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Periodontal-disease-associated biofilm: A reservoir for pathogens of medical importance

Ana Paula Vieira Colombo [a, *], Clarissa Bichara Magalhães [a], Fátima Aparecida Rocha Resende Hartenbach [a, b], Renata Martins do Souto [a], Carina Maciel da Silva-Boghossian [a, c]

[a] Institute of Microbiology, Department of Medical Microbiology, Federal University of Rio de Janeiro, Av. Carlos Chagas Filho, 373/CCS/ Bloco I, lab. I2-03, Cidade Universitária – Rio de Janeiro, RJ CEP: 21941-902, Brazil
[b] School of Dentistry, Department of Clinics, Federal University of Rio de Janeiro Rua Professor Rodolpho Paulo Rocco, 325, Cidade Universitária – Rio de Janeiro, RJ CEP: 21941-617, Brazil
[c] School of Dentistry, University of Grande Rio, R. Prof. José de Souza Herdy, 1160, Jardim Vinte e Cinco de Agosto, Duque de Caxias, RJ CEP: 25071-202, Brazil

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ABSTRACT

The ecological diversity of the periodontal microenvironment may provide suitable conditions for the colonization of species not usually considered members of the oral microbiota. In this investigation, we aimed to determine the prevalence and levels of pathogenic species of medical relevance in the microbiota of individuals with distinct periodontal clinical status. Subgingival biofilm was obtained from patients with periodontal health (H, n = 81), gingivitis (G, n = 55), generalized aggressive (AgP, n = 36) or chronic periodontitis (CP, n = 98), and analyzed for 39 microbial taxa using a checkerboard DNA–DNA hybridization technique. Microbial differences among groups, as well as associations between clinical and microbiological parameters were sought by non-parametric and univariate correlation tests. *Neisseria* spp., *Peptostreptococcus anaerobius*, *Candida albicans*, enterobacteria, *Pseudomonas aeruginosa*, *Eubacterium saphenum*, *Clostridium difficile* and *Olsenella uli* were detected in high mean prevalence and counts in the subgingival microbiota of the study population. Species that were more related to periodontal inflammation and tissue destruction at the patient and site levels included enterobacteria, *C. albicans*, *Neisseria* spp., *P. aeruginosa*, *O. uli*, *Hafnia alvei*, *Serratia marcescens* and *Filifactor alocis* ($p < 0.05$). In contrast, *Fusobacterium necrophorum*, *Lactobacillus acidophilus*, *Staphylococcus aureus* and *Streptococcus pneumoniae* were associated with periodontal health ($p < 0.05$). Pathogenic species of medical importance may be detected in high prevalence and levels in the periodontal microbiota. Regardless of their role in periodontal health or disease, the periodontal biofilm may be a source for dissemination and development of systemic infections by these pathogenic microorganisms.

1. Introduction

Periodontal diseases are among the most common oral infectious diseases associated with the establishment of a highly pathogenic biofilm that triggers an immune/inflammatory host response, leading to the destruction of supporting periodontal tissues and eventual tooth loss [1,2]. In addition to the substantial economic burden and negative impact of these diseases on quality of life [3,4], oral bacteria and periodontal infections have been indicated as potential risk factors for several systemic diseases [5–8]. Due to the anatomical proximity of the periodontal biofilm to the gingival blood stream, periodontal pockets may act as reservoirs of microbial pathogens and their products, as well as inflammatory mediators and immunocomplexes that may disseminate to other sites of the human body [7,9]. The highly complex periodontal microbiota plays a major role in the establishment of periodontal health as well as the development of periodontal diseases. This microbiota comprises mostly commensal

* Corresponding author. UFRJ/CCS – Instituto de Microbiologia Paulo de Góes – Bloco I, lab. I2-03, Av. Carlos Chagas Filho, 373 Cidade Universitária – Rio de Janeiro, RJ CEP: 21941-902, Brazil.
E-mail addresses: apcolombo@micro.ufrj.br (A.P. Vieira Colombo), clari_bmag@yahoo.com.br (C.B. Magalhães), fathimarocha@gmail.com (F.A.R.R. Hartenbach), renatamsouto@yahoo.com.br (R. Martins do Souto), carinabogho@hotmail.com (C. Maciel da Silva-Boghossian).

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resident members of oral species that have co-evolved to colonize the human oral cavity [10–12]. However, the existence of a large variety of ecological determinants in the oral ecosystem may provide optimal conditions for the establishment of microorganisms not usually considered residents of the normal oral microbiota. Although there is still controversy whether these species are merely contaminants or transient members, strong evidence has been showing that they may indeed colonize the oral microbiota [13–17]. In normal conditions of oral health, one should not expect these microorganisms to overcome in proportions the very well-adapted oral species. On the other hand, these pathogenic species may increase significantly in frequency and counts in individuals presenting oral infections such as periodontitis, poor hygiene and/or immunosuppression [18–22]. The same way oral pathogens have been implicated in extra-oral infections [6,7,9], high levels of medically important pathogens in the periodontitis-associated microbiota may pose a risk for systemic dissemination and development of infections at distant body sites, particularly in immune-compromised individuals [13,14,20,21,23,24]. In the current investigation, the frequency of detection and levels of pathogenic species commonly related to relevant systemic infections were determined in the subgingival biofilm of individuals with various periodontal clinical conditions.

