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Levels of *Selenomonas* species in generalized aggressive periodontitis

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Background and Objective: To compare the levels of *Selenomonas sputigena* and uncultivated/unrecognized *Selenomonas* species in subgingival biofilms from periodontally healthy subjects and from subjects with generalized aggressive periodontitis.

Material and Methods: Fifteen periodontally healthy subjects and 15 subjects with generalized aggressive periodontitis were recruited and their clinical periodontal parameters were evaluated. Nine subgingival plaque samples were collected from each subject and all were individually analyzed for the levels of 10 bacterial taxa, including cultured and uncultivated/unrecognized microorganisms, using the RNA-oligonucleotide quantification technique. Between-group differences in the levels of the test taxa were determined using the Mann–Whitney *U*-test.

Results: Subjects with generalized aggressive periodontitis showed significantly higher mean counts of *Porphyromonas gingivalis*, *S. sputigena* and the *Mitsuokella* sp. Human Oral Taxon (HOT) 131 (previously described as *Selenomonas* sp. oral clone CS002), while higher mean counts of *Actinomyces gerencseriae* and *Streptococcus sanguinis* were found in periodontally healthy subjects ($p < 0.01$). *Selenomonas* sp. HOT 146 was only detected in the generalized aggressive periodontitis group. In the generalized aggressive periodontitis group, the levels of *P. gingivalis* and *S. sputigena* were higher in deep sites (probing depth ≥ 5 mm) than in shallow sites (probing depth ≤ 3 mm) ($p < 0.01$). Furthermore, in subjects with generalized aggressive periodontitis, sites with probing depth of ≤ 3 mm harbored higher levels of these two species than sites with the same probing depth in periodontally healthy subjects. There were positive correlations between probing depth and the levels of *P. gingivalis* ($r = 0.77$; $p < 0.01$), *S. sputigena* ($r = 0.60$; $p < 0.01$) and *Selenomonas* dianae (previously described as *Selenomonas* sp. oral clone EW076) ($r = 0.42$, $p < 0.05$).

Conclusion: S. sputigena and Mitsuokella sp. HOT 131 may be associated with the pathogenesis of generalized aggressive periodontitis, and their role in the onset and progression of this infection should be investigated further.

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The role of the oral microbiota in the etiology of periodontal diseases has been well established, and specificity may exist among certain bacterial species or groups and the various forms of periodontal disease (1–5). The complexity and diversity of the periodontal microbiota has been confirmed by numerous studies (6–8). However, only a few species have been recognized as periodontal pathogens, namely *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia* (9).

Aggressive periodontitis is characterized by a rapid rate of tissue destruction and progression, early age of onset, specific patterns of periodontal breakdown and familial aggregation. These features are quite distinct from those observed in chronic periodontitis (10). The microbiota of the localized and generalized forms of aggressive periodontitis contain higher proportions of *A. actinomycetemcomitans* compared with that of chronic periodontitis, whereas the proportions of red complex pathogens (5) do not differ between these periodontal conditions, despite their clinical differences (7). These observations suggest that further analyses of the microbiota are necessary in order to explain differences in clinical outcomes.

The use of open-ended culture-independent approaches has expanded the breadth of knowledge of the oral microbiota. The oral cavity may harbor more than 700 bacterial species (6,11,12), of which 35% remain uncultivated (13,14). Even though those taxa have remained undetected in most studies, it is possible that specific uncultivated/unrecognized taxa may play a role in the etiology of oral diseases, or alternatively, they may represent beneficial bacterial species.

Using 16S ribosomal RNA (rRNA) sequencing analysis, Faveri *et al*. (11) observed that *Selenomonas* species dominated the disease sites of subjects with generalized aggressive periodontitis. *Selenomonas sputigena* was the most frequently detected bacterial species, whereas high levels of other species of *Selenomonas* were often also present, including *Selenomonas noxia*, *Selenomonas dianae*, *Selenomonas* sp. Human Oral Taxon (HOT) 146 e *Mitsuokella* sp. HOT 131 (these taxa were previously described as *Selenomonas* sp. oral clone EW076, *Selenomonas* sp. oral clone EW084 and *Selenomonas* sp. oral clone CS002, respectively) (6,11). Recent efforts on taxonomic classification have led to an update of the genus/species initially proposed (13,14). A high prevalence of *Selenomonas* species has also been observed in subgingival samples from subjects with chronic periodontitis (6,15–17). However, none of these studies were able to quantify the levels of multiple uncultivated/unrecognized taxa in a large number of individual biofilm samples simultaneously. Information on the absolute numbers and proportions of organisms in samples is important in distinguishing species associated with periodontal health or disease and to evaluate the effects of periodontal therapy (5,18).

