

Leukotoxicity of *Aggregatibacter actinomycetemcomitans* in generalized aggressive periodontitis in Brazilians and their family members

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ABSTRACT

Objective: The purpose of this study was to examine the leukotoxin promoter types of *Aggregatibacter actinomycetemcomitans* clones in subjects with generalized aggressive periodontitis (GAgP) and in their family members (FM). Material and Methods: Thirty-five patients with GAgP (33.9±7.1 years), 33 of their FM (22.8±11.4 years), and 41 patients with chronic periodontitis (CP) (44.1±9.4 years) were clinically analyzed using the plaque index, gingival index, probing depth (PD), and clinical attachment level (CAL). Subgingival biofilm samples were collected from four interproximal periodontal sites (>PD and >CAL) of each patient. The presence of *A. actinomycetemcomitans* and its leukotoxic clone was confirmed by polymerase chain reaction (PCR). Results: *A. actinomycetemcomitans* was observed in 23 (51.1%) GAgP patients and 16 (30.1%) CP patients. Thirty-seven (94.8%) patients showed minimally leukotoxic strains and 2 (5.1%) showed highly leukotoxic strains. In the FM group, 10 (30.3%) had aggressive periodontitis (AgP), 12 (36.3%) had CP, 11 (33.3%) were periodontally healthy or had gingivitis, and 12.2% were *A. actinomycetemcomitans* positive. Greater full mouth PD and CAL were observed in GAgP patients positive for the bacteria than those negative for it ($p < 0.05$), and the presence of *A. actinomycetemcomitans* positively correlated with GAgP (Odds ratio, 3.1; confidence interval, 1.4-7.0; $p = 0.009$). Conclusions: The presence of *A. actinomycetemcomitans* was associated with the clinical condition of GAgP, with most patients exhibiting a generalized form of the disease and minimally leukotoxic clones. Most of the relatives of GAgP patients presented either CP or AgP.

Keywords: *Aggregatibacter actinomycetemcomitans*. Leukotoxin. Periodontitis.

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a gram-negative, capnophilic coccobacillus¹³, that is strongly associated with aggressive periodontitis (AgP)¹⁴. Despite being considered a periodontopathogen, *A. actinomycetemcomitans* can frequently be detected in periodontally healthy individuals, suggesting variability in its virulence²⁰. The microorganism produces several virulence

factors that may be involved in the destruction of periodontal tissues, the most important among them being the leukotoxic activity^{8,13}.

Development of techniques to detect the genetic variability of microorganisms has allowed for the observation of genetic differences in the leukotoxin promoter region between various *A. actinomycetemcomitans* strains, which are directly correlated with their leukotoxicity³⁴. Strains that are highly leukotoxic have a deletion of 530 base pairs in the leukotoxin promoter region, while those that are

minimally leukotoxic contain the complete leukotoxin promoter region. Thus, the highly leukotoxic strains (strain JP2) can produce 10 to 20-fold more toxin than the others, providing them with the potential to interfere with the host's innate immune defense⁴. Some studies have shown that individuals harboring highly leukotoxic strains have a higher risk of AgP^{5,15,16}, resulting in further progression of clinical attachment level (CAL)^{17,18}. However, most of these studies assessed patients with localized AgP^{5,15,28,29,35}, while only a few evaluated generalized AgP (GAgP)^{8,15,16}.

Some studies have examined the transmission of *A. actinomycetemcomitans* between family members^{11,17,26,28}. Besides genetic predisposition, transmission of *A. actinomycetemcomitans* can be considered a favorable hypothesis to explain the familial aggregation of individuals with AgP^{15,18}. Patients who carried the JP2 clone were from families in which at least one other individual exhibited the disease⁵.

AgP is more prevalent in subjects of African ancestry, and it is hypothesized that besides host susceptibility, specific *A. actinomycetemcomitans* clones with enhanced leukotoxic activity are associated with the disease in these populations⁸. These particular clones could have been disseminated worldwide over centuries and might be related to the onset of AgP in different ethnic groups^{10,15}. A study has shown that, in Brazil, skin color is not directly related to genomic ancestry²³; the immense heterogeneity of the Brazilian population may make it difficult to predict race-related diseases or microorganisms. Indeed, the present study evaluated individuals from one of the three vast metropolitan areas of northeastern Brazil. Distinct from other populations that are predominantly African-American, this population is miscegenous, a blend over the years of white European, African-American, and some indigenous populations, with no clear predominance of any race³.

