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Oral Microbial Burden of Periodontal Pathogens among Orthodontic Patients

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ORAL MICROBIAL BURDEN OF PERIODONTAL PATHOGENS AMONG

ORTHODONTIC PATIENTS

By

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Abstract

Oral Microbial Burden of Periodontal Pathogens among Orthodontic Patients

By

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Objectives

Many research studies involving orthodontic patients have a natural inclination to focus on

changes in levels of cariogenic pathogens after bracket placement, and very few studies examine

the role of changes of periodontal pathogens – particularly among adult patients. Interestingly,

recent evidence suggests that increased levels of a specific periodontal pathogen, *Fusobacterium*

nucleatum, may elevate risk for development of colon cancer in adults through direct pathways.

Based upon this new evidence, the objective of the current study was to screen saliva samples

taken from orthodontic patients to determine the prevalence of periodontal pathogens, including

F. Nucleatum, *T. denticola, and P. gingivalis.*

Methods

Following an OPRS (human subjects) approved protocol, saliva samples were collected at

random from orthodontic and non-orthodontic patients over the course of several weeks. DNA was subsequently isolated from these samples and screened using polymerase chain reaction (PCR) for the presence of *Fusobacterium nucleatum*, *Treponema denticola* and *Porphyromonas gingivalis*, using primers designed specifically to distinguish these micro-organisms.

Results

A total of 310 samples were collected and analyzed. The 159 orthodontic samples revealed lower overall levels of the three oral pathogens tested, compared to the 151 non-orthodontic samples. More specifically, the levels of *F. nucleatum*, *T. denticola, and P. gingivalis* were detected in 38.4%, 27.7% and 36.5% of orthodontic patients compared with 39.1%, 35.8%, and 40.4% in non-orthodontic patients respectively.

Conclusions

These findings support previous evidence that a significant proportion of orthodontic clinic patients may harbor periodontal pathogens at high levels. These results are much higher than previous studies which found periodontal pathogens including *P. gingivalis in* about 39.1% of clinic patients. Although high levels of periodontal pathogens were observed in the orthodontic sample, interestingly, even higher levels were observed in the non-orthodontic sample, when comparing the two. These findings are important when determining oral health changes that adult patients within this population may face during orthodontic treatment. These findings suggest that orthodontic patients could benefit from not only routine dental and periodontal treatment, but also from increased education and awareness regarding the possibility of increased risk for the development of colon cancer among some patients.

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Chapter 1: Introduction

Background and Significance

Fusobacterium nucleatum is a common bacterium of the human oral flora [1]. *F. nucleatum* is an obligate anaerobic bacterium that can be observed as fusiform or spindle-shaped rods. It is mostly found during dental plaque formation, and is known primarily for its synergistic ability to act as a bridge, through coaggregation, of a wide range of Gram-positive and Gram-negative plaque microorganisms [7,8].

Although *F. nucleatum* is most frequently associated with gingivitis and periodontal disease [2], new clinical interest has been gained due to increased correlations being linked to *F. nucleatum's* pathogenic potential involving invasive human anaerobic infections of the head and neck, chest, lung, liver and abdomen [5,6,13]. A major clinical concern, is *F. nucleatum*'s potential to reach vital organs and other body cavities, through the oropharyngeal portal, causing serious diseases outside of the mouth [9,10]. Due to *F. nucleatum*'s adherence ability, it can adhere to host tissue cells and inhibiting human T-cell responses to mitogens and antigens, thus modulating the host's immune response [3,12]. Studies within the last 10 years have discovered *F. nucleatum*'s pathogenicity was underestimated due to its ability to adapt to oxidative stresses [4]. Over time research is trending towards identifying disorders involving disseminated *F. Nucleatum* from the oral cavity, such as *Fusobacterium nucleatum* Pericarditis [11], Intestinal Dysbiosis, Colorectal Neoplasia development, and most recently Colorectal Cancer [14]. More research is needed to evaluate the potential link between intra-oral periodontal pathogens, increased health-risk, and orthodontic therapy.

Research Question

1. Does the prevalence of *F. nucleatum* vary between orthodontic and non-orthodontic patients, at levels high enough for detection from unstimulated saliva samples?

   Ho: Microbacterial assays will not show an increase in *F. nucleatum* concentrations in pre-teen, teen, and adult orthodontic patient's, due to an altered oral environment with fixed orthodontic appliances.

   HA: Microbacterial assays will show an increase in *F. nucleatum* concentrations in pre-teen, teen, and adult orthodontic patients, due to an altered oral environment with fixed orthodontic appliances.

2. Is the health status or oral health parameters using UNLV School of Dental Medicine orthodontic patients differ from those of age-matched non-orthodontic patients?

   Ho: Orthodontic and non-orthodontic, age-matched patients will have the same health and oral health parameters to controls

   HA: Orthodontic and non-orthodontic, age-matched patients will not have the same health and oral health parameters to controls

Research Design

The design of this study is non-randomized retrospective analysis of previously collected saliva samples from orthodontic patients and non-orthodontic patients. Saliva samples were collected, at random, from orthodontic patients over many weeks spanning three years in total. Following the OPRS (human subjects) approved protocol, these saliva samples will be used to create an oral health profile for each patient, based on different factors that have been collected during this

saliva sample collection period, which includes: existing health conditions, DMFT index/score (cariogenic profile), pocket depth (periodontal profile), other health conditions. A comparison will then be performed with age and gender matched samples from orthodontic and non-orthodontic patients.

A microbial profile will also be created by isolating DNA from these saliva samples using high fidelity polymerase chain reaction (PCR) using primers designed specifically to distinguish the periodontal pathogens *Porphyromonas gingivalis*, *Treponema denticola*, and *Fusobacterium nucleatum*. The objective is to do the first comprehensive oral health and systemic health profile on orthodontic and non-orthodontic patients of similar age and gender. These findings are important to determine the changes to oral health that adult patients within this population may face during orthodontic treatment and may suggest these patients could benefit from not only from dental care and periodontal disease treatment, but also from increased education or awareness regarding the possibility of increased risk for the development of colon cancer among some patients. This research is novel, and will provide insight as information is being gathered to form clinical health parameters to safeguarding at-risk individuals, who may need to take precautions against dissemination of this organism into their body, which could result in a life-threatening Fusobacterium infection.

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Chapter 2

Oral Microbial Prevalence of Periodontal Pathogens among Orthodontic Patients

Role of Authors:
Dr. David Jolley designed the study, was the primary author, data collector and analyzer, and graphics generator. Dr Karl Kingsley was secondary author and assisted with data analysis.

Abstract

Changes in the oral microbial flora are commonplace during Orthodontic therapy, although some evidence suggests these alterations may extend for some time after. Many studies have screened for changes in cariogenic pathogen levels, and more evidence is accumulating to demonstrate significant changes among periodontal pathogens within these patients. Although several studies at this predominantly low-income, dental-school based Orthodontic clinic have screened for cariogenic pathogens – none to date have provided multi-organismal screening for periodontal pathogens. This goal of this study was to complete a retrospective, cross-sectional study of saliva samples to screen for *Fusobacterium nucleatum*, *Treponema denticola*, and *Porphyromonas gingivalis* among the Orthodontic and non-Orthodontic patient populations (n=125). PCR screening was performed on the isolated DNA from these, revealing pathogens in nearly half of Orthodontic patient samples and more than half of non-Orthodontic samples. This data also demonstrated females exhibited greater prevalence than males, while the overall prevalence among non-Orthodontic samples was greater, and this may be associated with higher average age, larger body mass index (BMI) and greater periodontal pocked depth (PPD) and

decayed-missing-filled teeth (DMFT) scores. These findings suggest the strong need to plan and implement a prospective study to determine the baseline prevalence of these pathogens among this patient population as they begin Orthodontic therapy and how these levels change over time. This may provide more relevant clinical information for oral health scientists and local epidemiologists to determine the most vulnerable populations, as well as the best methods and timing for interventions to prevent poor oral health outcomes and long-term consequences associated with acute periodontal disease.

Key words: Periodontal pathogen, Orthodontics, Saliva Screening

Introduction

Although many studies of oral microbial changes during Orthodontic therapy have necessarily focused on cariogenic pathogens [1,2], fewer studies have closely examined the changes to other oral flora, including periodontal pathogens [3,4] . Studies have demonstrated that orthodontic treatment alters the oral microbiome and can both directly and indirectly alter the oral microbial composition, thereby dramatically increasing the potential for both cariogenic and periodontal disease [5-7]. Recent evidence has suggested that microbial alterations during orthodontic treatment may outlast the duration of therapy and influence long-term oral health outcomes [8-10].

Many studies have demonstrated normal, baseline ranges for levels of potential periodontal pathogens in the oral biofilm and subgingival crevices that may trigger disease if homeostasis is disrupted [11,12]. These pathogens, include *Fusobacterium nucleatum* (FN), *Treponema denticola* (TD), and *Porphyromonas gingivalis* (PG) – the major etiologic agent implicated in chronic and persistent periodontitis [12,13]. Although modern materials and Orthodontic

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techniques have improved oral health outcomes in recent years, all current treatments are associated with increased levels of periodontal pathogen levels to some degree in many patients [14,15].

