QUANTITATIVE ANALYSIS OF AGGREGATIBACTER ACTINOMYCETEMCOMITANS IN DENTAL PLAQUE SAMPLES OF MOROCCAN SCHOOL CHILDREN WITH AND WITHOUT PERIODONTITIS.

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by
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ABSTRACT

Objectives: Microbial infection and the host response to the infection play a significant role in the etiology of periodontal diseases. Previous studies reported a relatively high prevalence of periodontitis among adolescents in Morocco. The importance of the composition of subgingival plaque and the presence and proportion of *Aggregatibacter actinomycetemcomitans* to the total plaque bacteria in the pathogenesis of periodontitis is not well understood. The purpose of this study is to compare the relative abundance of *A. actinomycetemcomitans* in subgingival dental plaque from young Moroccans with aggressive periodontitis, chronic periodontitis, and those without periodontitis, and to construct a multivariable model to investigate the effect of demographic attributes of age, gender and relative ratio of *A. actinomycetemcomitans* in dental plaque with periodontal disease status.

Methods: Sample population includes 984 subjects, aging from 12-20 years old, who were surveyed and examined for periodontal disease status. 82 subjects were selected consisting of 26% aggressive periodontitis, 12% chronic periodontitis, and 62% without periodontitis. Subgingival plaque was collected from these 82 subjects. Whole DNA was extracted and purified, and real-time PCR was used employing a primer for eubacteria, and specific primer for *A. actinomycetemcomitans*. PCR assays confirmed the amplification and quantification of DNA of total bacterial and *A. actinomycetemcomitans*.

Results: 73% of the subjects harbored *A. actinomycetemcomitans*: 63% in aggressive periodontitis, 90% in chronic periodontitis, and 73% in controls. The percentage *A. actinomycetemcomitans* to total bacterial load increased with age, was

similar among males and females, and was somewhat higher in persons with periodontitis than the controls. Using a logistic regression analysis that included age, gender and *A. actinomycetemcomitans* ratio showed that only age is significantly correlated with the diagnosis of periodontitis in this population.

Conclusions: A. actinomycetemcomitans is prevalent among this young Moroccan group and is somewhat more prevalent in subjects with periodontitis than the controls. However, the presence and ratio of this species to the total bacteria in subgingival plaque explained only a small proportion of the variance in periodontitis in this group.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Periodontitis is a disease characterized by a microbially-initiated, host-mediated inflammation that results in the loss of periodontal attachment and alveolar bone support around teeth (Page & Schroeder 1981, Tonetti et al. 2018). It is one of the most prevalent oral diseases with an overall prevalence of approximately 50% (Albandar & Rams 2002, Albandar 2005). It is estimated that 30% to 35% of all tooth loss is caused by periodontitis (Brown et al. 1989).

Microbial infection and the host response play a significant role in the etiology of periodontal diseases (Page & Schroeder 1981, Bosshardt 2018). The subgingival microbial flora in humans is complex and includes a large number of bacterial species that comprises pathogenic and non-pathogenic microorganisms (Könönen & Müller 2014). In 1976, Page and Schroeder described the four stages of the pathogenesis of periodontal disease: initial, early, established and advanced, relating to the host's periodontal status from healthy to periodontitis, respectively. With the growth and accumulation of microbial species in a subgingival environment, the initiation of periodontal disease occurs with increased inflammatory cellular infiltrates. Although Page and Schroeder's model was a popularly accepted understanding of the pathogenesis of periodontitis, it was limited by the knowledge regarding the identities of pathogenic bacteria and their influence on the host response (Hajishengallis et al. 2017).

There are two major phenotypes of periodontitis: a chronic and aggressive form. Chronic periodontitis is the more common phenotype and it is associated with older age groups, whereas the aggressive form is much less common and is associated with young

age population (Albandar 2014). Although the prevalence of aggressive periodontitis is low, it has a profound impact on the quality of life of affected individuals (Llanos et al. 2018) as it causes progressive loss of the tissue supporting teeth, and this leads to loss of permanent teeth at early age (Nibali et al. 2013, Graetz et al. 2017).

