Prevalence of Periodontopathic Bacteria in Aggressive Periodontitis Patients in a Chilean Population

Marta Gajardo,* Nora Silva,* Leyla Gómez,* Rubén León,† Beatriz Parra,‡ Adolfo Contreras,§ and Jorge Gamonal‖

Background: *Actinobacillus actinomycetemcomitans* is considered a major etiologic agent of aggressive periodontitis (AgP). Other periodontopathic bacteria such as *Porphyromonas gingivalis* are also suspected of participating in aggressive periodontitis although the evidence to support this is controversial. The aim of the present study was to determine the prevalence of eight periodontopathic bacteria in Chilean patients with AgP.

Methods: Subgingival plaque samples were collected from 36 aggressive, 30 localized, and six generalized periodontitis patients. Samples from 17 advanced chronic periodontitis (CP) patients were taken as controls. Samples collected from the four deepest periodontal pockets in each patient were pooled in prereduced transport fluid (RTF) and cultured. Periodontal bacteria were primarily identified by colony morphology under stereoscopic microscope and rapid biochemical tests. The identity of some bacterial isolates was confirmed by colony polymerase chain reaction (PCR).

Results: AgP showed a significantly higher prevalence of *C. rectus* than CP ($P = 0.036$). The only statistical difference found was for *C. rectus*. Patients with AgP showed a higher, but not statistically significant, prevalence of *P. gingivalis, E. corrodens, P. micros*, and *Capnocytophaga* sp. A similar prevalence in both groups of patients was observed for *F. nucleatum* and *P. intermedia/nigrescens,* and *A. actinomycetemcomitans* was less prevalent in AgP than CP patients. In localized AgP, *P. intermedia/nigrescens, E. corrodens, F. nucleatum*, and *P. micros* were the more prevalent pathogens in contrast to generalized AgP patients who harbored *A. actinomycetemcomitans, P. gingivalis*, and *Capnocytophaga* sp. as the most prevalent bacteria.

Conclusions: *C. rectus, P. gingivalis, E. corrodens, P. micros*, and *Capnocytophaga* sp. were the most predominant periodontopathic bacteria of AgP in this Chilean population, but the only statistical difference found here between AgP and CP was for *C. rectus*, suggesting that the differences in clinical appearance may be caused by factors other than the microbiological composition of the subgingival plaque of these patients. In this study, the prevalence of *A. actinomycetemcomitans* was much lower than that of *P. gingivalis*. *J Periodontol 2005;76:289-294.*

KEY WORDS

Chileans; periodontitis, aggressive/microbiology; periodontitis, aggressive/pathogenesis.

* Area of Microbiology, Faculty of Dentistry, University of Chile, Santiago, Chile.
† Area of Biochemistry, Faculty of Dentistry, University of Chile.
‡ Area of Microbiology, School of Dentistry, University of Valle, Cali, Colombia.
§ Department of Periodontology, School of Dentistry, University of Valle.
‖ Area of Periodontology, Laboratory of Periodontal Biology, Faculty of Dentistry, University of Chile.

Aggressive and chronic periodontitis are caused by an overgrowth of putative periodontal pathogens in subgingival plaque followed by an immunoinflammatory response in a susceptible host. As a result of this interaction, attachment loss, periodontal pocket formation, active bone resorption, and inflammation occur.[1] The infectious etiology of periodontitis is well documented, as is the role of the host immune response in the pathogenesis of the disease.[2,3] Dental plaque is a complex microbial biofilm comprised of as many as 500 different bacterial species organized in the supragingival and subgingival locations.[4] As opposed to the supragingival plaque attached to the tooth surface and dominated by *Streptococci* and *Actinomyces*, Gram-positive facultative species,[5] the subgingival plaque is tooth and tissue associated and contains a great variety of Gram-negative anaerobic bacteria.[6] From supra- to subgingival plaque, there is a significant decrease of *Streptococci* and *Actinomyces* species accompanied by an increase of *Tannerella forsythensis* (*Bacteroides forsythus*), *Porphyromomas gingivalis*, and *Treponema denticola*.[7] Interestingly, *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis*, and *Tannerella forsythensis* have been strongly associated

