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*Original Research Article*

# PREVALENCE OF *AGGREGATIBACTER ACTINOMYCETEMCOMITANS AMONG CLINICAL 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 Fuso*bacterium 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) age-matched 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 harbored 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.

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34 Conclusions: These results are significant as *Aggregatibacter actinomycetemcomitans* has
35 been traditionally observed as facilitating heterotypic communities of overtly pathogenic
36 organisms, compared with other gram-negative oral microbes. These heterotypic biofilm
37 communities exhibit greatly increased capacities to resist antimicrobial drugs and other host
38 immune factors and the capacity to facilitate heterotypic associations within the biofilm may
39 be restricted to a few key species. This project successfully demonstrated evidence that non-
40 invasive salivary screening of orthodontic patients may be sufficient to assess and detect
41 changes to this periodontal pathogen – thereby increasing the potential quality and efficiency
42 of Orthodontic dental treatment among this patient population
43

44 Key words: *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum,* saliva
45 screening, microbial prevalence, Orthodontic treatment
46

47 Abbreviations: *Aggregatibacter actinomycetemcomitans* (AA), *Fusobacterium nucleatum*
48 (FN), Institutional Review Board (IRB), Office for the Protection of Human Subjects
49 (OPRS), University of Nevada, Las Vegas – School of Dental Medicine (UNLV-SDM),
50 Polymerase chain reaction (PCR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH),
51 deoxyribonucleic acid (DNA),
52

## 53 **1. Introduction**

54 The oral flora is a complex ecosystem characterized by numerous bacterial species and
55 changes to the levels of these bacteria in health, disease, and dental treatments such as
56 orthodontics [1.2]. Many studies of the oral flora are centered around consensus bacteria
57 responsible for caries and chronic periodontal disease [3-6]. Other virulent bacterial strains
58 may receive less attention because their mere presence is not strictly correlated with the
59 presence of chronic periodontal disease [7-10].
60

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

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68 presence in adults is associated with risk of pre-diabetes, metabolic syndrome, and coronary
69 artery disease [20-23].
70
71 Although some evidence has demonstrated changes to subgingival periodontal microbes such
72 as AA, little is known regarding whether orthodontic treatment will result in changes to the
73 salivary levels of this bacterial species – a non-invasive and more readily assessed measure of
74 risk [7-9,24,25]. Fixed orthodontic appliances introduces new surfaces for plaque
75 accumulation and obstacles to removing daily plaque on and between teeth while reducing
76 the efficiency of natural plaque removal mechanisms, such as salivary flow accompanied by
77 movement of the oral mucosa and tongue [26,27]. Although some studies have documented
78 the change in periodontal pathogen burden during orthodontic treatment using saliva, most
79 have focused on traditional cariogenic bacteria and some periodontal pathogens, such as
80 *Porphyromonas gingivalis* - but not *Aggregatibacter* [8,28-30].
81
82 Based upon this paucity of evidence, the main objective of this study was to evaluate the
83 prevalence of AA among Orthodontic and non-Orthodontic patients from a public dental
84 school clinic. The main research question was to assess if there is variation in the prevalence
85 of AA between adult orthodontic and non-orthodontic patients that is detectable in salivary
86 samples taken from these patients. Successful completion of this project would provide
87 preliminary evidence that non-invasive salivary screening of orthodontic patients may assess
88 changes to this periodontal pathogen – thereby increasing the quality and efficiency of dental
89 treatment among this patient population.
90

