

**Quantification of *Aggregatibacter aphrophilus* in  
Saliva Samples and Correlation to Carriage of  
*Aggregatibacter actinomycetemcomitans***

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## ABSTRACT

*Aggregatibacter actinomycetemcomitans* is a gram-negative bacterium frequently associated with the development of aggressive periodontitis. *Aggregatibacter aphrophilus* is closely related to *A. actinomycetemcomitans* but despite this, it does not seem to be involved in any oral infectious disease. Preliminary data from *in vitro* studies of unpublished observations indicate that *A. aphrophilus* might have negative impact on the survival of *A. actinomycetemcomitans*. This could possibly depend on toxins secreted via the type VI secretion system (T6SS) in *A. aphrophilus*. The aim of this study was to compare quantities between these species in saliva samples collected from a cohort in Kenya (n = 116) and examine whether there exist any inverse correlation. Two sets of primers were tested by using PCR to determine their specificity in detecting *A. aphrophilus*. To examine an eventual correlation, samples were analysed by qPCR and thereafter compared with previously determined amounts of *A. actinomycetemcomitans* in these samples. Results from this study indicated that primers targeting the T6SS, i.e. the *hcp* gene were more specific for detection of *A. aphrophilus* than the *rpoB* primers. A majority of the samples contained both *Aggregatibacter* species, supporting the idea that *A. aphrophilus* is a common member of the oral microbiota. However, according to our data there was no general association between high amounts of *A. aphrophilus* and low quantities of *A. actinomycetemcomitans*.

## INTRODUCTION

Periodontitis is a highly prevalent inflammatory disease induced by commensal oral microbiota. The infection causes destruction of the tissues surrounding the tooth by initiating the host immune system. Untreated periodontitis causes gradual breakdown of the tooth anchoring system, consequently leading to tooth loss (Henderson *et al.*, 2010; Höglund Åberg *et al.*, 2015). The disease is divided into chronic and aggressive periodontitis, the first one is most common and mainly affects middle-aged individuals, while the second one debuts in young adolescence. Another difference is that aggressive periodontitis frequently correlates to certain microbiota such as *Aggregatibacter actinomycetemcomitans* (Höglund Åberg *et al.*, 2015; Armitage and Cullinan, 2010).

The genus *Aggregatibacter* consists of three facultative anaerobic, non-motile, gram-negative and rod-shaped or coccobacilli bacteria. *A. actinomycetemcomitans*, *Aggregatibacter segnis*, and *Aggregatibacter aphrophilus* are part of the indigenous oral microbiota but only the first is considered a periodontal pathogen and for that reason it is most studied (Nørskov-Lauritsen and Kilian, 2006).

*A. actinomycetemcomitans* colonizes the oral cavity initially by specific adhesins that bind to the oral epithelium (Fine *et al.*, 2010). The bacterium can further establish itself in the subgingival pocket where it could cause periodontal disease in susceptible individuals (Höglund Åberg *et al.*, 2015). *A. actinomycetemcomitans* has several virulence mechanisms such as leukotoxin and cytolethal distending toxin (CDT), secreted exclusively by *A. actinomycetemcomitans* in the oral biofilm (Johansson, 2011). The virulence differs between strains of *A. actinomycetemcomitans*, for instance the JP2 clone that expresses large amounts of leukotoxin (Höglund Åberg *et al.*, 2014; Haubek *et al.*, 2008). There is strong evidence that this clone transmits intra-familially and the prevalence therefore frequently depends on ethnicity and geographic location. The JP2 clone is more common in North and West Africa while a less virulent type dominates in Northern Europe (Henderson *et al.*, 2010; Doğan *et al.*, 2008; Haubek *et al.*, 2007).

*A. actinomycetemcomitans* is closely related to *A. aphrophilus*, genome sequencing indicates a difference of 15-24 % in genetic content between the species (Kittichotirat *et al.*, 2015). *A. aphrophilus* colonizes dental supragingival biofilm, saliva and oropharynx but is more uncommon in subgingival biofilm (Nørskov-Lauritsen, 2014; Di Bonaventura *et al.*, 2009). *A. aphrophilus* possesses genes that express several adhesion molecules (EmaA, Aae, YadA) that could contribute to attachment in the host (Di Bonaventura *et al.*, 2009). Unlike *A. actinomycetemcomitans*, *A. aphrophilus* does not seem to be involved in periodontitis or any other oral infectious disease. Both species are however known to be an etiologic factor external of the oral cavity, for instance in infectious endocarditis and cerebral abscesses (Moazzam *et al.*, 2015; Yew *et al.*, 2014; Nørskov-Lauritsen, 2014).