The RNA-oligonucleotide quantification technique (ROQT) allows the quantification of uncultivated unrecognized and cultured taxa in multiple subgingival biofilm samples in parallel (19). In addition, the method does not require sample pooling, amplification or dilution, procedures that may introduce bias in the composition of the microbial communities under study (15,18,20). Therefore, the purpose of the present study was to evaluate the levels of *Selenomonas* species, as well as their relationship with *P. gingivalis* and some host-compatible species (*Actinomyces gerensceriae, Streptococcus anginosus/gordonii* and *Streptococcus sanguinis*) in subgingival biofilm samples from patients with generalized aggressive periodontitis and periodontally healthy control subjects using the ROQT.

Material and methods

Subject population

Fifteen periodontally healthy subjects and 15 individuals presenting with generalized aggressive periodontitis were included in this investigation. All study participants were systemically healthy and were recruited at the Guarulhos University School of Dentistry (Guarulhos, SP, Brazil). The medical and dental histories were obtained and a full-mouth periodontal examination was performed. Based on these data, the periodontal diagnosis was made, and subjects who fulfilled the inclusion/exclusion criteria were invited to participate in the study. The study protocol was explained to each subject, and a signed Informed Consent was obtained. This study protocol was approved by the Ethics Committee in Clinical Research of Guarulhos University.

Clinical examination

One trained and calibrated examiner performed the clinical examination in all subjects. Visible plaque [scored as 0 (absent) or 1 (present)], gingival bleeding (0/1), bleeding on probing (0/1), suppuration (0/1), probing depth (mm) and clinical attachment level (mm) were measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) in all teeth, excluding third molars, at the baseline visit. Probing depth and clinical attachment level measurements were recorded to the nearest millimeter using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA).

Inclusion criteria

Generalized aggressive periodontitis and periodontal health were diagnosed based on the periodontal classification of the American Academy of Periodontology (10). Subjects had to have at least 20 teeth and needed to meet the following criteria in order to be included in this study.

Generalized aggressive periodontitis — $\leq$ 35 years of age; a minimum of six permanent incisors and/or first molars with at least one site each with probing depth and clinical attachment level of $\geq$ 5 mm; a minimum of six teeth (other than first molars and incisors) with at least one site each with probing depth and clinical attachment level of $\geq$ 5 mm; and familial aggregation (at least one other member of the family presenting, or with a history of, periodontal disease) (7).

Periodontally healthy — $\leq$ 35 years of age; no sites with probing depth and clinical attachment level measurements of $\geq$ 3 mm; and $\leq$ 10% of sites exhibiting bleeding on probing.

Exclusion criteria

Exclusion criteria were pregnancy, lactation, smoking, subgingival periodontal therapy in the past 12 months, any systemic condition that could affect the progression of periodontal disease (e.g. diabetes and immunological disorders),

long-term administration of anti-inflammatory medication and antibiotic therapy in the previous 6 months.

Microbiological examination

Sample collection— Individual subgingival plaque samples were collected from nine noncontiguous interproximal sites per subject. For the generalized aggressive periodontitis group, three sites with a probing depth of ≤ 3 mm and six sites with a probing depth of ≥ 5 mm were selected. Nine sites with a probing depth of ≤ 3 mm were collected from the periodontally healthy group. The selected sites were randomized in different quadrants. After the clinical parameters had been recorded, the supragingival plaque was removed and the samples were taken with individual sterile Gracey curettes and immediately placed in separate polypropylene tubes containing 100 μL of RNAse-free TE (10mM Tris–HCl, 1 mM EDTA, pH 7.6) and kept at −80°C until required for extraction of total nucleic acids (TNA).

