Original Research Article

PREVALENCE OF *AGGREGATIBACTER ACTINOMYCETEMCOMITANS* AND *FUSOBACTERIUM NUCLEATUM* AMONG CLINICAL ORTHODONTIC AND NON-ORTHODONTIC SALIVA SAMPLES

Abstract

Objectives: The oral flora is a complex ecosystem characterized by numerous bacterial species and changes to the levels of these bacteria in health, disease, and dental treatments such as orthodontics. Although some studies have documented changes in periodontal pathogen burden during orthodontic treatment using saliva, most have focused on traditional cariogenic bacteria and some periodontal pathogens, such as *Porphyromonas gingivalis* or *Fusobacterium nucleatum*– far fewer have focused on *Aggregatibacter actinomycetemcomitans* – commonly associated with aggressive periodontitis. Therefore, the main objective of this study was to evaluate the prevalence of this organism among orthodontic and non-orthodontic patients from a public dental school clinic.

Experimental Methods: Using an approved protocol, samples were taken from orthodontic (n=39) and non-orthodontic (n=45) patients. DNA was extracted and screened for *Aggregatibacter actinomycetemcomitans.* 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 isolated from these patient samples revealed that more than half (54%) of the orthodontic samples harboured significant levels of *Aggregatibacter actinomycetemcomitans*, compared with only one-quarter (25%) of samples from non-orthodontic patients. In addition, screening for *Fusobacterium nucleatum* revealed a slightly increased prevalence among orthodontic patients (27%) compared with non-orthodontic patients.

Conclusions: These results are significant as *Aggregatibacter actinomycetemcomitans* has been traditionally observed as facilitating heterotypic communities of overtly pathogenic organisms, compared with other gram-negative oral microbes. These heterotypic biofilm communities exhibit greatly increased capacities to resist antimicrobial drugs and other host immune factors and the capacity to facilitate heterotypic associations within the biofilm may be restricted to a few key species. This project successfully demonstrated evidence that non-invasive salivary screening of orthodontic patients may be sufficient to assess and detect changes to this periodontal pathogen – thereby increasing the potential quality and efficiency of orthodontic dental treatment among this patient population

Key words: *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum,* saliva screening, microbial prevalence, orthodontic treatment

Abbreviations: *Aggregatibacter actinomycetemcomitans* (AA), *Fusobacterium nucleatum* (FN), Institutional Review Board (IRB), Office for the Protection of Human Subjects (OPRS), University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM), Polymerase chain reaction (PCR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), deoxyribonucleic acid (DNA),

1. Introduction

The oral flora is a complex ecosystem characterized by numerous bacterial species and changes to the levels of these bacteria in health, disease, and dental treatments such as orthodontics [1.2]. Many studies of the oral flora are centred around consensus bacteria responsible for caries and chronic periodontal disease [3-6]. Other virulent bacterial strains may receive less attention because their mere presence is not strictly correlated with the presence of chronic periodontal disease [7-10].

One of these bacterial strains is *Aggregatibacter actinomycetemcomitans* (AA), a commensal bacterium found among the oral flora [7,11,12]. This organism is a facultative non-motile, gram-negative, bacillus commonly associated with aggressive periodontitis, but is also found commonly in the oral flora not suffering from that severe periodontal condition [13,14]. In addition to oral infections, its several serotypes have a variety of virulence factors enable to evade defence mechanisms of many tissues and is capable of being found in infections of the skin, GI tract, sinus and reproductive systems [15-19]. Recent evidence indicates that its

presence is associated with risk of pre-diabetes, metabolic syndrome, and coronary artery disease [20-23].

Although some evidence has demonstrated changes to subgingival periodontal microbes such as AA, little is known regarding whether orthodontic treatment will result in changes to the salivary levels of this bacterial species – a non-invasive and more readily assessed measure of risk [7-9,24,25]. Fixed orthodontic appliances introduce new surfaces for plaque accumulation and obstacles to removing daily plaque on and between teeth while reducing the efficiency of natural plaque removal mechanisms, such as salivary flow accompanied by movement of the oral mucosa and tongue [26,27]. Although some studies have documented the change in periodontal pathogen burden during orthodontic treatment using saliva, most have focused on traditional cariogenic bacteria and some periodontal pathogens, such as *Porphyromonas gingivalis* - but not *Aggregatibacter* [8,28-30].

Based upon this paucity of evidence, the main objective of this study was to evaluate the prevalence of AA among orthodontic and non-orthodontic patients from a public dental school clinic. The main research question was to assess if there is variation in the prevalence of AA between orthodontic and non-orthodontic patients that is detectable in salivary samples taken from these patients. Successful completion of this project would provide preliminary evidence that non-invasive salivary screening of orthodontic patients may assess changes to this periodontal pathogen – thereby increasing the quality and efficiency of dental treatment among this patient population.

