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Aggregatibacter actinomycetemcomitans and *Fusobacterium nucleatum* prevalence correlates with salivary microbial burden in Orthodontic patients.

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ABSTRACT

Objectives: Many factors influence the presence and growth of oral microbial flora, including the use of orthodontic appliances. Although much research has focused on classical oral pathogens, much less information is available to determine the relationship between *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* among these patients. The primary objective of this study was to determine the relationship between oral prevalence of *Aggregatibacter* and *Fusobacterium* among orthodontic and non-orthodontic patient saliva samples.

Experimental Methods: This study was a retrospective study of previously collected saliva samples from orthodontic (n=55) and non-orthodontic (n=55) patients using an approved protocol. DNA was extracted and screened for *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*. Males and females were equally represented, although a majority of patients participating in this study were Hispanics and ethnic minorities.

Results: PCR analysis of the DNA revealed that 54.5% of orthodontic samples harbored significant levels of *Aggregatibacter Actinomycetemcomitans*, while 29.1% of non-orthodontic samples harbored significant levels of *Aggregatibacter actinomycetemcomitans (p=0.0068)*. In addition, screening for *Fusobacterium* revealed 38% of orthodontic samples harbored this organism, compared with 33% of non-orthodontic samples *(p=0.4599)*. Screening of these samples using the 16S universal primer

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revealed AA-positive orthodontic samples had the highest PCR band intensity, with similar band intensity of AA-Negative orthodontic samples AA-positive non-orthodontic samples, AA-negative non-orthodontic samples. While screening for *Fusobacterium* using the 16S universal primer revealed higher band intensity (microbial burden) among the FN-positive samples among both the orthodontic and non-orthodontic samples. In brief, although microbial burden was lower among the corresponding non-orthodontic samples in general, the FN-positive samples were found to harbor the highest band intensity and microbial burden.

Conclusions: This study provides significant data that clearly suggest a correlation between overall microbial oral burden and *Aggregatibacter* presence in orthodontic patients. Both AA and FN were more prevalent among orthodontic patient samples than non-orthodontic samples, although the difference in the prevalence of FN was not statistically significant. In addition, it was demonstrated the AA was more prevalent than FN overall, and among each of the categories evaluated (orthodontic, non-orthodontic). AA appears to be more prevalent among patients with orthodontic brackets than FN, although both organisms appear to have similar characteristics. This may suggest that although both organisms facilitate heterotypic associations between varying species of oral bacteria, AA may be an earlier or more significant organism in this process in orthodontic patients.

Keywords: *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, Orthodontics, Salivary screening

Introduction

Many factors influence the presence and growth of oral microbial flora, including the use of orthodontic appliances [1,2]. Many studies have evaluated different methods for reducing the overall microbial burden among this patient population, with a specific focus on cariogenic and periodontal-related bacteria [3,4]. Although much research has focused on classical oral pathogens, such as *Streptococcus mutans* and *Lactobacillus acidophilus*, much less information is available to determine the relationship between *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* among these patients [5,6].

More specifically, some previous studies have demonstrated that the presence of *Aggregatibacter* (but not *Fusobacterium*) was more prevalent in the saliva of orthodontic patients [7,8]. Although many studies have evaluated the role of *Aggregatibacter* in oral pathogenesis, more efforts have recently focused on the role of this organism to influence and modulate oral ecology [9-11].

A recent review has suggested that changes in *Streptococcus* or *Aggregatibacter* prevalence among the oral microbial flora may be related to the growth and complexity of the oral bacterial community in orthodontic patients [12]. However, direct evidence of this type of relationship between oral microbial species and the effects on microbial burden remain unresolved [13,14].

Based upon this information, the objective of this study was to determine the relationship between oral prevalence of *Aggregatibacter* and *Fusobacterium* among orthodontic and non-orthodontic patient saliva samples.

Methods

Protocol and approval

This study was a retrospective study of previously collected saliva samples that were originally collected under a protocol that was approved by the Institutional Review Board (IRB) and Office for the Protection of Human Subjects (OPRS) at the University of Nevada, Las Vegas OPRS#1502-506M titled "The Prevalence of Oral Microbes in Saliva from the University of Nevada, Las Vegas – School of Dental Medicine pediatric and adult clinical population". Inclusion criteria included current patients of record at the University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM) orthodontic and main patient clinics. Exclusion criteria included any patients that declined to participate in the study and any patients not being treated at the UNLV-SDM clinics.

