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...
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/μL 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 GAC TTG GCA GT-3’; 20 nt, 55% GC, Tm=70°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
- 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
- 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

**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/μL) 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/μL 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    |
| Orthodontic samples (n=55)   |                               |
| 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%)| $\chi^2=1.300$, 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%)| $\chi^2=1.819$ 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 1](http://escipub.com/international-journal-of-dental-research-and-reviews/)

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.

![Figure 2](http://escipub.com/international-journal-of-dental-research-and-reviews/)

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).

IJDRR: http://escipub.com/international-journal-of-dental-research-and-reviews/
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).
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.
References

1. Andruccioli MC, Faria G, Nelson-Filho P, Romano FL, Matsumoto MA. Influence of resin-modified glass ionomer and topical fluoride on levels of Streptococcus mutans in saliva and biofilm adjacent to metallic brackets. J Appl Oral Sci. 2017 Mar-Apr;25(2):196-202. doi: 10.1590/1678-77572016-0231. PMID: 28403360

2. El-Patal MA, Asiry MA, AlShahran I, El Bayoumy SY, Ahmed Wakwak MA, Mohamed Khalil MA. The effect of fiber-reinforced composite versus band and loop space maintainers on oral Lactobacillus acidophilus and Streptococcus mutans levels in saliva. J Indian Soc Pedod Prev Dent. 2018 Jul-Sep;36(3):301-307. doi: 10.4103/JISPPD.JISPPD_155_18. PMID: 30246754

3. Alp S, Baka ZM. Effects of probiotics on salivary Streptococcus mutans and Lactobacillus levels in orthodontic patients. Am J Orthod Dentofacial Orthop. 2018 Oct;154(4):517-523. doi: 10.1016/j.ajodo.2018.01.010. PMID: 30268262

4. Aydinbelge M, Cantekin K, Herdem G, Simsek H, Percin D, Parkan OM. Changes in periodontal and microbial parameters after the space maintainers application. Niger J Clin Pract. 2017 Sep;20(9):1195-1200. doi: 10.4103/1119-3077.180070. PMID: 29072246

5. Davis JE, Freel N, Findley A, Tomlin K, Howard KM, Seran CC, Cruz P, Kingsley K. A molecular survey of S. mutans and P. gingivalis oral microbial burden in human saliva using relative endpoint polymerase chain reaction (RE-PCR) within the population of a Nevada dental school revealed disparities among minorities. BMC Oral Health. 2012 Aug 27;12:34. doi: 10.1186/1472-6831-12-34. PMID: 22925755

6. Jolley D, Wonder K, Chang E, Kingsley K. Oral microbial prevalence of periodontal pathogens among orthodontic patients. International Journal of Dentistry and Oral Health (IJDOH) 2016, 1(6): doi http://dx.doi.org/10.16966/2378-7090.159

7. Nelson-Filho P, Carpio-Horta KO, Andruccioli MC, Feres M, Bezerra da Silva RA, Garcia Paula-Silva FW, Romano FL. Molecular detection of Aggregatibacter actinomycetemcomitans on metallic brackets by the checkerboard DNA-DNA hybridization technique. Am J Orthod Dentofacial Orthop. 2012 Oct;142(4):481-6. doi: 10.1016/j.ajodo.2012.04.021. PMID: 22999671

8. Klinger J, Shen C, Kingsley K. Prevalence of Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum among Clinical Orthodontic and Non-Orthodontic Saliva Samples. Journal of Advances in Microbiology 2018, 11(3): 1-9, 2018; DOI: 10.9734/JAMB/2018/42698

9. Gholizadeh P, Pormohammad A, Eslami H, Shokouhi B, Fakhrazadeh V, Kafil HS. Oral pathogenesis of Aggregatibacter actinomycetemcomitans. Microb Pathog. 2017 Dec;113:303-311. doi: 10.1016/j.micpath.2017.11.001. Epub 2017 Nov 5. Review. PMID: 29117508

10. Whitmore SE, Lamont RJ. The pathogenic persona of community-associated oral streptococci. Mol Microbiol. 2011 Jul;81(2):305-14. doi: 10.1111/j.1365-2958.2011.07707.x. Epub 2011 Jun 3. Review. PMID: 21635580