RNA-Oligonucleotide Quantification Technique (ROQT)

Counts of the 10 bacterial species/phylotypes (Table 1) were determined in each sample using the ROQT (19). In brief, oligonucleotides targeting the 16S rRNA gene of selected bacterial species/phylotypes were designed, synthesized and labeled. These probes were hybridized with the total nucleic acids (TNA) of subgingival biofilm samples. Sequences complementary to the probes were used as standards for quantification. Chemiluminescent signals generated by hybrids were visualized using film. The procedures associated with the technique are described in detail below. Microbiological analysis was performed at the Laboratory of Microbiology of Guarulhos University.

Extraction of TNA

Extraction of TNA from all samples was performed using a Masterpure RNA & DNA purification kit (Epicentre, Madison, WI, USA). Cells were pelleted by centrifugation and resuspended in 25 μL of TE. One microliter of proteinase K (50 μg/μL) and 300 μL of tissue and cell lysis buffer were added followed by incubation at 65°C for 15 min. After 5 min on ice, 175 μL of MCP protein precipitation reagent (Epicentre) was added to each sample. After centrifugation, 500 μL of isopropanol was added to the supernatant and the TNA were precipitated by centrifugation. The pellets were rinsed twice with 70% ethanol, air dried and resuspended. The TNA samples were kept at −80°C until analysis.

Preparation of probe and standards

Oligonucleotide probes targeting the *16S rRNA* gene of eight cultivated and two uncultivated/unrecognized bacterial taxa were synthesized (Table 1). The probe panel also included a universal (eubacterial) probe, which was based on a conserved region of the bacterial *16S rRNA* gene. In the present study, the universal probe was used as a positive control of the assays performed and levels were not considered in the data analysis. Probe sequences were 18–22 nucleotides in length and had minimal secondary structure. The sequences employed in the ROQT were those routinely used in the Human Oral Microbial Identification Microarray and have been published previously (20). One-hundred picomoles of each oligonucleotide probe was labeled using a digoxigenin 3'-end labeling kit (Roche, Indianapolis, IN, USA). The standards for detection of the oligonucleotide probes were a mixture of sequences complementary to each oligonucleotide probe (Table 1). The final mixtures of standards had 0.004 and 0.04 pM of each sequence, corresponding to 10^5 and 10^6 bacterial cells, respectively. The oligonucleotide probes and their

Table 1. Sequences of oligonucleotide probes and of the standards used for quantification of the species/phylotypes evaluated

Species/phylotypes	Sequence (5′–3′)	Control (3′–5′)
Selenomonas sputigena	CCGTCACCCAAACTCAAT	GGCAGTGGGTTTGAGTTA
Selenomonas noxia	CTATTCGCATTAGGCACG	GATAAGCGTAATCCGTGC
Selenomonas sp. HOT 146	GGACTCATCTCTGAGTCT	CCTGAGTAGAGACTCAGA
Selenomonas dianae[a]	CTCTGCATGCTTCAGTCA	GAGACGTACGAAGTCAGT
Mitsuokella sp. HOT 131[b]	GGCGCAACATTCGGTATT	CCGCGTTGTAAGCCATAA
Aggregatibacter actinomycetemcomitans	TTAAAGGTCCGCCTACGT	AATTTCCAGGCGGATGCA
Porphyromonas gingivalis	GTGGAAGCTTGACGGTAT	CACCTTCGAACTGCCATA
Actinomyces gerensceriae	ACCCCAGAAGCCCGTT	TGGGGTCTTCGGGCAA
Streptococcus anginosus/gordonii	CAACTCACAGTCTATGGTGTAG	GTTGAGTGTCAGATACCACATC
Streptococcus sanguinis	CAATAATCAATTTTATGCGGT	GTTATTAGTTAAAATACGCCA
16S rRNA Universal	CTGCTGCCTCCCGTAGG	GACGACGGAGGCATCC

The *S. anginosus/gordonii* probe was a 'combination probe', in that it could not distinguish the two species.
HOT, Human Oral Taxon. Oral taxon designations for uncultivated/unrecognized taxa are provided in accordance with the Human Oral Microbiome Database (http://www.homd.org) when available; GenBank assession numbers can also be found in the Human Oral Microbiome Database website.
[a]*Selenomonas dianae* was initially classified as Selenomonas sp. oral clone EW076.
[b]*Mitsuokella* sp. HOT 131 was initially classified as a member of the genus *Selenomonas*. Due to further revision and validation, it has recently been recognized as a member of the genus *Mitsuokella*.