2. Material and methods

2.1. Subject population

A total of 270 sequential subjects who sought dental treatment between 2007 and 2014 at the Dental School of the Federal University of Rio de Janeiro (UFRJ) were enrolled in the present study. Research was conducted according to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects. All subjects were informed about the nature of the study and a signed consent form was obtained from each individual. The study protocol was reviewed and approved by the Review Committee for Human Subjects of the Clementino Fraga Filho University Hospital/UFRJ.

2.2. Clinical examination and periodontal diagnosis

Subjects were submitted to medical/dental anamnesis, and information regarding age, gender, ethnicity/color, and smoking status was obtained. Smoking status was recorded as non-smoker and smoker (current or former smokers). All subjects had at least 14 teeth and ≥18 years of age. Exclusion criteria included pregnancy, nursing, periodontal therapy and use of antibiotics within the previous six months, as well as any systemic condition that could affect the progression of periodontitis. Individuals who required antibiotic coverage for routine periodontal procedures were also excluded. Clinical examination was performed by trained and calibrated examiners (C.M.S-B; C.B.M; F.A.R.H.). Full-mouth periodontal clinical measurements included presence/absence of visible supragingival biofilm (PL) and bleeding on probing (BOP), as well as probing depth (PD) and clinical attachment level (CAL) recorded in millimeters using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA). After clinical examination, subjects were diagnosed as having periodontal health (H, n = 81), gingivitis (G, n = 55), generalized aggressive (AgP, n = 36) or chronic periodontitis (CP, n = 98) according to criteria described by the American Academy of Periodontology [25], with modifications [26]. Briefly, H patients presented ≤10% of sites with BOP, no PD or CAL >3 mm, although PD or CAL = 4 mm without BOP in up to 5% of the sites was allowed; G patients had >10% of sites with BOP, no PD or CAL >3 mm, although PD or CAL = 4 mm without BOP in up to 5% of the sites was allowed; CP patients presented >10% of teeth with PD and/or CAL ≥ 5 mm and BOP; AgP presented ≥30% of teeth with PD and/or CAL ≥5 mm with BOP, including at least one incisor and one first molar, and ≤39 years of age.

2.3. Biofilm sampling

Subgingival biofilm samples were obtained from 7 healthy sites (PD and/or CAL < 4 mm, no BOP) and 7 sites with the greatest PD (PD and/or CAL > 4 mm with BOP) from periodontitis patients; 7 sites with gingivitis (PD and/or CAL < 4 mm with BOP) from G patients, and 7 healthy sites from H patients. After removal of supragingival biofilm with a sterile gauze, subgingival biofilm samples were individually collected using sterile Gracey curettes (Hu-Friedy), and placed into microtubes containing TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.6).

2.4. Microbiological assessment

Microbial analyses were performed by the checkerboard DNA–DNA hybridization technique according to Heller et al. [27]. The samples in TE were lysed by adding 0.5 M NaOH and boiling for 10 min. Denatured DNA was neutralized with 5 M $C_2H_3O_2NH_4$ and fixed in individual lanes on a nylon membrane (Hybond-N+, GE Healthcare Life Sciences, Piscataway, NJ) using the Minislot 30 (Immunetics, Cambridge, MA). The Miniblotter45 apparatus (Immunetics) was used to hybridize 26 (Appendix A) whole genomic digoxigenin-labeled probes (Roche Molecular Systems, Alameda, CA). DNA from *Enterobacter agglomerans*, *Enterobacter cloacae*, *Enterobacter gergoviae*, *Enterobacter sakazakii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae* was combined in an enterobacteria probe, whereas DNA from *Neisseria subflava*, *Neisseria polysaccharea*, *Neisseria meningitidis*, and *Neisseria lactamica* was pooled in a *Neisseria* spp. probe. Bound probes were detected using anti-digoxigenin phosphatase-conjugated antibody (Roche Molecular Systems) and fluorescence (AttoPhos®, Promega Corporation, Madison, WI) by an imaging capture system (Storm TM 860 and ImageQuant version 5.2, Molecular Dynamics, GE Healthcare Life Sciences). Signals were evaluated visually by comparison with the standards at 10^5 and 10^6 cells for the test species on the same membrane, and recorded as: 0 = not detected; 1 = $<10^5$ cells; 2 = ~10^5; 3 = 10^5–10^6 cells; 4 = ~10^6; 5 = $>10^6$ cells.

2.5. Statistical analysis

Data entry in a database was carried out two investigators (C. M. S-B. and C. B. M.) and error proofed by a senior investigator (A. P. V. C.). A statistical program (SPSS, Statistical Package for the Social Sciences 21.0, IBM Brasil, SP, Brazil) was used for all analyses. Regarding demographic data, mean age, frequency of gender, ethnicity and non-smokers or smokers were computed for each group. Full-mouth clinical measurements were computed for each subject and averaged across subjects within groups. Nominal data were dichotomized as presence (1) or absence (0), and their frequencies computed for each patient and averaged within groups. Normality distribution of all variables was verified using the Kolmogorov–Smirnov test. Significant differences in demographic and clinical parameters among groups were determined by Mann–Whitney, Kruskal–Wallis and χ^2 tests. Statistical significance was set at α level of 5%. Microbiological data were expressed as mean % of colonized sites (prevalence) and mean counts (levels) of colonization, calculated for each species in each subject, and then within each group. In the prevalence analysis, only the presence of the microorganism was considered. The levels (scores 0 to 5) of