New diagnostic methods involving salivary biomarkers have improved the ability to monitor oral and periodontal diseases in recent years [16,17]. These advances facilitate studies investigating salivary screening for oral microbial changes during Orthodontic treatment [18,19]. In fact, studies from this school have utilized salivary biomarkers to screen for cariogenic pathogen changes among Orthodontic clinic patients – although no large-scale screening for periodontal pathogen levels has yet been attempted within this patient population [20-22].

Our studies have informed us that oral health status among Orthodontic patients, particularly at this dental school-based clinic, may be of particular concern due to the large number of low-income and Minority patients who may face greater barriers and challenges to receive high quality healthcare [23,24]. The higher prevalence of these cariogenic pathogens, combined with increased barriers, and lowered access to care may explain some of these observations – although the full spectrum of changes within the oral microbial flora remains incomplete. These data serve as the basis for the current study objective to screen Orthodontic and non-Orthodontic patients from this dental school patient clinic and determine the relative prevalence of periodontal pathogens.

Materials and Methods

Human Subjects

The protocol submission "Retrospective investigation of oral microbes from the UNLV-SDM patient population" (OPRS#762911-1) was approved by the UNLV Biomedical IRB on August

3, 2015. Saliva samples were originally collected and appropriately archived from a convenience sample of eligible patients. Exclusion criteria included patients that chose not to participate, patients aged seven or younger, and adult patients with oral cancer. The approval for the original study "The prevalence of oral microbes in saliva from the UNLV School of Dental medicine pediatric and adult clinical population" was granted in May 2013 by the Office of Research Integrity and Protection of Research (Human) Subjects (OPRS#1305-4466M). This project will retrospectively examine a number of these samples (n = 125).

Saliva Collection Protocol

Although this is a retrospective study, the original protocol involved in-clinic saliva collection. As samples were collected, each was assigned a unique, non-duplicated number generated at random to preserve patient confidentiality and prevent research bias.

Patient demographics

In addition to the saliva collection, some demographic data was also obtained from each patient. This included the sex, age and self-reported race or ethnicity, as well as some biometric data, including body mass index (BMI) parameters such as height and weight, as well as some clinic observations regarding score for decayed, missing, or filled teeth (DMFT), and depth of periodontal pockets (PPD).

Cell counting and DNA isolation

Following the saliva collection, each sample was kept cool (using ice) until laboratory processing. All samples were processed using a standard aliquot (500 uL) and the GenomicPrep DNA isolation kit from Amersham Biosciences (Buckinghamshire, UK) as previously described

[20-22]. The quality and quantity of DNA was determined using absorbance readings of 260/280 nm.

PCR: Polymerase chain reaction

To screen for the pathogen of interest (FN, TD or PG), a standard amount of isolated DNA was processed using the exACTGene complete PCR kit from Fisher Scientific (Fair Lawn, NJ, USA) and primers for TD, FN, PG and the human enzyme (control) glyceraldehyde- 3- phosphate dehydrogenase (GAPDH), which were made by SeqWright (Houston, Texas, USA):

TD primer (forward); 5'-TAATACCGAATGTGCTCATTTACAT-3'

TD primer (reverse); 5'-CTGCCATATCTCTTGTCATTGCTCTT-3'

FN primer (forward); 5'-CGCAGAAGGTGAAAGTCCTGTAT-3'

FN primer (reverse); 5'-TGGTCCTCACTGATTCSCACAGA-3'

PG primer (forward); 5'-TACCCATCGTCGCCTTGGT-3'

PG primer (reverse); 5'-CGGACTAAAACCGCATACACTTG-3'

GAPDH primer (forward); 5'-ATCTTCCAGGAGCGAGATCC -3'

GAPDH primer (reverse); 5'-ACCACTGACACGTTGGCAGT -3'

Each PCR reaction had an identical setup, using a standardized amount of DNA (1mg). The basic parameters were denaturation at 94C for three minutes, then 30 amplification cycles that consisted of denaturation at 94C for 20 seconds , annealing at varying temperatures (based upon the primer sequence) for 60 seconds, extension at 72C for 30 seconds with a final extension at 72C for five minutes. Results were visualized using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, New York, USA) following gel

electrophoresis using Reliant agarose gels (Lonza: Rockland, Maine, USA) and UV illumination using ethidium-bromide.

DNA standard: GAPDH

A DNA standard was creating using an existing human cell line, HGF-1 to determine the minimum cell number needed for relative endpoint or RE-PCR comparison. This DNA allowed for the determination of the PCR conditions, also known as the minimum cycle threshold or CT that is the minimum number of PCR cycles needed to visualize a known quantity of DNA amplified by PCR and the maximum cycle saturation point or CS, as was described in previous work [20-22]. Using this standard and method, CT was determined to be twenty cycles (C20) with saturation C35.

DNA standard: PG

Porphyromonas gingivalis or PG was purchased from ATCC (FDC-381; Manassas, VA), as previous described [20,21]. Using overnight growth suspensions, absorbance readings at 650 nm with an optical density (OD) reading of 0.8 were found to approximate 107 CFU/mL. Dilutions of this were made to yield cell number f 5.0 x 10^6, 10^5, 10^4 and 10^3 CFU/mL, which represent salivary microbial concentrations that correspond to disease risk ranging from 10^6 CFU/mL representing very high risk and 10^3 CFU/mL which represents normal or average risk. Threshold or CT for PG was found to require twenty five cycles (C25) and saturation was found to be C45. Combining the data from the GAPD and PG experiments, CT was C20 and C25, respectively, while CS was C3 and C45, respectively [20, 25, 26]. Based upon this information, RE-PCR was performing using an intermediate cycle within those ranges at C30, which was in between the detection and saturation limits for both organisms.

Statistical analysis

The sample size was initially determined using the lower estimated DNA recovery rate from the DNA extraction kit (90%) to provide a minimum expected difference of 0.10. To obtain statistical power of $p = 0.80$ and significance level, a = 0.05 – a sample size (n = 50) was necessary [27]. Chi square analysis was used to determine any differences in categorical data regarding patient demographics (Sex, Race), as well as any differences in TD, PG or FN between groups (based on Sex, Race).

Results

Saliva samples were grouped based upon the clinic from which the patients were originally recruited, which included the Orthodontic clinic and (non-Orthodontic) Main Patient clinics (Table 1). The Orthodontic sample reflected an overall distribution, to the overall distribution within this clinic population. For example, the samples derived from patients in the Orthodontic clinic (n=54) contained more females (59.3%) than males (40.7%), which was roughly similar to their overall distribution within the overall Orthodontic clinic (p=0.1941). Moreover, the percentage of samples from minority patients (66.7%) reflected approximately the same percentages within the Orthodontic clinic overall (64.9%) and not statistically significant (p=0.2330). In addition, the vast majority of these minority patients self-identified as Hispanic (n=28/36=77.8%).

The samples collected from the non-Orthodontic or Main patient clinic were nearly equally distributed among females (50.7%) and males (49.3%), which was similar to their percentages within the overall main clinic population (49.4%, 50.6%, p=0.4109). The majority of patients

identified themselves as racial or ethnic minorities (60.6%), which was also similar to the overall clinic patient composition (59.2%, *p*=0.3677). As with the Orthodontic clinic samples, the overwhelming majority of these minority patients were Hispanic (n=34/43 or 79.1%).

Table 1. Patient sample and clinic characteristics

	Orthodontic sample (n=54)	Orthodontic clinic	Statistics
Sex			
Male	40.7% (n=22)	38.7%	c2=1.686, d.f.=1
Female	59.3% (n=32)	61.3%	*p*=0.1941
Race or Ethnicity			
Caucasian	33.3% (n=18)	35.1%	c2=1.422, d.f.=1
Non-Caucasian	66.7% (n=36)	64.9%	*p*=0.2330
Hispanic/Latino	51.9% (n=28)	53.9%	
Black/Afr. Am.	11.1% (n=6)	9.8%	
Asian/Other	3.7% (n=2)	1.3%	
	Non-Orthodontic sample (n=71)	**Main clinic**	**Statistics**
Sex			
Male	49.3% (n=36)	50.6%	c2=0.676, d.f.=1
Female	50.7% (n=35)	49.4%	*p*=0.4109
Race/Ethnicity			
Caucasian	39.4% (n=28)	40.8%	c2=0.811, d.f.=1
Non-Caucasian	60.6% (n=43)	59.2%	*p*=0.3677
Hispanic/Latino	47.9% (n=34)	39.3 %	
Black/Afr. Am.	8.5% (n=6)	13.1%	
Asian/Other	4.2% (n=3)	6.8%	

	Combined samples (n=125)
Sex	
Male	46.4% (n=58)
Female	53.6% (n=67)
Race/Ethnicity	
Caucasian	36.8% (n=46)
Non-Caucasian	63.2% (n=79)
Hispanic/Latino	49.6% (n=62)
Black/Afr. Am.	9.6% (n=12)
Asian/Other	4.0% (n=5)

These corresponding patient samples were then subjected to the DNA isolation procedure prior to screening and analysis (Table 2). These data revealed a recovery rate of 98.4% (n=123/125), comparable to previous studies [20,21,28,29]. DNA concentrations averaged 474.5 ng/uL, which on average ranged from 578.5 ng/uL in the Orthodontic samples, to 393.2 ng/uL in non-Orthodontic patient samples. Purity of DNA ranged between 1.61 and 2.0, allowing for the screening by PCR that demonstrated the presence of both human (GAPDH) and bacterial (16S rRNA) DNA.

