Research Paper

Prevalence of *Pseudomonas aeruginosa* and *Acinetobacter* spp. in subgingival biofilm and saliva of subjects with chronic periodontal infection

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Abstract

P. aeruginosa and *Acinetobacter* spp. are important pathogens associated with late nosocomial pneumonia in hospitalized and institutionalized individuals. The oral cavity may be a major source of these respiratory pathogens, particularly in the presence of poor oral hygiene and periodontal infection. This study investigated the prevalence of *P. aeruginosa* and *Acinetobacter* spp. in subgingival biofilm and saliva of subjects with periodontal disease or health. Samples were obtained from 55 periodontally healthy (PH) and 169 chronic periodontitis (CP) patients. DNA was obtained from the samples and detection of *P. aeruginosa* and *Acinetobacter* spp. was carried out by multiplex and nested PCR. *P. aeruginosa* and *Acinetobacter* spp. were detected in 40% and 45% of all samples, respectively. No significant differences in the distribution of these microorganisms between men and women, subgingival biofilm and saliva samples, patients ≤ 35 and > 35 years of age, and smokers and non-smokers were observed regardless periodontal status ($p > 0.05$). In contrast, the frequencies of *P. aeruginosa* and *Acinetobacter* spp. in saliva and biofilm samples were significantly greater in CP than PH patients ($p < 0.01$). Smokers presenting *P. aeruginosa* and high frequencies of supragingival plaque were more likely to present CP than PH. *P. aeruginosa* and *Acinetobacter* spp. are frequently detected in the oral microbiota of CP. Poor oral hygiene, smoking and the presence of *P. aeruginosa* are strongly associated with periodontitis.

Key words: *Pseudomonas aeruginosa*, *Acinetobacter* spp., subgingival biofilm, saliva, periodontitis, PCR.

Introduction

Periodontal diseases are bacterial infections associated with a complex microbiota of the dental biofilm that induces a local and systemic inflammatory response, leading to periodontal tissue destruction (Page and Kornam, 1997; Paster *et al.*, 2006; Socransky *et al.*, 1998). The microbial diversity of the human oral cavity has been recognized for decades, and over 700 species have been identified in this habitat (Paster *et al.*, 2006). In addition to the resident oral species, studies have shown that the oral cavity harbours high proportions of various medically important pathogens, particularly in individuals with poor oral hygiene, periodontal diseases and/or immunosuppression (Ali *et al.*, 1994, 1996; Botero *et al.*, 2007; Colombo *et al.*, 2002, 2009; Da Silva-Boghossian *et al.*, 2011; Fritschi *et al.*, 2008; Gonçalves *et al.*, 2009; Persson *et al.*, 2008; Slots *et al.*, 1988, 1990; Souto and Colombo 2008; Souto *et al.*, 2006). Conceivably, these species may disseminate to distant body sites increasing the risk for systemic infectious conditions such as bacterial pneumonia (Paju and Scannapieco, 2007; Scannapieco, 1998; Scannapieco *et al.*, 1998).

Pseudomonas aeruginosa and *Acinetobacter* spp. are major respiratory pathogens associated with late-onset nosocomial pneumonia in hospitalized and institutionalized individuals (Chastre and Fagon, 2002). *P. aeruginosa* can be identified in a range of infections, especially those with a tendency to become chronic such as lung infections