Little is known about the etiopathogenesis of aggressive periodontitis. Findings in studies of the microbial flora of aggressive periodontitis suggest that the profile of the subgingival bacteria in these patients is diverse (Könönen & Müller 2014). A recent study used the bacterial culture method to investigate the composition of the subgingival microbiota in Moroccan aggressive periodontitis patients and reported high frequency detection of *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia*, and *Fusobacterium nucleatum* (Chahboun et al. 2015).

There is evidence implicating *A. actinomycetemcomitans* as a causative agent of destructive periodontal disease (Henderson et al. 2010). This bacterium selectively kills human leukocytes through expression of RTX (repeat in toxin) leukotoxin (Johansson 2011). It also expresses cytolethal distending toxin (CDT) which is cytotoxic to eukaryotic cells (Smith & Bayles 2006). *A. actinomycetemcomitans* employs CDT, leukotoxin, and LPS to evade host innate defense mechanisms and induce an inflammatory response. Cell-wall components of *A. actinomycetemcomitans*, including lipopolysaccharide and cell-surface capsular-like polysaccharide, can promote osteoclast formation and alveolar bone loss (Herbert et al. 2016).

The percentage of individuals harboring *A. actinomycetemcomitans* seems to vary significantly between populations in various geographical areas world-wide, and between

ethnic groups. In addition, this microorganism may also show different colonization patterns in different types of periodontal diseases and in periodontally healthy subjects.

Objectives

This study investigated the relationship between the detection of the periodontal pathogen *A. actinomycetemcomitans* in subgingival dental plaque and periodontal disease status in Moroccan schoolchildren. The specific aims of the study were to:

1-Compare the prevalence and relative abundance of positively identifying *A. actinomycetemcomitans* in subgingival dental plaque samples of aggressive periodontitis patients relative to the prevalence of this microorganism in controls without periodontitis.

2-Study the effect of demographic attributes (age, gender) and bacterial load on periodontal disease status.

CHAPTER 2

MATERIALS AND METHODS

Study Population

A survey was undertaken in 2015 to study the dental health of school children in the city of Tiznit, Morocco. Four schools were selected using a probability sampling scheme to yield a sample representative of the metropolitan area of Tiznit. Demographic data, dental and medical history, health-related behavior evaluation, and dental clinical examinations were completed on 984 children 13-20 years old. The subjects received a standard full-mouth periodontal examination that consisted of probing measurements, gingival recession, and gingival bleeding on probing at six sites per tooth on all permanent teeth. The clinical attachment level was calculated from the pocket and recession measurements. The subjects were classified into three groups according to their periodontal status using the criteria described previously (Albandar 2014, Kissa et al. 2016), and included aggressive periodontitis, chronic periodontitis, and without periodontitis groups. Two years following the baseline survey examination subjects with aggressive periodontitis or chronic periodontitis, and a group of subjects without periodontitis from the same schools as the cases, and matched on gender, were invited to participate in a follow up examination. 82 subjects agreed to participate in the follow up examination comprising 21 in the aggressive periodontitis group, 10 in the chronic periodontitis group, and 51 without periodontitis. The subjects were examined clinically as described above, and were interviewed to collect demographic data and information about their dental and medical health history.

Plaque Sample Collection

In each subject subgingival dental plaque samples were collected from four sites with the deepest probing depths. Sites were isolated with cotton rolls, and any supragingival plaque was removed, then a sterile paper point was inserted into the depth of the pocket and left in place for 30 seconds. The paper points from each subject were placed in an Eppendorf tube containing TE buffer and stored at -20°C until analyzed.

Bacterial DNA Extraction and Purification

The plaque samples were thawed and the vial containing the paper points and TE buffer were vortexed thoroughly. The entire volume of suspension was transferred to a new 1.5ml Eppendorf tube. The suspension was centrifuged at 13,000 rpm for 10 minutes and the supernatant was discarded. The cellular pellet was re-suspended in 162 µl PBS with 18 µl Metapolyzme (Sigma, USA), dissolved well with vortex, and incubated at 35°C for 4 hours. DNA was then extracted using the Invitrogen Purelink Genomic DNA extraction kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. After first incubation, 20 µl of proteinase K was added, 200 µl Genomic Lysis/ Binding Buffer, and vortexed well and incubated for 30 mins at 55°C. After second incubation, 200 µl of 96% Ethanol was added.