with periodontal disease, disease progression, and unsuccessful therapy.[7] The American Academy of Periodontology revised the classification of periodontal diseases in 1999.[8] The consensus report for the new classification[9] confirmed that *Actinobacillus actinomycetemcomitans* can cause aggressive periodontitis, and is present in an elevated proportion in aggressive periodontitis (AgP). *Porphyromonas gingivalis* was also mentioned as an etiologic agent of AgP, although its importance in the pathogenesis of this disease has been generally thought to be less than that of *A. actinomycetemcomitans*. In a previous study in Chile, low prevalence of *A. actinomycetemcomitans* was observed in AgP patients.[10] Supposing that the prevalence of *A. actinomycetemcomitans* is low in AgP, other periodontopathic bacteria may play an important role. Other species such as *Porphyromonas gingivalis, Fusobacterium nucleatum, Campylobacter* species, *Prevotella intermedia/nigrescens, Peptoestreptococcus micros*, and various spirochetes have been implicated in destructive periodontal disease.[3] The role of these bacteria in the pathogenesis of human periodontal disease is based on their high frequency of isolation and pathogenic potential that includes a myriad of virulence factors that enables them to subvert the host defense systems. These include the ability to attach to epithelial cells and extracellular matrix proteins, proteases, collagenases, endotoxins (LPS), antibiotic resistance, bacteriocins, the production of chemotactic inhibitors, leukotoxins, cytotoxins, toxic metabolic substances (H_2S, putrecins), immunosuppressive proteins, etc.[11-13]

In the present study, we investigated the prevalence and proportions of periodontopatic bacteria in aggressive periodontitis patients in a Chilean population. Bacterial culture was used for detection and quantitative evaluation of *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia/nigrescens, Eikenella corrodens, Fusobacterium nucleatum, Capnocytophaga* species, *Campylobacter rectus,* and *Peptostreptococcus micros*.

MATERIALS AND METHODS

Study Population

Fifty-three periodontal patients were divided into three groups:[8] 1) localized aggressive periodontitis (N = 30); 2) generalized aggressive periodontitis (N = 6); and 3) chronic periodontitis (N = 17) (Table 1). Patients were selected from among those attending the Faculty of Dentistry Graduate Periodontal Clinic, University of Chile and the Eloisa Díaz Diagnostic and Treatment Center of the North Metropolitan Health Services in Santiago, Chile. None of the subjects had received any periodontal treatment. Subjects did not suffer from systemic illness and had not received antibiotics or non-steroid anti-inflammatory therapy in the 6-month period prior to the study. The protocol was clearly explained to all patients, and Institutional Reviews Board-approved informed consents were signed. The protocol stated that, within 2 weeks of the detection of disease, all patients would be provided with periodontal treatment.

Table 1.

Clinical Criteria Used to Determine Periodontal Status

Localized aggressive periodontitis	• The age of disease onset was estimated at <20 years old by medical examination and interview • ≥4 mm loss of attachment on more than two or more first molars and/or incisors, and no more than two affected cuspids, premolars, or second molars
Generalized aggressive periodontitis	• The age of disease onset was estimated at <30 years old by medical examination and interview • ≥4 mm loss of attachment on more than two or more first molars and/or incisors, and three or more affected cuspids, premolars, or second molars
Generalized chronic periodontitis	• ≥4 mm loss of attachment was observed in at least 30% of residual teeth

Clinical Measurements

Clinical parameters were taken by a skilled clinician (JG) at all teeth excluding third molars and included clinical attachment level (CAL), probing depth (PD) measured with an automated probe,[¶] supragingival plaque accumulation (PI), and bleeding on probing (BOP). Six sites were examined in each tooth: mesio-buccal, buccal, disto-buccal, disto-lingual, lingual, and mesio-lingual.