in cystic fibrosis patients (Wagner and Iglewski, 2008). This species presents many virulence properties including the ability to produce and secrete extracellular enzymes and toxins (Pihl *et al.*, 2010; Smith and Iglewski, 2003, Woods *et al.*, 1982) to adhere to and form biofilms on tissues and abiotic surfaces (Pihl *et al.*, 2010; Smith and Iglewski, 2003), as well as to present resistance to many antibiotics (Slots *et al.*, 1988, 1990). *Acinetobacter* spp. are a major concern in nosocomial infections due to their rapid development of multi-drug resistance, surviving desiccation and persistence in the environment for long periods of time (Fourrier *et al.*, 1998; Karlowsky *et al.*, 2003; Luna *et al.*, 2007). These organisms are associated with bacteremia, pulmonary infections, meningitis, diarrhea and notorious nosocomial infections with mortality rates of 20 to 60% (Luna *et al.*, 2007). Transmission is via person-to-person contact, contaminated water, food and hospital equipment (Agodi *et al.*, 2007; Luna *et al.*, 2007). The prevalence of *Pseudomonas* and *Acinetobacter* spp. in subjects with periodontal diseases may vary widely among different populations (Ali *et al.*, 1994, 1996; Colombo *et al.*, 2002; Persson *et al.*, 2008; Slots *et al.*, 1988, 1990; Slots *et al.*, 1991; Souto *et al.*, 2006). These pathogens have also been associated with treatment failure in patients with refractory periodontitis (Colombo *et al.*, 1998, 2009). The role of these microorganisms in the etiology and pathogenesis of periodontal diseases is unclear. Regardless, we hypothesized that the existence of disease may increase colonization of the oral microbiota by these species. The aim of this study was to determine the carriage rate of *P. aeruginosa* and *Acinetobacter* spp. in the subgingival biofilm and saliva of subjects with periodontal health or chronic periodontitis.

Methods

Subject population and clinical diagnosis

Two hundred and twenty four adult subjects who sought dental treatment at the Dental School of the Federal University of Rio de Janeiro, Brazil were recruited for the study. Informed consent was obtained from all enrolled individuals. The study protocol was approved by the Review Committee for Human Subjects of the Clementino Fraga Filho University Hospital. Exclusion criteria included pregnancy, use of local or systemic antimicrobial agents within 6 months prior to the entry into the study, diabetes and other systemic conditions that could affect the periodontal *status*. All subjects had at least 10 natural teeth and were over 18 years of age. During the first visit, subjects were submitted to an anamnesis questionnaire, and information regarding age, gender and smoking status was obtained. Smoking was recorded as never-having-smoked and smoker (current or former smokers). Periodontal clinical measurements were performed at six sites per tooth and included probing depth (PD), clinical attachment level

(CAL), presence or absence of supragingival biofilm (SB), bleeding on probing (BOP) and suppuration (SUP). After initial clinical evaluation, subjects were categorized as periodontally healthy (PH, n = 55) or chronic periodontitis (CP, n = 169). The PH controls had no sites with PD and/or CAL $>$ 3 mm and no more than 10% of sites with BOP. CP patients presented at least 10% of teeth with PD and/or CAL 3 5 mm, or at least 15% of teeth with PD and /or CAL 3 4 mm, and $>$ 10% of sites with BOP. After clinical examination and sampling, subjects with evidence of destructive periodontal disease were treated for periodontitis by full mouth scaling and root planning under local anesthetic and instructions in proper home care procedures in the Department of Dental Clinic at the Federal University of Rio de Janeiro.

Sampling

After removal of supragingival biofilm with sterile gauze, subgingival biofilm samples were taken of up to 6 sites with PD $\geq$ 4 mm and BOP from CP patients, and from 3 randomly selected sites with PD $<$ 4 mm and no BOP from PH individuals using sterile periodontal curettes (Hu-Friedy, Chicago, IL). For each patient, samples were pooled and placed in Eppendorf tubes containing 500 µL of TE (Tris-HCl 10 mM, EDTA 1 mM, pH 7.6). For saliva samples, patients were not allowed to clean their teeth or to eat 30 min before sampling. They rinsed out their mouths with 10 mL of 0.9% sterile saline for 60 s, and the mouthwashes were collected in sterile plastic recipients. All samples were stored in a freezer at -20 °C.