Therefore, the evaluation of the periodontal and microbiological status of individuals with AgP in this population is relevant. The purpose of this study was to examine the leukotoxin promoter types of *A. actinomycetemcomitans* clones in subjects with GAgP and in their family members.

MATERIAL AND METHODS

Selection of subjects

This study was conducted in 35 patients with GAgP (test group) and one to four of their immediate family members (FM). A total of 33 relatives of 26 GAgP patients were included. A group of 41 patients with chronic periodontitis (CP) were used as the control group. Subjects were selected among patients who sought care at the Periodontology

Clinic, Faculty of Pharmacy, Dentistry and Nursing, at the Federal University of Ceará, Brazil.

AgP patients were classified according to the clinical criteria suggested by the American Academy of Periodontology (AAP)³⁰ as follows:

Localized AgP: interproximal attachment loss on at least two permanent teeth, involving no more than two teeth other than the incisors and the first molars;

GAgP: generalized interproximal attachment loss affecting three permanent teeth other than the first molars and incisors. The disease affects systemically healthy individuals and results in rapid attachment loss and bone destruction;

CP patients were also classified according to the criteria of the AAP^{14,21,22} as follows:

Slight to moderate destruction: characterized by the presence of periodontal sites with a probing depth (PD) of up to 6 mm and CAL of up to 4 mm;

Advanced destruction: characterized by the presence of a PD greater than 6 mm and CAL greater than 4 mm.

Exclusion criteria were as follows: periodontal treatment within the previous 6 months, antibiotic therapy within the previous 3 months, and systemic alterations that could have interfered with the periodontal conditions. The relatives of GAgP patients should have at least the first permanent molars.

The research protocol was approved by the Ethics Committee, Federal University of Ceará, Brazil. All participants or guardians were informed about the purpose of the study and provided written consent.

Clinical measurements

Clinical measurements were made on all the completely erupted permanent teeth, except the third molars, using a periodontal probe (PCP-UNC 15, Trinity, São Paulo, SP, Brazil). The following parameters were evaluated: plaque index¹, gingival index¹, PD, and CAL. PD and CAL were taken at six sites *per* tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual). A single examiner evaluated all the clinical periodontal parameters, and the measurement reproducibility was calculated by using the intraclass correlation coefficient (ICC) for PD and CAL. The agreement between replicate measurements was high (ICC>0.80).

Microbiological sampling

The supragingival plaque was removed with curettes and sterile cotton pellets, and the area was isolated with sterile cotton rolls. Subgingival plaque samples were collected by means of two sterile paper points (size 35) at each site, for 20 seconds (Dentsply Maillefer 35, Dentsply, Rio de Janeiro, RJ, Brazil) and stored separately. They were collected from four proximal tooth sites with greater CAL and increased PD. In FM without

periodontal disease, the subgingival plaque samples were collected from four proximal sites of the first permanent molars or incisors. All the samples were immersed in microtubes containing 1 mL of Ringer's sterile solution⁹ and stored at -80°C until they were processed.

Microbiological evaluation

The microtubes containing the samples were thawed on ice. The bacterial cells or suspension were dispersed by vortexing at maximal setting for 1 min and centrifuged at 12,000x g for 10 min. Genomic DNA was extracted from the pellet (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA, USA) and 20 µL aliquot of the resultant supernatant was added to 30 µL of the reaction mixture containing 25 µM polymerase chain reaction (PCR) buffer (Promega Corporation, Madison, WI, USA), 25 µM MgCl₂ (Promega Corporation, Madison, WI, USA), 0.2 µM dNTP mix (Promega Corporation, Madison, WI, USA), 1.25 U Taq polymerase (Promega Corporation, Madison, WI, USA), and 100 ng of each primer (Invitrogen, São Paulo, SP, Brazil), resulting in a final volume of 50 µL. Negative and positive controls were included in each reaction.