2. Methodology

2.1 Project approval

This project was reviewed and 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 all current patients of record at UNLV-SDM clinics. Exclusion criteria included any patient who declined to participate and any subject who was not a patient of record at UNLV-SDM. In brief, clinic patients were randomly asked to participate in three, randomly selected days per week for a set period of three months.

2.2 Sample collection

In brief, all adult patients were asked to provide Informed Consent, while pediatric patients were asked to provide Pediatric Assent and their parent or guardian was asked to provide Parental Permission. Each sample and corresponding demographic information intake sheet was assigned a randomly generated, non-duplicated identifier that was designed to protect patient information. Demographic information included only basic information, such as Sex, Age, and Race or Ethnicity.

2.3 DNA isolation

Patient saliva samples were brought to the biomedical laboratory for storage at -80C until processing. In brief, patient samples were processed using the GenomicPrep DNA isolation kit from Amersham Biosciences (Little Chalfont, UK). Quantification and quality of DNA was assessed using spectrophotometric UV absorbance readings at 260 and 280 nm (A260, A280). DNA with a ratio of A260:A280 greater than 1.65 was subsequently screened using PCR and primers specific for *Aggregatibacter actinomycetemcomitans* (AA).

2.4 PCR screening

Polymerase Chain Reaction (PCR) screening of the isolated DNA was accomplished using the exACTGene complete PCR kit from Fisher Scientific (Fair Lawn, NJ) and an Eppendorf MasterCycler (Hamburg, Germany). A positive control for human DNA was used – glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme from the glycolytic pathway. In addition, a positive control for bacterial DNA was also used – 16S rRNA universal primer, to confirm the presence of bacterial DNA. Primers for *Aggregatibacter actinomycetemcomitans* (AA) and *Fusobacterium nucleatum* (FN) were also synthesized by Eurofins Genomics (Louisville, KY):

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: 67C

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: 63C

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

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

2.5 Statistical analysis

Using the IRB-approved protocol, saliva samples were obtained from orthodontic and non-orthodontic patients of record. Simple descriptive statistics of the study sample and the clinic population were provided and Chi-Square analysis was used to determine any differences among the demographic groups (Sex, Age, Race or Ethnicity). Following PCR screening, differences between demographics of positive and negative samples also were assessed using Chi-Square analysis

3. Results

A total of thirty-nine (n=39) orthodontic samples and forty-five (n=45) non-orthodontic samples were collected from clinic patients, yielding a total study sample size of eighty-four (n=84) (Table 1). Analysis of these demographics revealed that the percentages of females in the study samples (both orthodontic and non-orthodontic) was slightly greater than males (56.4%, 57.8%, respectively). This was similar to the demographic distribution of females in the orthodontic and main patient clinics (60.4% and 56.4%, respectively), and not statistically significant (p=0.4142).

An evaluation of self-reported Race/Ethnicity revealed approximately one-fourth of the study sample (both orthodontic and non-orthodontic) identified as White or Caucasian, which was similar to the overall percentage from the orthodontic and main patient clinics, p=0.6532. The greatest proportion of non-White or minority patients were Hispanic in both the study samples (51.3%, 51.1%) and the Orthodontic clinic (52.3%), which was also not significantly different, p=0.6532. Finally, the proportion of patients under 18 years of age was approximately half in both the study samples (51.2%, 51.1%), which was similar to the overall percentage in the orthodontic clinic (56.7%), p=0.2255.

Table 1. Demographic analysis of study participants

	Orthodontic sample (n=39)	Non-orthodontic sample (n=45)	Statistical analysis	Orthodontic clinic population (n=1,463)	Main clinic population (n=73,024)
Sex					
Female	56.4 % (n=22)	57.8% (n=26)	$\chi 2$=0.667 d.f.=1	60.4% (n=884)	56.4% (n=41,185)
Male	43.6% (n=17)	42.2% (n=19)	p=0.4142	39.6% (n=579)	43.6% (n=31,839)
Race/Ethnicity					
White	25.6% (n=10)	24.4% (n=11)	$\chi 2$=1.627 d.f.=3	24.7% (n=361)	24.1% (n=17,599)
Hispanic	51.3% (n=20)	51.1% (n=23)	p=0.6532	52.3% (n=765)	49.5% (n=36,147)
Black	15.4% (n=6)	13.3% (n=6)		11.8% (n=172)	13.1% (n=9,566)
Asian	7.7% (n=3)	11.1% (n=5)		7.9% (n=117)	11.5% (n=8,398)
Other				3.3% (n=48)	1.8% (n=1,314)