Isolation of bacterial DNA

Bacterial DNA was extracted from saliva and subgingival biofilm samples according to the methodology described by Laine *et al.* (2000). Briefly, samples were centrifuged at 300 x *g* for 10 min. The pellet was washed twice in 0.9% saline, re-suspended in 100 µL of 50 mM NaOH and boiled for 10 min. Samples were then neutralized with 14 µL of 1 M Tris (pH 7.5) and centrifuged at 14,000 x *g* for 3 min. Supernatants were collected and stored at -20 °C until PCR amplification was performed.

Detection of *P. aeruginosa* and *Acinetobacter* spp. by multiplex and nested PCR

For PCR detection of *P. aeruginosa*, two sets of primers were used: one set (PS1, 5'-ATGAACAACGTTCTGAAATTCTCTGCT-3' and PS2, 5'-CTTGCGGCTGGCTTTTTCCAG-3') consisted of primers corresponding to the beginning and the end of the open reading frame of the OprI gene (De Vos *et al.*, 1997), while the other set of primers (PAL1, 5'-ATGGAAATGCTGAAATTCGGC-3' and PAL2, 5'-CTTCTTCAGCTCGACGCGGACG-3') corresponded to the open reading frame of the OprL gene. A negative

control with no DNA template and a positive control consisting of 20 µL of DNA obtained from the *P. aeruginosa* strain ATCC 27853 were used in all reactions. The PCR reaction was performed in a 100 µL reaction mixture containing 30 pmol of each primer, 10 µL of 10 X PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of desoxynucleotide triphosphate mixture (final concentration 0.2 mM each dATP, dCTP, dTTP and dGTP), 2 U of Platinum® Taq DNA polymerase (Invitrogen, Grand Island, NY) and 20 µL of DNA template. The reaction was performed in a thermal cycler Primus 25/96, MWG (Biotech, Ebersberg, Germany). The initial denaturation step occurred at 95 °C for 2 min, and was followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 57 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 2 min. The products obtained were analyzed on a 1.5% agarose gel electrophoresis performed at 4 V/cm in Tris-acetate-EDTA buffer (TAE). The gel was stained with 0.5 µg/mL ethidium bromide and visualized on an ultraviolet transilluminator (Bioamerica, Miami, USA) A 100 bp ladder digest (Invitrogen) was used as a standard molecular weight. The expected products after amplification were 250 bp and 510 bp in length. A pair of ubiquitous bacterial primers (5'-AGA GTT TGA TCC TGG CTC AG-3' and 5'-ACG GCT ACC TTG TTA CGA CTT- 3') designed by Willis *et al.* (1999) was used to indicate the presence of bacterial DNA in the clinical samples, particularly the ones that were *P. aeruginosa*-negative. For the reaction using the universal 16S rDNA primers, PCR amplification included a 25 µL reaction mixture containing 0.2 mM of forward and reverse universal primers, 2.5 µL of 10x PCR buffer, 2 mM $MgCl_2$, 1.25 U of Platinum® Taq DNA polymerase, and 200 mM of each deoxynucleosidetriphosphate (Invitrogen). The PCR program included an initial denaturation step at 97 °C for 1 min, followed by 30 cycles of a denaturation step at 97 °C for 45 s, a primer annealing step at 55 °C for 45 s, an extension step at 72 °C for 1 min and a final step of 72 °C for 4 min. The presence of bacterial DNA was determined by an amplicon of 1,505 bp in size visualized on a 1.5% agarose gel. *Acinetobacter* spp. detection was carried out by a nested PCR (Broderick *et al.*, 2004; Vanbroekhoven *et al.*, 2004). The first amplification was performed with the bacterial 16S rRNA universal primers 27f, 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492r, 5'-TAC GGC TAC CTT GTT ACG ACT T-3' (amplicon of 1450 bp). Approximately 100 ng of sample DNA was added into a 50 µL PCR mixture containing 0.5 pmol of each primer, 400 µM of each dNTP, 3 mM $MgCl_2$, 10x PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), and 1.5 U Platinum® Taq DNA polymerase (Invitrogen). The amplification program included an initial step of 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and a final step of 72 °C for 5 min [34]. The second amplification was performed