Table 2. Recovery and isolation of DNA

	DNA recovery	Unsuccessful	Analysis/Recovery
Orthodontic samples	n=54	n=0	100% (n=54/54)
			ave.= 578.5 ng/uL
			A260/A280: 1.61-2.0
	n=54; GAPDH		
	n=54; 16S rRNA		

Non-orthodontic samples	n=69	n=2	97.2% (n=69/71)
			ave.= 393.2 ng/uL
			A260/A280: 1.62-2.0
n=69; GAPDH			
n=69; 16S rRNA			

As described in the Materials and Methods section, DNA standards were generated to find the threshold and saturation PCR cycles (CT, CS) generally used to compare relative starting DNA concentrations in relative endpoint PCR (Figure 1). Using these standards and methods, CT for GAPDH was observed at C20 and for PG at C25, with the corresponding CS at C35 and C45, respectively, RE-PCR was subsequently completed at C30, which was higher than the lower detection limit (CT), but still below the limits of saturation (C35-C45) for both.

Dilutions standardized cell numbers 10^6, 10^5, 10^4 and 10^3 cells/mL (human) or CFU/mL (bacteria) were processed accordingly. These numbers approximate research demonstrating salivary microbial concentrations and disease risk associations [20, 25,26]:

10^6 CFU/mL indicates very high risk;

10^5 CFU/mL indicates high risk;

10^4 CFU/mL indicates moderate risk;

$< 10^3$ CFU/mL indicates normal or average risk

These serial dilutions were prepared to establish PCR standard curves for both GAPDH and PG (Figure 1B). These data indicate that signal band intensity (SBI) at cycle 30 (C30) is nearly

perfectly correlated with the starting cell number for both PG (R^2=0.9945) and GAPDH (R^2=0.9797).
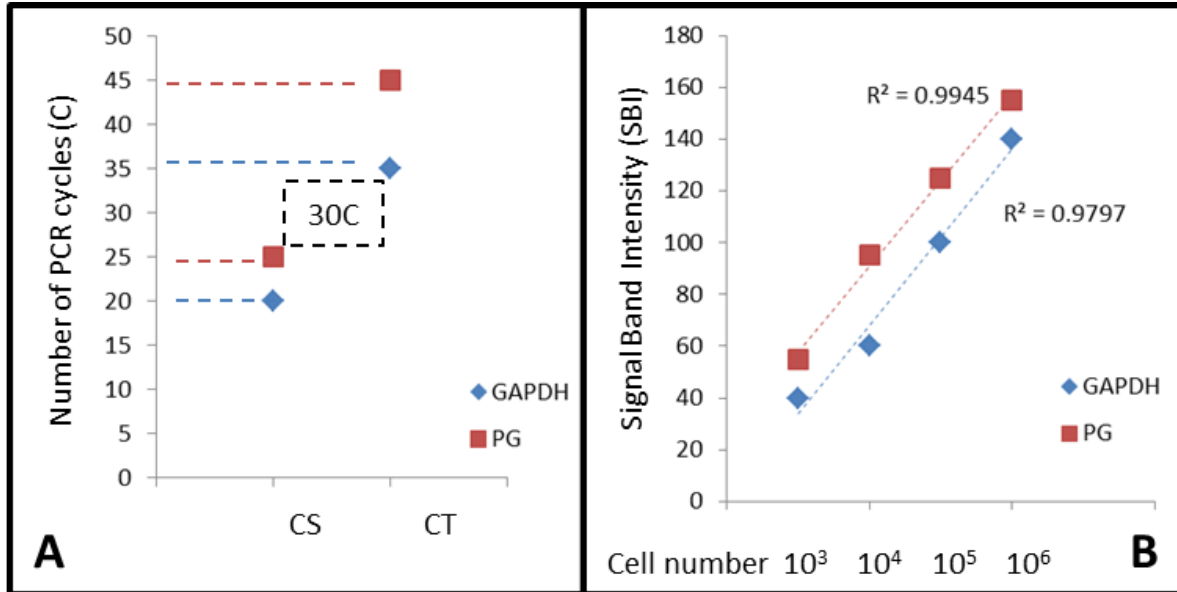
Figure 1. DNA standards and quantitative analysis. PCR Cycle Threshold (CT) or detection limit and Cycle Saturation (CS) were determined for human (GAPDH) and bacterial (PG) cells, revealing the optimal screening cycle between C25 and C35. PCR signal band intensity (SBI) was strongly correlated with starting cell number (R^2>0.97) at C30, which will allow for an approximation of starting cell number from the saliva samples screened.

Following DNA isolation, all samples were screened for the presence of *F. nucleatum* (FN), *T. denticola* (TD) and *P. gingivalis* (PG) at levels at or above pre-determined disease-risk levels (>10^4 CFU/mL) as described by previous saliva-based PCR screening studies (Figure 2) [20-22]. These data revealed that FN, TD and PG were present at or above these pre-determined levels in 52%, 41.6% and 48% of all samples, respectively. More specifically the prevalence of FN, TD and PG within the Orthodontic samples (46.3%, 38.9%, 44.4%) was significantly lower than the control, non-Orthodontic samples (56.3%, 43.7%, 50.7%)
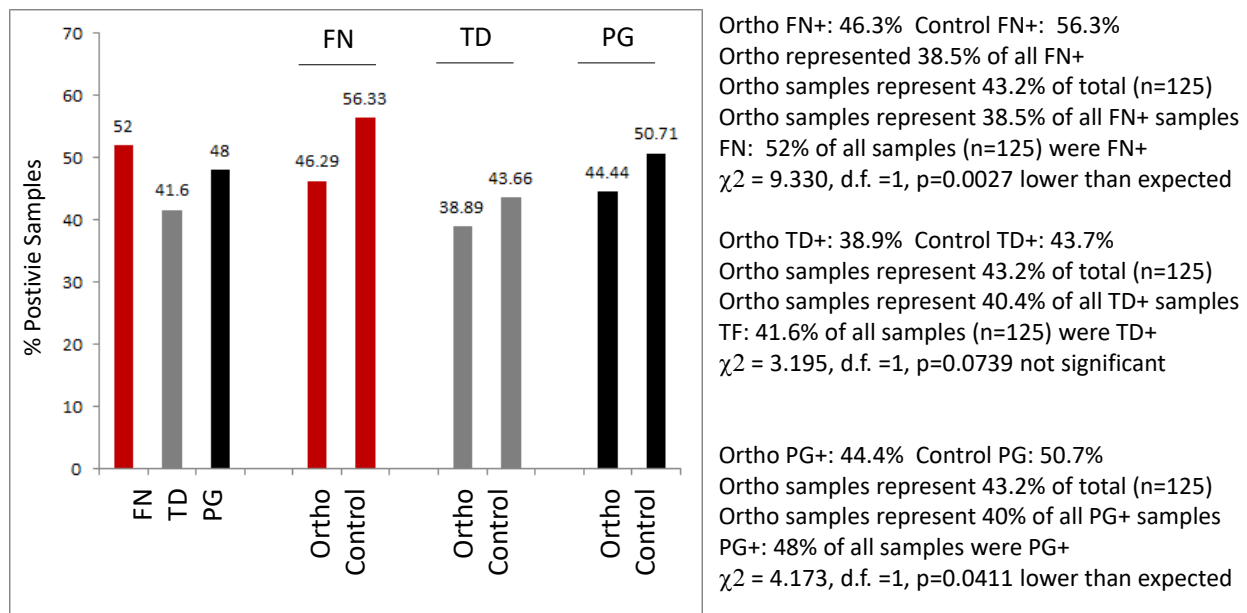
Figure 2. PCR screening of DNA isolated from saliva. Using previously established DNA standards to determine the PCR cycle threshold detection standards for >10^4 CFU/mL, nearly half of all samples were found to harbor FN, PG and TD. More detailed analysis revealed the Orthodontic samples had significantly lower prevalence of FN (*p*<0.01) and PG (*p*<0.05), as well as lower prevalence of TD (*p*=0.07) than non-Orthodontic samples.

To determine if the differences in prevalence of FN, TD and PG between the Orthodontic and non-Orthodontic clinic samples were due to other factors, more detailed analyses were performed to evaluate any possible influence by Sex/Gender (Figure 3). Although a general pattern of significantly lower periodontal pathogen prevalence was found among all the Orthodontic samples, FN prevalence among Male Orthodontic patients, specifically, was significantly higher than expected (*p*<0.01). Moreover, although a higher prevalence of periodontal pathogens was observed in the non-Orthodontic (control) samples – a gender / sex specific pattern was also evident with females exhibiting significantly higher levels of all periodontal pathogens than males, but proportionally much higher levels of FN and PG (*p*<0.01). However, no significant differences were observed between Racial or Ethnic categories.