First, PCR with specific primers (5'-GGACTAYAGGGTATCTAAT-3'; 5'-AGAGTTTGATCMTGG-3') for the 16S ribosomal DNA (16S rDNA) was performed to confirm the presence of bacterial DNA³³. Subsequently, the samples were evaluated by PCR with specific primers for the presence of *A. actinomycetemcomitans* (5'-AAACCCATCTCTGAGTTCTTCTTC-3'; 5'-ATGCCAACTTGACGTTAAAT-3')². Once confirmed, the samples were analyzed for the presence of the leukotoxin operon using the primers ltx3 (5'-GCCGACACCAAAGACAAAGTCT-3') and ltx4 (5'-GCCATAACCAAGCCACATAC-3') as previously described²⁴. The primers resulted in amplicons of 686 and 1.216 bp from strains of the JP2 clone and non-JP2 clone respectively, used as controls.

Amplification (Biocycler, Biosystems, Curitiba, PR, Brazil) of the 16S rDNA and *A. actinomycetemcomitans* DNA was performed at an initial cycle at 94°C for 10 min, followed by 30 cycles at 96°C for 30 s, 55°C for 30 s and 72°C for 30 s, with the final extension at 72°C for 10 min¹⁵. For the leukotoxin promoter region, amplification was performed at an initial cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, followed by a final extension at 72°C for 8 min²⁴.

The amplification products were analyzed by electrophoresis on an agarose gel and stained with ethidium bromide (10 µg/mL). A 1.5% gel was used to assess the 16S rDNA and the presence of *A. actinomycetemcomitans*, while a 2.0% gel was used to assess the *A. actinomycetemcomitans* clonal diversity. The gels were stained with ethidium

bromide and photographed (Canon Powershot A640, Canon, USA) under ultraviolet light (LTA/LTB GE, Loccus Biotecnologia, São Paulo, SP, Brazil).

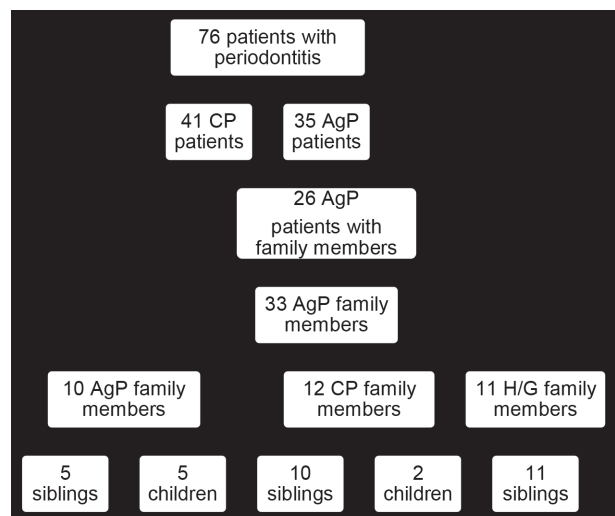
PCR was repeated three times for each sample. JP2 (*A. actinomycetemcomitans* JP2) and non-JP2 (*A. actinomycetemcomitans* HK1605) reference strains were used to determine the detection levels of the PCR method. The strains were serially diluted 10-fold, followed by PCR as with the samples. The detection limits for *A. actinomycetemcomitans* and leukotoxin promoter region primers were approximately 10² and 10³ colony-forming units (CFU) of the microorganism, respectively. These detection levels were similar to that described previously^{2,24}.

Statistical analysis

The clinical data set was tested for normality using the Kolmogorov-Smirnov test. Comparisons between three groups (GAgP, CP, and RM) were made by ANOVA or Kruskal-Wallis. Comparisons between two groups (GAgP and CP) were made by the unpaired *t*-test or Mann-Whitney U test. Odds ratio (OR) analysis was used to assess the relationship between the presence of *A. actinomycetemcomitans* and the occurrence of AgP. A difference of *p*<0.05 was considered statistically significant. All data analysis was performed using the GraphPad InStat software (Graph Pad Software Inc, San-Diego, CA, USA).