DNA isolation

All previously collected saliva samples had DNA extracted using the GenomicPrep DNA isolation kit (Amersham Biosciences), as previously described [6,8]. The quantity and purity of the extracted DNA was determined using UV absorbance readings at 280 and 260 nm, as previously described [13,14]. Samples deemed acceptable for this study had a minimum DNA concentration of 100 ng/uL and purity (A260:A280 ratio) of 1.65 or higher.

PCR screening

DNA was screened using polymerase chain reaction (PCR) using the exACTGene complete PCR kit (Fisher Scientific) and a thermocycler (Eppendorf), as previously described [15]. To verify the presence of control (human) DNA, a positive control was used - glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme from the glycolytic pathway. Primers for bacterial DNA, including 16S rRNA universal primer, *Aggregatibacter actinomycetemcomitans* (AA), and *Fusobacterium nucleatum* (FN) were synthesized by Eurofins Genomics:

GAPDH forward primer, 5'-ATC TTC CAG GAG CGA GAT CC-3'; 20 nt, 55% GC, Tm=66°C
GAPDH reverse primer, 5'-ACC ACT GAC ACG TTG GCA GT-3'; 20 nt, 55%GC, Tm=70°C
Annealing temperature: 67°C

16S rRNA universal primer, 5'-ACG CGT CGA CAG AGT TTG ATC CTG GCT-3'; 27 nt, 56% GC, Tm=76°C
16S rRNA universal primer, 5'-GGG ACT ACC AGG GTA TCT AAT-3'; 21 nt, 48% GC, Tm=62°C
Annealing temperature: 63°C

AA forward primer, 5'-ATT GGG GTT TAG CCC TGG T-3'; 19 nt, 53% GC, Tm=67°C
AA reverse primer, 5'-GGC ACA AAC CCA TCT CTG A-3'; 19 nt, 53%GC, Tm=65°C
Annealing temperature: 66°C

FN primer (forward); 5'-CGC AGA AGG TGA AAG TCC TGT AT-3'; 23 nt, 48% GC, Tm 67°C
FN primer (reverse); 5'-TGG TCC TCA CTG ATT CAC ACA GA-3'; 23 nt, 48% GC, Tm 68°C
Annealing temperature: 68°C

Statistical analysis

Basic average statistics were compiled for the DNA parameters associated with these samples and compared using two-tailed Students t-tests. Demographic analysis was facilitated using Chi Square, which was used to determine any demographic differences among the orthodontic and non-orthodontic groups.

Results

A total of one hundred ten (n=110) patient saliva samples were identified with sufficient DNA (>100 ng/uL) and purity (A260:S280 ratio) for inclusion in this study (Table 1). More specifically fifty five (n=55) samples from non-orthodontic patients were identified, with an average DNA concentration of 712.3 ng/uL and purity of 1.69. These samples were matched with orthodontic patient samples, with an average DNA

concentration of 722.1 ng/uL and a purity of 1.71. No significant differences were found between the average DNA concentrations in each group (*p*=0.742).

Table 1. DNA analysis of selected samples.

	DNA analysis	Statistical analysis
Non-orthodontic samples	(n=55)	
DNA concentration	Average = 712.3 ng/uL	
DNA concentration	Range (223.1 – 1411 ng/uL)	
DNA purity	(A260:A280) average = 1.69	Students t-test
		(two-tailed)
Orthodontic samples	(n=55)	*p*=0.742
DNA concentration	Average = 722.1 ng/uL	
DNA concentration	Range (199.4 – 998.2 ng/uL)	
DNA purity	(A260:A280) average = 1.71	

The demographic analysis of these patients revealed a nearly equal distribution of males and females within each sample (Table 2). The majority of patients from each sample were Hispanic, which reflects the overall patient population of UNLV-SDM [16]. No significant differences were identified between these two samples (orthodontic, non-orthodontic) in either sex or racial/ethnic background.

Table 2. Demographic analysis of study sample population.