Age					
Under <18 yrs.	51.2% (n=20)	51.1% (n=23)	$\chi 2$=1.469 d.f.=1	56.7% (n=830)	N/A (Pediatric clinic)
Over > 18 yrs.	48.7% (n=19)	48.9% (n=22)	*p*=0.2255	43.3% (n=633)	100% (n=73,024)

Each saliva sample was processed to isolate DNA, both bacterial and human (Table 2). In total, DNA was successfully isolated from n=81/84 samples (96.4%), which is well within the expected recovery range (95-100%). The average concentration of DNA from the orthodontic samples was 699.1 ng/uL that ranged between 550 – 885 ng/uL, which is lower but comparable to the average of the non-orthodontic samples of 804.7 ng/uL that ranged between 571 – 980 ng/uL, *p*=0.0018.

Table 2. DNA isolation and analysis

	DNA analysis	Statistical analysis
Orthodontic samples (n=39)		
DNA concentration	ave.= 699.1 ng/uL	Students t-test
DNA concentration	range=550-885 ng/uL	(two-tailed)
		p=0.0018
Non-orthodontic samples (n=45)		
DNA concentration	ave.= 804.7 ng/uL	
DNA concentration	range=571-980 ng/uL	

The DNA from each sample was then screened using PCR for the presence of *Aggregatibacter actinomycetemcomitans* or AA above the threshold limit of detection from saliva at 30 cycles, which roughly approximates 10^4 CFU/mL (Figure 1). These results revealed that more than half of the orthodontic samples (56.4%) had detectable levels of AA in saliva, compared with only 25% of the non-orthodontic samples. Correspondingly, less

than half of orthodontic samples tested negative for AA, while three-quarters (75%) of the non-orthodontic samples were found to have no AA above the threshold limit of detection.

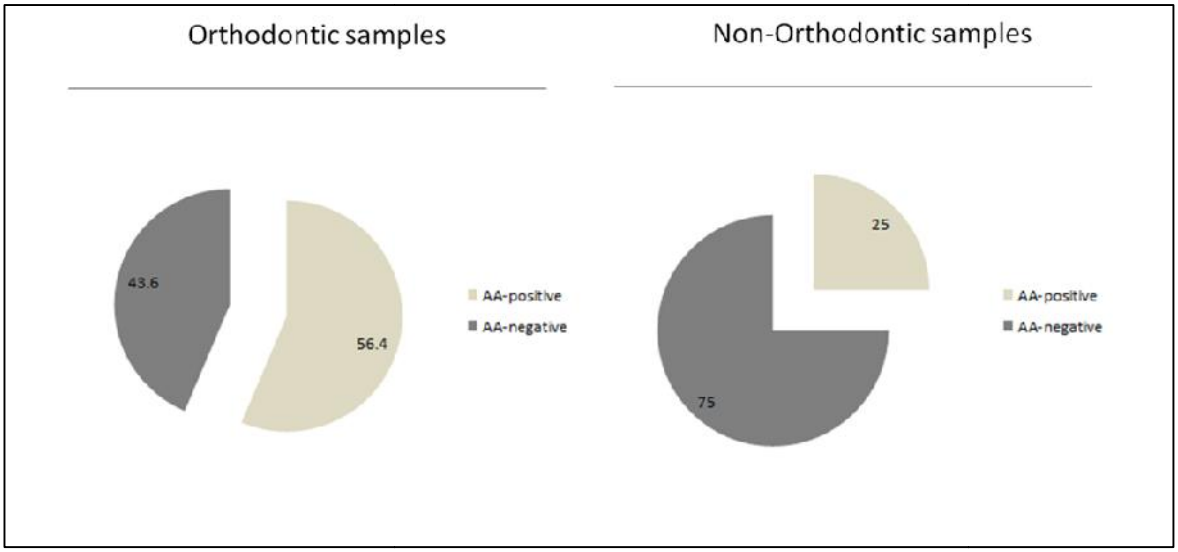

Figure 1. PCR screening of DNA isolates. PCR screening revealed 56.4% of orthodontic samples harboured detectable levels of *Aggregatibacter actinocetemcomitans* (AA) in saliva, compared with only 25% of non-orthodontic samples. This was statistically significant, p=0.036.

To determine if this phenomenon was restricted to AA, another gram-negative organism was selected for screening – *Fusobacterium nucleatum* or FN (Figure 2). PCR screening of the DNA isolated from the orthodontic and non-orthodontic samples revealed significant levels of FN (above the limit of detection) in one fourth (27.7%) of the orthodontic saliva samples and only one-fifth (19%) of non-orthodontic samples tested, which was also statistically significant.