The prepared lysate was transferred to PureLink Spin Column in a first Collection Tube, centrifuged at 10,000g for 1 minute at room temperature. The first collection tube was discarded, and the spin column was placed into a new second collection tube. 500 µl Wash Buffer 1 with ethanol was added to the spin column, and centrifuged at 10,000g for 1 minute at room temperature. The second collection tube was discarded and placed in a new third collection tube. 500 µl Wash Buffer 2 with ethanol

was added to the spin column, and centrifuged at 14,000 rpm for 3 minutes at room temperature. The third collection tube was discarded and the spin column was placed in a sterile 1.5ml microcentifuge tube. 80µl PureLink Genomic Elution Buffer was added to the spin column, incubated at room temperature for 1 minute, and centrifuged at 14,000 RPM for 1 minute for final purified DNA.

Qubit dsDNA High Sensitivity Assay (ThermoFisher Scientific, USA) were used to quantify DNA following manufacturer's protocol. 5 µl sample DNA with 195 µl Qubit working solution were added to 0.5ml tubes. 10 µl Qubit Standard #1 and #2 with 190 µl Qubit working solution were added to 0.5ml tubes and were measured against the sample DNA for quantficiation. Remaining sample DNA were stored at -80°C.

Laboratory Analysis

The primers used in the PCR analysis are shown in Table 2. Total bacteria and *A. actinomycetemcomitans* were detected and quantified in DNA extracts using TaqMan real-time technology. The amplification reaction mixture consisted of 5 ul DNA temple, 3.96 DEPC water, 0.04 ROX ref dye, 1 primer/probe mix, and 10 Platinum qPCR supermix-UDG. Specific products were amplified and detected using Quant-Studio 3 Real-Time PCR System (ThermoFisher Scientific, USA), with the following thermal conditions: 50°C for 2 min, 95°C for 2 min, followed by 45 cycles of 95°C for 10 seconds, and 60°C for 1 min for both *A. actinomycetemcomitans* and total 16S rRNA bacterium. The data capture of the amplified products was obtained after the second step of the PCR stage.

A. actinomycetemcomitans will be detected and quantified in the DNA extracts using a Taqman real-time PCR assay as described previously (Al-Hebshi et al. 2014).

Briefly, a previously validated primers/probe set targeting the hgpA gene were obtained as an optimized, ready to use kit from PrimerDesign (Plymouth Meeting, USA). The sequences of probes and primers used in the study (Table 1).

Table 1. Sequence of Primers and Probes Used in the Quantitative PCR Assays

Test species	Sequences 5'-3'	Target gene	Product size	Ref
Aggregatibacter actinomycetemco-mitans	F-primer: GGRAGAATGGATGGCGATAT R-primer: ATCAGAATGAACATAACCTATACCA Probe: FAM-ATGAACGCAATTCAGCCCAGA ACTG-BHQ	hgpA	81 bp	Al-Hebshi 2014
Total bacteria	F-primer: AAACTCAAAGGAATTGACGGGG R-primer: TTGCGCTCGTTGCGGGACT Probe: TTGCGCTCGTTGCGGGACT	16S rRNA	205 bp	Al-Hebshi 2010

The kit includes a plasmid-based positive control that is used for construction of a standard curve for absolute quantification of the total. For relative quantification, the absolute count was normalized to that of total bacteria using a similar kit with universal bacterial primers/probe set.

Statistical Analysis

The percentage of *A. actinomycetemcomitans* count to total bacterial count (A.a. relative abundance) was calculated and compared between demographic and disease classification groups. The comparisons used the Mantel-Haenszel Chi Square method, and used p-value of 0.05.

The *A. actinomycetemcomitans* counts for 2 subjects were out of range, and these subjects were regarded as outliers and were excluded from the analyses. A multivariable logistic regression analysis that adjusted for study design (subjects clustered within schools) was performed using the SAS software v9.4.

