

# Distribution of *Prevotella intermedia*, *Porphyromonas gingivalis* and *Actinobacillus actinomycetencomitans* in venezuelan population with chronic periodontitis

*Distribución de Prevotella intermedia, Porphyromonas gingivalis and Actinobacillus actinomycetencomitans en una población venezolana diagnosticada con Periodontitis crónica*

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## Resumen:

**Objetivo:** Determinar la asociación de *Prevotella intermedia* (Pi), *Porphyromonas gingivalis* (Pg) and *Actinobacillus actinomycetencomitans* (Aa) con profundidad sondeable y pérdida de inserción. **Materiales y Métodos:** 256 muestras de placa dental obtenidas de 16 pacientes con periodontitis crónica y sacos periodontales con profundidades variables, fueron analizadas por RCP. Los pacientes fueron evaluados clínicamente para determinar sus índices de placa dental, índice gingival, sangramiento al sondaje, profundidad del saco y pérdida de inserción. Las muestras de placa subgingival fueron obtenidas y evaluadas para determinar la presencia de Pi, Pg and A.a. **Resultados:** La distribución de las especies fue 100%, 69% y 19% para Pi, Aa y Pg respectivamente. Pi fue observada independientemente de las otras especies analizadas en 31% de los pacientes, la combinación de Pi + Aa fue detectada en 50%, y Pi+Aa+Pg en 19%, mientras la asociación de Pi+Pg no fue observada. Pi fue asociada con profundidades de saco  $\leq 3$  mm y pérdida de inserción entre 3.1-5 mm. La asociación de Pi+Aa fue mas alta en pacientes con profundidades de saco  $> 3$  mm y pérdida de inserción entre 3.1-5 mm. **Conclusiones:** Podemos concluir que la presencia de Pi fue asociada con sacos menos profundos mientras los sacos mas profundos fueron asociados con coinfección entre las especies evaluadas.

**Palabras Claves:** *Actinobacillus actinomycetencomitans*, *Prevotella*, *Porphyromonas gingivalis*, Periodontitis Crónica

## Abstract

**Objective:** To determine the association of *Prevotella intermedia* (Pi), *Porphyromonas gingivalis* (Pg) and *Actinobacillus actinomycetencomitans* (Aa) with pocket depth and attachment loss. **Methods:** 256 dental plaque samples obtained from periodontal pockets with variable depths of 16 patients diagnosed with chronic periodontitis, were analysed by PCR. The patients were clinically assessed for plaque and gingival index, bleeding on probing, pocket depth and attachment level. Subgingival plaque samples were obtained and were evaluated for the presence of P.i, P.g and A.a. **Results:** The distribution of the species was 100%, 69% and 19% for Pi, Aa and Pg respectively. Pi was observed independently from the other species analyzed in 31% of the patients, the combination of Pi+Aa was detected in 50 %, and Pi+Aa+Pg in 19%, while the association with Pi+Pg was not observed. Pi was associated with pocket depth  $\leq 3$ mm and attachment loss between 3.1-5mm. The Pi+Aa association was higher in patients with pockets depth  $> 3$ mm and attachment loss between 3.1-5 mm. Pg was only observed in combination with Aa+Pi in patients with a pocket depth  $> 3$ mm and attachment loss between 3.1-5mm. **Conclusions:** We may conclude that Pi presence was associated with shallow pockets while the deepest pockets were related with coinfection between the evaluated species.

**Key Words:** *Actinobacillus actinomycetencomitans*, *Prevotella*, *Porphyromonas gingivalis*, Chronic Periodontitis.

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## Introduction

Periodontitis is a multifactorial inflammatory disease process, leading to destruction in the periodontium. Periodontitis is caused by mixed infections with the subgingival microbiota in a state of continual flux [2]. The flora found in adult subjects with periodontitis is very heterogeneous, as far as the species found are concerned [26,16,25], but it remains dominated by anaerobic micro-organisms and Gram-negative capnophilic bacteria [8].

The identification of bacterial pathogens in periodontal diseases has been difficult because of a number of factors [36]. The periodontal microbiota is a complex community of microorganisms, many of which are still difficult or impossible to isolate in the laboratory. Currently, it is apparent that multiple species function as pathogens, and these may be present in a low number at healthy sites.

Haffajee and Socransky[31], suggested the following subgingival species: *Actinobacillus actinomycetencomitans* (A.a), *Porphyromonas gingivalis* (P.g), *Prevotella intermedia* (P.i), as putative periodontal pathogens. *A. actinomycetencomitans* have been associated with progressive adult periodontitis [28], and is unfrequently found in periodontally healthy subjects [22], whereas *P. intermedia* has been found in healthy subjects and is commonly found in patients with periodontal diseases [37]. *P. gingivalis* has not been found in either healthy subjects or in patients with gingivitis [4]. Consequently, elevated levels of these putative pathogens may be useful indicators of both active periodontitis and increase risk of gingival attachment loss. However, the knowledge

of how their numbers are related to disease progression is still unclear, therefore accurate assessment of their numbers in clinical samples is needed for longitudinal studies [7].

