Association of three putative periodontal pathogens with chronic periodontitis in Brazilian subjects

Cristiane GONÇALVES, Geisla Mary S. SOARES, Marcelo FAVERI, Paula Juliana PÉREZ-CHAPARRO, Eduardo LOBÃO, Luciene Cristina FIGUEIREDO, Gustavo Titonele BACCELLI, Magda FERES

Universidade de Guarulhos, Departamento de Periodontia, Guarulhos, SP, Brasil.

Corresponding address: Magda Feres - Centro de Pós-Graduação e Pesquisa - CEPPE - Universidade Guarulhos - Praça Tereza Cristina, 229 - Centro - Guarulhos - SP - Brazil - 07023-070 - e-mail: mferes@ung.br

Submitted: December 06, 2015 - Modification: February 15, 2016 - Accepted: March 1º, 2016

ABSTRACT

Objective: The aim of this study was to evaluate the association of Porphyromonas endodontalis, Filifactor alocis and Dialister pneumosintes with the occurrence of periodontitis. Material and Methods: Thirty subjects with chronic periodontitis (ChP) and 10 with periodontal health (PH) were included in the study. Nine subgingival biofilm samples were collected as follows: i) PH group - from the mesial/buccal aspect of each tooth in two randomly chosen contralateral quadrants; ii) ChP group - from three sites in each of the following probing depth (PD) categories: shallow (≤3 mm), moderate (4-6 mm) and deep (≥7 mm). Checkerboard DNA-DNA hybridization was used to analyze the samples. Results: We found the three species evaluated in a higher percentage of sites and at higher levels in the group with ChP than in the PH group (p<0.05, Mann-Whitney test). We also observed these differences when the samples from sites with PD≤4 mm or ≥5 mm of subjects with ChP were compared with those from subjects with PH (p<0.05, Mann-Whitney test). In addition, the prevalence and levels of D. pneumosintes, and especially of F. alocis were very low in healthy subjects (0.12x10^5 and 0.01x10^5, respectively). Conclusion: F. alocis and D. pneumosintes might be associated with the etiology of ChP, and their role in the onset and progression of this infection should be further investigated. The role of P. endodontalis was less evident, since this species was found in relatively high levels and prevalence in the PH group.

Keywords: Chronic periodontitis. Etiology. Pathogens. Microbiology.

INTRODUCTION

The oral cavity naturally hosts hundreds of species that together constitute the oral microbiome. Most of these microorganisms are compatible with a good oral health status, while some of them are considered pathogens that might trigger infectious processes, such as periodontitis. Knowledge about the periodontal pathogens associated with the onset and progression of periodontal diseases has been largely concentrated on the microorganisms that comprise the “subgingival microbial complexes” previously described by Socransky, et al.24,25 (2002, 1998). Nonetheless, a recent systematic review has indicated the existence of other candidate periodontal pathogens20.

The first line of evidence to define a microorganism as a true pathogen is to determine if the organism is present in a higher prevalence and/or levels and/or proportions/abundance in disease than in health (association studies)22. The increased levels of a bacterial species during disease is one of the most important parameters to evaluate its role as a periodontal pathogen, and is more meaningful than the mere presence or absence of the microorganism in the subgingival environment22,24. Some investigations have shown that Porphyromonas endodontalis, Filifactor alocis and Dialister pneumosintes are present in a higher prevalence and/or abundance in patients with periodontitis than in periodontally healthy...
subjects\textsuperscript{1,2,5,7,10,14,15,18,21}. However, to our knowledge, no studies to date have compared the prevalence as well as the levels of these three species in subjects with good periodontal health and with disease.

Therefore, the aim of this study was to evaluate the association of three putative periodontal pathogens, \textit{P. endodontalis}, \textit{F. alocis} and \textit{D. pneumosintes} with periodontitis, by comparing their levels and prevalence in subjects with chronic periodontitis (ChP) and those in good periodontal health (PH). This knowledge might help to establish more effective preventive and treatment strategies for these infections.

\textbf{MATERIAL AND METHODS}

\textbf{Study population}

Ten periodontally healthy subjects and 30 subjects with ChP were selected from the population referred to the Periodontal Clinic of Guarulhos University (Guarulhos, SP, Brazil) for treatment. Detailed medical and dental records were obtained, and one trained and calibrated examiner performed a full-mouth periodontal examination. Subjects who fulfilled the inclusion criteria were invited to participate in the study. All eligible subjects were informed of the nature, potential risks and benefits of their participation in the study and signed an informed consent document. The Guarulhos University’s Clinical Research Ethics Committee approved the study protocol.