"complementary" sequences were synthesized by Invitrogen (São Paulo, SP, Brazil).

Hybridization

Ninety microliters of 2% glutaraldehyde (Ted Pella, Redding, CA, USA) and 910 μL of 6 × SSC (1 × SSC = 150mM NaCl, 15 mM sodium citrate, pH 7.0) were added to each sample of TNA and to the standards. The final solutions were deposited in individual lanes of a Minislot (Immunetics, Cambridge, MA, USA), concentrated onto a nylon membrane (Boehringer Mannheim, Indianapolis, IN, USA) and fixed to the membrane. The membranes were prehybridized into a plastic hybridization bag at 42°C for at least 90 min in 35 mL of a prehybridization solution containing 50% formamide, 5 × SSC, 1% casein (Vetec, Rio de Janeiro, RJ, Brazil), 5 × Denhardt's reagent (Sigma, St Louis, MO, USA), 25 mM sodium phosphate (pH 6.5) and 0.5 mg/mL of yeast RNA (Boehringer Mannheim). Then, the membranes were placed in a Miniblotter 45 (Immunetics), with the "lanes" containing the TNA at 90° to the channels of the device. The digoxigenin-labeled oligonucleotide probes were diluted in hybridization buffer (*ULTRAhyb*® Oligo buffer; Ambion, Austin, TX, USA), and added to individual lanes of the Miniblotter 45 at final concentrations of 2–40 pM. The membranes were hybridized at 42°C for 80 min and then washed at 37°C for 60 min with 2 × SSC containing 0.5% sodium dodecyl sulfate. After blocking with maleic acid buffer containing 10% casein, hybrids were detected by incubation with a 1 : 2500 dilution of anti-digoxigenin antibody conjugated with alkaline phosphate (Roche) for 30 min. The membranes were washed three times with maleic acid buffer for 15 min each time and then with 200 mL "buffer 3" [0.04% $MgCl_2$ and 2.1% diethanolamine (pH 9.5), equal volumes] for 5 min. Finally, 1 mL of a chemiluminescent substrate (CDP Star; Tropix, Bedford, MA, USA) diluted in "buffer 3" was deposited onto the membrane surface, which was then exposed to an X-ray film (IBF-Medix; AGFA, Rio de Janeiro, RJ, Brazil). The films were scanned and the signal intensities of the samples and the standards were measured using the TotalLab software (NonLinear USA, Durham, NC, USA). Signals were converted to absolute "counts" by comparison with standards on the membrane. Absence of signal detection was recorded as zero.

Statistical analysis

The mean age and the mean percentage of sites with visible plaque, gingival bleeding, bleeding on probing and suppuration, as well as mean probing depth and clinical attachment level were computed for each subject and then averaged across subjects in each group separately. Mean estimated "counts" ($\times 10^5$) of individual bacterial species were computed for each site individually, then averaged within each subject and then across subjects in each group separately. Prevalence of the test taxa was computed by determining the percentage of sites per subject colonized by $\geq 10^5$ cells of each species. The mean percentage of "positive" sites was calculated for each subject and then averaged across subjects in each group. The significance of differences between the two groups for age and the clinical and microbiological parameters was analyzed using the Mann–Whitney *U*-test. The Wilcoxon test was used to detect statistically significant differences within probing depth categories in the group of subjects with generalized aggressive periodontitis. The chi-square test was employed to compare the differences in the frequency of gender and prevalence of subjects colonized by bacterial species. Spearman correlation was used to assess possible associations between probing depth and mean "counts" of bacterial species.

Results

Clinical findings

The demographic characteristics and clinical parameters of the study population are presented in Table 2. No statistically significant differences were observed between groups for age and gender. The generalized aggressive periodontitis group displayed significantly higher mean probing depth, mean clinical attachment level and percentage of bleeding on probing, as well as levels of plaque and numbers of moderate and deep periodontal pockets ($p < 0.05$) in comparison with the periodontally healthy group.