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Statistical analysis

Orthodontic sample

Female: 59.3%
    FN, TD, PG lower

Male: 40.7%
    FN higher
    TD, PG lower

$\chi 2 = 34.596$
d.f. =3
$p<0.01$
Different than expected

Non-Orthodontic sample

Female: 49.3%
    FN, TD, PG higher

Male: 50.7%
    FN, TD, PG same or lower

$\chi 2 = 97.617$
d.f. =3
$p<0.01$
Different than expected
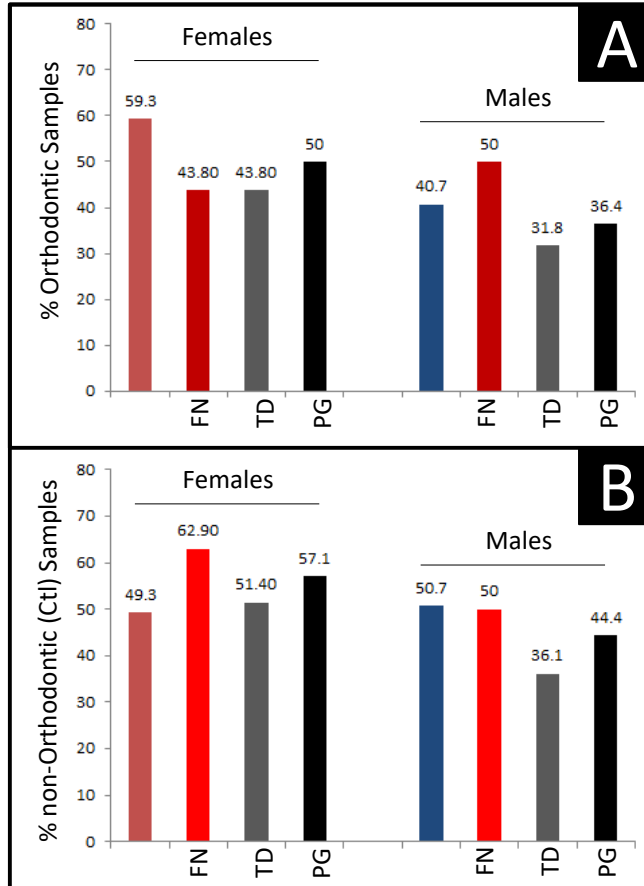
Figure 3. Analysis of PCR screening by sex. Sorting of Orthodontic samples into Females and Males revealed an overall pattern of lower pathogen prevalence except among a significantly higher proportion of Male Orthodontic patients ($p<0.01$). The analysis of non-Orthodontic (control) samples also revealed a sex-specific pattern with significantly higher proportions of Females exhibiting pathogen prevalence than Males ($p<0.01$).

Finally, the additional demographic and health data from each patient sample was also analyzed and reviewed (Table 3). This information included patient age, body mass index or BMI, periodontal pocket depth (PPD) and decayed, missing, and filled teeth (DMFT) score, which were grouped by clinic (Orthodontic or Main clinic) and then sorted by gender and ethnicity. Is analysis revealed that the average age of patients from the Orthodontic sample (24.4 years) was significantly lower than those from the Non-Orthodontic sample (28.3 years). Although no striking differences were found among the ages of males and females or minorities and non-minorities from the Orthodontic sample, there were much larger differences from the non-

Orthodontic sample. In addition, average BMI was also significantly higher within the non-Orthodontic sample (29.3) than the Orthodontic sample (25.7) with only minor differences observed between genders and by race or ethnicity.

Interestingly, PPD was much greater within the non-Orthodontic samples (4.11) compared with the Orthodontic samples (3.12), which varied widely. More specifically, males within the Orthodontic sample had much greater PPD (4.67) than females (2.66) while Minorities exhibited greater PPD (3.67) than Whites (2.21). These differences were not observed within the non-Orthodontic sample. As expected, DMFT score varied significantly with lower scores among the Orthodontic sample (10.75) compared with the non-Orthodontic samples (23.56) and with higher DMFT scores among Minorities from either clinic.

Table 3. Analysis of study sample demographic and health parameters.

	Orthodontic (n=54)	Non-Orthodontic (n=69)	Statistics
Age	**24.39 +/-4.71**	**28.34 +/- 3.79**	*p*<0.001
Males	23.64 +/-5.02	30.44 +/-2.65	Two-tailed t-test
Females	25.77 +/-3.66	26.71 +/-3.44	t=5.1545
Non-Minority	24.61 +/-3.53	31.2 +/-5.66	SED=0.766
Minority	26.2 +/-3.11	24.47 +/-7.11	
BMI	**25.67 +/-6.36**	**29.31+/-6.22**	*p*=0.0018
Males	28.17+/-2.83	29.01 +/- 5.99	Two-tailed t-test
Females	24.01+/-4.78	29.85 +/- 6.35	t=3.1893
Non-Minority	26.34+/-6.72	31.32 +/-5.94	SED=1.141
Minority	24.34+/-6.05	27.66 +/-7.28	
PPD	**3.12+/-0.78**	**4.11 +/-2.86**	*p*=0.0149
Males	4.67+/-0.52	4.34 +/-1.93	Two-tailed t-test

Females	2.66+/-0.88	3.12 +/-2.63	t=2.4708
Non-Minority	2.21+/-1.84	3.45 +/-1.66	SED=0.401
Minority	3.76+/-1.15	3.62 +/-1.94	
DMFT	**10.75+/-1.21**	**23.56 +/-7.56**	*p*<0.001
Males	11.4+/-1.23	24.65 +/-6.25	Two-tailed t-test
Females	10.1+/-1.63	22.29 +/-7.65	t=12.318
Non-Minority	9.40+/-1.08	20.78 +/-5.71	SED=1.040
Minority	12.1+/-0.99	25.26 +/-8.69	

Discussion

The objective of this study was to determine the oral microbial burden of specific periodontal pathogens among Orthodontic patients for comparison with non-Orthodontic controls. As recent evidence has suggested, many studies of changes to the oral microbial flora among Orthodontic patients have focused largely on cariogenic pathogens, while fewer studies have examined the potential changes associated with specific periodontal pathogens, such as *T. denticola*, *F. nucleatum* and *P. gingivalis* – particularly among adult patients. The outcomes of this study clearly demonstrated observable differences found between samples from Orthodontic and non-Orthodontic patients.

Unlike previous studies of this Orthodontic patient clinic, which demonstrated much higher prevalence or oral cariogenic pathogens [20,22], the results of this study found significantly lower levels within this patient sample compared with the main patient clinic. One potential explanation for these observations could be the disproportionately high percentage of very low income, first-time dental visits among the main clinic population, which may be considerably

different from Orthodontic patients that have been through several screening and follow-up appointments [20,22-24]. In addition, the current study sample size (n=125) is larger than any of the previous studies evaluated, ranging from n=52 to n=75, which may also have influenced these findings. Interestingly, the most recent study from this school found mostly cariogenic and one periodontal pathogen (PG) in nearly half of the Orthodontic samples, which roughly compares with the results of the current study. The non-Orthodontic samples from that previously study, however demonstrated only about 25% harbored PG at or above disease risk levels, which is far lower than the findings of this current study – suggesting that more research will be needed to further elucidate the disparate nature of these results.

This study has several limitations that must also be considered when evaluating the results and conclusions. The retrospective study design may have significantly affected the results through selection bias of the recruitment team or other confounding factors, such as self-selection bias [20-22]. In addition, collection of these samples at only one patient visit and time point suggests that no temporal conclusions can be made regarding the observations in periodontal pathogen prevalence from this type of cross sectional study. No attempt was made to standardize the amount of time a patient was in treatment within the Orthodontic treatment, which may have also influenced these results.

Despite these limitations, these findings are among the first to describe in detail the prevalence of periodontal pathogens among this patient population and the associated demographic factors. These findings suggest the strong need to plan and implement a prospective study to determine the baseline prevalence of PG, FN and TD among these patients as they begin Orthodontic therapy and how these levels change over time. This may provide more relevant clinical

information for oral health scientists and local epidemiologists to determine the most vulnerable populations, as well as the best methods and timing for interventions to prevent poor oral health outcomes and long-term consequences associated with acute periodontal disease.

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Chapter 3

Microbial Screening for Periodontal Pathogens in a Dental School-Based Orthodontic Clinic.

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Role of Authors:
Dr. David Jolley designed the study and was the primary author, data collector and analyzer, and graphics generator. Dr Karl Kingsley was the secondary author and assisted with data collection.

Abstract

Orthodontic treatment in the U.S. remains highly prevalent among youth and teenagers, although notable changes in demographics have more recently demonstrated increasing popularity among adults and minority patients. The majority of studies and research regarding changes to oral health during orthodontic treatment has traditionally and necessarily focused on the development of dental caries, although many studies demonstrate increased risk for periodontal disease among older and minority patients. Using this information, the main objective of this current study was to perform an analysis of specific periodontal pathogens and markers of periodontal health among adult orthodontic patients in a U.S. dental school clinic. Using previously collected saliva samples and oral health data, the total number of samples was n=310. DNA was isolated and further analysis and molecular screening was performed using relative endpoint (RE) polymerase chain reaction (PCR), which revealed lower prevalence of three key periodontal disease-associated pathogens - *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola* (*T. denticola*) and *Fusobacterium nucleatum* (*F. nucleatum*) among the Orthodontic samples

than among the non-Orthodontic samples. Unlike many other studies of Orthodontic patients, this study included a majority of patients that self-identified as racial or ethnic minorities – a group that has not been the tradition focus of Orthodontic treatment or research in the U.S.. Moreover, this study is among the first to examine periodontal pathogens and oral health markers among adult Orthodontic patients. As the demographics in the U.S. shift towards a higher percentage of racial and ethnic minorities, and the tendency of adults to seek Orthodontic care increases, this study provides critical information to evaluate and analyze the potential risks and oral health parameters that may influence treatment outcomes and long-term oral health within these populations.

