

Detection of periodontal pathogens in a Uruguayan population with aggressive periodontitis using conventional and molecular methods

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DOI: 10.22592/ode2018n32a9

Abstract

Periodontal diseases are a major health problem affecting tooth-supporting tissues. Among them, aggressive periodontitis is characterized by rapid progression, family aggregation, systemically healthy patients (¹), and is subdivided into localized and generalized according to the extent of the disease.

Microbiota plays a major role in the etiopathogenesis of these diseases, including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* and *Fusobacterium nucleatum*.

The aim of this work was to study the prevalence of these microorganisms in 50 Uruguayan patients with aggressive periodontitis. Detection was conducted with conventional bacteriological techniques and PCR. In the generalized disorders, a higher prevalence of *F. nucleatum* and *P. intermedia* was observed, although *P. gingivalis* and *T. forsythia* were also important. In the localized disorders, *P. intermedia*, *F. nucleatum* and *A. actinomycetemcomitans* were the main ones. A similar flora to other geographical locations was present in our country.

Keywords: prevalence, microbiota, aggressive periodontitis.

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Introduction and background

Aggressive periodontitis is a destructive periodontal disease that can affect the health of the primary or permanent dentition and, over a short period, can lead to loss of tooth-supporting tissue. The term aggressive periodontitis was approved at the *Workshop for the Classification of the Periodontal Diseases and Conditions* organized by the *American Academy of Periodontology* (AAP) in 1999 to define a group of destructive periodontal diseases with a rapid progression rate, a classification that was valid at the time this study was conducted ⁽²⁻⁴⁾.

Aggressive periodontitis can appear in its localized form, localized aggressive periodontitis (LAP) or in its generalized form, generalized aggressive periodontitis (GAP) depending on the extent of periodontal destruction ⁽¹⁾.

According to the World Health Organization (WHO), severe periodontal disease, which may result in tooth loss, affects 15–20% of middle-aged (35–44 years) adults ⁽⁵⁾. As for the aggressive form, Albandar mentions that the prevalence of aggressive periodontitis is variable if different locations are considered, although many of these studies used different variables and methodologies. The prevalence of localized aggressive periodontitis varies considerably between continents, and differences in race/ethnicity seem to be a major contributing factor. Several studies have consistently shown that aggressive periodontitis is more prevalent in Africa and Afrodescendants and less prevalent in the Caucasian population of Europe and North America. The prevalence among children and young adults is higher in older than in younger age groups ⁽⁶⁾. The prevalence of aggressive periodontitis in Europe is low, between 0.1% and 0.2%, and prevalence is higher, between 3% and 10%, in Brazil and the United States ⁽⁷⁾. Studies show that in Latin America the preva-

lence of aggressive periodontitis is higher in this region than in industrialized countries. Prevalence ranges from 0.3% to 4.5%, and the localized form is the least prevalent one ⁽⁸⁾. Worldwide, the prevalence of LAP is lower than 1%, and the prevalence of GAP is 0.13% ⁽⁹⁻¹⁰⁾.

Aggressive periodontitis is a disorder of multifactorial origin, such as host response, genetic factors and the subgingival microbiota ^(6,9,11).

Some of the bacterial pathogenic species that have been linked to the development of aggressive periodontitis are: *Aggregatibacter actinomycetemcomitans* (*Aa*) ^(2,12) and *Porphyromonas gingivalis* (*Pg*) ⁽¹³⁾, the first being particularly important in the localized form ⁽¹⁴⁻¹⁵⁾ and the second one in the generalized form ⁽¹⁵⁻¹⁶⁾, but another group of periodontopathogens, including *Tannerella forsythia* (*Tf*), *Prevotella intermedia* (*Pi*) ⁽¹⁻¹⁵⁾ and *Fusobacterium nucleatum* (*Fn*) ⁽¹⁵⁾ have been linked to it.