Microbiological findings

The mean estimated 'counts' ($\times 10^5$) of the 10 species evaluated in the subgingival plaque samples from the periodontally healthy and generalized

Table 2. Demographic characteristics and full-mouth clinical parameters of both study groups

Clinical variables	Periodontally healthy ($n = 15$)	Generalized aggressive periodontitis ($n = 15$)
Age (years)^{NS}	26.6 ± 3.6	27.4 ± 4.3
Gender (M/F)^{NS}	9/6	8/7
Probing depth (mm)*	1.96 ± 0.6	4.62 ± 0.81
Clinical attachment level (mm)*	1.04 ± 0.5	4.53 ± 1.23
Percentage of sites with		
Plaque accumulation*	34.5 ± 9.9	74.1 ± 10.4
Gingival bleeding*	4.5 ± 2.9	24.2 ± 10.9
Bleeding on probing*	6.1 ± 3.2	74.1 ± 15.7
Suppuration*	0.0 ± 0.0	4.5 ± 6.2
Probing depth ≤ 3 mm*	100	37.3 ± 20.1
Probing depth 4–6 mm*	0	39.9 ± 28.2
Probing depth ≥ 7 mm*	0	22.8 ± 15.7

Results are given as mean ± standard deviation.
M/F, male/female; NS, not significant ($p > 0.05$).
*$p < 0.05$ (Mann–Whitney *U*-test).

Table 3. Mean estimated "counts" of 10 bacterial taxa in subgingival biofilm samples obtained from subjects with generalized aggressive periodontitis and in periodontally healthy subjects

Species/phylotypes	Experimental group: Periodontally healthy (n = 15)	Experimental group: Generalized aggressive periodontitis (n = 15)	MW
Selenomonas sputigena	0.3 ± 0.5	4.5 ± 2.9	0.001
Selenomonas noxia	0.2 ± 0.4	1.2 ± 2.0	NS
Selenomonas sp. HOT 146	0	0.1 ± 0.4	NS
Selenomonas dianae	0.05 ± 0.9	0.5 ± 1.0	NS
Mitsuokella sp. HOT 131	0.01 ± 0.02	0.12 ± 0.09	0.01
Aggregatibacter actinomycetemcomitans	0.06 ± 0.01	0.2 ± 0.4	NS
Porphyromonas gingivalis	0.1 ± 0.3	7.8 ± 3.9	0.001
Actinomyces gerencseriae	1.7 ± 0.6	0.3 ± 0.4	0.001
Streptococcus anginosus/gordonii	1.3 ± 1.0	1.4 ± 1.8	NS
Streptococcus sanguinis	1.3 ± 1.5	0.3 ± 0.01	0.01

Results are given as mean counts ($\times 10^5$) ± standard deviation.
HOT, Human Oral Taxon; NS, not significant.
MW (Mann–Whitney *U*-test).

aggressive periodontitis groups are shown in Table 3. Subjects with generalized aggressive periodontitis showed significantly higher mean "counts" of *P. gingivalis* ($p < 0.001$), *S. sputigena* ($p < 0.001$) and *Mitsuokella* sp. HOT 131 ($p < 0.01$). *Selenomonas* sp. HOT 146 was detected only in the generalized aggressive periodontitis group. Throughout this manuscript, HOT or human oral taxon designations for uncultivated/unrecognized taxa are provided in accordance with the Human Oral Microbiome Database (13, HOMD; http://www.homd.org) when available. GenBank accession numbers can also be found in the HOMD website.

The levels of *A. actinomycetemcomitans* were elevated more in subjects with generalized aggressive periodontitis than in periodontally healthy subjects; however, the difference between the groups did not reach statistical significance ($p > 0.05$). Mean "counts" of *A. gerencseriae* and *Streptococcus sanguinis* were significantly higher in periodontally healthy subjects ($p < 0.01$).

The mean estimated "counts" of the bacterial species detected in shallow and deep periodontal pockets (probing depth ≤ 3 mm and ≥ 5 mm) in subjects with generalized aggressive periodontitis are shown in Table 4. Significantly higher mean "counts" of *P. gingivalis* and *S. sputigena* were detected in deep pockets than in shallow sites. In addition, higher mean 'counts' of these two pathogens were present in both shallow and deep sites in subjects with generalized aggressive periodontitis, compared with the shallow sites of periodontally healthy subjects (Fig. 1).