Background and Introduction

Orthodontic treatment in the U.S. remains highly prevalent among youth and teenagers, although notable changes in demographics have more recently demonstrated increasing popularity among adults and minority patients (1,2). Although the average length of orthodontic treatment is approximately 24 months, depending on the age at which treatment begins, there may be considerable variation in treatment duration (3,4). For adult and other older orthodontic patients, increases in the duration of orthodontic treatment may often be associated with decreased oral health and other negative changes to the oral cavity (5-7).

The majority of studies and research regarding changes to oral health during orthodontic treatment has traditionally and necessarily been focused on the development of dental caries (8-10). However, recent evidence has suggested there may be significant changes to the periodontal status among adolescent orthodontic patients, which is of considerable scientific interest (11,12). Although new evidence has suggested that nearly half of all adults in the U.S. now have some

form of periodontitis, fewer studies have evaluated the effects of orthodontic treatment on the periodontal status of adults (13-15).

Using this information, the main objective of this current study was to perform an analysis of specific periodontal pathogens and markers of periodontal health among adult orthodontic patients in a U.S. dental school clinic. More specifically, this study sought to determine the prevalence and oral microbial burden of three key periodontal disease-associated pathogens - *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola* (*T. denticola*) and *Fusobacterium nucleatum* (*F. nucleatum*) from oral saliva samples, previously taken from adult orthodontic and non-orthodontic control patients in a U.S. dental school-based clinic (14-16). This data will contribute to an understanding of periodontal pathogen prevalence among adult orthodontic patients within this clinic and will expand the evidence regarding periodontal health and disease risk within this population.

Methods and Materials

Human subjects

The current study was retrospective in nature, analyzing previously collected patient saliva samples and oral health data. This project protocol "Retrospective investigation of oral microbes from the University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM) patient population" was approved by the Biomedical Institutional Review Board (IRB) and the Office of Research Integrity and the Protection of Human Subjects (OPRS protocol 762911-1) in August 2015. Three specific studies of orthodontic and non-orthodontic adult patient samples were selected to be used in this combined study (14-16) for a total sample size of n=310. The original

saliva and patient data collection was approved under the OPRS protocol 1002-3361 in April 2010 and collected on multiple, randomly selected dates between 2010 and 2015.

Original study design

Saliva samples and oral health data from adult patients were originally collected as part of a convenience sample of adult UNLV-SDM clinic patients. All patients previously provided Informed Consent. Exclusion criteria included patients with oral cancer and patients that declined to participate. Saliva samples and the corresponding patient demographic and oral health data were given unique, randomly generated numbers to prevent research bias and to prevent any identifying information from being disclosed. No self-identifying information regarding any specific patient was available to any member of the research team.

Patient demographic and oral health information

Basic demographic information regarding each patient sample was previously obtained at the time of consent and saliva collection, which included patient sex (gender) and self-reported racial identity (ethnicity), as well as patient age, height, and weight. Height and weight were then used to calculate Body Mass Index (BMI), which is an approximate measure of overall body composition. Basic oral health information, which included the decayed, missing and filled teeth (DMFT) score, as well as the average periodontal pocket depth (PPD) were also recorded at the time of the saliva collection.

DNA isolation and quantification

In brief, DNA was previously isolated from the saliva samples using a standard protocol and procedure using the Genomic Prep DNA isolation kit from Amersham Biosciences, as was previously described (14-16). The measure of DNA quality was previously obtained by the ratio

of spectrophotometric absorbance readings at 260 and 280 nm (A260/A280), which also facilitated quantification – based upon DNA standards.

PCR screening

From the repository of previously isolated DNA, each sample was then screened for each of three key periodontal pathogens for this study. The molecular screening for these pathogens included primers specific for *P. gingivalis* or PG, *F. nucleatum* or FN, and *T. denticola* or TD, as well as the positive control, human GAPDH gene – as previously described (14,15).

P. gingivalis: 5'-TACCCATCGTCGCCTTGGT-3' (forward)

P. gingivalis: 5'-CGGACTAAAACCGCATACACTTG-3' (reverse)

F. nucleatum : 5'-CGCAGAAGGTGAAAGTCCTGTAT-3' (forward)

F. nucleatum : 5'-TGGTCCTCACTGATTCSCACAGA-3' (reverse)

T. denticola: 5'-TAATACCGAATGTGCTCATTTACAT-3' (forward)

T. denticola: 5'-CTGCCATATCTCTTGTCATTGCTCTT-3' (reverse)

GAPDH: 5'-ATCTTCCAGGAGCGAGATCC -3' (forward)

GAPDH: 5'-ACCACTGACACGTTGGCAGT -3'(reverse)

Parameters for RE-PCR baseline detection specific to each pathogen were established, which required a minimum of twenty cycles (C20). Saturation (or PCR ceiling) limits were also determined at approximately forty five cycles (C45). RE-PCR was then performed at a mid-range point at thirty five cycles (C35) using standard aliquots of DNA isolated from serial dilutions of PG, FN and TD between 10^2 - 10^6 CFU/mL to establish a standard curves. These concentrations approximate the known estimates for saliva disease risk, which correspond with 10^2 (below average risk), 10^3 (normal or average risk), 10^4 (moderate increased risk), 10^5 (high

disease risk), 10^6 very high disease risk, as previously identified and used in similar studies for molecular screening of patient saliva (14,15,18,19).

Statistical analysis

Basic descriptive statistics were used to provide information and analysis about the study participants from the Orthodontic and non-Orthodontic clinics. Simple means (averages) and standard deviations (SD) were determined for DNA concentrations, as well as purity. Similarly, patient age, basic health measurements (BMI) and oral health information (DMFT, PPD) were averaged and t-tests were performed to determine any significant differences in the continuous data between groups (Orthodontic, non-Orthodontic; Below/normal disease risk, Elevated disease risk). However, Chi square (χ2) analysis was used to determine any overall differences between groups and periodontal pathogen prevalence, which is the most appropriate test for categorical data analysis (20).

Results

The total number of samples included in this analysis was n=310 (Table 1). An analysis of the overall sample demographics revealed a nearly equal distribution of males and females (*p*=0.8001), which closely resembled the overall patient population within the Main Dental Clinic (17). Furthermore, the distribution of patients from specific racial and ethnic (non-Caucasian) minorities (56.8%) was not significantly different from that of the Main Clinic patient registry (*p*=0.1225). The majority of non-White participants were Hispanic (46.8%). Further analysis of the sorted patient clinic samples (Orthodontic or non-Orthodontic) revealed a nearly even distribution among males and females (*p*=0.1883), and did not represent a significant proportional difference among racial or ethnic minorities (*p*=0.8481).

Table 1. Clinical sample demographic analysis

	Clinical samples (n=310)	Overall clinic	Statistical analysis
Gender / Sex			
Male	48.1% (n=149)	47.7%	$\chi2=0.064$, d.f.=1
Female	51.9% (n=161)	52.3%	$p=0.8001$
Ethnicity / Race			
Non-minority (White)	43.2% (n=134)	40.8%	$\chi2=2.385$, d.f.=1
Minority (non-White)	56.8% (n=176)	59.2%	$p=0.1225$
Hispanic	46.8% (n=145)		
Black	6.1% (n=19)		
Asian / Other	3.8% (n=12)		

	Orthodontic samples (n=159)	Non-Orthodontic samples (n=151)	Statistical analysis
Gender / Sex			
Male	49.1% (n=78)	47.0% (n=71)	$\chi2=1.770$, d.f.=1
Female	50.9% (n=81)	53.0% (n=80)	$p=0.1883$

Ethnicity / Race			
Non-Minority (White)	43.4% (n=69)	43.1% (n=65)	χ2=0.037, d.f.=1
Minority (non-White)	56.6% (n=90)	56.9% (n=86)	p=0.8481
Hispanic	45.3% (n=72)	48.3% (n=73)	
Black	6.3% (n=10)	5.9% (n=9)	
Asian / Other	5.0% (n=8)	2.6% (n=4)	

The analysis of the previous DNA isolation from each of the clinical samples revealed these procedures had an overall success rate of 98.9% (n=279/282) (Table 2). Examination of the yield from the clinical isolates revealed an overall average DNA concentration of approximately 443.59 +/- 125.3 ng/uL. The spectrophotometric ratio analysis revealed a range of A260:A280 between 1.59 and 2.05 for the successful DNA isolates, demonstrating adequate purity for RE-PCR screening.

