POSITIVE CORRELATIONS BETWEEN PRESENCE OF GRAM NEGATIVE ENTERIC RODS AND *PORPHYROMONAS GINGIVALIS* IN SUBGINGIVAL PLAQUE

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ABSTRACT

The association between Gram negative enteric rods and Porphyromonas gingivalis in periodontal diseases has received little attention in the literature. Thus, the aim of this study was to investigate the associations between Gram negative enteric rods, Porphyromonas gingivalis and clinical parameters of periodontal disease. The prevalence of Gram-negative enteric rods and P. gingivalis were examined in patients with chronic periodontitis. Chi-square and Mann-Whitney tests were used to determine differences in clinical variables versus the presence or absence of both microorganisms. Correlations of both organisms and clinical data were determined using Spearman rank correlation coefficient. Gram-negative enteric rods and P. gingivalis were detected in 20 (26.3%) and 51 (67.1%) subjects, respectively. A total 17 (22.4%) individuals harbored both microorganisms studied. There were significantly positive correlations between enteric rods and presence of P. gingivalis (r=0.531, P<0.0001). Both microorganisms were significantly and positively correlated with probing depth, clinical attachment level and bleeding on probing (P<0.0001). The mean probing depth (mm) of the sampled sites was significantly deeper in patients with presence of P. gingivalis and Gram-negative enteric rods. This study suggests that the presence of Gram negative enteric rods and P. gingivalis is related to adverse periodontal conditions. These results could have an impact on periodontal treatment and should be taken into account in the mechanical and antimicrobial treatment of periodontal disease in some populations.

Key words: Periodontal diseases, Porphyromonas gingivalis.

CORRELACIÓN POSITIVA ENTRE BACILOS ENTÉRICOS GRAM-NEGATIVOS Y *PORPHYROMONAS GINGIVALIS* EN PLACA SUBGINGIVAL

RESUMEN

La asociación entre bacilos entéricos Gram-negativos y Porphyromonas gingivalis en las enfermedades periodontales ha recibido poca atención en la literatura. Así, el objetivo de este estudio fue investigar la asociación entre bacilos entéricos Gram-negativos, Porphyromonas gingivalis y los parámetros clínicos de la periodontitis. Se evaluó la prevalencia de bacilos entéricos Gram-negativos y P. gingivalis en pacientes con periodontitis crónica. Se utilizaron pruebas de Chi-cuadrado y Mann-Whitney para determinar las diferencias en las variables clínicas frente a la presencia o ausencia de ambos microorganismos. Se empleo el coeficiente de correlación de Spearman para determinar las correlaciones de ambos organismos y los datos clínicos obtenidos. Se detectaron bacilos entéricos Gram-negativos y P. gingivalis en 20 (26,3%) y 51 (67,1%) sujetos, respectivamente. Un total de 17 (22,4%) individuos presentó los dos microorganismos estudiados. Se observó una correlación positiva significativa entre bacilos entéricos y la presencia de P. gingivalis (r = 0,531, P <0,0001). Se encontró una correlación positiva estadísticamente significativa de los dos microorganismos estudiados con la profundidad de sondaje (PS), pérdida de inserción clínica y sangrado al sondaje (P <0,0001). El promedio de la profundidad de sondaje (mm), de los sitios incluidos en la muestra, fue significativamente superior en pacientes con presencia de P. gingivalis y bacilos entéricos Gram-negativos. La presente investigación sugiere que la presencia de bacilos entéricos Gram negativos y P. gingivalis se relaciona con condiciones periodontales adversas. Estos resultados podrían tener un impacto en el tratamiento periodontal y se deben tener en cuenta en la terapia mecánica y antimicrobiana de la periodontitis en diferentes poblaciones.

Palabras clave: Enfermedades periodontales, Porphyromonas gingivalis.