\textbf{Inclusion and exclusion criteria}

Inclusion criteria for periodontally healthy subjects were: \(>30\) years, \(\geq24\) teeth, no sites with probing depth (PD) and/or clinical attachment level (CAL) \(\geq3\) mm and fewer than 20\% of sites with gingival bleeding and/or bleeding on probing (BOP). Inclusion criteria for subjects with periodontitis were: \(>30\) years, \(\geq20\) teeth, \(\geq8\) sites in different teeth with PD\(\geq5\) mm, CAL\(\geq3\) mm and BOP.

Exclusion criteria were pregnancy, lactation, currently being a smoker, antimicrobial therapies during the previous 6 months, medical conditions requiring prophylactic antibiotic coverage, continuous use of mouth-rinse containing antimicrobial agents in the preceding 3 months, systemic conditions that could affect the progression of periodontitis and long-term administration of anti-inflammatory and immunosuppressive medications.

\textbf{Clinical evaluation}

One examiner performed the clinical monitoring and carried out all clinical measurements in a given subject. Visible plaque (0/1), gingival bleeding (0/1), BOP (0/1), suppuration (0/1), PD and CAL were measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) in all teeth, excluding third molars. PD and CAL measurements were recorded to the nearest millimeter using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA).

\textbf{Microbiological evaluation}

\textbf{Sample collection}

After having recorded the clinical parameters, supragingival plaque was removed and then nine individual subgingival plaque samples were collected per subject using sterile 11/12 mini-Gracey curettes as follows: Periodontally healthy subjects: samples were collected from the mesial/buccal aspect of each tooth in two randomly chosen contralateral quadrants. Subjects with ChP: samples were collected from three sites in each of the following PD categories: shallow (PD\(\leq3\) mm), moderate (PD 4-6 mm) and deep (PD\(\geq7\) mm).

The samples were placed in separate plastic tubes containing 0.15 mL of TE (10 mM Tris-HCl, 1mM EDTA, pH 7.6). One hundred microliters of 0.5 M NaOH were immediately added to each tube and the samples were stored at -80°C.

\textbf{Checkerboard DNA–DNA hybridization}

The samples were evaluated by checkerboard DNA–DNA hybridization\textsuperscript{19,23} at the Guarulhos University Laboratory of Microbiology. The samples were boiled for 10 min and 0.8 mL of 5 M ammonium acetate was added to each sample. The DNA released was then placed into the extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA), concentrated on a 15/15 cm, positively charged, nylon membrane (Boehringer Mannheim, Indianapolis, IN, USA) and fixed to the membrane by baking it at 120°C for 20 min. The membrane was then placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at 90°C in the lanes of the device. Digoxigenin-labelled whole genomic DNA probes for \textit{P. endodontalis}, \textit{F. alocis} and \textit{D. pneumosintes} were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using the antibody to digoxigenin conjugated with alkaline phosphatase, and chemiluminescence detection. The last two lanes in each run contained standards at concentrations of \(10^{5}\) and \(10^{6}\) cells of each species. Signals were evaluated by comparison with the standards at \(10^{5}\) and \(10^{6}\) bacterial cells for the test species on the same membrane. The sensitivity of this assay was adjusted to allow detection of each DNA probe. This procedure was carried out in order to provide the same sensitivity.
of detection for each of the species.

Statistical analysis

Each individual clinical parameter as well as mean counts (x10^5) of each bacterial species evaluated and the percentage of sites colonized by these species were computed per subject and then across subjects in both groups. The Mann-Whitney test was used to seek significance of differences between the two groups for age, clinical and microbiological parameters, and applying Bonferroni’s correction for multiple comparisons. Thus, from this computation, a p value of <0.016 was considered to be statistically significant at p<0.05. Similarly, individual values of p<0.003 and p<0.0003 were considered statistically significant at p<0.01 and p<0.001, respectively. A Chi-square test was used to compare differences in the frequency of gender. Levels of the three bacterial species were averaged separately within two PD categories (PD ≥5 mm and PD ≤4 mm) per subject, and then across subjects with ChP. The Wilcoxon Test was used to find the significance of differences between these two categories. The level of significance was set at 5%.

RESULTS

Table 1 presents the demographic and clinical characteristics of the subjects with ChP and PH. As expected, we observed statistically significant differences between the two groups for all clinical parameters evaluated (p<0.05). The mean age and percentage of females did not differ between groups (p>0.05).