*A. aphrophilus* contains genes encoding the type VI secretion system (T6SS) (Di Bonaventura *et al.*, 2009). The T6SS exist in approximately 25 % of all gram-negative bacteria (Ho *et al.*, 2014). A typical T6SS consists of a set of necessary genes that together form a membrane-anchored organelle with a contractile needle (Cianfanelli *et al.*, 2016). Two of the required proteins are VgrG/PAAR (valine-glycine repeat G) and Hcp (haemolysin co-regulated protein). Hcp forms a tubular hexamer structure that allows transport of effector proteins while VgrG and PAAR together forms the sharp tip of the tube. When attacking competitors, like surrounding bacteria, the organelle contracts in a one-step manner and punctures the membrane of target cells (Ho *et al.*, 2014). Through the secretion system, *A. aphrophilus* translocates effector proteins into competitors or to the periplasm, which makes it an effective virulence factor (Cianfanelli *et al.*, 2016).

Preliminary data from *in vitro* studies in the laboratory of Oral Microbiology indicate that *A. aphrophilus* might have negative impact on the survival of *A. actinomycetemcomitans*. The aim of this study was therefore to compare quantities between the species in a collection of saliva samples and examine whether there exists any inverse correlation. Our hypothesis was that individuals with high levels of *A. aphrophilus* in saliva might have low levels of *A. actinomycetemcomitans*. A potential difference could possibly depend on effector proteins secreted via *A. aphrophilus* type VI secretion system.

## **MATERIALS AND METHODS**

### **Ethical considerations**

The saliva samples and bacterial strains used in this study were collected in Kenya (Lindholm and Johansson *et al.*, 2015, unpublished data). Ethical permit was issued by University of Nairobi and Kenyatta National Hospital (Ref: KNH-ER/A/30). The participants and their parents received both verbal and written information and approved partaking. No individual can be identified or linked to this study and consequently no ethical problems were recognized.

Increased knowledge of *A. actinomycetemcomitans* and its close relative *A. aphrophilus* might help us to understand why the first mentioned cause periodontal disease while the other does not. Further understanding about the mechanisms of *A. aphrophilus* and its eventual interaction with *A. actinomycetemcomitans* could possibly lead to developing new diagnostic methods and therapeutics in the future.

### **Search for literature**

Articles used for this study were found by the database of medicine PubMed with filters for human species and publication date of five or ten years. Key words used: *Aggregatibacter aphrophilus* (NJ8700), periodontitis, *Aggregatibacter actinomycetemcomitans* (D7S/D7SS), *Aggregatibacter aphrophilus*, hcp, T6SS, type VI secretion, aggressive periodontitis. Some articles were obtained from the tutor.

### **Isolation of DNA from saliva samples and bacterial strains used**

Stimulated saliva from a total of 118 individuals have been used in this study. Samples from 116 individuals had already been collected from the Maasai population in Kenya in purpose of another research project. For DNA isolation, 500  $\mu$ L of the saliva-mix were added to 500  $\mu$ L 10 mM Tris buffer with 1 mM EDTA (pH = 8,0) in 1,5 mL Eppendorf tubes. DNA from saliva were extracted using Arrow Bugs n' Beads<sup>TM</sup> extraction kit (nr.

120102, Diasorin) and the extraction robot Diasorin I.N.U.K Limited (Dublin, Ireland). Additionally, the two authors donated saliva and the DNA was isolated using the same extraction procedure.

In this study, two *A. aphrophilus* reference strains were used: NJ8700, a strain isolated from the oral flora of a healthy individual (Di Bonaventura *et al.*, 2009) and strain HK83, donated by Dr. Niels Nørskov-Lauritsen, Aarhus University (Nørskov-Lauritsen *et al.*, 2005). Finally, *A. actinomycetemcomitans* strain D7SS, a smooth colony type derivative of D7S, was used as a control strain. The D7S is a rough colony type strain that was originally isolated from a patient with aggressive periodontal disease (Wang *et al.*, 2002). D7S was donated by Dr. Casey Chen, University of Southern California.

### **PCR analysis**

Eight bacterial isolates from the Kenya population were used. We have referred to these *A. aphrophilus* isolates as 4K, 12K, 13K, 21K, 29K, 30K, 32K, and 53K. Bacterial colonies were taken from agar plates and boiled 5 min in 100 µL of sterilized water before being used as PCR templates.

To specifically detect *A. aphrophilus* in PCR, two sets of primers were tested: primers targeting genes encoding the T6SS protein Hcp and RNA polymerase gene beta-subunit (*rpoB*). The *rpoB* primers had been used in an earlier study to successfully detect *rpoB* in *A. aphrophilus* (Park *et al.*, 2013). *A. actinomycetemcomitans* strain D7SS and sterilized water were used as negative controls and DNA from *A. aphrophilus* strain HK83 and NJ8700 as positive controls. The *hcp1* primers and the *rpoB* primers were designed based on the genome sequence in NJ8700. All primers were obtained from Eurofin Genomics ([www.eurofinngenomics.eu](http://www.eurofinngenomics.eu)) and the sequences are listed below.