RESULTS

The sample comprised 109 individuals divided into three groups divided into GAgP, FM, and CP. Among the FM group, 10 had AgP, 12 had CP, and 11 were periodontally healthy or had gingivitis (Figure



AgP=aggressive periodontitis; CP=chronic periodontitis; H/G=periodontally healthy or gingivitis

Figure 1- Flowchart of the study population

1). Among all the subjects with AgP (n=45), only one subject in the FM group presented localized AgP - a 10-year-old boy, while others presented with GAgP.

Clinical data of the subjects are summarized in Table 1. A statistically significant difference between the ages of all groups was observed. The GAgP patients presented signs of more severe periodontal destruction and inflammation, as observed from the significantly higher mean PD than the CP and FM groups. CAL was also higher in the GAgP group followed by the CP group (Table 1).

In Table 2, all subjects, including the FM, were grouped based on their periodontal condition and the presence of *A. actinomycetemcomitans*.

Twenty-three (51.2%) GAgP and 16 (30.2%) CP patients presented the bacteria. The presence of *A. actinomycetemcomitans* was associated with the clinical condition of AgP (OR, 3.1; confidence interval, 1.4–7.0, $p=0.009$). Among the FM group, 3 patients with AgP and 1 with CP were positive for the bacteria. In healthy/gingivitis patients, the microorganism was not detected. Full mouth PD and CAL in GAgP patients positive for *A. actinomycetemcomitans* were higher than those in patients negative for it ($p<0.05$).

Among all the patients who were positive for *A. actinomycetemcomitans*, 37 (94.8%) exhibited minimally leukotoxic strains and 2 (5.1%), a man and a woman aged 37 and 31 years old, respectively,

Table 1- Clinical periodontal parameters (mean± standard deviation) of subjects with generalized aggressive periodontitis (GAgP), chronic periodontitis (CP), and their family members (FM)

Parameters	GAgP	FM	CP
N	35	33	41
Age (years)	33.9±7.1	22.8±11.4	44.1±9.4*
Number of teeth	25.0±2.3	24.2±5.3	22.0±3.5
PD fm (mm)	3.7±0.9*	2.4±0.5	2.9±0.5
CAL fm (mm)	4.3±1.2***	2.0±1.4	3.8±1.1
GI fm (%)	14.9±10.5	10.7±13.5	20.6±19.0**
PI fm (%)	32.3±19.7	24.8±19.6	33.5±14.0**
PD ss (mm)	9.8±2.1*	5.3±2.0	8.2±2.3
CAL ss (mm)	10.7±2.8***	4.6±3.3	9.4±2.9

*Statistically significant difference between the groups

**Statistically significant difference between the CP and FM groups

***Statistically significant difference between the groups, except GAgP and CP

PD: probing depth; CAL: clinical attachment level; GI: gingival index; PI: plaque index; fm: full mouth; ss: sampled sites for microbial analysis

Table 2- Clinical periodontal parameters (mean± standard deviation) and the presence of *A. actinomycetemcomitans* in all subjects with aggressive periodontitis (AgP), chronic periodontitis (CP) or periodontally healthy/gingivitis (H/G)

Parameters	AgP		CP		H/G	
	Aa+	Aa-	Aa+	Aa-	Aa+	Aa-
N	23*	22	16	37	-	11
%	51.2%	48.8%	30.2%	69.8%	-	100%
Age (years)	33.5±7.5	28.7±10.0	43.1±9.9	41.1±10.6	-	12.7±3.3
PD fm (mm)	3.8±0.9**	3.2±0.7	2.9±0.5	2.7±0.5	-	1.9±0.2
CAL fm (mm)	4.4±1.3**	3.6±0.9	3.7±1.1	3.5±1.2	-	-
PD ss (mm)	9.7±2.6	8.5±2.0	8.1±2.0	7.5±2.4	-	3.3 ± 0.9
CAL ss (mm)	10.5±3.5	9.1±2.5	9.4±2.8	8.4±3.0	-	-

*Statistically significant association (odds ratio, 3.1; confidence interval, 1.4–7.0; $p=0.009$). Groups that exhibited highly leukotoxin strains

** Statistically significant difference between AgP

PD: probing depth; CAL: clinical attachment level; fm: full mouth; ss: sampled sites for microbial analysis; Aa+: presence of *A. actinomycetemcomitans*; Aa-: absence of *A. actinomycetemcomitans*

exhibited highly leukotoxic strains with GAgP. The woman was screened subsequent to her brother being diagnosed with GAgP.