	Non-Orthodontic	Orthodontic	Statistical analysis
Sex			
Male	N=27 (49.1%)	N=26 (47.3%)	$\chi2$=1.300, d.f.=1
Female	N=28 (50.9%)	N=29 (52.7%)	p=0.2543
Race/Ethnicity			
White	N=14 (25.4%)	N=15 (27.3%)	$\chi2$=1.819 d.f.=1
Hispanic	N=30 (54.5%)	N=31 (56.4%)	p=0.1774
Black	N=6 (10.9%)	N=5 (9.1%)	
Asian/Other	N=5 (9.1%)	N=4 (7.3%)	

All samples were then screened for the presence of AA using primers specific for this organism (Figure 1). These data revealed that more than half (54.5%) of orthodontic samples harbored significant levels of AA. In contrast, approximately one third of non-orthodontic samples (29.1%) harbored significant levels of AA (*p*=0.0068).

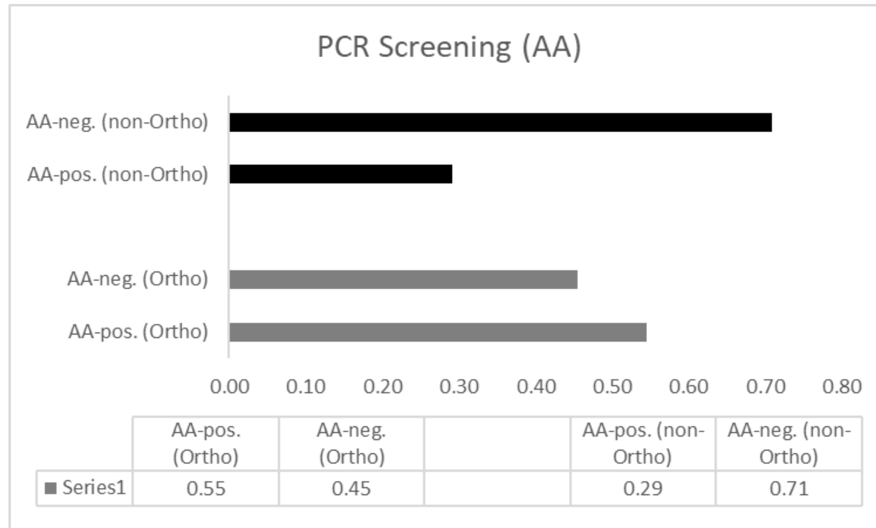

Figure 1. PCR screening of samples for AA. Salivary DNA samples screened for the presence of *Aggregatibacter* (AA) using PCR revealed 54.5% of orthodontic samples harbored this organism, compared with 29.1% of non-orthodontic samples (p=0.0068).

Screening of these samples using the 16S universal primer revealed the PCR band intensity was highest among the orthodontic samples which harbored AA (Figure 2). Although the PCR band intensity was higher among AA-positive than AA-negative non-orthodontic samples, these were comparable levels to the AA-negative orthodontic samples and significantly lower than the levels observed among the AA-positive orthodontic samples.