Figures 1 and 2 present the mean percentage of sites colonized by F. alocis, D. pneumosintes and P. endodontalis and the mean counts of these species, respectively, in the two clinical groups. All three species were present in a statistically significant, higher percentage of sites and mean counts in subjects with ChP than in those in PH (p<0.05). The mean levels of F. alocis, D. pneumosintes and P. endodontalis in the healthy subjects were 0.01x10^5, 0.12x10^5 and 1.89x10^5, respectively; and 1.77x10^5, 7.61x10^5 and 4.01x10^5, respectively, in the periodontitis subjects. Figure 3 shows the mean counts (x10^5) of the three microbial species in subjects with PH and in sites with PD ≥5 mm and ≤4 mm in subjects with ChP. F. alocis and D. pneumosintes were present in statistically significant, higher mean levels in shallow and deep sites of periodontitis subjects in comparison with the levels observed in healthy subjects (p<0.05). The levels of P. endodontalis did not differ significantly between shallow sites

**Table 1- Clinical parameters and demographic characteristics of the two groups**

|               | Healthy (n=10) | Periodontitis (n=30) | Mann-Whitney (p) |
|---------------|---------------|----------------------|------------------|
| Number of females | 9             | 22                   | 0.73889          |
| Age           | 38.3±6.8      | 42.2±6.4             | 0.26             |
| PD            | 1.8±0.2       | 3.7±0.7              | 0.0              |
| CAL           | 0.9±0.2       | 4.5±1.3              | 0.0              |
| % sites with: |               |                      |                  |
| Plaque        | 25.5±10.1     | 80.0±15.6            | 0.0              |
| Gingival bleeding | 2.0±0.8   | 27.7±15.3            | 0.0              |
| Bleeding on probing | 3.1±1.2 | 78.9±14.1            | 0.0              |
| Suppuration   | 0.0±0.0       | 1.1±0.8              | 0.00001          |

PD: probing depth; CAL: clinical attachment level; SD: standard deviation

**Figure 1- Mean percentage of sites colonized by the three bacterial species evaluated. The Mann-Whitney test was used to assess significance of differences between groups; **p<0.01 and ***p<0.001
DISCUSSION

We found the three candidate periodontal pathogens evaluated in this study in statistically significant higher percentage of sites and in higher levels in subjects with ChP than in periodontally healthy individuals. This data is in agreement with previous investigations that also showed an association of *P. endodontalis*1,6,10,15-17, *F. alocis*1,3,6,8,10,14,15,21 and *D. pneumosintes*7,8,14,18 with periodontal diseases. In addition, a recent Systematic Review, which evaluated the weight of evidence for the existence of novel periodontal taxa might be associated with the etiology of periodontitis. The authors proposed that there is moderate evidence in the literature to support the role of *P. endodontalis* and *F. alocis* as periodontal pathogens, and some evidence for *D. pneumosintes*20.

Although levels of the three bacterial species evaluated in this study were elevated in subjects with ChP in comparison with periodontally healthy individuals (Figures 1 and 2), their levels and prevalence in healthy subjects also provided essential information as regards their possible role in the etiology of the disease. *F. alocis* was present in only 8% of the sites of healthy individuals and *D. pneumosintes* was also present at very low levels in healthy individuals, but was detected in 42% of the sites evaluated. We found *P. endodontalis* in almost 50% of the sites of healthy subjects and at relatively high levels. This data might indicate that *P. endodontalis* is an opportunistic bacterial species or an accessory pathogen that may not trigger the disease...
process, but whose levels may increase when the inflammation process begins, thus contributing to the disease process\(^\text{12}\). Apparently, \textit{F. alocis} retains the characteristics of a so called “keystone pathogen”, microorganisms that have the potential to initiate periodontal destruction by causing a dysbiosis in the subgingival ecosystem\(^\text{3,13}\). Although its (\textit{F. alocis}) prevalence and levels in healthy subjects was very low in this study, Aruni, et al.\(^\text{3,4}\) (2014,2011) showed that \textit{F. alocis} has some unique virulence factors that favor its persistence in inflammatory environments and may mediate \textit{P. gingivalis} proliferation, suggesting that this species can play a pivotal role in microbial community dynamics.