with the primers Ac436f, 5'-TTT AAG CGA GGA GGA GG-3' and Ac676r, 5'-ATT CTA CCA TCC TCT CCC-3' (amplicon of 280 bp) in a reaction volume of 50 µL containing 2 pmol of primer Ac436f, 1 pmol of primer Ac676r, 200 µM of each dNTP, 2 mM $MgCl_2$, 10x PCR buffer, and 1.5 U Platinum® Taq DNA polymerase (Invitrogen). The amplification programme was carried out according to Vanbroekhoven *et al.* (2004).

Statistical analysis

All statistical tests were performed using the Statistical Package for the Social Sciences (SPSS Inc®.v.10 Chicago, IL, version 17). Full-mouth clinical measurements were computed for each subject and then average across subjects within the groups. Differences on clinical parameters between groups were sought using Mann-Whitney and χ^2 tests. The frequency of detection of *P. aeruginosa* and *Acinetobacter* spp. were computed for each subject, and significant differences between groups were sought using the χ^2 test. Associations between demographic and periodontal parameters, and the frequency of *P. aeruginosa* and *Acinetobacter* spp. were examined by the χ^2 test. Predictor variables for periodontitis were investigated using multivariate logistic regression analysis, from which ORs with 95% CI were reported. Statistical significance was reached at the 5% level.

Results

The demographic and periodontal clinical features of the subject groups are shown in Table 1. CP patients comprised of a significantly higher proportion of smokers ($p < 0.01$, Chi-square test) and older individuals ($p < 0.01$; Mann-Whitney test) than PH subjects. No significant differences between groups were observed for gender. As ex-

Table 1 - Demographic and full-mouth clinical parameters (mean ± SD) of Periodontally Healthy (PH) and Chronic Periodontitis (CP) subjects of the study population.

Clinical parameters	PH (N = 55)	CP (N = 169)
(%) Males	31	35
(%) Smokers [**]	6	25
Age (years) [*]	31.1 ± 11	40.2 ± 14
Number of missing teeth [*]	0.95 ± 1.6	5.6 ± 5.7
Probing depth (mm) [*]	1.9 ± 0.5	2.9 ± 1.1
Clinical attachment level (mm) [*]	2.0 ± 0.6	3.7 ± 1.5
% sites with:		
Bleeding on probing [*]	2.5 ± 4.7	43 ± 28
Supragingival biofilm [*]	11.5 ± 11	45 ± 30
Suppuration [*]	0	2 ± 7

[*] Refers to $p < 0.01$, Mann-Whitney test; [**] refers to $p < 0.01$, Chi-square test.

pected, all clinical parameters of periodontal tissue destruction and inflammation (tooth loss, PD, CAL, BOP, SB and SUP) were significantly greater in the CP group compared to controls (p < 0.01; Mann-Whitney test).

Overall, *P. aeruginosa* was detected in 40% and *Acinetobacter* spp. in 45% of all samples. The frequency of *P. aeruginosa* and *Acinetobacter* spp. in saliva and biofilm samples from both clinical groups are depicted in Figures 1 and 2. In saliva samples, a significant higher prevalence of *P. aeruginosa* and *Acinetobacter* spp. were observed in CP subjects (49.7% and 51%, respectively) compared to PH controls (14.6% and 24.4%, respectively) [p < 0.01; Chi-square test]. Likewise, *P. aeruginosa* and *Acinetobacter* spp. were detected significantly more often in subgingival
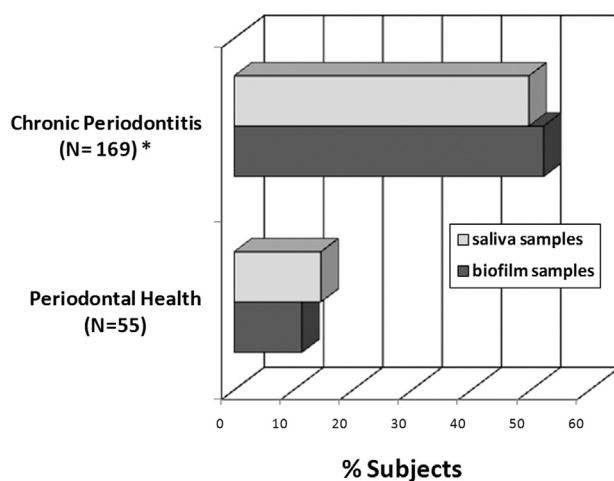