Studies have been conducted in Uruguay using traditional bacterial identification methods, but so far, there are no studies concerning the levels of simultaneous occurrence of the periodontal pathogens mentioned above in cases of aggressive periodontitis. In addition, traditional bacteriological techniques have certain limitations, such as maintaining the viability of the sample, bacterial growth time (7 to 15 days), as well as the difficulty of bacterial growth since they respond to microorganisms that require specific nutrients and are, mostly, obligate anaerobes. Bacterial recognition involves performing additional tests, such as biochemical tests, to identify the species ⁽¹⁷⁾.

The introduction of molecular analysis methods, such as polymerase chain reaction (PCR), has expanded and simplified studies of the composition of oral microbiota of patients with periodontal pathologies, improving their sensitivity, specificity and efficiency ⁽¹⁸⁾.

In this work, we analyzed the presence of five periodontal pathogens: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* and *Fusobacterium nucleatum* in the periodontal pockets of Uruguayan patients from the Clinic of Periodontics of the School of Dentistry, Universidad de la República, Uruguay. Microbiological identification techniques included traditional bacteriological techniques, as well as techniques to identify the genetic material present through PCR⁽¹⁷⁾.

In this study, we were able to identify the microorganisms that are present and more prevalent in cases of aggressive periodontitis in Uruguay, both in the generalized and localized forms, and to determine whether the prevalence rates are the same as those found in other geographical locations.

It also allowed us to correlate traditional bacteriological techniques and molecular techniques.

Work methodology

We selected 50 patients from the Clinic of Periodontics of the School of Dentistry with aggressive periodontitis, without prior periodontal treatment and who signed an informed consent. Patients with diabetes, arthritis, ulcerative colitis, HIV, cancer and cardiovascular disease, pregnant women and those who had been treated with antibiotics and/or anti-inflammatories three months before the study were excluded. The Ethics Committee of the School of Dentistry, UdelaR, approved the design of the study following the regulations of the MERCOSUR and the Helsinki Declaration on research involving human subjects.

Supragingival plaque was taken to obtain the samples, using sterile gauze to avoid bleeding. Medium sterile paper points (N° 25) were placed deeply into the pocket and left for 15 seconds

to collect the samples. They were then placed in 1.5 ml of RTF (*Reduced Transport Fluid*)⁽¹⁸⁾. Each sample included eight paper points from four sites selected in each quadrant.

We carried out the bacteriological diagnosis using the traditional methodology for *A. actinomycetemcomitans* and for the pigmented anaerobes *P. gingivalis* and *P. intermedia*. We also conducted the identification by PCR for *T. forsythia* and *F. nucleatum*. Samples were processed in the Microbiology Laboratory of the School of Dentistry, stirred vigorously for 45 to 60 seconds, and then serial dilutions in RTF were prepared. 100 µl was taken from the sample and placed in an *Eppendorf* tube with 900 µl more of RTF, thus achieving a 1:10 dilution. 100 µl was streaked, expanding the sample with a glass rod on a TSVB plate: tryptic soy serum, bacitracin (75 µg/ml), vancomycin (5.0 µg/ml), 10 ml of horse serum (10%) to isolate *A. actinomycetemcomitans*. The plates were incubated in a candle jar (environment rich in carbon dioxide) for 7 days at 37°C. In addition, 100 µl was taken from the RTF original sample and 1:10, 1:100 and 1:1000 dilutions in RTF were obtained. Then, 100 µl of the latter two was streaked in base agar medium with blood with menadione and hemin, and incubated for 14 days at 37°C in absolute anaerobiosis to recover anaerobes (*Porphyromonas* and *Prevotella*).

From the sample in RTF, 100 µl samples were taken, in addition to the original sample, stored at -30°C and then processed using the PCR technique. The following wild strains were used as positive controls: *Aggregatibacter actinomycetemcomitans* (ATCC 29522), *Porphyromonas gingivalis* (BAA-308), *Prevotella intermedia* (ATCC 25611), *Tannerella forsythia* (ATCC 43037) and *Fusobacterium nucleatum* (ATCC 25586).

Identification by culture

Presumptive colonies of *A. actinomycetemcomitans* were identified given the presence of a star-like structure inside the colonies observed using a stereoscopic magnifying glass (Fig. 1), Gram stain technique, catalase test (+) and MUG negative (4-Methylumbelliferyl- β -D-galactoside) to study lactose fermentation.