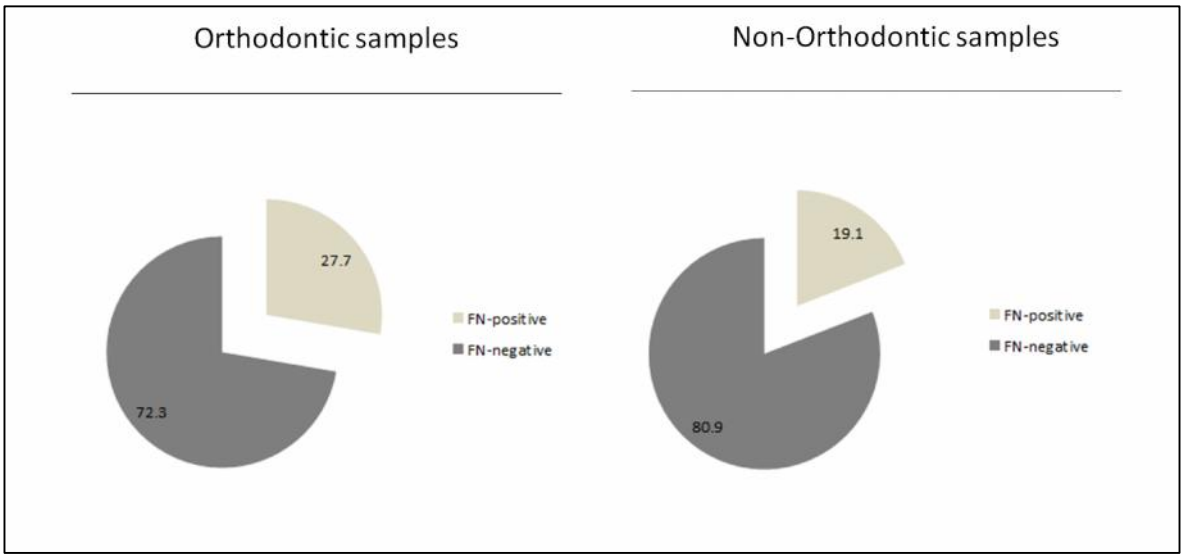

Figure 2. PCR screening of DNA isolates. PCR screening revealed 27.7% of orthodontic samples harboured significant levels of *Fusobacterium nucleatum* (FN), compared with only 19.1% of non-orthodontic samples. This was statistically significant, p=0.041.

4. Discussion

The main objective of this study was to evaluate the prevalence of *Aggregatibacter actinomycetemcomitans* or AA among orthodontic and non-orthodontic patients from a public dental school clinic. The results of this study demonstrate that AA is detectable in saliva samples from these patients. Moreover, the main finding was that more than half of the orthodontic subjects harboured significant levels of AA in unstimulated saliva, compared with only one-fourth of the non-orthodontic subjects. These results are significant as AA is mainly associated with localized aggressive periodontitis and chronic periodontitis [31,32].

These results are significant as AA has been traditionally observed as facilitating heterotypic communities of overtly pathogenic organisms, compared with other gram-negative oral microbes [33,34]. In fact, biofilm communities exhibit greatly increased capacities to resist antimicrobial drugs and other host immune factors [35,36]. The capacity to facilitate heterotypic associations within the biofilm may be restricted to a few key species, including AA [37,38].

For comparison, another gram-negative, periodontal pathogen was assessed in this study – *Fusobacterium nucleatum* or FN [39]. Although the results of this study demonstrated a difference between the prevalence of FN among orthodontic samples (27%) compared with non-orthodontic samples (19%), these differences were less dramatic and are more likely a secondary result due to the primary influx of AA among the orthodontic patients [7,24]. Although these results are significant and may provide some useful biometric indicators for non-invasive biofilm community assessment among orthodontic patients, there are some limitations associated with this type of study.

First, only non-invasively collected saliva was available for this study, which may limit the conclusions that can be made from these analyses. No corresponding direct biofilm collection was possible, therefore only inferential analyses can be made from these results. Second, and more importantly, this was a cross-sectional study that collected saliva from orthodontic and non-orthodontic patients at a single time point, which means no temporal information can be evaluated regarding the change in microbial prevalence over time. Finally, limited scope and duration of this study did not allow for the ability to screen for, select and evaluate patients based upon the presence of other dental prosthetics, fixed restorations or other factors, which may have influenced the potential for periodontal disease or other oral conditions that may have influenced these observations.

5. Conclusions

Despite these limitations, this project successfully demonstrated preliminary evidence that non-invasive salivary screening of orthodontic patients may be sufficient to assess and detect changes to periodontal pathogens, such as AA and FN – thereby increasing the potential quality and efficiency of orthodontic dental treatment among this patient population.

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