91 **2. Methodology**

92 *2.1 Project approval*

93 This project was reviewed and approved by the Institutional Review Board (IRB) and Office
94 for the Protection of Human Subjects (OPRS) at the University of Nevada, Las Vegas
95 OPRS#1502-506M titled "The Prevalence of Oral Microbes in Saliva from the University of
96 Nevada, Las Vegas – School of Dental Medicine pediatric and adult clinical population".
97 Inclusion criteria included all current patients of record at UNLV-SDM clinics. Exclusion
98 criteria included any patient who declined to participate and any subject who was not a
99 patient of record at UNLV-SDM.
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102 *2.2 Sample collection*
103 In brief, all adult patients were asked to provide Informed Consent, while pediatric patients
104 were asked to provide Pediatric Assent and their parent or guardian was asked to provide
105 Parental Permission. Each sample and corresponding demographic information intake sheet
106 was assigned a randomly generated, non-duplicated identifier that was designed to protect
107 patient information. Demographic information included only basic information, such as Sex,
108 Age, and Race or Ethnicity.
109
110 *2.3 DNA isolation*
111 Patient saliva samples were brought to the biomedical laboratory for storage at -80C until
112 processing. In brief, patient samples were processed using the GenomicPrep DNA isolation
113 kit from Amersham Biosciences (Little Chalfont, UK). Quantification and quality of DNA
114 was assessed using spectrophotometric UV absorbance readings at 260 and 280 nm (A260,
115 A280). DNA with a ratio of A260:A280 greater than 1.65 was subsequently screened using
116 PCR and primers specific for *Aggregatibacter actinomycetemcomitans* (AA).
117
118 *2.4 PCR screening*
119 Polymerase Chain Reaction (PCR) screening of the isolated DNA was accomplished using
120 the exACTGene complete PCR kit from Fisher Scientific (Fair Lawn, NJ) and an Eppendorf
121 MasterCycler (Hamburg, Germany). A positive control for human DNA was used –
122 glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme from the glycolytic
123 pathway. In addition, a positive control for bacterial DNA was also used – 16S rRNA
124 universal primer, to confirm the presence of bacterial DNA. Primers for *Aggregatibacter*
125 *actinomycetemcomitans* (AA) and *Fusobacterium nucleatum* (FN) were also synthesized by
126 Eurofins Genomics (Louisville, KY):
127
128 GAPDH forward primer, 5’-ATC TTC CAG GAG CGA GAT CC-3’; 20 nt, 55% GC,
129 Tm=66°C
130 GAPDH reverse primer, 5’-ACC ACT GAC ACG TTG GCA GT-3’; 20 nt, 55%GC,
131 Tm=70°C
132 Annealing temperature: 67C
133
134 16S rRNA universal primer, 5’-ACG CGT CGA CAG AGT TTG ATC CTG GCT-3’; 27 nt,
135 56% GC, Tm=76°C

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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 approved sampling protocol, saliva samples were obtained from Orthodontic and non-Orthodontic patients. 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 clinic at 60.4%, 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 clinic, $p$=0.6532. The greatest

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170 proportion of non-White or minority patients were Hispanic in both the study samples
171 (51.3%, 51.1%) and the Orthodontic clinic (52.3%), which was also not significantly
172 different, *p*=0.6532. Finally, the proportion of patients under 18 years of age was
173 approximately half in both the study samples (51.2%, 51.1%), which was similar to the
174 overall percentage in the Orthodontic clinic (56.7%), *p*=0.2255.
175
176 Table 1. Demographic analysis of study participants

| | Orthodontic sample (n=39) | Non-Orthodontic sample (n=45) | Orthodontic Clinic population (n=1,463) | Statistical analysis |
|---|---|---|---|---|
| **Sex** | | | | |
| **Female** | 56.4 % (n=22) | 57.8% (n=26) | 60.4% (n=884) | $\chi 2$=0.667 |
| **Male** | 43.6% (n=17) | 42.2% (n=19) | 39.6% (n=579) | d.f.=1 |
| | | | | *p*=0.4142 |
| | | | | |
| **Race/Ethnicity** | | | | |
| **White** | 25.6% (n=10) | 24.4% (n=11) | 24.7% (n=361) | $\chi 2$=1.627 |
| **Hispanic** | 51.3% (n=20) | 51.1% (n=23) | 52.3% (n=765) | d.f.=3 |
| **Black** | 15.4% (n=6) | 13.3% (n=6) | 11.8% (n=172) | *p*=0.6532 |
| **Asian** | 7.7% (n=3) | 11.1% (n=5) | 7.9% (n=117) | |
| **Other** | | | 3.3% (n=10) | |
| | | | | |
| **Age** | | | | |
| **Under <18 yrs.** | 51.2% (n=20) | 51.1% (n=23) | 56.7% (n=830) | $\chi 2$=1.469 |
| **Over > 18 yrs.** | 48.7% (n=19) | 48.9% (n=23) | 43.3% (n=633) | d.f.=1 |
| | | | | *p*=0.2255 |