INTRODUCTION

Periodontitis is a biofilm-related infection with mixed microbial aetiology. *Porphyromonas gingivalis*, a gram-negative anaerobe, is a major etiological agent in the initiation and progression of chronic periodontal disease[1] and has also been implicated as a contributory factor in the development of systemic diseases[2]. The virulence of *P. gingivalis* has been attributed to a variety of potential factors associated with its cell surface, including fimbriae, lipopolysaccharides, capsules, proteases, hemagglutinins and major outer membrane proteins[1,2]. *P. gingivalis* has shown the ability to invade human gingival fibroblasts and epithelial cells in cell culture[3]. On the other hand, the role of Gram-negative enteric rods in the pathogenesis of periodontal disease is unknown, but some investigators have suggested that they may have an impact on the progression and treatment of periodontal disease[4]. Subgingival Gram-

negative enteric rods often persist after periodontal debridement and surgery and have been implicated as key pathogens in cases of refractory periodontitis[5-9]. They were detected at higher frequency and in higher proportions in patients with failing implants[7]. Additionally, they show less susceptibility to chlorhexidine[4], and the fact that they exhibit *in vitro* resistance to most adjunctive antibiotics used to treat periodontitis[10-13] means that periodontal lesions associated with these organisms do not respond to conventional treatment modalities[5-9]. Additionally, enteric rods also have shown the capacity to invade human tissue and produce enterotoxins and endotoxins[14]. Invasiveness and ubiquitous intraoral distribution may be the main reasons for the reported observation of rather poor results after conventional, merely mechanical treatment of periodontal infections[15].

To our knowledge, there are no investigations that study the relationships between enteric rods and *P. gingivalis* with clinical parameters. Geographical differences in the presence of these microorganisms could have an impact on clinical parameters and periodontal treatment protocols, which may enable the establishment of specific therapeutic strategies. The aim of this study was to investigate the relationships between these organisms and clinical parameters of patients with chronic periodontitis.

MATERIALS AND METHODS

A total 76 adult patients in good general health (patients free of systemic diseases such as diabetes, arthritis, ulcerative colitis, Crohn's disease, HIV infection, cancer and heart disease) and with no current medication usage were included in the study after providing informed consent. Medical history and clinical and radiographic examination were conducted for each patient. The study design was approved by the Ethics Committee on Human Research of the University Research Department of the University of Antioquia according to the Declaration of Helsinki on experimentation involving human subjects. One of the authors carried out a complete periodontal examination. The following clinical parameters were recorded: probing depth (PD), clinical attachment level (CAL) and percentages of sites with bleeding on probing (BOP), suppuration and plaque. A marked probe (UNC-15, Hu-Friedy, Chicago, IL) was used in all instances. Periodontal diagnosis was established based on the consensus report of the American Academy of Periodontology (AAP)[16].

Subgingival microbial samples were taken from the six deepest pockets. After removing supragingival plaque with curettes and isolating the area with cotton pellets, paper points were inserted into each periodontal pocket for 20 seconds. The six paper points (Maillefer, Ballaigues, Switzerland) were pooled in screw cap vials containing Viability Medium Götenborg Anaerobically (VGMA) III medium[17]. All samples were labeled and processed within 4 hours after sampling. The samples were analyzed using microbial culture techniques for the presence of periodontopathic bacteria according to Slots[18]. Briefly, most samples were processed at room temperature (25ºC) and incubated in CO_2 and anaerobic culture systems. Brucella blood agar medium was incubated at 35ºC in an anaerobic jar for 7 days. The Trypticicase Soy Serum Bacitracin Vancomycin agar (TSBV) medium was incubated in 10% CO_2 at 37ºC for 4 days. Presumptive identification was performed according to the methods described[19], and using a commercial identification micromethod system (RapID ANA II, Remel, Norcross, GA, USA) for *P. gingivalis.* Total viable counts (TVC) were defined as the total number of colony-forming units obtained on non-selective media plates. Species found on selective media were counted and their percentage of TVC was calculated.

Isolation of Gram-negative enteric rods by culture: After placement for 20 s, the paper points were pooled in a vial containing 2.0 ml of VMGA III transport medium[17]. The sample vials were maintained at room temperature, transferred to the laboratory and processed within 4 h after sampling. After the vials were placed in an incubator for 30 min at 37ºC, bacterial plaque was mechanically dispersed with a test tube mixer at the maximal setting for 60 s. Serial 10-fold dilutions were prepared in pepton water, and aliquots were plated on MacConkey agar. The plates were incubated aerobically at 37ºC for 24 h. Each isolate was characterized according to colonial and cellular morphology and Gram-stain characteristics. Gram-negative enteric rods were speciated using a standardized biochemical test (API 20E, Bio-Merieux, Marcy L'Etoile, France). Total viable counts were defined as the total number of colony forming units obtained on non selective media plates. Species found on selective media were counted and presented as counts x 10^5. Each patient provided a pooled subgingival plaque sample according to the method described by Herrera et al[20]. Equal numbers of isolates were used from each subject.