Figure 1 - Frequency of detection of *P. aeruginosa* in saliva and subgingival biofilm samples from periodontally healthy (N = 55) and chronic periodontitis subjects (N = 169). * Significant difference between groups for saliva and biofilm samples (p < 0.05; χ^2 test).
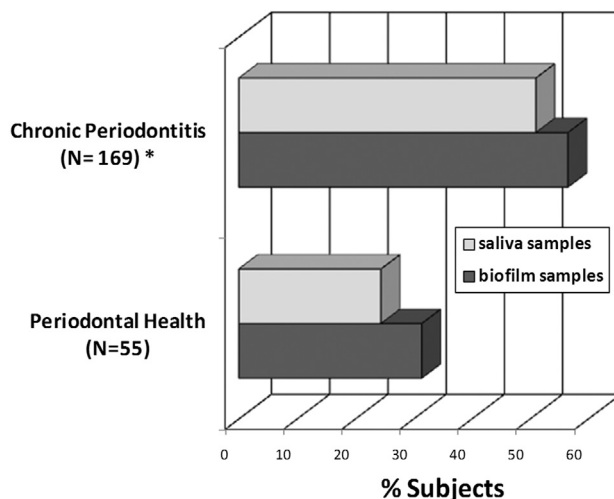

Figure 2 - Frequency of detection of *Acinetobacter* spp. in saliva and subgingival biofilm samples from periodontally healthy (N = 55) and chronic periodontitis subjects (N = 169). * Significant difference between groups for saliva and biofilm samples (p < 0.05; χ^2 test).

biofilm samples from CP (52.2% and 56.5%, respectively) than PH subjects (11.4% and 31.4%, respectively) [p < 0.01; Chi-square test]. Due to significant differences in smoking status and age between CP and PH, the prevalence of *P. aeruginosa* and *Acinetobacter* spp. were evaluated only in non-smokers and subjects with ≤ 35 years of age. Both pathogens were more frequently detected in CP than PH subjects (p < 0.05; Chi-square test, data not shown). Moreover, there were no significant differences in the distribution of *P. aeruginosa* and *Acinetobacter* spp. between men and women, subgingival biofilm and saliva samples, patients ≤ 35 and > 35 years of age, and smokers and non-smokers regardless periodontal status (p > 0.05, Chi-square test, Table 2). Associations between *P. aeruginosa* and *Acinetobacter* spp. and clinical parameters of disease were also examined (Table 2). For this analysis, only subgingival biofilm samples were considered. Patients with large proportions of visible supragingival biofilm, generalized BOP and periodontal destruction (PD and CAL > 4 mm) presented higher frequencies of *P. aeruginosa* and *Acinetobacter* spp. compared to individuals with less signs of disease (p < 0.01, Chi-square test, Table 2). In addition, a strong association between the detection of both species was found. Of the samples positive for *P. aeruginosa*, 76.4% were also positive for *Acinetobacter* spp., whereas of the samples negative for *P. aeruginosa,* 75.2% were negative for *Acinetobacter* spp. (p < 0.001, Chi-square test, data not shown).