CHAPTER 3

RESULTS

Quantitative Real-Time PCR Assay

The quantitative real-time polymerase chain reaction assays showed excellent linearity ($R^2 \geq 0.998$) over a dynamic range of 5-10^6 copies /reaction (Figures 1 and 2), achieving a sensitivity of 10 copies/sample. This indicates that the primers that were used for the PCR had high specificity in detecting for *A. actinomycetemcomitans* and total bacteria species.

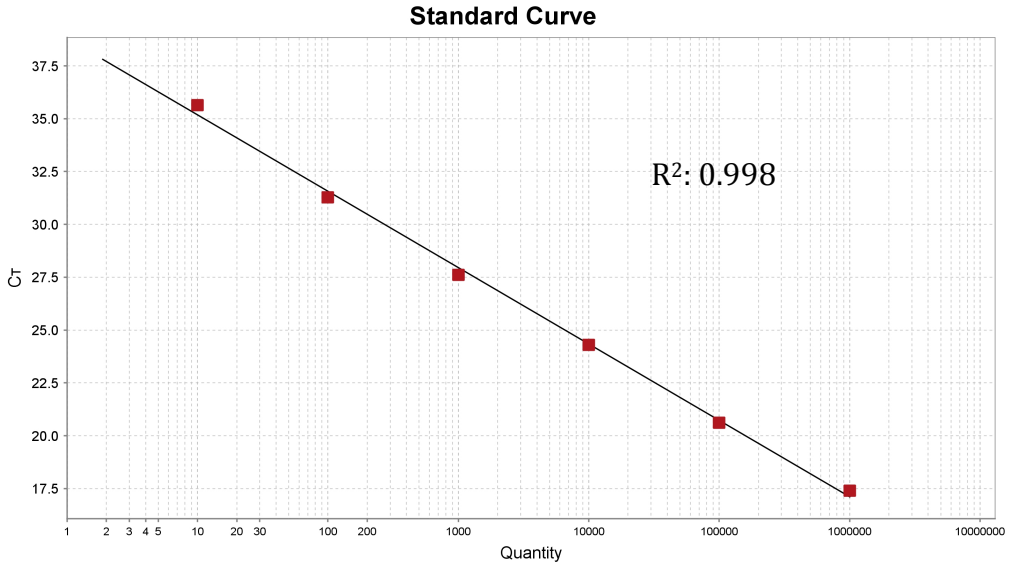

Figure 1. Real-Time PCR standard curves for *A. actinomycetemcomitans* primer used in this study. Serial dilutions of plasmid-based positive control were prepared with final concentrations of 5-10^6 copies/reaction. Assays were run as described in the text. The curves were obtained by plotting log DNA copies count against threshold cycle values (C_t).