Primers	Orientation	Sequence (5'-3')
rpoB	Reverse	ACCAAAACAACCCGTTATCAGAAGTC
	Forward	CATGTACGTACGCACCTCAAAAC
hcp1	Reverse	CTGAGGTTTACGCCAGTC
	Forward	CCTACACCAGCGTTTATTTC

The PCR reactions were performed using Illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare) and PCR programme JO1 (95 °C 10 min, 35 cycles of: 95 °C 1 min, 54 °C 1 min, 72 °C 1 min). Every tube contained 22 µL distilled water, 1 µL of each primer pair, 5 µL loading buffer and 1 µL DNA (final volume 30 µL). The DNA marker contained 10 µL Gene Ruler, 40 µL distilled water and 10 µL loading buffer.

A 15 µL sample of the PCR products were analysed by 1 % agarose (SeaKem®) gel electrophoresis in Tris-Borate-EDTA buffer (pH 8.3) and 5 µL GelRed™ (Bio-Nuclear AB) at 100 V for approximately 60 min. The amplification products were visualized using an UV transilluminator.

### Quantitative PCR analysis

The reaction mixture for the qPCR contained 1 µL each of the primer pairs (hcp1), 5 µL KAPA SYBR® FAST qPCR Mastermix 2x (Techum LAB AB) and 3 µL of extracted DNA (final volume 10 µL). The qPCR was carried out using Rotor-gene 6000 real time PCR machine (Corbett Life Science) and programme 2016-04-21 (95 °C 3 min. 45 cycles of: 95 °C 3 sec, 5 °C 20 sec, 72 °C 1 sec. Melt 50-95 °C).

Purified *A. aphrophilus* NJ8700 DNA (62.7 ng/µL) was ten-folded creating a serial dilution of 10<sup>1</sup>-10<sup>7</sup> and run according to the qPCR procedure previously mentioned to generate a standard curve. In every qPCR run, a minimum of two known concentrations from the standard curve were included and defined as standards in the software workspace.

## Analysis of qPCR results and statistical analysis

The data were analysed using the Rotor-Gene 6000 Series Software workspace version 1.7.87. The quantification was analysed using auto-find function and by importing the standard curve.

SYBR® FAST qPCR Mastermix 2x added in the qPCR reaction mixture is a fluorescence dye binding double-stranded DNA providing a signal that represents the amount of PCR products. The intersection point is defined as the threshold cycle ( $C_t$ ) and represents the number of cycles required for the fluorescent signal to reach the threshold level. Lower amount of initially DNA require more amplification cycles to reach threshold and therefore results in a higher  $C_t$ -value. Since the fluorescence dye will detect any double-stranded DNA, also contaminating DNA, a melt curve analysis was made to distinguish peaks specific for *A. aphrophilus* (78-84 °C).

Data was exported to Excel and organized by the presence of *A. aphrophilus* and the amount of the bacteria in ng/ $\mu$ L. The  $C_t$ -values were translated to the concentration unit ng/ $\mu$ L with the standard curve as reference. To evaluate the qPCR data, levels of *A. aphrophilus* were approximated in none (0) (including samples with very low amounts and incorrect melt peaks), low (1) (approximately  $9.41 \times 10^{-9}$ - $1.0 \times 10^{-3}$  ng DNA/ $\mu$ L) and high (2) ( $\geq 1.0 \times 10^{-3}$  ng DNA/ $\mu$ L).

Each tube run in the qPCR was loaded with 3  $\mu$ L DNA and 7  $\mu$ L of reaction mix i.e. the DNA was diluted 3/10. Thus, the actual concentrations of *A. aphrophilus* DNA in the saliva DNA preparations are approximately 3.3-fold higher than measured by qPCR. To estimate how many *A. aphrophilus* cells are measured in the qPCR, a conversion calculator (URI Genomics & Sequencing Centre University of Rhode Island, 2004) was used. For example, 3 ng DNA could be approximated to  $1.2 \times 10^6$  *A. aphrophilus* cells in total on basis of the size in basepairs of the chromosome of this species.

To compare the quantities between the species we got access to qPCR data from Oral Microbiology (Claesson *et al.*, personal communication), detecting *A.*

*actinomycescomitans* in the same saliva samples by using primers targeting the leukotoxin gene (*ltxA*) (Kirakodu *et al.*, 2008). The two saliva samples from the authors were also analysed using this procedure to quantitate the level of *A. actinomycescomitans*. These data were also divided into groups: none (0) (including samples with very low amounts), low (1) (<6 600 cells/mL) and high (2) (≥6 600 cells/mL). The quantities of the species in the samples were compared to each other through this division. The saliva samples donated by the authors were also analysed using the above procedures.