The percentage of sites colonized by the 10 species/phylotypes evaluated in subgingival plaque samples taken from subjects with generalized aggressive periodontitis and periodontally healthy subjects are presented in Table 5. Subjects with generalized aggressive periodontitis showed a higher mean percentage of sites colonized by *S. sputigena* ($p < 0.005$), *Mitsuokella* sp. HOT 131 ($p < 0.041$) and *P. gingivalis* ($p < 0.001$) in comparison with periodontally healthy subjects. No differences in the prevalence of subjects colonized by all species analyzed were observed between groups, except for *P. gingivalis*, which was more prevalent in subjects with generalized aggressive periodontitis (data not shown, $p < 0.05$). Spearman correlations revealed positive correlations between probing depth and mean "counts" of *P. gingivalis* ($r = 0.77$, $p = 0.0001$), *S. sputigena* ($r = 0.60$, $p < 0.009$) and *Selenomonas* dianae ($r = 0.42$, $p = 0.031$).

Table 4. Mean estimated "counts" of 10 bacterial taxa in subgingival biofilm samples obtained at baseline probing depth (≤ 3 mm) and at a probing depth of ≥ 5 mm in subjects with generalized aggressive periodontitis

Species/phylotypes	Generalized aggressive periodontitis: Probing depth ≤ 3 mm	Generalized aggressive periodontitis: Probing depth ≥ 5 mm	*p-value
Selenomonas sputigena	1.7 ± 1.7	6.7 ± 5.5	0.027
Selenomonas noxia	0.6 ± 0.9	1.3 ± 1.1	NS
Selenomonas sp. HOT 146	0.01 ± 0.01	0.3 ± 0.8	NS
Selenomonas dianae	0.04 ± 0.09	1.2 ± 2.9	NS
Mitsuokella sp. HOT 131	0.08 ± 0.01	0.02 ± 0.05	NS
Aggregatibacter actinomycetemcomitans	0.01 ± 0.04	0.3 ± 0.8	NS
Porphyromonas gingivalis	2.4 ± 2.7	12.2 ± 6.1	0.001
Actinomyces gerencseriae	0.2 ± 0.5	0.5 ± 0.8	NS
Streptococcus anginosus/gordonii	1.4 ± 2.8	1.4 ± 1.8	NS
Streptococcus sanguinis	0.5 ± 0.09	0.1 ± 0.02	NS

Results are given as mean counts ($\times 10^5$) ± standard deviation.
HOT, Human Oral Taxon; NS, not significant.
*$p < 0.05$ (Wilcoxon test).

Discussion

Previous investigations using the *16S rRNA* cloning and sequencing strategy showed that *Selenomonas* spp. are present at higher proportions in the subgingival biofilm samples of subjects with chronic (6,15,17,21,22) and aggressive (11) periodontitis. Recent studies have reported contrasting findings regarding the potential role of *Selenomonas* species in periodontitis.