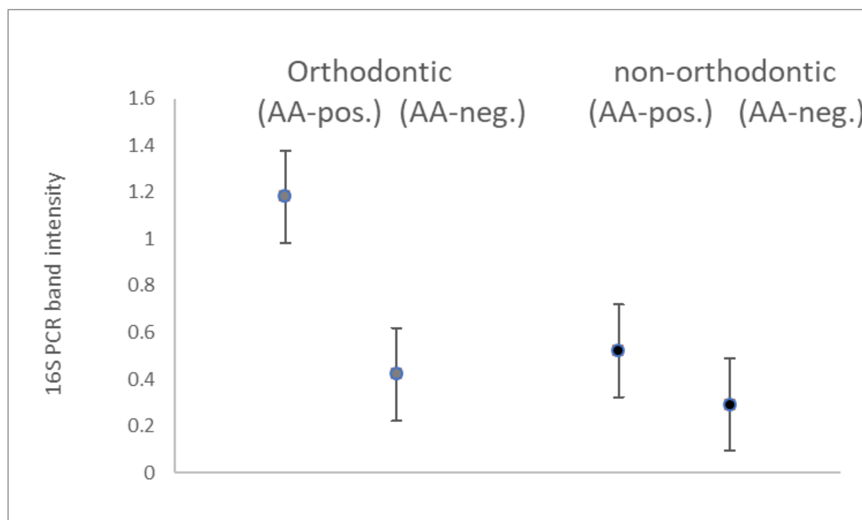

Figure 2. 16S universal primer PCR screening. Screening of samples using 16S rRNA universal primer revealed significantly higher band intensity (corresponding with bacterial levels) among the AA-positive orthodontic samples. In addition, 16S PCR band intensity was higher among AA-positive non-orthodontic samples but were significantly lower than observed among the AA-positive orthodontic samples.

Each of the samples were also screened for the presence of FN using primers specific for this organism (Figure 3). The analysis of these data revealed that slightly more than one third (38%) of orthodontic samples harbored significant levels of FN. Among non-orthodontic samples, approximately one third (33%) were positive for FN (p=0.4599).

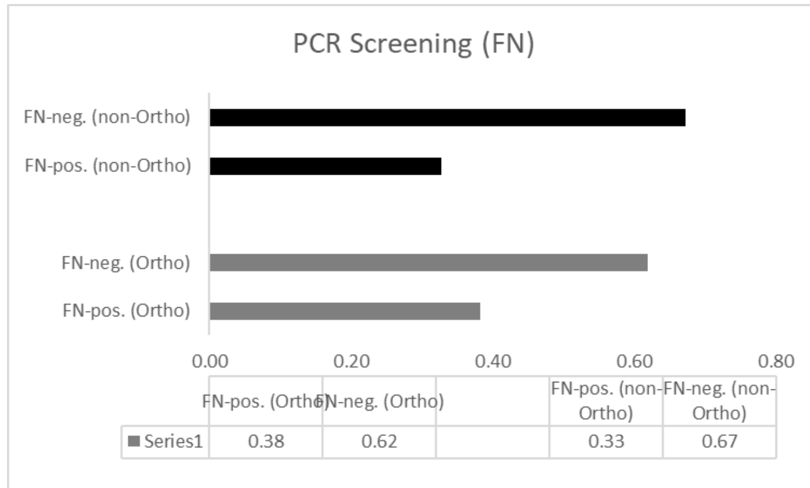

Figure 3. PCR screening of samples for FN. Salivary DNA samples screened for the presence of *Fusobacterium* (FN) using PCR revealed 38% of orthodontic samples harbored this organism, compared with 33% of non-orthodontic samples (p=0.4599).

Analysis of these samples into the categories of FN-positive and FN-negative using the 16S universal primer revealed higher band intensity (microbial burden) among the FN-positive samples among both the orthodontic and non-orthodontic samples (Figure 4). In brief, although microbial burden was lower among the corresponding non-orthodontic samples in general, the FN-positive samples were found to harbor the highest band intensity and microbial burden.

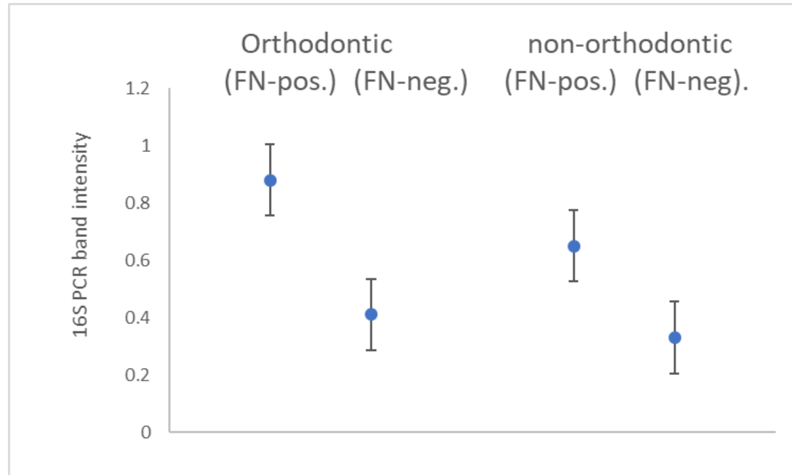

Figure 4. 16S universal primer PCR screening. Screening of samples using 16S rRNA universal primer revealed significantly higher band intensity (corresponding with bacterial levels) among the FN-positive orthodontic samples. In addition, 16S PCR band intensity was higher among FN-positive non-orthodontic samples but were significantly lower than observed among the corresponding FN-positive orthodontic samples.