DISCUSSION

This study aimed to evaluate the presence of *A. actinomycetemcomitans* along with high or low leukotoxicity clones in GAgP patients and their close relatives. The results indicate an association between the presence of *A. actinomycetemcomitans* and the occurrence of GAgP, since 51.1% of patients with this disease (mean age: 33.5 years) were positive for the microorganism. Several studies have described the occurrence of *A. actinomycetemcomitans* in periodontal disease conditions similar to GAgP. This microorganism was found in 55% of patients (age range: 14-52 years)²⁷, and in 20% of adults (range 25-35 years)¹⁹.

However, only two patients with GAgP presented the highly leukotoxic *A. actinomycetemcomitans* clone, while 21 patients (91.3%) with the disease harboring the microorganism exhibited minimally leukotoxic clones. In accordance with previous studies^{8,12,15,16,20,29} they also presented periodontal destruction. Surprisingly, both the patients who harbored the leukotoxic clone were over 30 years of age. Highly leukotoxic clones are strongly associated with localized AgP^{5,15,16,35}. Since this disease is associated with a young age, it could be hypothesized that the JP2 clone has a predilection for younger subjects. Patients harboring the highly leukotoxic strains were much younger (mean age: 12.7 years) than those harboring the minimally toxic *A. actinomycetemcomitans* (mean age: 25.5 years)³⁵. It was also observed that subjects with a mean age of 13.9 years were more frequently infected by the JP2 clone, while subjects with a mean age of 35.4 did not show the infection¹⁵; these results were corroborated by the observations of our present study (mean age: 33.9 years).

Highly leukotoxic strains were not found in patients with periodontitis with mean age of 33.6 years²⁰. In another study, 12 out of 94 *A. actinomycetemcomitans*-positive individuals exhibited the JP2 clone, but only one patient was over 30 years⁸. Among 131 Moroccan schoolchildren positive for *A. actinomycetemcomitans*, 11.5% presented the JP2 clone¹⁶. However, their age ranged between 14 and 19 years, much younger than those with AgP evaluated in our current study. Two specific studies revealed the prevalence of the leukotoxic clones in Brazil; both studies associated the young age of the subjects with the prevalence of highly leukotoxic clones of this microorganism^{10,29}.

Results from cross-sectional^{10,15} and cohort studies^{5,35} have demonstrated that the JP2 clone can serve as a risk marker for localized AgP in children

and adolescents. However, in the current study, the two JP2 clone positive patients were older than what has been described by other authors. Infection with the JP2 clone may have resulted in disease onset at a young age, but it may not necessarily be required for the maintenance of the disease¹⁷. One explanation for this is that for a majority of the patients evaluated in this study, their clinical periodontal examination was their first ever dental appointment. Lack of treatment resulted in the highly leukotoxic clone being present even at a later age. Another explanation is that the JP2 infection affects the immune tolerance, while other bacteria in the biofilm can sustain and maintain the inflammatory process, which manifests clinically as attachment and bone loss around the teeth. The JP2 clone may be constantly present below the limit of detection, while maintaining the activity of bone resorption¹⁷. On the other hand, we can hypothesize that the clones can cause periodontal destruction in older subjects as well. However, being a cross-sectional investigation, estimating the onset of the periodontal destruction is difficult.

The microbiological analyses of this study were performed by PCR that only provides information on the presence or absence of the microorganism's DNA, unlike the quantitative data obtained by real-time PCR. However, the detection level of *A. actinomycetemcomitans* and its leukotoxin operon was determined to be around 10² and 10³ CFU, respectively. The vast majority of studies on *A. actinomycetemcomitans* leukotoxin operon have been performed using the PCR technique^{4,8,12,14,16,17,29}, which has been found to be highly reproducible and specific for analysis of the JP2 genotype^{17,24}.