### **Statistical analysis**

Statistical data was analysed to estimate the relative risk (RR) for carriers and non-carriers of *A. aphrophilus* to also carry *A. actinomycescomitans* (95 % confidence interval and p-value). The exposed group (*A. aphrophilus*-positive) and control group (*A. aphrophilus*-negative) were compared through calculations according to Altman (1991), and available at MedCalc (MedCalc Statistical Software, 2017). Graphs were assembled using Excel and images were adjusted using Adobe Photoshop.

## **RESULTS**

### **Primer selection**

For PCR analysis, two different primer pairs (*rpoB* and *hcp1*) were tested to determine, which were more specific in detecting *A. aphrophilus*. The products obtained from PCR were visualized in electrophoresis (Fig. 1). According to the result, both primer pairs detected *A. aphrophilus* (lane 4, 12), however the *rpoB* primers also detected *A. actinomycescomitans* to some extent (lane 10) (Fig. 1). The *hcp1* primers were considered more specific since *A. aphrophilus* was easier to separate from both *A. actinomycescomitans* and the negative control compared to the results using *rpoB*. Therefore the *hcp1* primers were selected for further qPCR analysis.

### **Confirming the identity of tentative *A. aphrophilus* isolates from the Kenya collection**

To confirm the presence of *A. aphrophilus* in the Kenya material, a minor selection of eight saliva bacterial isolates from this collection were tested in PCR, analysed using the hcp1 primers and visualized in electrophoresis (Fig. 1). The DNA fragments from the samples (lane 5-8, 13-16) had the same size as the positive control *A. aphrophilus* strain HK83 (approximately 400-bp). Thus, all eight bacterial samples were confirmed to have *A. aphrophilus*. We therefore concluded that the Kenya material was suitable for additional qPCR analysis.

### **Generation of a qPCR standard curve for quantitation of *A. aphrophilus* DNA**

To find a method for quantification, a series of ten-fold dilutions of *A. aphrophilus* strain NJ8700 DNA ( $62.7-1.881 \times 10^{-6}$  ng/ $\mu$ L) were made and analysed in qPCR. This resulted in a standard curve with the known concentration for each dilution on the x-axis and the C<sub>t</sub>-value on the y-axis (Fig. 2). This functioned as a calibration curve to calculate the concentration of unknown samples run in qPCR. In every run, a minimum of two samples with known concentrations were included and defined as standards.

### **Quantification of *A. aphrophilus* in saliva samples from a study cohort in Kenya**

In order to examine whether there existed any inverse correlation between the quantities of the species in saliva, a selection of saliva samples collected from adolescents in Kenya (n = 116) were analysed by qPCR using the hcp1 primer pairs. When looking at the presence, 59 % of the samples contained both *Aggregatibacter* species, 16 % of the samples contained only *A. aphrophilus* and 13 % contained only *A. actinomycetemcomitans*. In 12 % of the samples none of the species were detected (Fig. 3A).

In samples where no *A. aphrophilus* was detected, 52 % contained *A. actinomycetemcomitans*. In samples where *A. aphrophilus* was detected, 78 % contained

*A. actinomycetemcomitans* (Fig. 4A). The relative risk was calculated to >1 (RR 1.5) indicating that individuals that had *A. aphrophilus*, had a significantly increased risk to also possess *A. actinomycetemcomitans* (p-value 0.03, 95 % CI 1.05-2.19).

In samples where *A. actinomycetemcomitans* was not detected, 58 % contained *A. aphrophilus*. In samples where *A. actinomycetemcomitans* was detected, 82 % also had presence of *A. aphrophilus* (Fig. 4A). The relative risk was calculated to >1 (RR 1.4) indicating that individuals that had *A. actinomycetemcomitans*, had a significantly increased risk to also possess *A. aphrophilus* (p-value 0.03, 95 % CI 1.04-1.94).

To extend the analysis to not only consider presence and absence of the species, bacterial levels were scored, based on the qPCR data as none (0), low (1), and high (2) respectively as described earlier (Fig. 3B). For the *A. aphrophilus* group 0, no *A. actinomycetemcomitans* was detected in 48 %, low quantities of *A. actinomycetemcomitans* in 48 % and high amounts in 3.4 % (Fig. 4B). In the *A. aphrophilus* group 2, no *A. actinomycetemcomitans* was detected in 29 % and low quantities was detected in 55 % while high amounts were detected in 17 % (Fig. 4B). The relative risk was calculated to >1 (RR 1.4) indicating that individuals that had high amounts of *A. aphrophilus* (2) had increased risk to also possess *A. actinomycetemcomitans* (p-value 0.1, 95 % CI = 0.93-2.06). The p-value was >0.05, indicating that this part of the result was not statistical significant.