Table 2. Analysis of DNA isolation

	DNA isolation	Expected range
Clinical samples	n=279/282 (98.9%)	90-95%
	DNA concentration	**Expected range**
Clinical samples	443.59 +/- 125.3 ng/uL	450-1000 ng/uL

	DNA purity	Expected range
	A260:A280 ratio	
Clinical samples	1.59 – 2.05	1.70 – 2.00

In order to more accurately quantify the results from this type of molecular screening, known quantities of *P. gingivalis* (PG), *F. nucleatum* (FN), and *T. denticola* (TD) were used to create DNA standards for RE-PCR and semi-quantitative analysis (Figure 1). More specifically, strong, positive curvilinear relationships were observed between CFU/mL and RE-PCR signal band intensity for PG (R^2=0.9665), FN (R^2=0.9268), and TD (R^2=0.9637) (Fig. 1A). Subsequently, all DNA isolates (Orthodontic, non-Orthodontic) from the clinical saliva samples were processed using RE-PCR and plotted based upon their signal band intensity, an approximate indirect measure of starting CFU/mL (Fig. 1B).
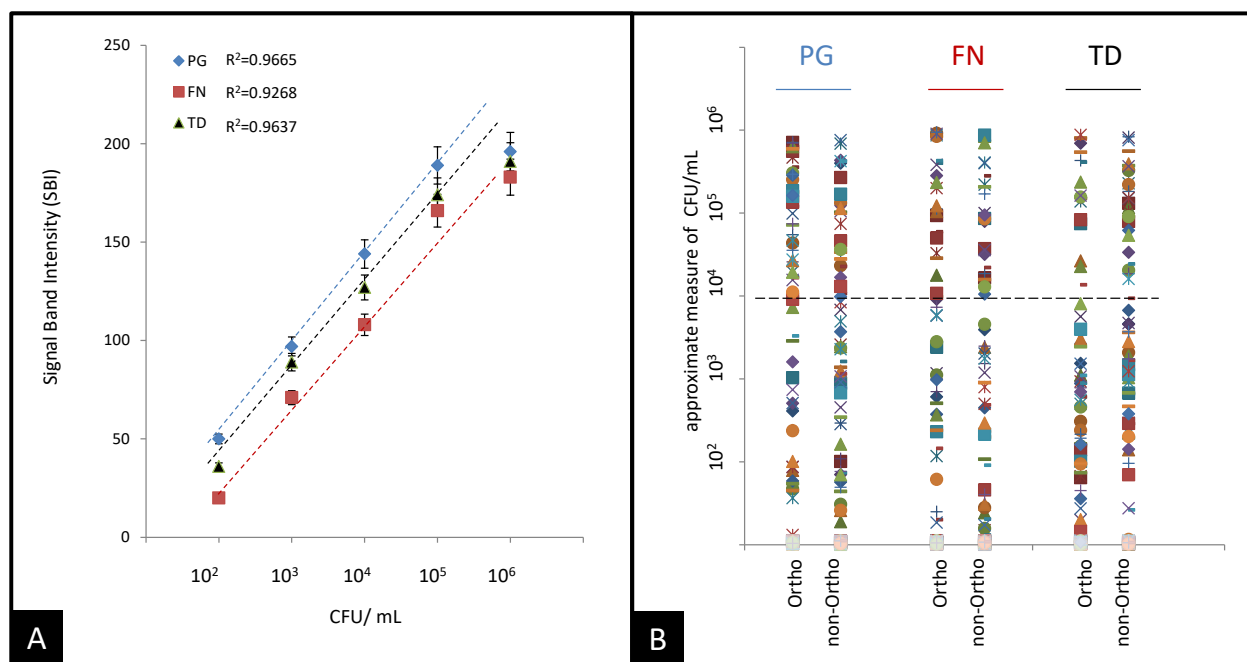

Figure 1. RE-PCR standards and screening results. A) RE-PCR signal band intensity (SBI) was measured for known quantities of PG, FN and TD (10^2-10^6 CFU/mL), revealing strong, positive linear correlations (R^2=0.9665, 0.9268,

0.9637, respectively). B) RE-PCR screening of clinical samples (Orthodontic, non-Orthodontic) revealed broad ranges of SBI for PG, FN and TD among both groups.

Further analysis of the RE-PCR molecular screening based upon the semi-quantitative results into categories using pre-determined disease risk values 10^3 CFU/mL (normal), 10^4 CFU/mL (moderate risk), 10^5 CFU/mL (high risk), and 10^6 CFU/mL (very high risk) revealed lower prevalence of elevated risk among the Orthodontic samples than the non-Orthodontic samples (15,16, 18,19) (Figure 2). More specifically, the percentage of Orthodontic samples with *P. gingivalis* above the elevated disease risk cutoff of 10^4 CFU/mL was 38.4%, which was lower than was observed among the non-Orthodontic samples (39.1%) although this difference was not statistically significant (*p*=0.7480). Similar results were observed with *T. denticola*, with lower prevalence found among Orthodontic samples (36.5% versus 40.4%) - although this was also not found to be statistically significant (*p*=0.0982). However, screening for *F. nucleatum* revealed the prevalence was significantly lower among Orthodontic samples (27.7%) than the non-Orthodontic controls (35.8%) (*p*<0.01).
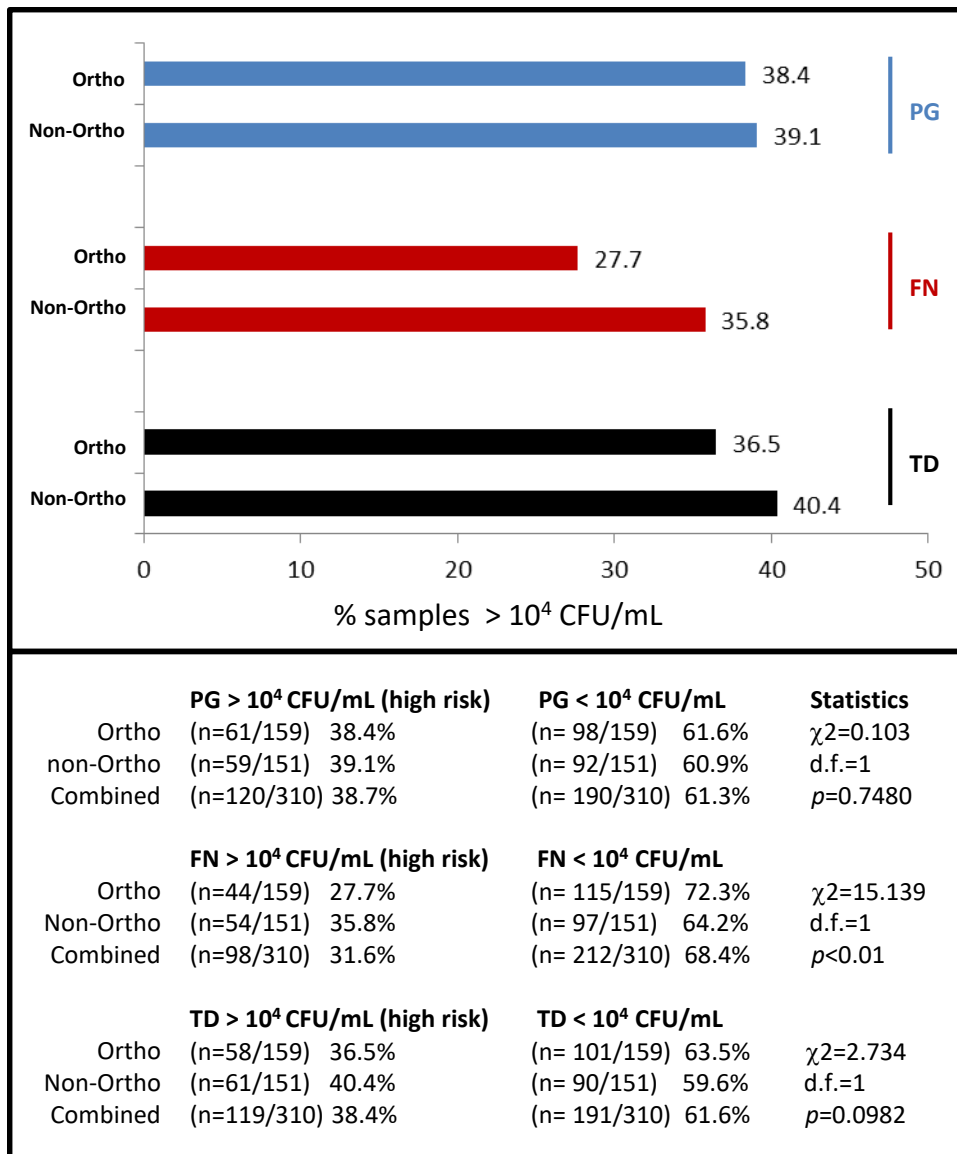
Figure 2. RE-PCR semi-quantitative analysis. Analysis of Relative endpoint (RE) Polymerase Chain Reaction (PCR) screening into higher than average disease risk ($>10^4$ CFU/mL) and normal or below average risk ($<10^4$ CFU/mL) categories revealed fewer Orthodontic samples harbored *P. gingivalis* or PG at levels of elevated disease risk or higher than non-Orthodontic samples (38.4% and 39.1%, respectively; *p*=0.7480). Similar results were found with *T. denticola* or TD (36.5% Orthodontic, 40.6% non-Orthodontic; p=0.0982). Significant differences were found with *F. nucleatum* or FN, however (27.7% Orthodontic, 35.8% non-Orthodontic; *p*<0.01).