In order to define the specific microorganism in the periodontal pocket, culture and/or immunological methods have been utilized [13,39]. However, there have many problems with sensitivity, specificity, reliability, and applicability. More recently, several methods for the rapid detection of selected periodontal pathogens have been reported, but the polymerase chain reaction (PCR) is the most sensitive method, which amplify a reduced number of bacterial DNA copies [19]. This method could be useful in detecting scarce limited periodontal pathogens in subgingival plaque samples, that could not be identified by culture and DNA probes. PCR is highly specific and it does not rely on viable cells.

The aim of this study was to determine the association of *Actinobacillus actinomycetencomitans* (A.a), *Porphyromonas gingivalis* (P.g) and *Prevotella intermedia* (P.i) with deepest pocket and severe attachment loss in patients diagnosed as Chronic Periodontitis using the PCR technique.

## Materials and Methods

### *Patients and clinical samples*

Sixteen patients age ranging from 20-59 year-old diagnosed as Chronic Periodontitis attended at the Faculty of Dentistry, Central University of Venezuela, were selected for the present study. 256 plaque samples were obtained from periodontal pockets with pocket depth > 3 mm and attachment level > 3 mm.

Exclusion criteria included presence of any poorly controlled systemic conditions that might affect the progression of periodontitis, periodontal therapy or use of antibiotics 6 months previous. Informed written consent was obtained in all cases. The diagnostic criteria used for chronic periodontitis were based in the 1999 International Workshop for Classification of Periodontal Diseases and Conditions [10].

All the patients showed clinical and radiographical evidence of alveolar loss and periodontal pockets depth equal or exceeding 3mm. All the subjects were clinically assessed for plaque index [24], gingival index [14], pocket depth, and attachment level [20]. The measurements were assessed at six sites per tooth, the mesiobuccal, midbuccal, distobuccal, distolingual, midlingual and mesiolingual surfaces.

Supragingival plaque was removed from the crown surface of four teeth per quadrant, with pocket depth and attachment level > 4mm, using sterile cotton rolls. Subsequently, subgingival bacterial samples were obtained by inserting sterile paper points into the depth of each study site (mesiobuccal, distobuccal, mesiolingual and distolingual) and left in place for 30 seconds and then pooled in 250µl of saline solution. Four samples per tooth for a total of sixteen samples from each subject were pooled for analysis. 50 µl of each tube (four tubes per patient) were taken and pooled to obtain an aliquot of 200 µl per patient were boiled for 5 minutes to disrupt the bacteria and stored at -70°C for PCR analysis.

#### *DNA extraction and PCR detection*

The aliquots of the plaque samples were performed following the protocol<sup>20</sup> using

the PCR Kit (Pharma Gen Test), to identify three periodontopathogenic bacteria species of *A. actinomycetencomitans*, *P. gingivalis* and *P. intermedia*.

Dental plaque samples were boiled for 10 min to disrupt the bacteria, and centrifuged in a microcentrifuge for 5 min to eliminate cells debris. The supernatant was removed and 10 µl of the supernatant was then added to a tube containing the PCR mix.

PCR amplifications reactions were carried out in a reaction mixture containing 200 µM dNTPs, 2.5 U Taq polymerase, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM MgCl<sub>2</sub>, 0.5µM of each of the three microorganisms specific primers: Primer AA2 (5' CTT TGC ACA TCA GCG TCA GTA CAT CCC CAA GG 3') is specific for *A. actinomycetencomitans*, producing an amplification product of 253 pb. Primer BGING (5' TAC ATA GAA GCC CCG AAG GAA GAC G 3') is specific for *P. gingivalis*, which produces an amplified fragment of 527 bp; Primer BINT (5' TCC GCA TAC GTT GCC TGC ACT CAA G 3') is specific for *P. intermedia* with an amplification product of 163 bp. 1.5µM of the Primer E, that is common to all three bacteria (5' CGT GCC AGC AGC CGC GGT AAT ACG 3') and 10<sup>-9</sup> µM of the positive control plasmid (pPG19) in a final volume of 50µl.

PCR cycling was carried out in an MJ Research thermal cycler. The temperature cycling conditions (30 in total) comprised an initial denaturation step at 94°C, 10 min, denaturing at 94°C, 1 min, annealing 70°C, 1min and extension at 72°C, 1min. In the final step, the PCR product was fully extended for 10 min at 72°C. The PCR amplified fragments were visualized in a 3% agarose gel stained with ethidium

bromide. If the clinical samples contained the microorganisms, the PCR products were 163, 253 and 527 bp length for *P.i.*, *A.a.* and *P.g.*, in addition to 1430 bp of the positive control amplification product.