Subgingival Plaque Samples

Subgingival plaque samples were collected from four periodontally affected sites, one in each quadrant, with a probing depth of >5 mm and an attachment loss of >3 mm in each patient. After isolating the area with cotton rolls and gently air drying, supragingival deposits were carefully removed with curettes.[#] Subgingival microbial samples were obtained by inserting two standardized No. 30 sterile paper points** into the deepest part of the periodontal pocket for 20 seconds. Samples from each patient were pooled in a vial containing 2 ml of cold sterilized prereduced transport fluid (RTF) without EDTA. Vials with samples were transported at 4°C to the Microbiological

¶ Florida Probe Corporation, Gainesville, FL.
# Hu Friedy, Gracey, Chicago, IL.
** Johnson & Johnson, Tokyo, Japan.

Laboratory of the Faculty of Dentistry, University of Chile, and processed immediately.

Microbiological Procedures

Subgingival plaque samples were dispersed by mixing†† for 45 seconds followed by a 10-fold serial dilution of the bacterial suspension in RTF. Aliquots of 100 µl of the appropriate dilution (10^{-3}, 10^{-4}, and 10^{-5}) were plated on non-selective Columbia blood-agar (5% defibrinated sheep blood, 1 mg/L hemin, and 0.5 mg/L menadione) for total anaerobic cell counts (colony forming units [CFU]/ml) and for detection and quantification of *P. gingivalis, P. intermedia/nigrescens, E. corrodens, Capnocytophaga* species, *C. rectus*, *F. nucleatum,* and *P. micros*. Plates were incubated anaerobically at 35°C for 5 to 7 days in a jar containing gas generator envelopes for the production of an anaerobic atmosphere.‡‡

Bacteria were primarily identified by colony morphology under a stereoscopic microscope,§§ pigment production, Gram stain and rapid enzymatic tests for oxidase, trypsin-like (BANA), and α-glucosidase detection. In addition, black pigmented colonies of *P. gingivalis* and *P. intermedia/nigrescens* were tested for red fluorescence under UV light (360 nm): negative for *P. gingivalis* and positive for *P. intermedia/ nigrescens*.[12] *A. actinomycetemcomitans* was also primarily identified by colony morphology (star-like inner structure or ridges) but also on selective TSBV medium (trypticase, 10% horse serum, bacitracin, and vancomycin).[14] One hundred µl of undiluted and 10^{-1} diluted samples were inoculated on the agar plates and incubated at 35°C for 2 to 3 days in CO_2 candle jars. The percentage of *A. actinomycetemcomitans* was obtained using the number of CFU on TSBV as a percentage of the total anaerobic counts. Gram stain and catalase production were also used for *A. actinomycetemcomitans* identification. To obtain *F. nucleatum* counts, selective CVE (trypticase, crystal violet, trytophane, erythromicin, 5% defibrinated sheep blood) medium was used.[12] Plates were incubated anaerobically, as mentioned above, for 5 days. Colony morphology, pigmentation, Gram stain, and direct sheep red blood cell hemaglutination were used for identification. The anaerobic isolates were further identified using an anaerobe system.‖‖

Table 2.

Clinical Data (Mean ± SD)

Measurement	CP (N = 17)	LAgP (N = 30)	GAgP (N = 6)
Age (range)	47.29 ± 7.48*† (36-59)	24.39 ± 3.77†‡ (17-26)	29.5 ± 6.12†‡ (21-32)
Females (%)	88.23	85.71	66
Sites with plaque (%)	78.77 ± 12.00§	53.53 ± 14.56§‖	72.10 ± 14.78‖
Bleeding on probing (%)	64.86 ± 17.05¶	40.47 ± 12.18¶#	57.10 ± 11.54#
Probing depth (mm)	3.67 ± 0.43**	3.79 ± 0.40††	4.30 ± 0.27**††
Probing depth at sampled sites (mm)	5.60 ± 0.32	5.96 ± 0.55	6.70 ± 0.41
Clinical attachment loss (mm)	3.39 ± 0.44‡‡	3.61 ± 0.50	4.00 ± 0.11‡‡