A further analysis of the demographic and oral health parameters associated with each sample was performed (Table 3). In brief, the overall age of the participants from the Orthodontic clinic samples (23.8 yrs.) was significantly lower than that of the non-Orthodontic patients (31.9 yrs.; $p<0.001$). Moreover, the average age of the patients with samples testing positive for any of the pathogens tested (FN, TD or PG) was higher in both the Orthodontic and non-Orthodontic samples than the samples that were found to be at normal or below average risk. Similarly, these patients were also significantly different in their overall average of BMI, with significantly higher BMI observed among the non-Orthodontic patients (27.51) than the Orthodontic patients (23.59). As with age, the patient samples that were found to exhibit periodontal pathogens at levels of elevated disease risk were found to have higher average BMI than those that did not, regardless of the clinic designation.

Table 3. Analysis of demographic and oral health parameters.

	Orthodontic samples (n=159)	Non-Orthodontic samples (n=151)	Statistical analysis
Age (average)	23.79	31.87	t=26.497
Negative samples	22.31	29.70	SE=0.305
Positive samples	24.93	34.55	$p<0.001$
BMI	23.59	27.51	t=13.489
Negative samples	23.18	23.05	SE=0.291
Positive samples	25.98	31.82	$p<0.001$

DMFT	19.44	24.29	t=19.469
Negative samples	16.78	22.31	SE=0.249
Positive samples	23.15	26.61	*p*<0.001
PPD	3.18	3.61	t=11.128
Negative samples	3.05	3.02	SE=0.049
Positive samples	3.56	4.75	*p*<0.001

Discussion

Although many studies of oral health and disease among Orthodontic patients have been published, few of these studies have focused on periodontal pathogens and periodontal disease among this population (5, 7, 8, 12). Some research has explored the relationship between periodontal health and disease within this population, although most of these studies were primarily focused on teenage and adolescent patients with only a minority percentage derived from adults (21-24). In addition, some of these studies had small sample sizes (range, n=19-54), and although this group has made some preliminary efforts to examine these relationships (14-16) this study may be among the largest studies of this nature to date.

One key difference in the current study, involved the demographics of a specific patient population. Unlike many other studies of Orthodontic patients, this study included a majority of patients that self-identified as racial or ethnic minorities – a group traditionally not associated with Orthodontic treatment or research (25, 26). Although some previous work focusing on this majority-minority patient population has examined oral health (27-29), few studies have focused more specifically on orthodontic patients and periodontal health (14-16).

In concordance with the most recent published work, these results provide corroborating evidence that the prevalence of periodontal pathogens and elevated disease risk among Orthodontic patients at this clinic was lower than that of non-Orthodontic patients (15). This may seem contradictory to published studies regarding increased risk and decreased oral health associated with Orthodontic treatment (14, 16, 17). This may be an indicator of two separate, distinct phenomenon. First, is that the patients seeking Orthodontic care may have a different and higher level of oral hygiene and health-promoting behaviors than non-Orthodontic patients (30, 31). However, the second possibility is that these patients may also be subject to more frequent dental visits, increased oral health awareness during treatment, and shorter time intervals between oral-hygiene visits (17, 32). Beyond these differences there are several other possible factors that may have influenced the findings of this study, which may also be considered as part of the study limitations.

For example, although the primary limitations of this study were the retrospective and cross-sectional nature of the samples collected for analysis, other limitations related to the sample must also be considered. One of the most important of these is the patient demographics, which must be considered a confounding variable due to the fact that the overwhelming majority of patients in the non-Orthodontic or control group are low-income, non-White and Medicaid patients (17,28,29). Recent evidence has confirmed oral health disparities among both adolescent and adults from low-income and minority patients, which may explain (in part) the observations of higher BMI, DMFT scores and PPD within these data (33-35).

Despite these limitations, this study is among the first to examine periodontal pathogens and oral health markers among adult Orthodontic patients. As the demographics shift in the U.S. to

include more racial and ethnic minorities and the tendency for adults and older patients to seek Orthodontic care, this study provides critical information to evaluate and analyze the potential risks and oral health parameters that may influence treatment outcomes and long-term oral health within these populations. Although these results confirm previous observations of lower periodontal pathogen prevalence among the Orthodontic patients, the retrospective and cross-sectional nature of this study does not allow any conclusions to be made about the temporal nature of these findings – suggesting that prospective studies of oral health and periodontal disease within this patient population may be needed to determine any temporal or longitudinal effects associated with Orthodontic treatment.

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Chapter 4: Summary and Conclusions.

New research is focusing on the ability of periodontal pathogens to infect the head and neck, chest, lung, liver and abdomen due to their invasive anaerobic ability. This study was initiated on the premise that a positive correlation may exist between orthodontic therapy using fixed orthodontic appliances (braces) and periodontal pathogens, such as *F. nucleatum*, which has been the primary periodontal pathogen implicated in some invasive infections. To date, there are few studies that review periodontal pathogens as anything other than risk factors for periodontal disease, and even fewer that correlate orthodontic therapy to elevated levels of these pathogens. For this reason this, this study was carried out to increase knowledge and awareness, along with providing invaluable information about specific periodontal pathogens and markers of periodontal health among orthodontic patients.

The first publication titled "Oral Microbial Prevalence of Periodontal Pathogens among Orthodontic Patients" is a retrospective, cross-sectional study of previously collected saliva samples from orthodontic and non-orthodontic patients in a U.S. dental school clinic. This study's primary purpose was to analyze the prevalence of *Fusobacterium nucleatum*, *Treponema denticola*, and *Porphyromonas gingivalis* among the Orthodontic and non-Orthodontic patient populations. The demographic distribution of the patient population in this study consisted mainly of low-income and minority treatment recipients. Several parameters of general-health were recorded such as BMI, age, and sex, along with several parameters of oral-health such as oral periodontal pocket depths and decayed, missing, and filled teeth scores. The results of this study revealed:

- The average age of patients from the Orthodontic sample was significantly lower than those from the Non-Orthodontic sample.

- Nearly half of Orthodontic patient samples were positive for the periodontal pathogens in analyzed in this study, and more than half of non-Orthodontic samples were positive for those same pathogens.

- Orthodontic samples - when sorted categorically into Female and Male, revealed an overall pattern of lower pathogen prevalence, except among a larger proportion of Male Orthodontic patients

- Non-Orthodontic samples - when sorted categorically into Female and Male, revealed a sex-specific pattern with significantly higher proportions of Females exhibiting pathogen prevalence than Males, but proportionally much higher levels of FN and PG.

- No significant differences were observed in periodontal pathogen levels between Racial or Ethnic categories in either clinic.

- The overall prevalence of periodontal pathogens was greater among non-Orthodontic samples.

- No statistical significant deviation of TD and PG levels between clinic populations

- Orthodontic clinic samples had a significantly lower prevalence of FN

- Data analysis of this study may suggest that there is no conclusive correlation between orthodontic bracket placement and elevation of periodontal pathogens, when compared against non-Orthodontic patients within this patient population.

- The average BMI was significantly higher within the non-Orthodontic sample than the Orthodontic sample, with only minor differences observed between genders and by race or ethnicity.

- PPD was much greater within the non-Orthodontic samples, compared with the Orthodontic samples, which varied widely. Males within the Orthodontic sample had much greater PPD than females. Minorities exhibited greater PPD than Whites. These differences were not observed within the non-Orthodontic sample.

- DMFT scores were lower among the Orthodontic sample compared with the non-Orthodontic samples, and with higher DMFT scores among Minorities from either clinic.

The data collected in this study helps to provide initial evidence that orthodontic therapy does affect the oral condition resulting in changes in periodontal microflora levels. This study may be among the first to indicate that orthodontic treatment may disrupt the oral periodontal condition in a way that produces a significant decrease in some periodontal pathogen levels, when compared to a non-Orthodontic population. This data is valuable when establishing a baseline for further studies into oral periodontal ecology changes within orthodontic patient populations.

This study's analysis of the general health status and oral health condition of non-Orthodontic patients manifests an overall inferior condition when compared to those of age-matched non-orthodontic patients. Many data variables included in this study involving periodontal pathogen levels in a predominantly low-income and minority population undergoing orthodontic therapy are the first of its kind and will be an important reference during future causation and correlation studies.

The second manuscript "Microbial Screening for Periodontal Pathogens in a Dental School-Based Orthodontic Clinic" screened a much larger number of previously collected saliva samples from a U.S. dental school clinic, than the initial published study. The principle objective of this analysis was to evaluate specific periodontal pathogens and markers of periodontal health among

adult orthodontic patients to determine if the results of the first study could be confirmed with a larger sample size. The results of the analysis exhibited:

- The overall age of the participants from the Orthodontic clinic samples was significantly lower than that of the non-Orthodontic patients.

- The patient distribution in this study was nearly equal to the distribution of males and females and racial-ethnic patients in the dental school clinical registries.