Fig. 1: Colony of *A. actinomycetemcomitans*

Pigmented anaerobic bacteria (*Porphyromonas* and *Prevotella*) were identified by pigment production (Fig. 2). They were also observed under ultraviolet light (360 nm), for differentiation based on fluorescence, in which colonies of *P. gingivalis* do not fluoresce and colonies of *P. intermedia* do (Fig. 3). To avoid any doubt, the identification was performed with biochemical tests for anaerobes using the API® kit.



Fig. 2: Colonies of pigmented bacteria in selective medium for obligate anaerobes.

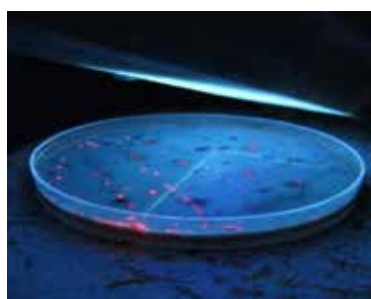


Fig. 3. Fluorescence in pigmented anaerobes
Fluorescent colonies of *Prevotella intermedia*
under ultraviolet light

A control group of 10 selected patients who did not have periodontitis was created to validate the study. As a result, no growth was obtained in 8 out of the 10 cases in the selective medium for *A. actinomycetemcomitans*. The remaining two did show growth, but it was not *A. actinomycetemcomitans*. As for pigmented anaerobes, growth was obtained in 4 out of the 10 cases, but with a low count.

Identification by PCR

Extraction of microbial genomic DNA

The samples were defrosted and homogenized for 30 seconds rigorously with a vortex. The content was then transferred to another *Eppendorf* tube and centrifuged at 13500 rpm for 3 minutes. The supernatant was discarded and the pellet was suspended again by pipetting in 500 μ l of RTF for 5 minutes at 100°C. They were then placed in ice for 5 minutes and then centrifuged at 13500 rpm for 5 minutes at 4°C. Finally, the supernatant with the microbial genomic DNA was taken and stored at -30°C until the PCR was performed.

The genome of each wild control strain was extracted using the ZymoBead™ Genomic DNA Kit (as described by the manufacturer). DNA concentration (ng/ μ l) was quantified by NanoDrop 2000.

The NCBI (*National Center of Biotechnology Information*) database was used to design the primers in order to search for and select 16S rRNA sequences to be targeted by the specific primers for *T. forsythia* (GenBank: AP013045.1) and *F. nucleatum* (GenBank: MH078456.1). Bibliographic information of studies with validated oligonucleotides for the species was also used (19).

PCR was performed with each pair of oligonucleotides using the genomes extracted from the wild strain. The primers used to detect bacterial species *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia* and *F. nucleatum* are shown in Table 1. The conditions of the multiplex PCR used for detection of these microorganisms are shown in Tables 2 and 3.

Table 1. Design of primers for detection of bacterial species present in periodontal samples

Primers (5'-3')	Species	Length (bp)
CGTGCCAGCAGCCGCGTAATACG	<i>P. gingivalis</i>	527
CTTTGCACATCAGCGTCAGTACATCCCAAGG	<i>P. intermedia</i>	163
TCCGCATACGTTGCGTGCACTCAAG	<i>A. actinomycetemcomitans</i>	253
TACATAGAAGCCCCGAAGGAAGACG		
GATGCTCCTGCTGGGTCTGTT	<i>T. forsythia</i>	394
CCGACACTTCGGATCGTTTAG		
AGGCGATGATGGGTAGCC	<i>F. nucleatum</i>	214
AGCCGTCACTTCTCTGTTG		

Table 2. Conditions of the multiplex PCR for detection of *T. forsythia* and *F. nucleatum*

35 cycles	Cycling	1X KCl buffer (Bioron) supplemented with 1 mM of MgCl ₂ , 0.5 uM of dNTPs, 0.5 uM of each primer, 1.2 units of de High Taq (Bioron) and 40-100 ng of DNA.
	95°C for 2 minutes	
	94°C for 30 seconds 57°C for 1 minute 72°C for 2 minutes	
	72°C for 5 minutes	