Statistical Analysis

Data were entered into an Excel (Microsoft Office 2007) database and proofed for entry errors. The

database was subsequently locked, imported into SPSS for Windows (SPSS, Statistical Package for the Social Sciences, version 15, Chicago, IL), formatted and analyzed. Indicators of Descriptive Statistics were used, such as frequencies, percentage, average, variance, and standard deviation. The presence of *P. gingivalis* and Gram-negative enteric rods-positive individuals were described as the percentage of individuals with at least one infected pocket. The chi-square test was used to assess differences between BOP versus the presence or absence of *P. gingivalis* and Gram-negative enteric rods. PD and CAL differences and the presence or absence of *P. gingivalis* and Gram-negative enteric rods were determined by the Mann-Whitney test. Association among both microorganisms was expressed through a non-parametric correlation coefficient (Spearman rank). Only sites presenting concomitantly CAL and PD of 4mm or more at baseline were considered in the analyses of CAL, PD, and BOP. The significance level was set at 0.05 for all tests.

RESULTS

Table 1 shows the clinical characteristics of the study subjects. A total 45 women (59.2%) and 31 men (40.8%) with chronic periodontitis were studied (age: 46±8.08 years), of whom 21.05% (16 subjects) were current smokers.

Among the 76 patients examined, Gram-negative enteric rods and *P. gingivalis* were detected in 20 (26.3%) and 51 (67.1%) individuals, respectively. A total 17 (22.4%) patients harbored both microorganisms studied.

Our previous paper[13] reported four species of Gram-negative enteric rods in subgingival plaque in 20 (26.31%) of 76 patients: *Klebsiella pneumoniae* occurred in 12 patients, *Pseudomonas aeruginosa* in four patients and three other species were recovered with lower prevalence.

Table 1: Clinical data at sampled sites.

Clinical Parameter	Mean ± SD
PD	5.14±1.33
CAL	5.61±1.83
% BOP	70±16
% PI	56±19
% SUP	4.3±3.1

Mean expressed in millimeters; SD=standard deviation.
PD: probing depth ; CAL: clinical attachment level; %BOP: percentage of sites with bleeding on probing; %PI: percentage of sites with plaque; %SUP percentage of sites with suppuration.

Table 2: Correlations among Gram negative enteric rods and Porphyromonas gingivalis.

Parameter	Presence enteric and *P. gingivalis*	Statistics
PD	r= 0.655	Spearman P<0.0001
CAL	r= 0.606	Spearman P<0.0001
% BOP	r= 0.502	Spearman P<0.0001

PD: probing depth ; CAL: clinical attachment level; %BOP: percentage of sites with bleeding on probing.

Gram-negative enteric rods in periodontal pockets were highly significantly and positively correlated with presence of *P. gingivalis* (r=0.531, *P*<0.0001); and both organisms were highly significantly and positively correlated with PD, CAL and BOP (Table 2).

Patients with presence or absence of *P. gingivalis* and Gram-negative enteric rods showed significantly different clinical conditions, as assessed by the clinical parameters studied (*P*<0.0001) (Table 3). The mean PD (mm) of the sampled sites was significantly deep-

Table 3: Comparison of clinical data at sampled sites.

Parameter	Presence enteric and *P. gingivalis* (Mean ± SD)	Absence enteric and *P. gingivalis* (Mean ± SD)	Statistics
PD	5.94±1.5	5.08±1.3	Mann Whitney P<0.001
CAL	6.76±2.1	5.47±1.7	Mann Whitney P<0.001
% BOP	84±14	65±17	X^2 P<0.001

Mean expressed in millimeters; SD=standard deviation.
PD: probing depth ; CAL: clinical attachment level; %BOP: percentage of sites with bleeding on probing.

er in patients with presence of *P. gingivalis* and Gram-negative enteric rods than in patients with absence of both microorganisms. Similar results were found for the CAL data. The proportion of sites with BOP was significantly higher in patients with presence of *P. gingivalis* and Gram-negative enteric rods than in patients without them.