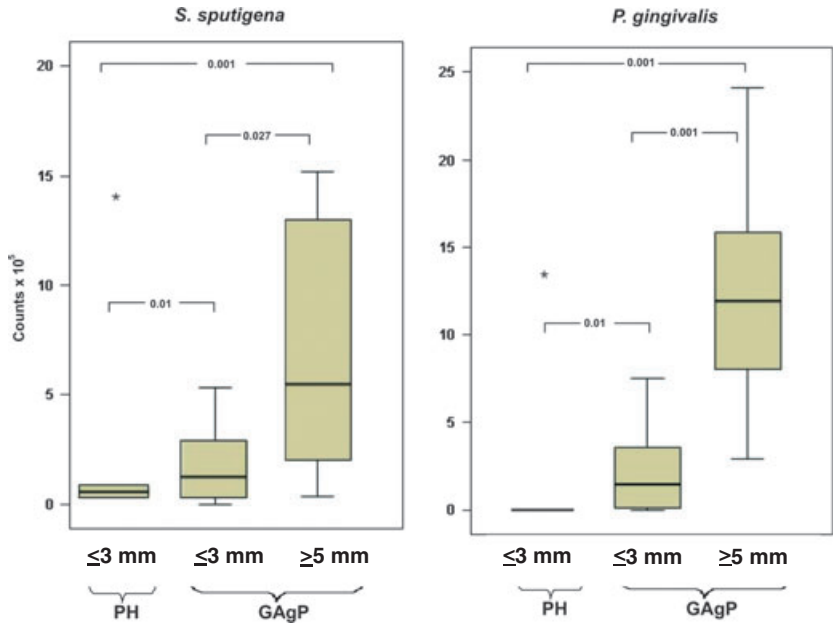

Fig. 1. Mean estimated "counts" ($\times 10^5$) of *Selenomonas sputigena* and *Porphyromonas gingivalis* in periodontally healthy subjects (PH) and in sites with probing depths of ≤ 3 mm or ≥ 5 mm in subjects with generalized aggressive periodontitis (GAgP). Box-plots show the median, minimum and maximum values. Between-group differences were analyzed using the Mann–Whitney *U*-test and the Wilcoxon test. The asterisk (*) represents extreme outliers.

Whilst a high level of *S. noxia,* in association with other bacterial species, was observed in a subset of young periodontitis patients (22), the use of a specific oligonucleotide probe targeting the majority of all oral *Selenomonas* spp. indicated that members of this genus were ubiquitous both in periodontitis-resistant subjects and in patients with periodontal disease (23). Therefore, the association between species of *Selenomonas* and periodontal disease remains unclear. Hence, the main goal of the present study was to determine the levels of selected *Selenomonas* spp., including uncultivated/unrecognized phylotypes, in subgingival biofilm samples from subjects with generalized aggressive periodontitis and from periodontally healthy individuals.

The present study used the ROQT (19) to determine the levels of the test taxa in subgingival biofilm samples from subjects with generalized aggressive periodontitis and from periodontally healthy subjects. The ROQT can overcome some of the limitations of other molecular biology techniques, including checkerboard DNA–DNA hybridization, real-time PCR and *16S rRNA* cloning analysis. None of these techniques has the ability to quantify the levels of multiple uncultivated species in large numbers of individual biofilm samples simultaneously. The ROQT is a high-throughput method for bacterial enumeration in clinical biofilm samples. In addition, it does not require sample pooling, amplification or dilution, because an entire individual sample is laid onto the membrane. Therefore, introduction of bias in the microbial profiles of the ecosystem under study from PCR-associated procedures can be eliminated (15,24,25).

In agreement with previous studies that used other microbiological techniques, such as culture techniques (4,26–28), PCR (29,30) and checkerboard DNA–DNA hybridization (5,7,31), the data from the present study obtained using the ROQT support the notion that *P. gingivalis* plays an important role in the etiology of generalized aggressive periodontitis, as well as *A. gerensceriae* and *S. sanguinis*, which were elevated in healthy subjects compared with periodontally diseased subjects.

The results of the present study reinforce the association of *S. sputigena* with the etiology of generalized aggressive periodontitis. This species was found at significantly higher levels (Table 3) and with a significantly higher prevalence (percentage of sites colonized) in subjects with generalized aggressive periodontitis than in periodontally healthy subjects (Table 5; $p < 0.01$), as well as at higher levels in

Table 5. Percentage of sites colonized by 10 bacterial taxa at ≥ 10^5 organisms in subgingival plaque samples taken from subjects with generalized aggressive periodontitis and from periodontally healthy subjects

Species/phylotypes	Experimental groups: Periodontally healthy (n = 15)	Experimental groups: Generalized aggressive periodontitis (n = 15)	MW p-value
Selenomonas sputigena	15.4 ± 18.9	51.4 ± 32.7	0.005
Selenomonas noxia	12.4 ± 19.4	27.8 ± 28.4	NS
Selenomonas sp. HOT 146	0	7.3 ± 10.7	NS
Selenomonas dianae	2.9 ± 8.0	15.4 ± 13.7	NS
Mitsuokella sp. HOT 131	6.1 ± 5.7	22.1 ± 11.9	0.041
Aggregatibacter actinomycetemcomitans	3.6 ± 6.7	13.9 ± 16.8	NS
Porphyromonas gingivalis	4.4 ± 8.1	65.5 ± 30.9	0.001
Actinomyces gerencseriae	38.2 ± 29.6	22.9 ± 15.0	NS
Streptococcus anginosus/gordonii	41.8 ± 30.4	27.8 ± 28.4	NS
Streptococcus sanguinis	27.9 ± 29.2	24.9 ± 26.1	NS