Stepwise logistic regression was performed in order to examine associations between demographic, clinical and bacterial parameters and periodontal status. Table 3 shows the predictor variables entered in the final model to distinguish between periodontal health and chronic periodontitis. Smokers (OR 10.22, CI 2.61-40.0, p < 0.01) presenting *P. aeruginosa* (OR 7.68, CI 3.13-18.85, p < 0.001) and high frequencies of supragingival plaque (OR 3.77, CI 1.12-12.62, p < 0.05) were more likely to present chronic periodontitis than periodontal health.

Discussion

Over the last few years, studies have indicated that the presence of non-oral species in the oral microbiota is not a transitory event or a result of contamination during sampling, and that the oral cavity may be a reservoir for medically important pathogens (Barbosa *et al.*, 2001; Botero *et al.*, 2007; Colombo *et al.*, 2002; Da Silva-Boghossian *et al.*, 2011; Fourrier *et al.*, 1998; Fritschi *et al.*, 2008; Gonçalves *et al.*, 2009; Slots *et al.*, 1988, 1990; Souto and Colombo, 2008; Souto *et al.*, 2006). It is possible that in addition to putative periodontal pathogens, species such as *Acinetobacter* spp. and *P. aeruginosa*, either associated with oral pathogens or not, may also play a role in the etiopathogenesis of periodontal diseases. The current investigation examined the subgingival biofilm and saliva

Table 2 - Association between the frequency (%) of *P. aeruginosa* and *Acinetobacter* spp. and demographic and periodontal clinical parameters in all subjects.

Demographic and clinical parameters	% *P. aeruginosa*	% *Acinetobacter* spp.
Gender		
males	37	44
females	42	46
Smoking status		
non-smokers	39	43
smokers	46	58
Age		
≤ 35 years	35	43
> 35 years	44	47
Type of sample		
Saliva	42	45
Subgingival biofilm	35	46
Probing depth *		
≤ 4 mm	16	28.4
> 4 mm	55	65
Clinical attachment level *		
≤ 4 mm	16.3	29
> 4 mm	50	57
Bleeding on probing *		
≤ 30% of sites	15	29
> 30% of sites	54	56
Supragingival biofilm *		
≤ 30% of sites	14	27
> 30% of sites	50	56

* Refers to $p < 0.01$, Chi-square test.

from patients with chronic periodontitis and periodontal health for the presence of *P. aeruginosa* and *Acinetobacter* spp. Data correlating periodontal infection and *P. aeruginosa* or *Acinetobacter* spp. colonization have indicated that, in general, the prevalence of these species in the oral cavity gets greater proportions in individuals with periodontitis (Barbosa *et al.*, 2001; Botero *et al.*, 2007; Colombo *et al.*, 1998, 2002, 2009; Da Silva-Boghossian *et al.*, 2011; Fourrier *et al.*, 1998; Fritschi *et al.*, 2008; Gonçalves *et al.*, 2009; Persson *et al.*, 2008; Slots *et al.*, 1988, 1990; Souto and Colombo, 2008; Souto *et al.*, 2006). Likewise, our results showed a significantly higher frequency of *P. aeruginosa* and *Acinetobacter* spp. in saliva and subgingival biofilm samples from periodontitis patients compared to periodontally healthy control. These species have also been associated with treatment failure in patients with refractory periodontitis treated with mechanical and antimicrobial therapy (Colombo *et al.*, 1998, 2009). Conceivably, the high rates of resistance to multiple prescribed broad spectrum antibiotics, as well as the ability to produce

Table 3 - Final model of the stepwise logistic regression analysis including demographic, clinical and bacterial parameters as predictor variables for chronic periodontal disease.