*A. actinomycetemcomitans* levels were also scored as none (0), low (1) and high (2) respectively (Fig. 3B). For the *A. actinomycetemcomitans* group 0, no *A. aphrophilus* was detected in 42 %, low amounts of *A. aphrophilus* in 21 % and high quantities in 36 % (Fig. 4C). In the category with high amounts of *A. actinomycetemcomitans* (2) 6.7 % contained no detectable *A. aphrophilus*, whereas both low and high amounts were detected in 47 % (Fig. 4C). The relative risk was calculated to >1 (RR 1.6) indicating that individuals that had high amounts of *A. actinomycetemcomitans* (2) had a significantly increased risk to also possess *A. aphrophilus* (p-value 0.003, 95 % CI = 1.17-2.24).

In the saliva samples donated from the authors, high amounts of *A. aphrophilus* were detected and categorized as the group with high quantities (2). Presence of *A. actinomycetemcomitans* was only detected in one of the samples and categorized as high (2).

Taken together, from these experiments we concluded that no apparent inverse correlation existed between the amounts of *A. aphrophilus* and *A. actinomycetemcomitans* in these saliva samples. The relative risk analysis rather indicated that the presence of *A. aphrophilus* also correlates to the presence of *A. actinomycetemcomitans*. However, as pointed out above, we found individuals possessing only one of the species, i.e. *A. aphrophilus* (n = 19) or *A. actinomycetemcomitans* (n = 15) (Fig. 3B).

## DISCUSSION

The aim of this study was to compare quantities between *A. aphrophilus* and *A. actinomycetemcomitans* in saliva and examine whether there exists any inverse correlation. As far as we know, this study is the first of its kind to investigate this relationship. This was of interest since earlier observations from *in vitro* studies demonstrated that *A. aphrophilus* might have a negative effect on the survival of *A. actinomycetemcomitans*. Our hypothesis was that this could depend on toxic proteins secreted via T6SS and *A. aphrophilus* could thereby possibly compete with *A. actinomycetemcomitans* in an early stage of colonization. Reduced amounts of *A. actinomycetemcomitans* in the subgingival biofilm could therefore lead to minimized risk of developing aggressive periodontitis.

The saliva samples collected from Kenya were suitable for this study since the quantities of *A. actinomycetemcomitans* already had been analysed. *A. actinomycetemcomitans* was present at variable levels in the majority of the samples and were therefore appropriate for further analysis referring to the presence of *A. aphrophilus*.

To test our hypothesis it was essential to find suitable primers for detecting *A. aphrophilus*. Primers targeting *hcp* were selected since the T6SS gene appears to be conserved in *A. aphrophilus* strains and as far known absent in *A. actinomycetemcomitans*. A previous study suggested that primers detecting the *rpoB* gene are adequate for studies related to oral infections (Park *et al.*, 2013). The *rpoB* gene exists in all bacteria but the gene sequence differs between species. According to our results, both *hcp1* and *rpoB* detected *A. aphrophilus* in PCR but the first mentioned was considered more specific for this study. This can be due to that the species are closely related and therefore hard to distinguish with the *rpoB* primers, while the *hcp* gene is only present in bacteria with the T6SS. There would have been an increased risk for false positive results with the *rpoB* primers.

Both species were confirmed in a majority of the samples, *A. aphrophilus* was confirmed in 75 % of the samples and *A. actinomycetemcomitans* in 72 %. This indicates that these are quite common bacteria of the oral microbiota in the Kenyan material. Studies show that the prevalence of *A. actinomycetemcomitans* in the population varies between different geographic areas (Rylev and Kilian, 2008). The presence of *A. aphrophilus* in the samples donated from the authors indicates that it could be a common bacterium also in other locations. As *A. actinomycetemcomitans* was only detected in one of these samples, it is apparent that individuals possessing only one of the species may be rather frequent in other locations as well.

From our study, we concluded that no apparent inverse correlation in quantities existed; rather presence and/or high amounts of *A. aphrophilus* in a sample was also commonly associated with higher levels of *A. actinomycetemcomitans*. The reason for this result is unclear but high bacterial levels in the subjects could be a possible explanation. The oral cavity is a complex environment and difficult to reproduce; this could possibly explain why the hypothesis based on *in vitro* data was not supported by analysis of the clinical material.

Interestingly though, there were individuals that according to qPCR carried only *A. aphrophilus* (n = 19) or only *A. actinomycetemcomitans* (n = 15). Theoretically, these

samples could cohere with our initial hypothesis and it would be of interest to analyse bacterial samples from these individuals to study their virulence characteristics such as their T6SS activity. Perhaps individuals not carrying *A. actinomycetemcomitans* are colonized by a special strain(s) of *A. aphrophilus*, which is more virulent and therefore more effective in inhibiting *A. actinomycetemcomitans*?