11. Tsai CC, Ho YP, Chou YS, Ho KY, Wu YM, Lin YC. Aggregatibacter (Actinobacillus) actinomycetemcomitans leukotoxin and human periodontitis - A historic review with emphasis on JP2. Kaohsiung J Med Sci. 2018 Apr;34(4):186-193. doi: 10.1016/j.kjms.2018.01.014. Epub 2018 Feb 17. Review. PMID: 29655406

12. Luccheses A, Bondemark L, Marcolina M, Manuelli M. Changes in oral microflora due to orthodontic appliances: a systematic review. J Oral Microbiol. 2018 Jul 3;10(1):1476645. doi: 10.1080/20002297.2018.1476645. eCollection 2018. Review. PMID: 29988826

13. McDaniel S, McDaniel J, Tam A, Kingsley K, Howard KM. Oral Microbial Ecology of Selenomonas noxia and Scardovia wiggsiae. Microbiology Research Journal International 2017, 21(3) 1-8. DOI : 10.9734/MRJI/2017/36110

14. McDaniel J, McDaniel S, Tam A, Kingsley K, Howard KM. Screening a Saliva Repository for Scardovia wiggsiae and Streptococcus mutants: A Pilot Study. Journal of Advances in Microbiology 2017, (5) 1: 1-8. doi: 10.9734/JAMB/2017/36111

15. Quan K, Kingsley K. Effect of dental sealants on oral microbial burden of Scardovia wiggsiae within a pediatric population. A pilot study. Microbiology Research Journal International. 2018, 24(6) 1-10. DOI: 10.9734/MRJI/2018/42947

16. Papageorgiou SN, Xavier GM, Cobourne MT, Eliades T. Effect of orthodontic treatment on the subgingival microbiota: A systematic review and meta-analysis. Orthod Craniofac Res. 2018 Nov;21(4):175-185. doi: 10.1111/ocr.12237. Epub 2018 Jul 20. Review. PMID: 30028077

17. Guo R, Lin Y, Zheng Y, Li W. The microbial changes in subgingival plaques of orthodontic patients: a systematic review and meta-analysis of clinical trials. BMC Oral Health. 2017 Jun 2;17(1):90. doi: 10.1186/s12903-017-0378-1. Review. PMID: 28576147

18. An JS, Kim K, Cho S, Lim BS, Ahn SJ. Compositional differences in multi-species
biofilms formed on various orthodontic adhesives. Eur J Orthod. 2017 Oct 1;39(5):528-533. doi: 10.1093/ejo/cjw089. PMID: 28339597

19. Bao K, Bostanci N, Thurnheer T, Grossmann J, Wolski WE, Thay B, Belibasakis GN, Oscarsson J. Aggregatibacter actinomycetemcomitans H-NS promotes biofilm formation and alters protein dynamics of other species within a polymicrobial oral biofilm. NPJ Biofilms Microbiomes. 2018 May 22;4:12. doi: 10.1038/s41522-018-0055-4. eCollection 2018. PMID: 29844920

20. Wong BK, McGregor NR, Butt HL, Knight R, Liu LY, Darby IB. Association of clinical parameters with periodontal bacterial haemolytic activity. J Clin Periodontol. 2016 Jun;43(6):503-11. doi: 10.1111/jcpe.12554. Epub 2016 May 4. PMID: 27105613

21. Paolantonio M, Festa F, di Placido G, D'Attilio M, Catamo G, Piccolomini R. Site-specific subgingival colonization by Actinobacillus actinomycetemcomitans in orthodontic patients. Am J Orthod Dentofacial Orthop. 1999 Apr;115(4):423-8. PMID: 10194288

22. de Freitas AO, Alviano CS, Alviano DS, Siqueira JF Jr, Nojima LI, Nojima Mda C. Microbial colonization in orthodontic mini-implants. Braz Dent J. 2012;23(4):422-7. PMID: 23207860

23. Kim SH, Choi DS, Jang I, Cha BK, Jost-Brinkmann PG, Song JS. Microbiologic changes in subgingival plaque before and during the early period of orthodontic treatment. Angle Orthod. 2012 Mar;82(2):254-60. doi: 10.2319/030311-156.1. Epub 2011 Aug 9. PMID: 21827233

24. Verrusio C, Iorio-Siciliano V, Blasi A, Leuci S, Adamo D, Nicolò M. The effect of orthodontic treatment on periodontal tissue inflammation: A systematic review. Quintessence Int. 2018;49(1):69-77. doi: 10.3290/j.qi.a39225. Review. PMID: 29114647