- The majority of Orthodontic patients in this study self-identified as racial or ethnic minorities.

- There is a lower prevalence of the three key periodontal disease-associated pathogens among the Orthodontic samples than among the non-Orthodontic samples.

- Although statistically insignificant, fewer Orthodontic samples harbored *P. gingivalis* or *T. denticola* at levels of elevated disease risk or higher than non-Orthodontic samples.

- A statistically significant lower prevalence of *F. nucleatum* was found among orthodontic patient samples.

- The average age of the patients with samples testing positive for any of the pathogens tested (FN, TD or PG) was higher in both the Orthodontic and non-Orthodontic samples than the samples that were found to be at normal or below average risk.

- The average BMI of patient samples testing positive for any of the pathogens was also significantly different, with significantly higher BMI observed among the non-Orthodontic patients than the Orthodontic patients.

- Patient samples that were found to exhibit periodontal pathogens at levels of elevated disease risk were found to have higher average BMI and/or age than those that did not, regardless of the clinic designation.

- Overall, the prevalence of periodontal pathogens and elevated disease risk among Orthodontic patients at this clinic was lower than that of non-Orthodontic patients

The data collected in this study is critical and may be among the first to examine and provide more depth regarding periodontal pathogens and oral health markers among adult Orthodontic patients. Now that a higher percentage of adults and racial and ethnic minorities are seeking orthodontic care, ongoing research will be necessary to fully analyze the potential risks and oral health parameters that may influence treatment outcomes and long-term oral health within these populations. The information provided in this study is valuable because not only does it analyze a non-traditional sample population, but it also indicates that some aspect of orthodontic therapy positively disrupts the oral ecology, reducing harmful periodontal pathogens.

Limitations and Recommendations

As one of the first studies to analyze periodontal pathogens levels in orthodontic patients, a pilot study design was appropriately chosen to evaluate and analyze existing saliva samples within a non-traditional population of orthodontic treatment recipients. Although the information gathered in this study provides the groundwork for continued research, it is evident that there were some limitations, which future studies of this nature would need to improve upon to establish a solid baseline to inferred correlations. Most of the limitations of this study are derived from, but not limited to, the use of an existing saliva repository, which restricted some of the conclusions that could be drawn. First, although the samples were all assigned a unique, non-duplicated number generated at random to preserve patient confidentiality and prevent research bias, most of the samples relied upon willing participants that were not randomly

selected, which may have imposed self-selection and cultural bias. Second, since the study is retrospective and cross-sectional in nature, and all samples were gathered at a single time-point, no defining conclusions can be made regarding causation of observed oral microbial prevalence. Third, some dental and health history information was insufficient to create and support an adequate patient profile to support the findings of this study is incomplete, which make it very difficult to define the study results. Lastly, the majority on non-orthodontic patient samples derived from the main dental clinic from the dental school consisted of low-income, minority patients, who lack adequate oral health education and were visiting a dental professional for the first time. The listed limitations suggest further planning and implementation of prospective studies to evaluate conditions in a more controlled manner.

To improve upon this study and to address the limitations described above, the following suggestions are recommended for a future prospective study. First, an effort needs to be made to establish a method where participants are randomly selected to avoid potential bias. Second, multiple samples must be taken to create a baseline before bracket placement, and at several points with set time-intervals during treatment. Third, a thorough health and dental history should be requirement for participation in the study. Dental recommendations might include participants having an established two-year minimum comprehensive dental-care history, along with following an established hygiene home-care regimen with recommended oral care products, and documenting any previous periodontal diagnosis or treatments. Medical recommendations might include annotating any history of antibiotic use along with previous medical conditions or limitations to medical care. Lastly, at some point, it might be possible to consider obtaining samples from a sample population with different demographics, to further compare and contrast results. It also may be helpful to categories sample age groups by decade. These are a few of the

many recommendations that could be implemented in future prospective studies in an effort to shed further light on the concept that some aspect of orthodontic therapy and adequate comparison periodontal pathogen changes as a result of, and during orthodontic therapy.

UNLV

UNLV Biomedical IRB - Administrative Review
Notice of Excluded Activity

DATE:	August 3, 2015
TO:	Karl Kingsley
FROM:	UNLV Biomedical IRB
PROTOCOL TITLE:	[762911-1] Retrospective investigation of Oral Microbes from the UNLV-SDM patient population
SUBMISSION TYPE:	New Project
ACTION:	EXCLUDED - NOT HUMAN SUBJECTS RESEARCH
REVIEW DATE:	August 3, 2015
REVIEW TYPE:	Administrative Review

Thank you for your submission of New Project materials for this protocol. This memorandum is notification that the protocol referenced above has been reviewed as indicated in Federal regulatory statutes 45CFR46.

The UNLV Biomedical IRB has determined this protocol does not meet the definition of human subjects research under the purview of the IRB according to federal regulations. It is not in need of further review or approval by the IRB.

We will retain a copy of this correspondence with our records.

Any changes to the excluded activity may cause this protocol to require a different level of IRB review. Should any changes need to be made, please submit a Modification Form.

If you have questions, please contact the Office of Research Integrity - Human Subjects at IRB@unlv.edu or call 702-895-2794. Please include your protocol title and IRBNet ID in all correspondence.

Appendix B

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International Journal of Dentistry and Oral Health

Oral Microbial Prevalence of Periodontal Pathogens among Orthodontic Patients

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Abstract

Background: Changes in the oral microbial flora are commonplace during orthodontic therapy, although some evidence suggests these alterations may extend for some time after. Although many studies have screened for changes in cariogenic pathogen levels, more evidence is accumulating to demonstrate significant changes among periodontal pathogens within these patients. Although several studies at this predominantly low-income, dental school-based Orthodontic clinic have screened for cariogenic pathogens-none to date have provided multi-organism screening for periodontal pathogens.

Objective: This goal of this study was to complete a retrospective, cross-sectional study of saliva samples to screen for *Fusobacterium nucleatum*, *Treponema denticola*, and *Porphyromonas gingivalis* among orthodontic and non-orthodontic patients (n=125).

Methods: Using previously collected saliva samples, DNA was isolated and screened using PCR using primers specific for each pathogen of interest. Differences in prevalence between groups (Orthodontic, non-Orthodontic) were measured using Chi-square analysis.

Results: This analysis revealed the presence of these pathogens in nearly half of orthodontic patient samples and more than half of non-Orthodontic samples. These data also demonstrated females exhibited greater prevalence than males, while the overall prevalence among non-orthodontic samples was greater. This may be associated with higher average age, larger body mass index (BMI) and greater periodontal pocket depth (PPD) and decayed-missing-filled teeth (DMFT) scores.

Conclusion: These findings suggest the strong need to plan and implement a prospective study to determine the baseline prevalence of these pathogens among this patient population as they begin orthodontic therapy and how these levels change over time. This may provide more relevant clinical information for oral health scientists and local epidemiologists to determine the most vulnerable populations, as well as the best methods and timing for interventions to prevent poor oral health outcomes and long-term consequences associated with periodontal disease.

Keywords: Periodontal pathogen; Orthodontics; Saliva screening

Introduction

Although many studies of oral microbial changes during Orthodontic therapy have necessarily focused on cariogenic pathogens [1,2], fewer studies have closely examined the changes to other oral flora, including periodontal pathogens [3,4]. Studies have demonstrated that orthodontic treatment alters the oral microbiome and can both directly and indirectly alter the oral microbial composition, thereby dramatically increasing the potential for both cariogenic and periodontal disease [5-7]. Recent evidence has suggested that microbial alterations during orthodontic treatment may outlast the duration of therapy and influence long-term oral health outcomes [8-10].

Many studies have demonstrated normal, baseline ranges for levels of potential periodontal pathogens in the oral biofilm and subgingival crevices, which may trigger disease if homeostasis is disrupted [11,12]. These pathogens, include *Fusobacterium nucleatum* (FN), *Treponema denticola* (TD), and *Porphyromonas gingivalis* (PG)-the major etiologic agent implicated in chronic and persistent periodontitis [12,13]. Although modern materials and Orthodontic techniques have improved oral health outcomes in recent years, all current treatments are associated with increased levels of periodontal pathogen levels to some degree in many patients [14,15].

New diagnostic methods involving salivary biomarkers have improved the ability to monitor oral and periodontal diseases in recent years [16,17]. These advances facilitate studies investigating salivary screening for oral microbial changes during Orthodontic treatment [18,19]. In fact, studies from this school have utilized salivary biomarkers to screen for cariogenic pathogen changes among Orthodontic clinic patients-although no large-scale screening for periodontal pathogen levels has yet been attempted within this patient population [20-22].

Our studies have informed us that oral health status among orthodontic patients, particularly at this dental school-based clinic, may be of particular concern due to the large number of low-income and Minority patients who may face greater barriers and challenges to receive high quality healthcare [23,24]. The higher prevalence of these cariogenic pathogens, combined with increased barriers and lowered access to care, may explain some of these observations -although the full spectrum of changes within the oral microbial flora remains incomplete. These data serve as the basis for the current study objective, which is to screen orthodontic and non-orthodontic patients from this dental school patient clinic and determine the relative prevalence of specific periodontal pathogens, such as FN, TD and PG.

Appendix C

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