177
178
179 Each saliva sample was processed to isolate DNA, both bacterial and human (Table 2). In
180 total, DNA was successfully isolated from n=81/84 samples (96.4%), which is well within
181 the expected recovery range (95-100%). The average concentration of DNA from the
182 Orthodontic samples was 699.1 ng/uL that ranged between 550 – 885 ng/uL, which is lower

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183 but comparable to the average of the non-Orthodontic samples of 804.7 ng/uL that ranged
184 between 571 – 980 ng/uL, $p$<0.01.
185
186 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) |
| | | $p$<0.01 |
| **Non-Orthodontic samples (n=45)** | | |
| **DNA concentration** | ave.= 804.7 ng/uL | |
| **DNA concentration** | range=571-980 ng/uL | |
| | | |

187
188 The DNA from each sample was then screened using PCR for the presence of
189 *Aggregatibacter actinomycetemcomitans* or AA (Figure 1). These results revealed that more
190 than half of the Orthodontic samples (56.4%) had significant and detectable levels of AA,
191 compared with only 25% of the non-Orthodontic samples. Correspondingly, less than half of
192 age-matched Orthodontic samples tested negative for AA, while three-quarters (75%) of the
193 non-Orthodontic samples were found to have no AA above the threshold limit of detection.
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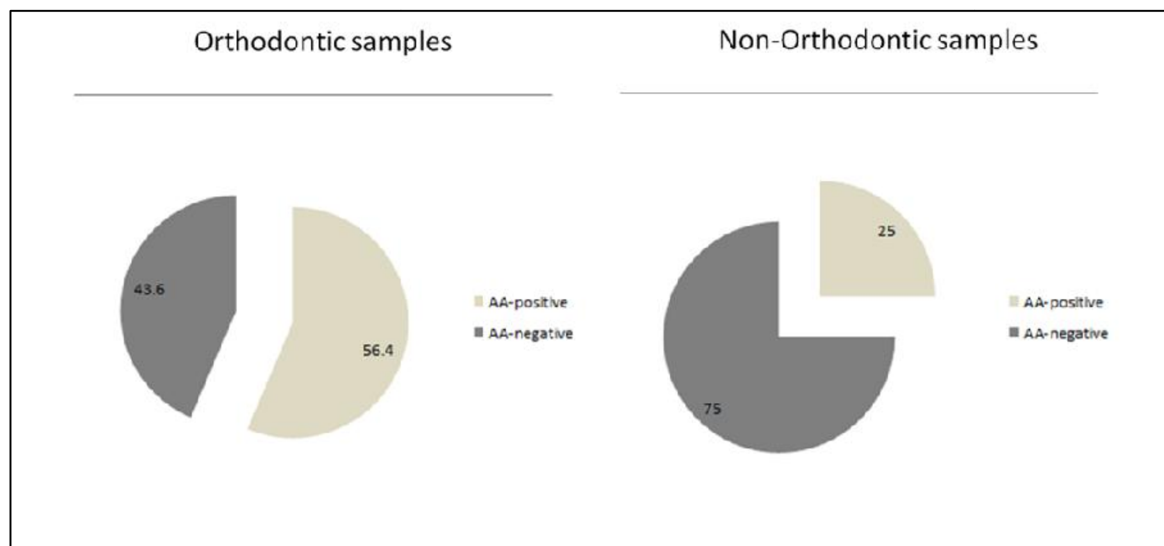


197

198 Figure 1. PCR screening of DNA isolates. PCR screening revealed 56.4% of Orthodontic
199 samples harbored significant levels of *Aggregatibacter actinocetemcomitans* (AA), compared
200 with only 25% of non-Orthodontic samples. This was statistically significant, $p<0.05$.

