	n = 32
Kitada et al.,	2009 / Japan	<i>S. mutans</i>	n = 58
Sanpei et al.	2010 / Japan	<i>S. mutans</i> <i>L. acidophilus</i>	n = 42
Fischer et al.	2008 / Switzerland	<i>P. gingivalis</i>	n= 40
Demling et al.	2010 / Germany	<i>P. gingivalis</i>	n = 20

In addition, the analysis of these data provided the opportunity to consider additional, secondary factors that may also be mediators of oral microbial burden within minority populations. Based upon research from the evidence base, it is clear that differences in oral hygiene (i.e. brushing, flossing) and oral health literacy vary greatly in the U.S. between White patients and minorities. Even more interesting, many nutritional and dietary preferences within these population subgroups may also influence the possibility for differences in oral microbial burden. This type of orthodontic research is so recent that virtually no studies have explored the possibility that endogenous, pre-existing oral microbial differences among some population subgroups may influence the course of orthodontic treatment and therapy, which may further complicate the existing barriers these populations face in obtaining health care, insurance and treatment.

Interdisciplinary research: Because health disparities among minority populations remain an on-going concern of healthcare professionals in the US, and more specifically within the dental and orthodontic professions, it is critical that clinical dentists and oral healthcare specialists become more familiar with biomedical research, public health investigations, and epidemiologic research methods. Furthermore, it is important for dentists and orthodontists to collaborate with, and integrate, their efforts to treat minority patients with social and public health professionals, as well as biomedical research scientists. This study incorporated basic biomedical research involving DNA extraction and PCR to evaluate the oral health of a changing, evolving patient population – comprised mainly of adults, females, and minorities.



This research is important to use in dental curricula, oral health education programs, and public service announcements in order to better serve the educators, oral health specialists, and patient population of Southern Nevada, particularly at UNLV-SDM. Little time is spent analyzing the demographic profile of patients, and reviewing the relevant evidence-based literature that might help these professionals-in-training provide higher quality and more appropriate levels of care for their patients.

APPENDIX

IRB Approval



**Biomedical IRB – Exempt Review  
Deemed Exempt**

**DATE:** June 22, 2011

**TO:** Dr. Karl Kingsley, School of Dental Medicine

**FROM:** Office of Research Integrity – Human Subjects

**RE:** Notification of review by /Cindy Lee-Tataseo/Ms. Cindy Lee-Tataseo, BS, CIP, CIM  
Protocol Title: **Retrospective Investigation Of Community Outreach By UNLV-  
SDM (Dental) Students: An Analysis of Participant Demographics At Community  
Dental Clinics**  
Protocol # 1106-3848M

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This memorandum is notification that the project referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46 and deemed exempt under 45 CFR 46.101(b)4.

*Any changes to the application may cause this project to require a different level of IRB review. Should any changes need to be made, please submit a **Modification Form**. When the above-referenced project has been completed, please submit a **Continuing Review/Progress Completion report** to notify ORI – HS of its closure.*

If you have questions or require any assistance, please contact the Office of Research Integrity - Human Subjects at [IRB@unlv.edu](mailto:IRB@unlv.edu) or call 895-2794.

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## VITA

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### Publications:

Jay Ericksen Davis, Nicholas Freel, Allison Findley, Keaton Tomlin, Katherine Howard, Clifford C. Seran, Patricia Cruz, and Karl Kingsley. A molecular survey of *S. mutans* and *P. gingivalis* oral microbial burden in human saliva using RE-PCR within the minority population of a Nevada dental school. Manuscript in preparation.

### Thesis Title:

Retrospective Evaluation of Microbial Presence in Existing Saliva Repository: A PCR Based Molecular Survey of Oral Microbial Populations from Existing Saliva Samples

### Thesis Examination Committee:

Chairperson, Clifford C. Seran, DDS  
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