*Statistical analysis*

Statistical analysis: The data was statistically analyzed using SPSS, version 10.1.

**Results**

A total of 256 plaque samples from 16 venezuelan patients diagnosed as chronic periodontitis were included in this study. The mean PI score observed was 1.2 ±0.4, distributed as follow: 63%(10/16) showed moderate plaque level, 31%(5/16) low and 6%(1/16) high. The mean GI score was 1.54±0.4 with 88%(14/16) of the study group showing moderated inflammation and 12%(2/16) mild inflammation (p<0.05). The mean of the pocket depht was 3.5 mm±0.6 of these 81%(13/16) showed pocket depth >3 mm. and 19% ≤ 3mm(3/16) (p<0.05). The mean of the clinical attachment loss observed was 3.85 mm±0.73, distributed as: 6% (1/16) with 0-3mm, 81%(13/16) 3.1-5 mm and 13%(2/16) >5mm. 44% (7/16) of the patients were diagnosed as Localized Chronic Periodontitis (LCP) and 56% (9/16) as Generalized Chronic Periodontitis (GCP).

The prevalence of each specie was recorded for each subject. *P.i.* was detected in all patients, *A.a.* en 69% (11/16) y *P.g.* en 19% (3/16), statistical significant differences were observed between *P.i.* and *A.a.*, *P.i.* and *P.g.* and *Aa* and *Pg* (p<0.0001). It is noteworthy that in 31% (5/16) of these cases *P.i.* was found alone, in 50% (8/16) coinfectd with *A.a.* and 19%(3/16) associated to *P.g.* + *A.a.*

Table 1. Relation between the bacterial sp, gingival inflammation and dental plaque index.

Species	Plaque Index			Gingival Index		
	Low	Moderate	High	Mild	Moderate	Severe
A.a	3	7	1	1	10	0
P.g	0	3	0	0	3	0
P.i	5	10	1	2	14	0

Table 1. shows the relation between the bacterial species analyzed and the means of plaque and gingival index. The detection of periodontopathogen distribution and frequency differs according to plaque score and gingival inflammation. The highest prevalence was observed in subjects with moderate plaque level and moderate gingival index. *A.a.* was detected in 3 patients with low index plaque, in 7 moderate plaque levels and in only 1 patient with high plaque score. *P.g.* was positive in 3 patients with moderate plaque and *P.i.* was detected in 5, 10 and 1 patients with low, moderate and high plaque index respectively. The relation observed was higher in patients with moderate inflammation for all species analyzed.

Table 2. A.a, P.g. and P.i. presence related to probing depth and clinical attachment loss in patients with chronic periodontitis.

Species	Pocket Depth		Clinical attachment loss		
	≤ 3 mm	> 3 mm	0-3 mm	3.1-5m m	>5 mm
P.i	2/5	3/5	0	4/5	1/5
A.a + P.i	1/8	7/8	1/8	6/8	1/8
P.g + P.i	0	0	0	0	0
A.a + P.i+ P.g	0	3/3	0	3/3	0

We analyzed the frequency of detection of *P.intermedia*, *P.gingivalis* and *A.actinomyetemcomitans* related to periodontal pocket depth and clinical attachment loss. The three pathogens were

detected more frequently in subjects with a mean periodontal pocket depth >3 mm and clinical attachment loss between 3.1-5 mm. In patients with pocket depth ≤ 3mm *P.i* alone was detected in two patients and in association with *A.a* in only one patient. The association *A.a* + *P.i* + *P.g* were not found in this group.(Table 2).

In the patients with GCP and LCP the detection frequencies of *A. actinomycetemcomitans* were 64% (7/11) and 36% (4/11) respectively. *P.g* was observed only in 3 patients diagnosed as GCP.(Table 3)

Table 3. *A.a*, *P.g*. and *P.i*. presence related to periodontal diagnosis.

	<i>A.a</i>	%	<i>P.g</i>	%	<i>P.i</i>	%
<b>GCP*</b>	7	64	3	100	9	100
<b>LCP**</b>	4	36	0	0	7	100

\* Generalized Chronic Periodontitis

\*\* Localized Chronic Periodontitis

## Discussion

After 20 years of worldwide research on the composition of periodontal flora, it is still difficult to establish a global view of the detection frequencies and proportions of the present species of the subgingival flora and their relationship to various periodontal conditions [6]. The difficulties derive from a variety of differences among the studies related with the purpose, materials and methods used. Additionally, the criteria used to define various types of periodontitis or periodontal health have varied or have been insufficiently described to enable comparisons. These elements reflect the inherent difficulties in studying a chronic infection in a body site with a normal flora [6].