Another limitation of this study is the lack of a healthy group not related familiarly to the AgP patients. However, it has been shown that in the gingival sulcus of patients without clinical signs of inflammation there is a clear predominance of gram-positive bacteria^{7,25}. Even when a more sensitive and specific technique was used (i.e. Human Oral Microbe Identification Microarray) and more subgingival sites sampled (14 teeth *per* patient), *A. actinomycetemcomitans* was not present among the species most commonly found in healthy subjects⁷.

In the present study, almost all the AgP patients were adults with a generalized disease and exhibited minimally leukotoxic *A. actinomycetemcomitans* clones. These patients, when younger, may have been colonized by highly leukotoxic strains and presented a localized disease, which may have begun with periodontal destruction. This is consistent with the hypothesis that there has been a change in the virulence of *A. actinomycetemcomitans* over time^{10,15}. Haubek, et al.¹⁸ (2007) suggested that highly leukotoxic *A. actinomycetemcomitans* strains comprise a single clone. But, regarding minimally

leukotoxic strains, Wahasugui, et al.³² (2013) found that such strains belonged to 8 different biotypes.

A recent study in Sudanese adolescents (14-19 years) with AgP showed that the *A. actinomycetemcomitans* JP2 clone was not detected in any of the subgingival plaque samples, while non-JP2 clones were detected in 70.6% of patients with the disease. It can also be suggested that the JP2 clone does not show a general dissemination pattern among various African populations as previously believed¹². The clone dispersed in America during the slave trade although not from all heritages; it mainly originated from the Mediterranean part of Africa¹⁸. This can help explain why some patients with the same pattern of disease do not present the JP2 clone. In the present study we evaluated a miscegenous population composed mainly of mixed afrodescendants, Caucasian Europeans and indigenous Brazilians, with no predilection of race. Even during the slave trade, identifying the African region that the subjects originated from was difficult. On arrival, subjects of different origins were sent to different regions of Brazil, which might explain the vast extent of mixed heritage among the Brazilian afrodescendants. A study has shown that in Brazil the race of a person and their genomic ancestry are not correlated²³. This genetic variability in the Brazilian population may result in differences in susceptibility for colonization by certain microorganisms. This may also explain the low frequency of the highly leukotoxic *A. actinomycetemcomitans* clones found in these subjects.

Another point that can be identified as important in the etiology and spread of the disease is familial transmission of *A. actinomycetemcomitans*^{11,15,28}; 64% of the FM of AgP patients carried the microorganism¹¹. On assessing Brazilian families of individuals with localized AgP, it was observed that 41.2% of parents and 58% of siblings were also colonized by this bacteria and that 5% had the JP2 clone²⁸. Another study⁶ observed that in FM with localized AgP, the parents were diagnosed with CP and GAgP. Both parents and children of the subjects were always colonized by minimally leukotoxic *A. actinomycetemcomitans* clones⁶. In a Brazilian indigenous population, all *A. actinomycetemcomitans* strains were non-JP2 clones, even those originated from patients with periodontitis³¹.

The FM group consisted of 18 children and 15 siblings of AgP patients. With regard to their periodontal status, 30.3% had AgP, 36.3% had CP, and 33.3% were periodontally healthy or had gingivitis. Among them, 12.2% were positive for *A. actinomycetemcomitans*, 10 had AgP, and 12 had CP. Only one (3%) exhibited a highly leukotoxic clone and three (9%) had minimally leukotoxic clones. Familial aggregation is one important characteristic of the various forms of AgP⁶. There is a consensus

among researchers that genetic factors, with the possible influence of the environment, play a role in this disease⁶. In the present study, most of the close relatives had manifestations of AgP (30.3%) and CP (36.3%). By evaluating periodontal status and intrafamilial transmission of AgP, it was found that 38% of FM of AgP had some form of periodontal disease, 19% had AgP and 19% had CP¹¹. According to these investigators, the parents and siblings of a patient with AgP who are positive for *A. actinomycetemcomitans* may exhibit increased susceptibility to destructive periodontal disease¹¹.

CONCLUSION

In conclusion, the presence of *A. actinomycetemcomitans* was associated with GAgP. Most subjects with this disease harboring *A. actinomycetemcomitans* exhibited minimally leukotoxic clones, while most of the relatives of AgP patients presented either CP or GAgP.

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