DISCUSSION

In the present investigation, we studied the associations between *P. gingivalis* and Gram-negative enteric rods and clinical parameters from subjects with chronic periodontitis. Information from this study may have therapeutic implications for the treatment of non-oral infections caused by oral pathogens. Dissemination of periodontal pathogens to other body sites frequently occurs and may cause serious diseases[21]. For these reasons, the study of the subgingival microbiota in a particular country is relevant for identifying its possible impact on outcomes after treatment[20].

This study identified Gram negative enteric rods in 20 (26.31%) of 76 patients. In Latin America; similar frequencies to those encountered in our study have been reported among Brazilians[11] and Colombians[12,20]. Subgingival Gram-negative enteric rods often persist after periodontal debridement and surgery[4-9]. Additionally, they exhibit less susceptibility to chlorhexidine[4] and *in vitro* resistance to most adjunctive antibiotics used to treat periodontitis[4,10-13]. Moreover, their high pathogenic potential may represent a cause of failure in periodontal therapy[4-9]. Further studies are required in order to clarify the effect of enteric rods on clinical parameters and response to periodontal treatment.

In the present investigation *P. gingivalis* was observed in 51 (67.1%) individuals. Our values are similar to the frequencies reported in South American populations[12,20]. Invasion by *P. gingivalis* has been proposed as a possible mechanism of pathogenesis in periodontal and cardiovascular diseases[22]. *P. gingivalis* has direct access to the systemic circulation and the endothelium in periodontitis patients, as transient bacteremias are common, and the ability of *P. gingivalis*, detected at the sites of atherosclerotic disease, to invade host cells has been demonstrated[23]. Mombelli et al.[24] observed that *P. gingivalis* is a pathogen which is able to invade periodontal tissues but evade mechanical-chemical therapies. Future studies will address the influence of multiple species of subgingival bacteria on the patients' responsiveness to periodontal therapy.

To the best of our knowledge, there are no studies on the association of periodontal Gram-negative enteric rods and *P. gingivalis* and relating these microorganisms with clinical parameters. In this study, a significantly positive correlation between Gram-negative enteric rods and *P. gingivalis* was observed ($P<0.0001$). Herrera et al.[20] and Botero et al.[12] found that Gram negative enteric rods were isolated from approximately one-third of the patients whose cultures were positive for subgingival *P. gingivalis*. In this regard, Botero et al.[12] noted that colonies of enteric rods are larger, indicating that they could colonize the periodontal pockets in high proportions. On the other hand, PCR detection does not take into consideration whether the sample is viable, and thus may yield to a higher frequency[12]. D'Ercole et al.[25] recently compared conventional culture methods and multiplex PCR for the detection of periodontopathogenic bacteria and observed that for both methods, there was a good degree of accuracy in the determination of *P. gingivalis.* In the present investigation, presumptive identification of microorganisms was performed according to the methods described by Slots and Reynolds[19] and using a commercial identification micromethod system for *P. gingivalis.* Several authors have also used these two methods to identify *P. gingivalis*[20,26,27]. Like Botero et al.[12], the present study reports the occurrence of the microorganisms detected based on culture techniques because it allows us to work subsequently with the cultured microorganisms. Like Barbosa et al.[11], in this study each patient was classified as positive for the presence of the two microorganisms studied when he/she presented at least one pocket infected with these organisms. However, a more extensive investigation evaluating the presence of these microorganisms in all pockets would be more appropriate to study correlations between Gram-negative enteric rods and *P. gingivalis* further.

The clinical parameters studied increased significantly in presence of *P. gingivalis* and Gram-negative enteric rods, compared to patients with absence of both microorganisms (Tables 2 and 3). This evidence indicated that *P. gingivalis* and Gram-negative enteric rods are closely associated with the process of periodontal breakdown and both microorganisms may be involved in the course of tissue destruction such as pocket deepening or active attachment loss. Differences in host response, oral hygiene habits, oral health care access and microbial composition may help explain these differences in the clinical expression of periodontitis in the population studied[28]. More exhaustive investigations addressing the association between periodontitis and environmental, economic and genetic variables are needed[20].

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