We are aware of a number of experimental limitations in our study. We observed a discrepancy in  $C_t$ -values between some analyses of the same sample despite that they were analysed multiple times in qPCR. This could possibly depend on a potential error in reagents in the DNA extraction kit (Arrow Bugs n' Beads™). The qPCR data on *A. actinomycetemcomitans* that we got access to in this study were extracted using the same kit. A different DNA extraction kit has been used on the same samples and it will be interesting to compare these results.

Another limitation was that *A. aphrophilus* was initially detected in two of the samples (4K, 12K) using PCR, but not according to our qPCR results. A possible explanation for this is that the cultivation technique is more sensitive and theoretically one cell is enough to give a positive result.

Another problem of this experiment was that the exact quantities of *A. aphrophilus* in 7 of the 116 saliva samples could not be determined. The melt curve analysis of these samples could however confirm the presence of *A. aphrophilus*, so they were therefore only categorized as positive or negative.

A fourth issue was whether to include or exclude some peaks in the melt curve analysis, theoretically these peaks might represent other bacteria that also possess an *hcp* gene. Genome sequencing of *A. aphrophilus* NJ8700 indicates that its genome encodes T6SS (Di Bonaventura *et al.*, 2009) and our PCR results support that *hcp* is common among *A. aphrophilus* strains, but T6SS also exists in other gram-negative bacteria (Ho *et al.*, 2014).

To summarize, our study demonstrates that the *hcp1* primers targeting the gene encoding the Hcp component of the T6SS could be useful in routine analyses for detecting *A.*

*aphrophilus*. This study also supports that *A. aphrophilus* is a frequent bacterium in the oral microbiota, with a high prevalence in our tested material. Further, our work did not indicate any association between high amounts of *A. aphrophilus* and low quantities of *A. actinomycetemcomitans*.

It would be of interest to further investigate the mechanisms of interaction of *A. aphrophilus* with other bacteria and if it could prevent an early colonization of *A. actinomycetemcomitans* and thereby the development of aggressive periodontitis. More research in this area could perhaps lead to development of new diagnostic and therapeutic methods.

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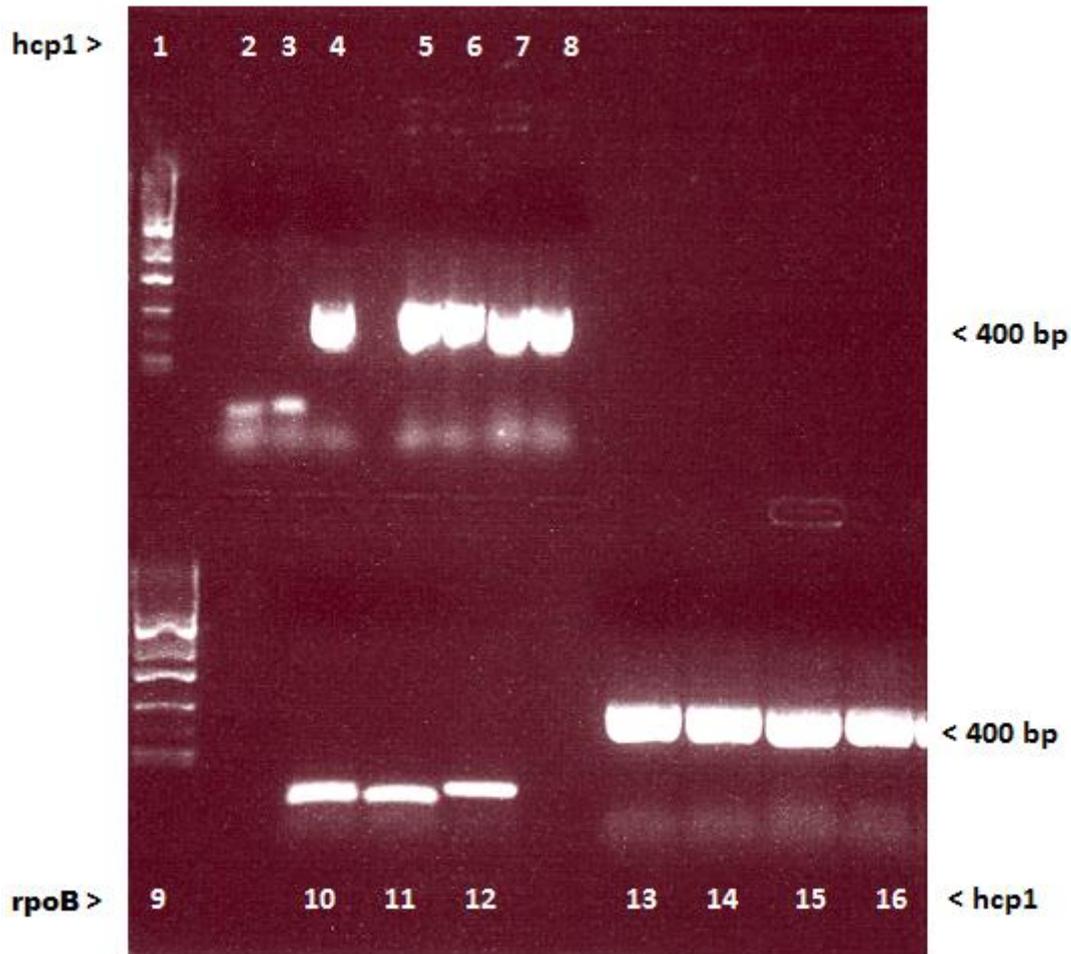
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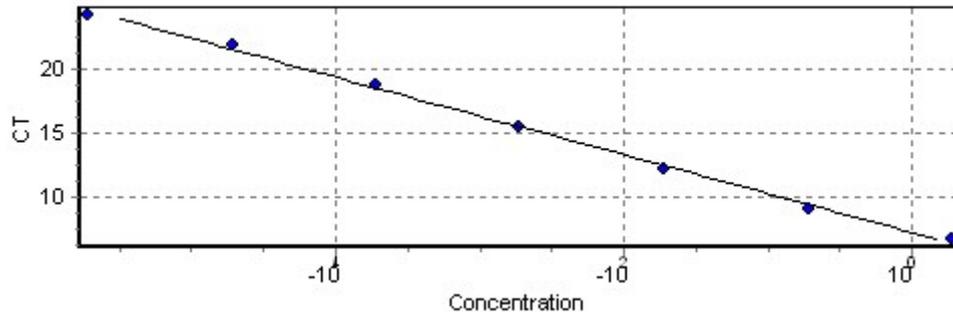
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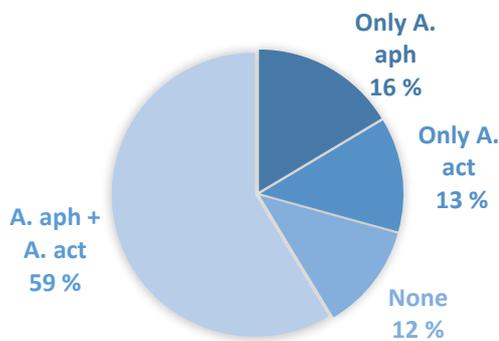
## FIGURES