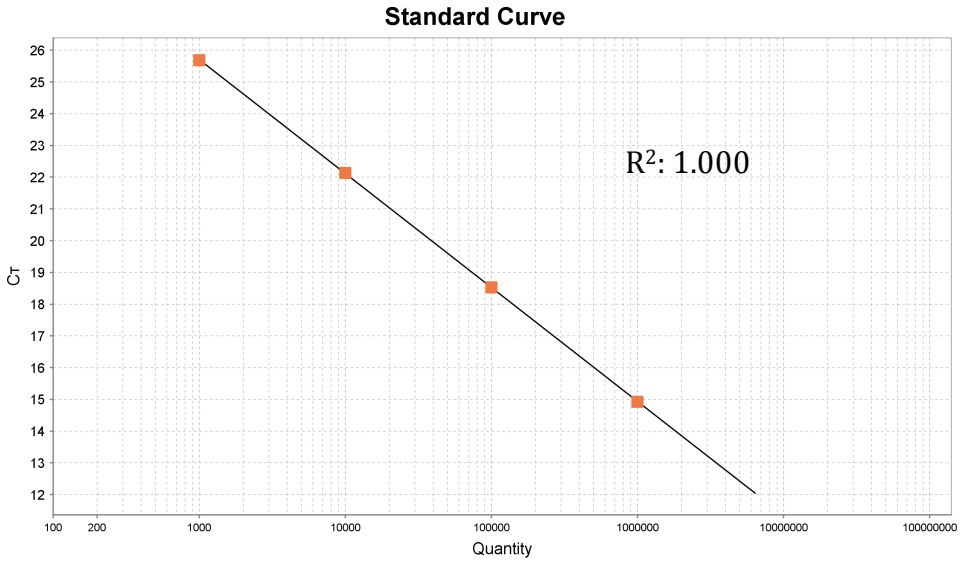

Figure 2. Real-Time PCR standard curves for total bacteria species primer used in this study. Serial dilutions of plasmid-based positive control were prepared with final concentrations of 5-10^6 copies/reaction. Assays were run as described in the text. The curves were obtained by plotting log DNA copies count against threshold cycle values (C_t).

A. actinomycetemcomitans

Two subjects had *A. actinomycetemcomitans* detection above 2% and were regarded as outliers and excluded from the analyses. Among the total study group (N=80) 73% of the subjects harbored *A. actinomycetemcomitans*. *A. actinomycetemcomitans* was detected in 63.2% in the aggressive periodontitis group, 90.0% in the chronic periodontitis group, and 72.5% in the control group as seen in Table 2. The amount of *A. actinomycetemcomitans* found amongst the different categories were not significantly different with p-value being 0.596 (Mantel-Haenszel Chi Square).

Table 2. Percentage of Tiznit Moroccan School Children with *A. actinomycetemcomitans* in Subgingival Plaque Categorized by Demographic Characteristics and Periodontal Disease Status

Variables	**Categories**	**A.a. (+) Patient Quantity**	**Total Patient Quantity**	**Percent with A.a.**	**Standard Error**
Periodontal Status	Aggressive Periodontitis	12	19	63.2	11.33
	Chronic Periodontitis	9	10	90.0	5.41
	No Periodontitis	37	51	72.5	7.91
Age	13-15 years old	22	26	84.6	4.18
	16-17 years old	23	37	62.2	5.55
	18-20 years old	13	17	75.5	9.09
Gender	Males	26	35	74.3	7.60
	Females	32	45	71.1	4.21

The mean percentage of *Aggregatibacter actinomycetemcomitans* relative to total bacteria (A.a ratio) were calculated based on demographic characteristics and periodontal disease status is observed in Table 3.

Table 3. Mean Percentage of *Aggregatibacter actinomycetemcomitans* Relative To Total Bacteria by Demographic Characteristics and Periodontal Disease Status in Tiznit Moroccan School Children

Variables	**Categories**	**Total Patient Count**	**A.a./ Total Bacteria (%)**	**Standard Error**
Periodontal Status	Aggressive Periodontitis	19	0.16	0.09
	Chronic Periodontitis	10	0.29	0.11
	No Periodontitis	51	0.11	0.03
Age	13-15 years old	26	0.12	0.007
	16-17 years old	37	0.14	0.05
	18-20 years old	17	0.20	0.09
Gender	Males	35	0.14	0.03
	Females	45	0.14	0.04

The percentage *A. actinomycetemcomitans* to total bacterial load increased with age (Figure 3), was similar among males and females (Figure 4), and was somewhat higher in persons with periodontitis than the controls (Figure 5).

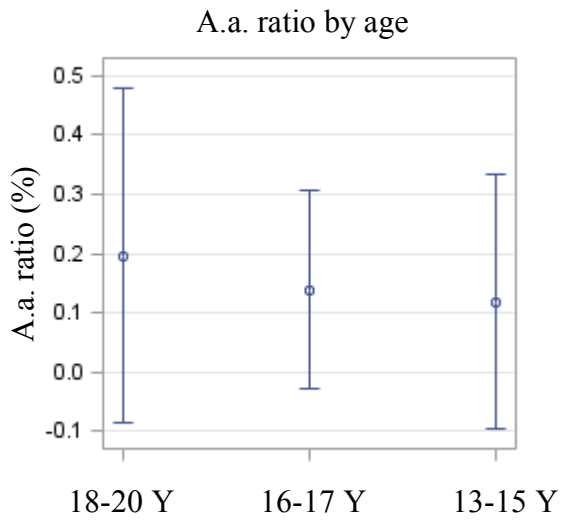

Figure 3. *A. actinomycetemcomitans* ratio by age. Percentage of *A. a.* to total bacterial load increased with age. Y= years.