Representative gel images were taken from four select patient samples to demonstrate the differences in PCR band signal intensity (Figure 5). These data demonstrated the range of signal band intensities, which ranged from low (Sample 1) to very high (Sample 4). Corresponding PCR screening for AA revealed three positives (Samples 2 – 4), while FN screening revealed only a single positive (Sample 1).

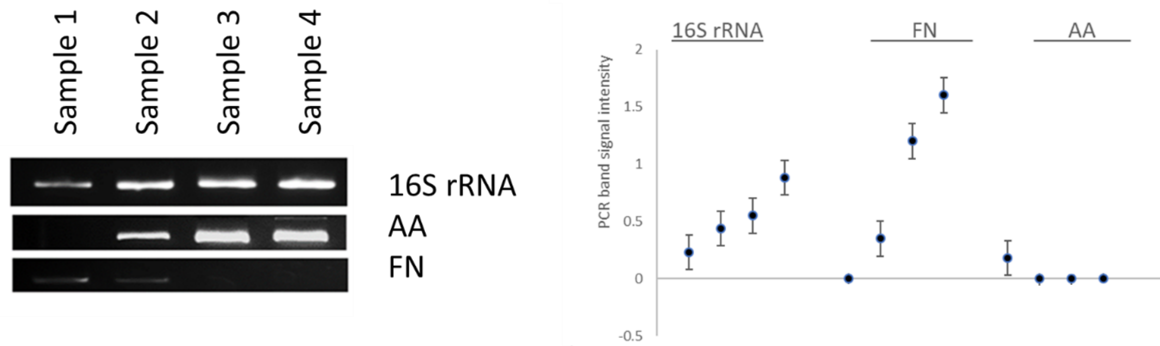

Figure 5. PCR signal band intensity. Screening of samples using PCR revealed not only the presence or absence of a particular microbial constituent, but also the relative microbial burden may be assessed using the signal band intensity of the 16S rRNA. The range of signal band intensities are shown from low (Sample 1) to high (Sample 4).

Discussion

The primary objective of this study was to determine the relationship between oral prevalence of *Aggregatibacter* and *Fusobacterium* among orthodontic and non-orthodontic patient saliva samples. These data revealed that AA was more prevalent among orthodontic patient samples than non-orthodontic samples. Although the prevalence of FN was slightly higher among orthodontic patient samples than non-orthodontic samples, this difference was not statistically significant. In addition, it was demonstrated the AA was more prevalent than FN overall, and among each of the categories evaluated (orthodontic, non-orthodontic).

These data support previous observations from this clinical population, which demonstrated orthodontic patients were more likely than non-orthodontic patients to harbor one of these organisms in significant numbers [6,8]. These observations are also supported by clinical studies and systematic review that confirm the effects of orthodontic treatment may trigger significant changes in the composition of subgingival microbes, including AA and FN [16-18].

One significant finding is that AA appears to be more prevalent among patients with orthodontic brackets than FN, although both organisms appear to have similar characteristics [19,20]. This may suggest that although both organisms facilitate heterotypic associations between varying species of oral bacteria, AA may be an earlier or more significant organism in this process in orthodontic patients [21].

This study had intrinsic limitations that must also be considered when reviewing these findings. For example, no temporal data was available to the study authors – which may limit the inferences about microbial composition changes that might be drawn from these analyses [22,23]. In addition, due to the study limitations (time and financial) only AA and FN were evaluated for this project although many other organisms may contribute to the overall microbial composition and sub-population prevalence [24].

Conclusions

Despite these limitations, this study provides significant data that clearly suggest a correlation between overall microbial oral burden and *Aggregatibacter* presence in orthodontic patients. Whether this relationship is unidirectional or bidirectional could not be established without more detailed longitudinal studies. These data provide strong evidence that more research is needed and that continued focus in this area may provide clinical guidelines for assessment of risk for patients undergoing orthodontic treatment.

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