201

202 To determine if this phenomenon was restricted to AA, another gram-negative organism was
203 selected for screening – *Fusobacterium nucleatum* or FN (Figure 2). PCR screening of the
204 DNA isolated from the Orthodontic and non-Orthodontic samples revealed significant levels
205 of FN (above the limit of detection) in one fourth (27.7%) of the Orthodontic saliva samples
206 and only one-fifth (19%) of non-Orthodontic samples tested, which was also statistically
207 significant, $p<0.05$.

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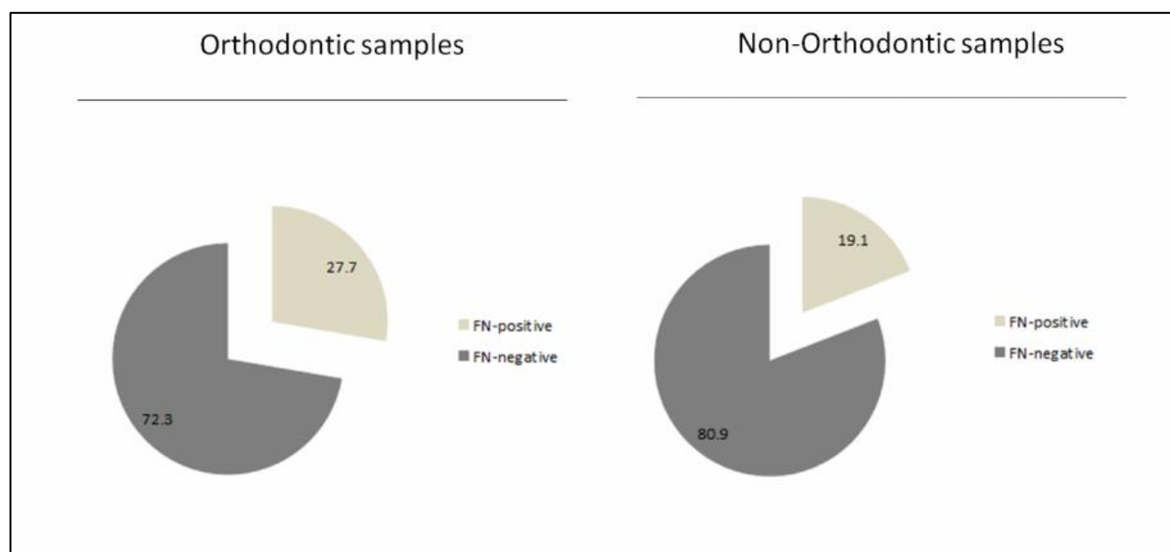


210
211
212 Figure 2. PCR screening of DNA isolates. PCR screening revealed 27.7% of Orthodontic
213 samples harbored significant levels of *Fusobacterium nucleatum* (FN), compared with only
214 19.1% of non-Orthodontic samples. This was statistically significant, $p<0.05$.
215
216
217 **4. Discussion**
218 The main objective of this study was to evaluate the prevalence of *Aggregatibacter*
219 *actinomycetemcomitans* or AA among Orthodontic and non-Orthodontic patients from a
220 public dental school clinic. The results of this study demonstrate that AA is detectable in
221 saliva samples from these patients. Moreover, the main finding was that more than half of the
222 Orthodontic subjects harbored significant levels of AA in unstimulated saliva, compared with
223 only one-fourth of the non-Orthodontic subjects. These results are significant as AA is mainly
224 associated with localized aggressive periodontitis and chronic periodontitis [31,32].
225
226 These results are significant as AA has been traditionally observed as facilitating heterotypic
227 communities of overtly pathogenic organisms, compared with other gram-negative oral
228 microbes [33,34]. In fact, biofilm communities exhibit greatly increased capacities to resist
229 antimicrobial drugs and other host immune factors [35,36]. The capacity to facilitate
230 heterotypic associations within the biofilm may be restricted to a few key species, including
231 AA [37,38].
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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.

## 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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