Microorganisms such *A. actinomycetemcomitans*, *P. intermedia* and *P. gingivalis*, have been considered to play a role in periodontal diseases [17,29].

Studies have demonstrated a strong correlation between the presence of putative periodontal organism and the destruction of periodontal tissues [18,27], and ongoing periodontal attachment loss in maintenance patients has been related to the persistence of, among others, *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*. [38]

In this study, PCR analysis was successfully used to identify three different periodontopathogens associated with human chronic periodontitis such as *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*.

In the present investigation, we analysed 256 subgingival plaque samples from 16 Venezuelan patients diagnosed as chronic periodontitis obtained from pockets > 3mm. The frequency of detection of the test species was correlated with plaque and gingival index, periodontal pocket depths and clinical attachment loss. Comparison of ours results with those of other studies is complicated by the fact that different methods and cut-off were applied in each study.

The present work demonstrated that the prevalence of *Prevotella intermedia* (100%) and *A. actinomycetemcomitans* (69%) was higher than *P. gingivalis* (19%). *P.i* was observed independently from the other species analyzed in 31% of the patients, however, the combination of *P.i* + *A.a* was detected in 50 %, and *P.i* + *A.a* + *P.g* in 19%, while the association with *P.i* + *P.g* was not observed. Positive correlation between pocket depth and the frequency of periodontopathogenic bacteria has been detected for *P.g* and *P.i* [9]; although, we did not observe this combination in our patients.

The correlation between the periodontopathogenic species with plaque and gingival index, demonstrated that all the species evaluated were found to be more frequent in patients with moderate plaque and gingival index.

The association of *P.i*+ *A.a* was highest in patients with pockets depth > 3mm and attachment loss between 3.1-5mm. *P.g* was only observed in combination with *A.a*+ *P.i* in patients with pocket depth >3mm and attachment loss between 3.1-5mm. Our results are in accordance with other studies where plaque samples from deep periodontal pockets obtained by a nucleic acid method were similar for *P.g*, and *A.a* [9,3].

Zambon et al [40], suggested that *P. gingivalis* and *A. actinomycetemcomitans* are closely associated with the pathogenesis of periodontitis, as well as the loss of connective tissue and severe resorption of alveolar bone; while some studies reported an association between depth pockets and the higher numbers of *P. gingivalis*, suggesting an etiological role in periodontal progression [15,36,12], however, others studies have not found this correlation [31].

The prevalence of *A. actinomycetemcomitans* was lower compared with others studies [9]. This might be explained by the fact that the patients in our study were older than those in other studies, thereby confirming an association of *A.a* with age [23]. In adults suffering chronic periodontitis, the presence of *A.a* may or not be associated with *P. gingivalis* and *P. intermedia* in the active phases of periodontitis [29].

Suzuki et al. [34] evaluated the distribution of different serotypes of *A.a* and *P.g* in Japanese adults. *A. a* serotype c

was detected more frequently in deep pockets that were positive for both *A.a* and *P.g*. This combination (*A.a*+ *P.g*) was not found in this study. Our results are in agreement with others investigators that have been reported that *A.a* and *P. g* are rarely detected in the same periodontal site [1,21,32].

Socransky et al. [33] also compared the pocket microflora with different depths and found a higher prevalence of *P. gingivalis* and *P. intermedia* in deep pockets than in shallow pockets. However, in our study *P.i* was associated with shallow pockets while the deepest pockets were associated with coinfection between the evaluated species.

The detection of *P. gingivalis* in large proportion is often associated with forms of rapidly progressing periodontitis, or with some of its more severe forms. The main clinical forms associated are: rapidly progressing periodontitis, the active phase of periodontitis in adults, generalized juvenile periodontitis and a recently described tobacco-associated periodontitis. In individuals affected by periodontal condition, *P. gingivalis* is considered a major pathogen. Sixou [25], consider the presence of *P. gingivalis* a major risk for the evolution of lesions.

The use of clinical parameters in sample site selection, particularly probing depth measurements, is likely to be considered to detect *P. gingivalis*. Similarly, sample site selection based on loss of clinical attachment increase the chance of detecting this microorganism [5]. In this study, we observed *P.g* only in combination with *A.a*+*P.i*.

We may conclude from the present study that *P. intermedia* was more significantly associated with chronic periodontitis in a venezuelan population.

The presence of *P.i* was associated with shallow pockets while the deepest pocket were associated with coinfection between the evaluated species.

Although PCR is sensitive, quantification of the target sequence is needed, knowledge of numbers of these pathogens and their relation with disease progression is important to establish a more effective and accurate therapy [35]. Besides, understanding the relationship among bacterial species could be useful in the comprehension of the biology of subgingival plaque and in developing strategies for its control.[31]

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