The strength of this study was the number of subgingival plaque samples analyzed for the levels and prevalence of the three bacterial species. Although the number of subjects included in this study was relatively small, 360 plaque samples were individually analyzed. To date, most of the association studies that have investigated \textit{F. alocis}, \textit{D. pneumosintes} or \textit{P. endodontalis} evaluated fewer samples\(^\text{1,6,9,10,15,16,18,21}\). In addition, these studies have generally only determined the prevalence or abundance (proportion) of these microbial species in the subgingival biofilm. Indeed, studies have recognized that evaluating a large number of plaque samples and quantifying the microorganisms are critical requisites when trying to establish an association between certain microorganisms and the onset and progression of periodontitis\(^\text{11}\).

It is important to mention that association studies only provide the initial information necessary to suggest a possible link between a microorganism and an infection. Further evaluations are necessary to confirm this type of association, such as clinical (i.e. risk assessment and interventional studies); host-response studies, and investigations into the mechanisms of pathogenicity of the suspected pathogens.

**CONCLUSION**

In conclusion, the results of this investigation suggested an association of \textit{D. pneumosintes}, and especially of \textit{F. alocis} with the etiology of periodontitis. The role of \textit{P. endodontalis} was less evident, since we found this species in relatively high levels and prevalence in periodontally healthy subjects. This data might guide future studies on the actual role of these three bacterial species in the etiology of periodontitis and help to establish a more effective treatment for these infections.

**ACKNOWLEDGMENTS**

This work was partly supported by the São Paulo Research Foundation, Grant 2012/20915-0. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

**REFERENCES**

1- Abusleme L, Dupuy AK, Dutza NN, Silva N, Burleson JA, Strasbaugh LD, et al. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. ISME J. 2013;7:1016-25.
2- Al-hebshi NN, Al-Alimi A, Taiyeb-Ali T, Jaafar N. Quantitative analysis of classical and new putative periodontal pathogens in subgingival biofilm: a case-control study. J Periodontal Res. 2015;50(3):320-9.
3- Aruni AW, Chioma O, Fletcher MH. Filifactor alocis: the newly discovered kid on the block with special talents. J Dent Res. 2014;93(8):725-32.
4- Aruni AW, Roy F, Fletcher MH. Filifactor alocis has virulence attributes that can enhance its persistence under oxidative stress conditions and mediate invasion of epithelial cells by Porphyromonas gingivalis. Infect Immun. 2011;79:3872-86.
5- Camelo-Castro AJ, Mira A, Pico A, Niballi L, Henderson B, Donos N. Subgingival microbiota in health compared to periodontitis and the influence of smoking. Front Microbiol. 2015;6:119.
6- Dahlen G, Leonhardt A. A new checkerboard panel for testing bacterial markers in periodontal disease. Oral Microbiol Immunol. 2006;21:6-11.
7- Ferraro CT, Gornic C, Barbosa AS, Peixoto RJ, Colombo AP. Detection of Dialister pneumosintes in the subgingival biofilm of subjects with periodontal disease. Anaerobe. 2007;13:244-8.
8- Fine DH, Markowitz K, Fairlie K, Tischio-Berseri D, Ferrendiz J, Furgang D, et al. A consortium of Aggregatibacter actinomycetemcomitans, Streptococcus parasanguinis, and Filifactor alocis is present in sites prior to bone loss in a longitudinal study of localized aggressive periodontitis. J Clin Microbiol. 2013;51(9):2850-61.
9- Ghayourni N, Chen C, Slots J. Dialister pneumosintes, a new putative periodontal pathogen. J Periodontal Res. 2002;37:75-8.
10- Griffen AL, Beall CJ, Campbell JH, Firestone ND, Kumar PS, Yang ZK, et al. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. ISME J. 2012;6:1176-85.
11- Huffajee AD, Socransky SS. Introduction to microbial aspects of periodontal biofilm communities, development and treatment. Periodontol 2000. 2006;47:7-12.
12- Hajishengalis G, Darveau RP, Curtis MA. The Keystone-pathogen hypothesis. Nat Rev Microbiol. 2012;10:717-25.
13- Hajishengalis G, Lamont RJ. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Mol Oral Microbiol. 2011;27:409-19.
14- Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML, Leys EJ. New bacterial species associated with chronic periodontitis. J Dent Res. 2003;82:336-44.
15- Kumar PS, Griffen AL, Moeschberger ML, Leys EJ. Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. J Clin Microbiol. 2005;43:3944-55.
16- Lombardo Bedran TB, Marcantoni RA, Spin Neto R, Alves Mayer MP, Grenier D, Spolidorio LC, et al. Porphyromonas endodontalis in chronic periodontitis: a clinical and microbiological cross-sectional study. J Oral Microbiol. 2012;4:10123.