* Age GAgP versus CP, *P* value 0.0001.
† Age CP versus LAgP, *P* value 0.0001.
‡ Age GAgP versus LAgP, *P* value 0.006.
§ Plaque index LAgP versus CP, *P* value 0.001.
‖ Plaque index LAgP versus GAgP, *P* value 0.012.
¶ Bleeding on probing LAgP versus CP, *P* value 0.0001.
\# Bleeding on probing LAgP versus GAgP, *P* value 0.029.
** Probing depth CP versus GAgP, *P* value 0.006.
†† Probing depth LAgP versus GAgP, *P* value 0.020.
‡‡ Clinical attachment loss CP versus GAgP, *P* value 0.020.

Data Analysis

Clinical parameters and presence of periodontal pathogens were calculated for each subject (mean ± standard deviation). Patients were used as the experimental unit of observation. Chi square test and Student *t* test were employed to compare the parameters between the clinical periodontitis groups and microbiological data. The difference in the prevalence of bacteria among groups was tested by Fisher's exact test. *P* values <0.05 were considered statistically significant.

RESULTS

Clinical data of subjects and sites selected for bacterial sampling are summarized in Table 2. No statistically significant differences in gender existed between groups. A significantly higher percent of sites with plaque and sites with BOP could be observed in the chronic periodontitis patients (*P* <0.05). Significantly higher PD and levels of loss attachment could be observed in patients with generalized aggressive compared to generalized chronic periodontitis (*P* <0.05).

†† Thermolyne maxi mix II type 37600, Dubuque, IA.
‡‡ Oxoid Limited, Wade Road, Basingstoke, Hampshire, U.K.
§§ Stmi 2000-C, Zeiss, Jena, Germany.
‖‖ BBL, Gaspak, Becton Dickinson, Cockeysville, MD.

Table 3.

Microbiological Findings in Subgingival Plaque Samples from Each Patient Group

	Chronic Periodontitis (N = 17)				Localized Aggressive Periodontitis (N = 30)				Generalized Aggressive Periodontitis (N = 6)			
	Isolation Frequency		% of Total Count		Isolation Frequency		% of Total Count		Isolation Frequency		% of Total Count	
Bacteria	N	%	Mean ± SD	Range	N	%	Mean ± SD	Range	N	%	Mean ± SD	Range
A. actinomycetemcomitans	6	35.29	1.63 ± 3.37	0.01-12.00	5	16.67	0.67 ± 1.97	1.5-10.0	2	33.33	0.25 ± 0.55	1.5-1.5
P. gingivalis	13	76.47	13.65 ± 13.39	0.4-36.00	26	86.67	12.19 ± 15.42	0.3-50.9	6	100	17.2 ± 10.00	6.1-31
P. intermedia/nigrescens	6	35.29	9.62 ± 18.26	2.5-51.20	12	40.00	2.12 ± 3.79	0.3-15.0	1	16.66	0.83 ± 1.86	0.5-5.0
E. corrodens	6	35.29	4.54 ± 10.79	2.1-43.00	16	53.33	2.07 ± 3.78	0.3-19.0	2	33.33	1.23 ± 2.00	1.2-5.4
F. nucleatum	7	41.18	1.10 ± 1.80	0.1-6.00	14	46.67	2.42 ± 5.55	0.07-28.5	2	33.33	1.25 ± 2.23	1.0-6.7
Capnocytophaga sp.	2	11.76	0.30 ± 0.99	0.7-4.00	8	26.67	0.69 ± 1.98	0.13-10.0	2	33.33	2.00 ± 3.05	1.5-8
C. rectus	4	23.52*	0.65 ± 1.41	0.1-4.40	15	50.00*	2.09 ± 3.64	0.13-16.6	3	50.00*	2.00 ± 2.68	0.9-7.1
P. micros	6	35.29	4.19 ± 11.10	0.3-43.00	15	50.00	4.56 ± 9.63	0.8-50.0	2	33.00	4.26 ± 7.88	4.0-21.6

* *C. rectus* CP versus LAgP and GAgP, *P* value = 0.036.