Variables [**]	β	OR [*]	Lower 95% CI	Upper 95% CI	p
Constant	-1.35	0.26			< 0.001
Smoker	2.32	10.22	2.61	40.0	< 0.01
Pseudomonas aeruginosa	2.04	7.68	3.13	18.85	< 0.001
Supragingival biofilm	1.33	3.77	1.12	12.62	< 0.05

[*]Reference: Periodontally healthy; [**]Variables entered on step 1: gender (male 1/female 0), smoking (smokers 1/non-smokers 0), age (> 35 years 1/ ≤ 35 years 0), PD and CAL (> 4 mm 1/ ≤ 4 mm 0), supragingival biofilm and BOP (> 30% of sites 1/ ≤ 30% of sites 0), *P. aeruginosa* and *Acinetobacter* spp. (positive 1/negative 0).

biofilms may result in the persistence of these pathogens in the periodontal pocket after therapy (Pihl *et al.*, 2010; Smith *et al.*, 2003; Woods *et al.*, 1982). Associations between the presence of *P. aeruginosa* and *Acinetobacter* spp. and demographic and periodontal clinical parameters were also examined. Both species were detected in significantly higher frequencies in subjects with larger proportions of visible supragingival biofilm, BOP, PD and CAL, reinforcing the relationship of these pathogens with inflammation, tissue destruction and poor oral hygiene (Abe *et al.*, 2001; Colombo *et al.*, 2002, 2009; Da Silva-Boghossian *et al.*, 2011; Paju and Scannapieco, 2007; Scannapieco *et al.*, 1998; Souto and Colombo, 2008; Souto *et al.*, 2006). Indeed, these microorganisms produce virulence factors of relevance to the pathogenesis of periodontitis. *P. aeruginosa* is tissue invasive, elaborates extracellular leukotoxins, suppresses lymphocyte proliferation, inactivate complement components, degrades basement membrane laminin and releases potent enterotoxins and endotoxins (Pihl *et al.*, 2010; Smith *et al.*, 2003; Woods *et al.*, 1982). *Acinetobacter* spp. are considered major nosocomial pathogens (Bergogne-Berezin *et al.*, 1996) accounting for about 80% of reported infections. Members of the genus seem to have a remarkable ability to develop resistance to even the most potent antimicrobial agents (Towner, 1997). Obana (1986) demonstrated that some clinical isolates had slime-producing ability, and that the slime also enhanced the virulence of other species, indicating the potential role of *Acinetobacter* in the enhancement of virulence in mixed infections.

In structured communities, coaggregation of different bacterial species seems to have evolved as an efficient strategy to optimize local opportunities. Studies have indicated that species of *Acinetobacter* and *Pseudomonas,* when inserted in highly organized mixed communities, may cooperatively interact with each other, exhibiting direct metabolic communications and frequent genetic exchanges of multi-drug-resistant genes due to the close evolutionary

proximity (Fournier *et al.*, 2006; Hansen *et al.*, 2007). We found that over 70% of sites colonized by *P. aeruginosa* were colonized by *Acinetobacter* spp. In a previous study with hospitalized individuals, our group also demonstrated a strong association between the oral colonization of these microorganisms (Zuanazzi *et al.*, 2010). Furthermore, *P. aeruginosa* and *Acinetobacter* spp. may interact with other species such as periodontal pathogens. Da Silva-Boghossian *et al.* (2011) demonstrated that *Aggregatibacter actinomycetemcomitans*, *A. baumannii* and the red complex (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*) associated with *P. aeruginosa* in the subgingival microbiota increased significantly the likelihood of a subject having aggressive periodontitis. *P. aeruginosa* also seemed to have a synergism with *A. actinomycetemcomitans* increasing the risk for periodontal disease. Likewise, Persson *et al.* (2008) showed that *P. aeruginosa* along with *T. forsythia* were independent predictors for periodontal disease. However, the exact mechanisms involved in these complex interactions are not elucidated yet.

In summary, our findings provide additional evidence for the oral cavity as a reservoir for *P. aeruginosa* and *Acinetobacter* spp., as well as of the association of these microorganisms with the presence of periodontal infection. Considering that eradication of this species from subgingival biofilm in deep periodontal pockets may be limited, close attention should be given to these patients in order to reduce the risk for periodontal breakdown and/or development of systemic diseases caused by these species in other areas of the body.

Acknowledgments

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