Table 3. Conditions of the multiplex PCR for detection of *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans*

30 cycles	Cycling	1X KCl buffer (Bioron) supplemented with 1.5 mM of MgCl ₂ , 0.5 uM of dNTPs, 0.5 uM of each primer, 2.5 units of de High Taq (Bioron) and 40-100 ng of DNA.
	94°C for 10 minutes	
	94°C for 1 minute 70°C for 1 minute 72°C for 1 minute	
	72°C for 10 minutes	

The results of amplifications (bands) were viewed in 1.2% agarose gel, 0.5X TBE (Tris-borate-EDTA) buffer, 5 µl of GoodView, 2 µl of Cyan/Orange loading buffer; 2–5 µl of the

PCR product for 2 h at 80 V. They were also photographed using a digital camera placed over an ultraviolet (UV) light transilluminator (Figs. 4 and 5).



Fig. 4 Detection of periodontal pathogens Tf=*Tannerella forsythia* (394 bp) and Fn = *Fusobacterium nucleatum* (214 bp) by classic PCR. Weight marker (Bioron # 304005). Fragment size (bp): 1000, 900, 800, 700, 500, 400, 300, 200.



Fig. 5 Detection of periodontal pathogens Pg = *Porphyromonas gingivalis* (527 bp), Aa = *Aggregatibacter actinomycetemcomitans* (253 bp) and Pi = *Prevotella intermedia* (163 bp) by classic PCR. Weight marker (Bioron # 304005). Fragment size (bp): 1000, 900, 800, 700, 500, 400, 300, 200.

Statistical analysis

Descriptive statistics were applied using absolute frequencies in percentages. The populations were compared using the Student's T-Test with a significance level of 0.05.

Results

The most prevalent microorganisms in the cases of aggressive periodontitis were *P. intermedia* and *F. nucleatum*. They were present in 93% and 83% of the patients, respectively. The next one in incidence was *A. actinomycetemcomitans*, a microorganism that is strongly correlated with aggressive periodontitis^(9,11-12,16). *A. actinomycetemcomitans* appeared in around 70% of the patients studied (Chart 1).

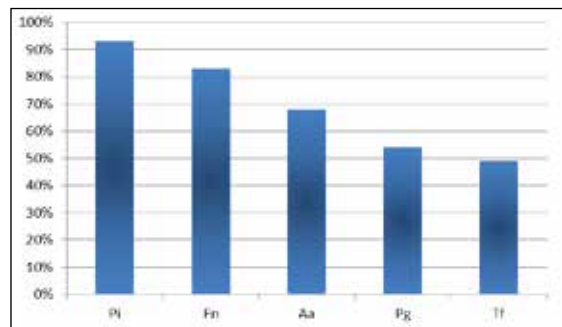


Chart 1: Prevalence of periodontal pathogens in aggressive periodontitis

**Pi=Prevotella intermedia 93%,
Fn=Fusobacterium nucleatum 83%,
Aa=Aggregatibacter actinomycetemcomitans 68%,
Pg=Porphyromonas gingivalis 54%,
Tf=Tannerella forsythia 49%**

When distinguishing between localized and generalized aggressive periodontitis, the microbiota is differentiated by emphasizing the

different microbiological etiopathogenesis of both forms. Of all the cases of aggressive disease studied, 60% were localized cases and 40% generalized cases. This is why in an analysis of the microbiota of aggressive cases of the disease without distinguishing between localized and generalized forms, prevalence percentages are conditioned by the larger number of localized cases.

Generalized aggressive periodontitis

Although *F. nucleatum* was a microorganism with a high prevalence in both forms, it mainly stood out in the cases of generalized aggressive disease, as it was present in almost 95% of the patients.

In the cases of the generalized form, the next microorganism in prevalence was *P. intermedia* (around 90%), followed by *P. gingivalis* in 75% of the samples and by *T. forsythia* in 63% of them. Moreover, although *A. actinomycetemcomitans* was recovered in several patients, it was the least prevalent of the microorganisms studied, being present in about 56% of cases (Chart 2).