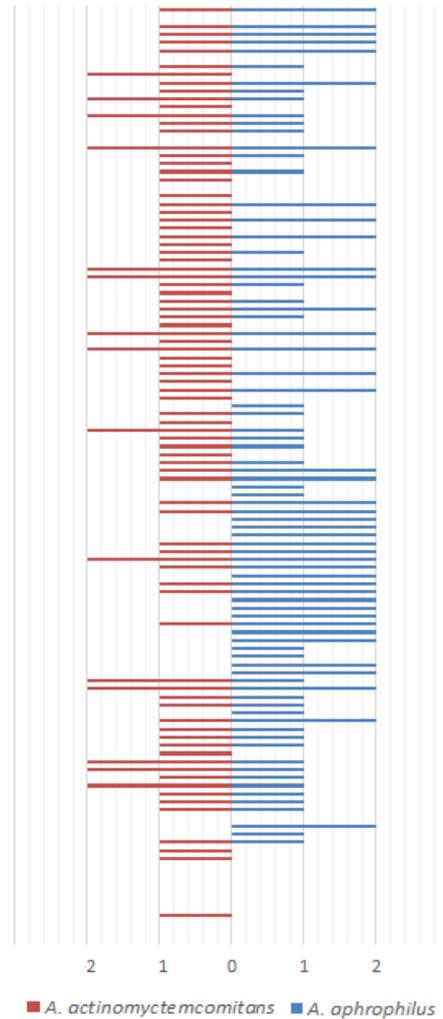
**Figure 1. Evaluation of PCR primers for detection of *A. aphrophilus*.** PCR analysis of boiled bacterial colonies on an 1 % agarose gel. Samples amplified with hcp1 primers are loaded in lane 2-8 and 13-16, and samples amplified with rpoB primers in lane 10-12. The following PCR products were loaded: DNA size marker (lane 1 and 9), *A. actinomycetemcomitans* D7SS (lane 2 and 10), control with no DNA (3 and 11), *A. aphrophilus* HK83 (4 and 12) and Kenyan saliva bacterial isolates (lane 5-8 and 13-16). Lane 5: 4K, lane 6: 12K, lane 7: 13K, lane 8: 21K, lane 13: 29K, lane 14: 30K, lane 15: 32K, lane 16: 53K.