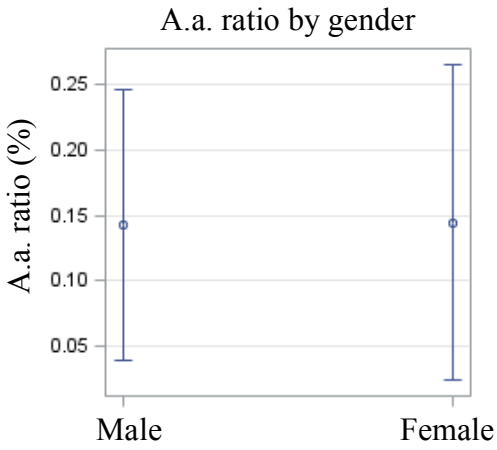

Figure 4. *A. actinomycetemcomitans* ratio by gender. The percentage of *A. a.* to total bacterial load was similar among males and females.

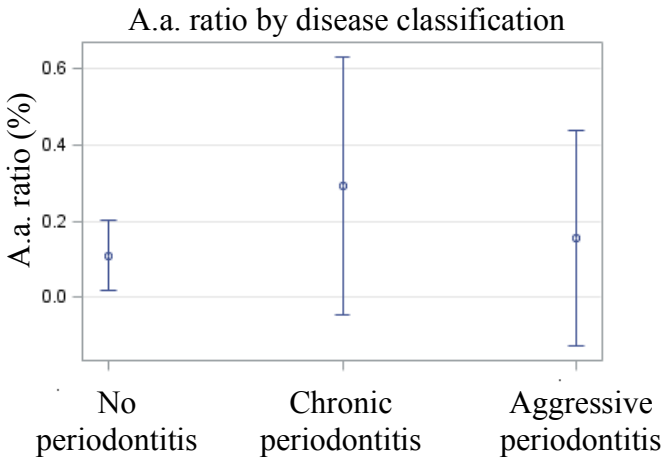

Figure 5. *A. actinomycetemcomitans* ratio by disease classification. The percentage *A.a.* to total bacterial load was somewhat higher in persons with periodontitis than no periodontitis.

Using a logistic regression analysis that included age, gender and *A. actinomycetemcomitans* ratio, it showed that only age was significantly correlated with the diagnosis of periodontitis in this population (Table 4).

Table 4. Logistic Regression Analysis of Demographic Characteristics and the Percentage of *Aggregatibacter actinomycetemcomitans* (Aa) as Predictors of Periodontitis in Tiznit Moroccan School Children. CI=confidence interval; OR=odds ratio

Variable	OR	C.I.	P
Age	2.0	1.2 - 3.4	0.01
Gender	3.5	0.6 - 19.7	0.15
Aa ratio	2.2	0.5 - 10.3	0.33

CHAPTER 4

DISCUSSION

Aggressive periodontitis is a rapidly progressive form of periodontal disease characterized by significant destruction of periodontal attachment at an early age. According to Rylev and Kilian (2008), various ethnic backgrounds have been reported with varying prevalence of aggressive periodontitis. Under Caucasian background, the prevalence of aggressive periodontitis in Finland, Switzerland, Denmark, Italy and the United States have been reported 0.1%, 0.1%, 0.1%, 0.5%, and 0.06%, respectively. In other ethnic backgrounds, studies from Japan, Chilean, Brazilian, Iraq, Kenya, Nigeria, and Saudi Arabia have been reported 0.47%, 0.32%, 2.6%, 1.8%, 0.29%, 0.8%, and 0.42%, respectively. From Haubek and colleagues (2001), their study, which included 301 Moroccan subjects aged from 14-19, reported the prevalence of aggressive periodontitis to be 7.6%.