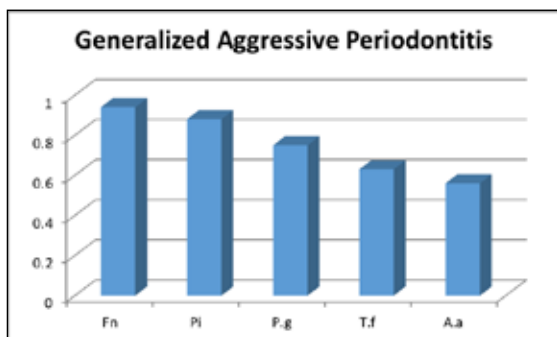


Chart 2: Prevalence of periodontal pathogens in generalized aggressive periodontitis

**Fn = *Fusobacterium nucleatum* 94%,
Pi = *Prevotella intermedia* 88%,
Pg = *Porphyromonas gingivalis* 75%,
Tf = *Tannerella forsythia* 63%,
Aa = *Aggregatibacter actinomycetemcomitans* 56%**

Localized aggressive periodontitis

As for the localized form, the most prevalent microorganism was *P. intermedia*, which was present in 96% of patients, followed by *F. nucleatum* and *A. actinomycetemcomitans*, both with a prevalence of around 75%. It is worth noting that there was a higher prevalence of *A. actinomycetemcomitans* than in the generalized form of the disease. This is consistent with the relevance that this microorganism has in localized cases of the disease⁽¹³⁻¹⁵⁾. Finally, the microorganisms *P. gingivalis* and *T. forsythia* had the lowest prevalence, being found in 40% of the patients studied (Chart 3).

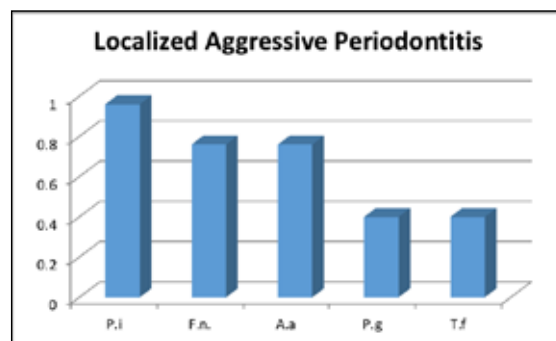


Chart 3: Prevalence of periodontal pathogens in localized aggressive periodontitis
Pi=Prevotella intermedia 96%
Fn=Fusobacterium nucleatum 76%
Aa=Aggregatibacter actinomycetemcomitans 76%
Pg= Porphyromonas gingivalis 40%
Tf= Tannerella forsythia 40%

Comparison of microbial prevalence between GAP and LAP

To facilitate the comparison, the respective prevalences of the different pathogens in the generalized and localized forms in our studies with Uruguayan patients are presented in a consolidated form below (Chart 4).

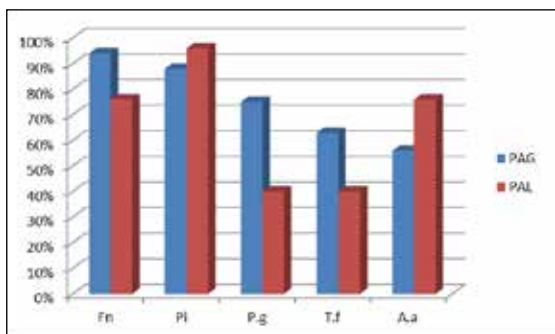


Chart 4: Comparison of the prevalence of periodontal pathogens between generalized (GAP) and localized aggressive periodontitis (LAP)

Fn = *Fusobacterium nucleatum*, Pi = *Prevotella intermedia*, Pg = *Porphyromonas gingivalis*, Tf = *Tannerella forsythia*, Aa = *Aggregatibacter actinomycetemcomitans*

Proportion of pathogens studied in the cases of aggressive periodontitis

Another way to analyze the relevance of the microorganism in the disease is to study it in connection to the detection of the rest of the pathogens studied. If we consider the detection of a certain pathogen in a patient as one occurrence, 100% of the occurrences would be represented by the total number of detections of the five pathogens studied. The following proportions can be found by studying the proportion of each microorganism in relation to the rest of the microorganisms (the total number of occurrences) (Chart 5).