DISCUSSION

This was a cross-sectional study of 53 subjects, 30 with localized aggressive periodontitis, six with generalized aggressive periodontitis, and 17 with chronic periodontitis. Our results show that *P. gingivalis* (88.8% versus 76.4%), *E. corrodens* (50.0% versus 35.2%), *P. micros* (47.2% versus 35.2%), and *Capnocytophaga* sp. (27.7% versus 11.7%) were isolated more frequently in aggressive than chronic periodontitis. In contrast, *A. actinomycetemcomitans* was found less frequently in aggressive (19.4%) than chronic (35.2%) periodontitis patients. *F. nucleatum* (44.4% versus 41.1%) and *P. intermedia/nigrescens* (36.1% versus 35.2%) were isolated with similar frequencies in both forms of periodontitis. The only statistical difference found here was for *C. rectus* (50.0% versus 23.5%). These results indicate that, as a whole, the subgingival microbiota differ but not significantly, between chronic and aggressive periodontitis patients. In addition, we also observed that *P. gingivalis, E. corrodens*, and *C. rectus* were present in ≥50% of AgP patients, making them the most prevalent bacteria in these subjects. In the CP group only *P. gingivalis* was found in more than 50% of patients. Similar results have been obtained in studies from Japan,[15] China,[16] the United Kingdom,[17] and in previous studies in Chile.[10,18]

When analyzing LAgP and GAgP groups of patients separately, the bacteria we found in at least 50% of the LAgP patients were *P. gingivalis* (86.6%), *E. corrodens* (53%), and *C. rectus* and *P. micros* (50.0%), while in GAgP patients, the most frequently found organisms were *P. gingivalis* (100%) and *C. rectus* (50%). The occurrence of *A. actinomycetemcomitans* in LAgP (16.6%) and GAgP (33%) patients was lower than that of *P. gingivalis*. Goodson et al.[19] tested for six probable periodontal pathogens in subgingival plaque by DNA probe analysis from 113 adult periodontitis patients in the United States and found that 70% of periodontitis sites were infected with *P. gingivalis*, 50% with *P. intermedia* and *E. corrodens*, 36% with *C. rectus*, and 11% with *A. actinomycetemcomitans*. In Kenya, the prevalence of the subgingival plaque from two sites in each of 20 adults from a rural area was 70% and 50% for *P. gingivalis*, 100% and 90% for *P. intermedia*, and 40% and 28% for *A. actinomycetemcomitans,* respectively.[20] In Japan, subgingival plaque was collected from 50 aggressive periodontitis patients (localized 10, generalized 40) and samples from 35 generalized chronic periodontitis.[15] *T. forsythensis* and *P. gingivalis* were detected more frequently at sites with severe loss of attachment (CAL ≥6 mm) compared to moderate loss of attachment (CAL <6 mm) in localized AgP patients. This tendency was also observed for *P. gingivalis* in generalized AgP patients. However, positive correlation between the presence of *C. rectus/T. denticola* and severity of clinical attachment level was not found in any of the three periodontitis groups.[15] It is possible that the differences in the detection percentages found in our study for these important periodontopathic organisms may be due, in part, to racial and geographical differences.

In the present study, pooled samples of four affected sites per patient were analyzed by bacterial culture. Although opinions differ as to whether microbiological data should be analyzed on individual sites or using the patient as the study unit, here we chose the latter approach based on the criterion that patients are being treated as a whole. Mombelli et al.[21] reported that four individual subgingival samples, each from the deepest periodontal pocket in each quadrant, should be included to detect *P. gingivalis*. Christersson et al.[22] also showed that sampling three pockets with ≥5 mm probing depth provided a 95% probability of detecting *P. gingivalis*. In contrast, they reported that the detection of *A. actinomycetemcomitans* may require a minimum of 25 samples from random sites in adult periodontitis patients.[22] To increase the likelihood of collecting the relevant periodontal pathogens, a subgingival specimen may be pooled from sites having the highest probability of harboring periodontal pathogens, such as pockets showing increased probing depth or undergoing active breakdown, or from three or four deep periodontal pockets.[21] In our study, the presence of *A. actinomycetemcomitans* was determined from a pooled subgingival sample of the four deepest periodontal pockets per patient and, therefore, the periodontal occurrence of this bacteria may have been underestimated.