The bacterium *A. actinomycetemcomitans* is strongly associated with aggressive forms of the disease (Henderson et al. 2010). *A. actinomycetemcomitans* is an oral commensal and opportunistist pathogen that has a distinct racial bias. In our study, the subgingival presence of *A. actinomycetemcomitans* was related to age, gender and periodontal disease status. One of the strengths of this study is having a high sensitivity and specificity quantitative real-time PCR assay, which preclude the need of viable samples and is less sensitive to the presence of other potentially dominating micro-organisms. Our group has previously validated the *A. actinomycetemcomitans*-specific primer/probe in confirming the presence of *A. actinomycetemcomitans*. From the qRT-PCR assay, we determined that a minimum of 10 copies of the DNA was sufficient to

amplify the DNA genome. Similar studies have been confirmed with Lau and colleagues (2004).

Based on age, there was a higher likelihood of subjects harboring *A. actinomycetemcomitans* when comparing subjects 18 to 20 years old with 13 to 15 years old. This finding is similar to Chahboun and colleagues' (2015) study, where they showed that the mean age in Moroccan population with localized aggressive periodontitis was 19.85 years old.

There was no difference between males versus females who harbored *A. actinomycetemcomitans.* According to Albandar (2014), the sex ratio for prevalence of aggressive periodontitis is inconsistent in the literature. According to Chahboun and colleagues (2015) study on the Moroccan population, 84.6% of females had localized aggressive periodontitis, but was not statistically significant.

Based on periodontal disease status, *A. actinomycetemcomitans* was observed 73% of all groups, with no significant difference. Chahboun and colleagues (2015) observed higher mean proportion of *A. actinomycetemcomitans* in localized aggressive periodontitis compared to chronic periodontitis. In our study, there were an uneven distribution of *A. actinomycetemcomitans* indicating a high prevalence among the Moroccan population. This is in agreement with a cross-sectional study by Chahboun and colleagues on school children in the same population (2015).

CHAPTER 5

CONCLUSION

The bacterial species *Aggregatibacter actinomycetemcomitans* is prevalent among the young school children of Tiznit, Moroccan. This study focused on three demographic characteristics, which were age, gender and periodontal disease status, to elucidate the etiopathogenesis of aggressive periodontitis. As age increased toward 18-20 years old, the odds ratio of having periodontitis was twice that of the 13-15 year old subjects (p-value = 0.01). The gender of the subjects and the *Aggregatibacter actinomycetemcomitans* ratio were not significant predictor of periodontitis.

The prevalence of *Aggregatibacter actinomycetemcomitans* was somewhat higher in subjects with periodontitis than in subjects without periodontitis, but the difference was not statistically significant. The presence and ratio of this bacterial species to the total count of bacteria in subgingival plaque explained only a small proportion of the variance in the data set, suggesting that other important etiological and risk factors remain to be determined.

CHAPTER 6

FUTURE DIRECTION

More research is required to understand the etiopathogenesis of aggressive periodontitis and the different colonization patterns in different types of periodontal diseases and in periodontally healthy subjects. A popular theory for periodontitis is dysbiosis where a low abundance of specific periodontal pathogens can coordinate a host response leading to attachment loss and alveolar bone loss (Hajishengallis et al. 2012). Microbial dysbiosis using microbiome analysis has not been thoroughly studied in aggressive periodontitis. Haubek and colleagues (2007) have also considered the possibility of competition between JP2 and non-JP2 strain that may prevent the more leukotoxic JP2 clone from causing bone loss. Our group aims to investigate the frequency of detection of the JP2 strain of *A. actinomycetemcomitans* compared to other strains of *A. actinomycetemcomitans* in subgingval dental plaque, correlate the detection frequency and levels of JP2 *A. actinomycetemcomitans* with the severity of periodontal disease status, and compare the JP2 strain with the non-JP2 strain. From the DNA extracted from this study, we plan to further examine the 2JP clone specific primer/ probe using quantitative real-time PCR assays. Understanding the abundance of the JP2 clone to non-JP2 clone is a major step forward to understanding pathogenesis and colonization pattern of aggressive periodontitis.

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