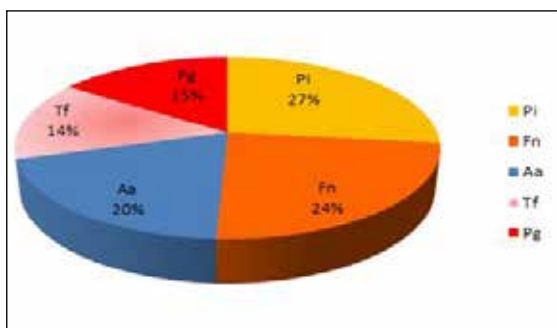


Chart 5: Proportion of pathogens in aggressive periodontitis

The most prevalent microorganism was *P. intermedia*, followed by *F. nucleatum*. *A. actinomycetemcomitans* was in third place with 20%.

As mentioned above, percentages are largely influenced by the larger number of localized over generalized cases.

No significant differences were found in the proportion of microorganisms when taking into account sex with a 0.05 significance level using the t-test for two samples, assuming equal variances (Table 4).

Table 4. Proportion of pathogens in cases of aggressive periodontitis according to sex (significance level $\alpha = 0.05$)

	Female	Male
Pi	26%	26%
Fn	25%	21%
Aa	20%	24%
Pg	15%	16%
Tf	14%	13%

Discussion

The microorganisms present in generalized aggressive periodontitis (GAP) in our study are similar to those found in cases of aggressive periodontitis in other countries⁽¹⁵⁻¹⁶⁾. According to the literature, the microorganisms usually detected in cases of GAP are *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythia*⁽¹⁶⁾. *P. gingivalis* is especially more prominent than *A. actinomycetemcomitans* in cases of generalized disease⁽¹⁵⁾.

As for the presence of the pathogens analyzed in our study, we found that in generalized aggressive periodontitis, *P. gingivalis* was more common than *A. actinomycetemcomitans*, which is consistent with the literature and other locations such as Chile and Colombia⁽²⁰⁻²¹⁾.

As for localized aggressive periodontitis, according to our results, *A. actinomycetemcomitans* exceeded the periodontopathogens *P. gingivalis* and *T. forsythia*, similarly to other locations such as Europa and North America^(2,11,15,21), where

A. actinomycetemcomitans is recovered particularly in cases of localized disease. However, in studies carried out in Latin America (Chile, Colombia), the recovery of *P. gingivalis* and *T. forsythia* exceeded that of *A. actinomycetemcomitans* in this form of the disease^(20, 22-23).

Furthermore, some authors highlight the importance of *A. actinomycetemcomitans* given its role in the pathogenesis of aggressive periodontitis, especially in the localized form, even though it has been suggested that it is probably of particular relevance at the beginning of the pathology and is then replaced by other pathogenic bacteria as the disease progresses^(11,13).

Conclusions

It was found that there is a difference in the flora between the localized and generalized forms of the disease in the Uruguayan patients studied.

The most prevalent microorganism in localized aggressive periodontitis was *P. intermedia*, while in generalized aggressive periodontitis it was *F. nucleatum*.

In addition, *P. gingivalis* and *T. forsythia* are more prevalent in the generalized form than in the localized form of the disease.

We also confirmed the significance of *A. actinomycetemcomitans* in aggressive periodontitis, which is especially significant in localized cases. A similar flora to other geographical locations was present in aggressive cases of periodontitis in our country, even though in some of those *P. gingivalis* is more common than *A. actinomycetemcomitans* in all forms of aggressive periodontitis^(20, 22). In our case, we only found this relation in generalized cases of the disease. In addition, *A. actinomycetemcomitans* was present in a higher proportion than *P. gingivalis* in cases of the localized form of the disease, which is consistent with the data reported in the literature⁽¹⁶⁾.

It was also possible to correlate the traditional bacteriological study with molecular study techniques.

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Received on: 14 Feb 2018 - Accepted on: 07 Aug 2018