**Figure 2. A qPCR standard curve for quantification of *A. aphrophilus*.** Ten-fold serial dilutions of *A. aphrophilus* strain NJ8700 DNA ( $10^1$ - $10^7$ ) run in qPCR to create a standard curve. The x-axis represents the concentration of each dilution for ( $\text{ng}/\mu\text{L}$ ) *A. aphrophilus* and the y-axis represents the  $C_t$ -value.

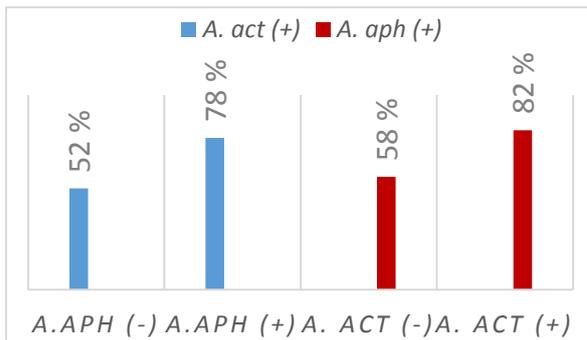


(A)

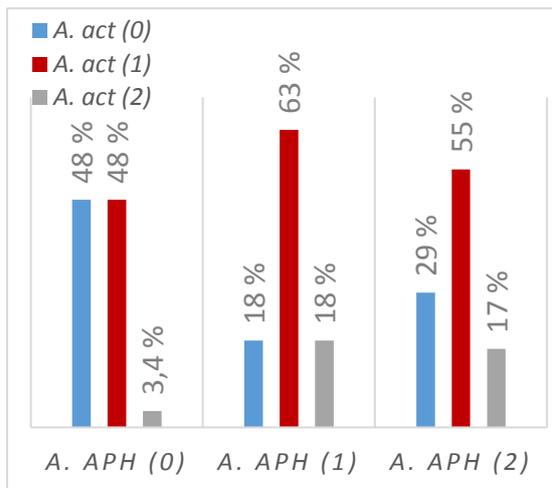


(B)

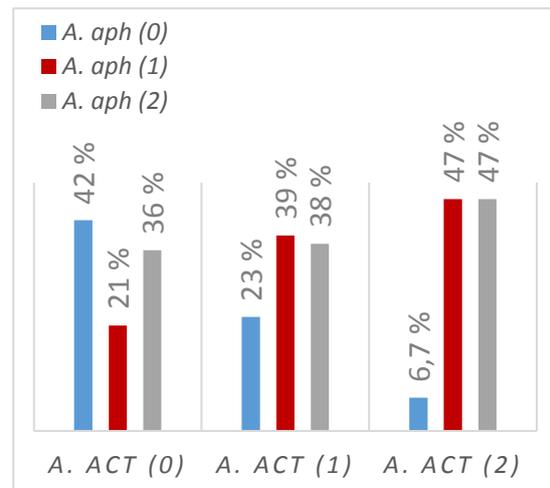
**Figure 3. Relative levels of the two species in the tested saliva DNA preparations as determined by qPCR.** (A) Proportion (%) of the saliva DNA samples (n = 116) divided into four categories. Samples containing only *A. aphrophilus* (16 %), only *A. actinomycetemcomitans* (13 %), none (12 %) and both species (59 %). (B) Based on the qPCR data, the quantities of *A. aphrophilus* and *A. actinomycetemcomitans* were compared for individual samples (n = 109). Both *A. aphrophilus* and *A. actinomycetemcomitans* were scored based on the threshold cycle value and categorized as none (0), low (1), and high (2) respectively as described in Materials and methods. The red bars (left) represent *A. actinomycetemcomitans*, and the blue bars (right) *A. aphrophilus*.



(A)



(B)



(C)

**Figure 4. Proportion (%) of the two species in the tested saliva DNA preparations as determined by qPCR. (A)** The blue bars (left) represents the presence of *A. actinomycetemcomitans* (%) in samples with *A. aphrophilus* (+) (n = 87) and without *A. aphrophilus* (-) (n = 29). The red bars (right) shows the presence of *A. aphrophilus* (%) in samples with *A. actinomycetemcomitans* (+) (n = 83) and without *A. actinomycetemcomitans* (-) (n = 33). **(B)** Proportion (%) of *A. actinomycetemcomitans* (0, 1, 2) in samples with *A. aphrophilus* categorized by 0 (n = 29), 1 (n = 38), 2 (n = 42). **(C)** Proportion (%) of *A. aphrophilus* (0, 1, 2) in samples with *A. actinomycetemcomitans* categorized by 0 (n = 33), 1 (n = 61), 2 (n = 15). Relative risk analysis indicated that carriage of one of the species have a significantly increased risk to also possess the other (see Results).