According to the criteria that a bacterium has to be above a certain minimal threshold with respect to the total cultivable subgingival microbiota to be associated with periodontal disease, it has been suggested that this threshold is 0.1% for *P. gingivalis*,[23] 0.01% for *A. actinomycetemcomitans*,[14,20] 2.5% for *P. intermedia*,[23-25] 5% for *F. nucleatum*,[24,25] 5% *Capnocytophaga* sp.,[24,25] 1% for *E. corrodens*,[23-25] and 2% for *C. rectus*.[25,26] Culture studies show that *A. actinomycetemcomitans* levels are lower than *P. gingivalis* levels among the subgingival cultivable microbiota of adult periodontitis patients.[27,28] The differences between *A. actimomycetemcomitans* and *P. gingivalis* levels were also demonstrated by Rams et al.[27] who examined subgingival samples of 1,800 periodontitis patients. The mean proportion of *A. actinomycetemcomitans* in the microbiota was 4%, whereas that of *P. gingivalis* was 23%. When compared with other periodontal bacteria, such as *P. intermedia/nigrescens, C. rectus*, and *P. micros*, the proportion of *P. gingivalis* was also the highest.

Our results showed little difference in the microbial component of different forms of periodontitis, the variances in clinical appearance may be caused by factors other than the microbiological composition of the subgingival plaque. Bacteria are essential, but insufficient to cause disease; a susceptible host is also essential and host factors are determinative.[30] Periodontitis is a family of related diseases that differ in etiology, natural history, disease progression, and response to therapy, but with common shared pathways of tissue destruction.[31] The shared events in the pathobiology are influenced by disease modifiers (also known as risk factors and indicators), both genetic and environmental or acquired, which may differ from one stage and form of disease to another.[32] The clinical picture observed is a result of the complex interplay among the microbial challenge, the shared events, and disease modifiers. The modifying factors are major determinants of the differences observed in different periodontal conditions.[30]

The results presented here indicate that on the basis of mean percentage of total counts, *P. gingivalis* (13.65%) and *P. intermedia/nigrescens* (9.62%) were strongly associated with chronic periodontitis, while *P. micros* (4.19%) and *E. corrodens* (4.54%) showed a moderate association. In this study the bacteria with mean percentages above the minimal threshold in chronic periodontitis patients were *P. gingivalis* (13.65%), *P. intermedia/nigrescens* (9,62%), *P. micros* (4.19%), *E. corrodens* (4.54%), and *A. actinomycetemcomitans* (1.63%). In aggressive periodontitis, *A. actinomycetemcomitans* was found at a higher level in the localized (0.67%) than in the generalized (0.25%) form, whereas *P. gingivalis* and *Capnocytophaga* sp. had higher levels in the generalized disease.

Taken together, the results presented here indicate that *P. gingivalis, C. rectus, E. corrodens*, and *Capnocytophaga* sp. were more strongly associated with aggressive periodontal disease, while *P. intermedia/nigrescens* and *F. nucleatum* presented a moderate prevalence in both clinical groups. In turn, the prevalence of *A. actinomycetemcomitans* was much lower than that of *P. gingivalis*. Studies are needed to further investigate the role of specific *A. actinomycetemcomitans* strains and other microbial species in the pathogenesis of aggressive periodontitis.

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Correspondence: Dr. Jorge Gamonal, Área de Periodoncia, Facultad de Odontología, Universidad de Chile, Avda. Santa Maria 0596 – Comuna de Providencia, Santiago, Chile. Fax: 56-2-7779724; e-mail: jgamonal@odontologia.uchile.cl.

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