Results are given as mean ± standard deviation.
HOT, Human Oral Taxon; NS, not significant.
MW (Mann–Whitney *U*-test).

deep sites compared with shallow sites in subjects with generalized aggressive periodontitis (Table 4). *S. sputigena* is a gram-negative, multiflagellated, motile, anaerobic rod (32). An early study showed that patients with periodontitis showed high titers of antibody against *S. sputigena* (33). Later, investigations demonstrated that this species was also associated with necrotizing ulcerative periodontitis (34), aggressive periodontitis (26), active periodontitis lesions (35,36) and generalized aggressive periodontitis (11). Studies using cloning and sequencing, as well as culture, also identified this species as a highly prevalent (37) and dominant member of the periodontal pocket microbiota of chronic periodontitis subjects (27,28,38,39).

Despite its possible association with disease, the components and the products of *S. sputigena* involved in the colonization of the oral cavity and the mechanisms of inducing tissue destruction are unknown. This species presented bone-resorption activity in experimentally colonized rats (40). It is noteworthy that lipopolysaccharide/lipid A of *S. sputigena* differs from that generally found in other gram-negative bacteria, which consisted mainly of fatty acids such as undecanoic, trideanoic, tridecenoic, 3-hydroxytridecanoic and 3-hydroxytetradecanoic acid. In addition, lipid A from *S. sputigena* is able to induce interleukin-1α and interleukin-6 in murine macrophages (41), suggesting its role in inducing inflammation. However, there is no evidence that lipid A from *S. sputigena* leads to a different inflammatory phenotype compared with other gram-negative bacterial species.

Members of the genus *Selenomonas* were frequently detected as part of the not yet cultivated/recognized species of the oral cavity (6,16,21,42). The present analysis of cultivated and uncultivated/unrecognized *Selenomonas* species/phylotypes revealed that these taxa are frequently part of the subgingival microbiota of subjects with generalized aggressive periodontitis. *Selenomonas* sp. HOT 146 was only detected in subjects with generalized aggressive periodontitis, and *Mitsuokella* sp. HOT 131 (initially described as *Selenomonas* sp. oral clone CS002) (6,11,16). was more prevalent and present in higher levels in subjects with generalized aggressive periodontitis than in periodontal healthy subjects. This species has also been associated with other anaerobic sites of the oral cavity, such as deep dentin caries (43). In addition, the levels of *Selenomonas dianae* (previously referred to as *Selenomonas* sp. oral clone EW076) (11,16). were positively correlated with the increase of probing depth, which is in accordance with the findings reported by Kumar *et al*. (16). Drescher *et al*. (23) analyzed the topography of the subgingival biofilm by fluorescence *in situ* hybridization and electron microscopy in subjects with generalized aggressive periodontitis, subjects with chronic periodontitis and periodontitis-resistant subjects, and revealed that *Selenomonas* spp. appeared in large numbers in all parts of the collected biofilms and seemed to make a relevant contribution to their structural organization. The authors reported that it is difficult to imagine that a group of organisms constituting an important part of the biomass does not contribute to the pathogenetic process of periodontal disease.

Overall, the data indicated that the *Selenomonas* species/phylotypes evaluated are heterogeneous in their ability to colonize oral sites and cannot be seen as a single taxonomic unit. Although their role in inducing periodontal destruction cannot be drawn unless longitudinal studies are performed and their phenotypic traits are evaluated, the data indicated that *S. sputigena* and *Mitsuokella* sp. HOT 131 are part of the subgingival microbiota associated with aggressive periodontitis and may play a role in the disease onset and progression.

Conclusion

Selenomonas sputigena and *Mitsuokella* sp. HOT 131 may be associated with the pathogenesis of generalized aggressive periodontitis and therefore their role in the onset and progression of this infection merits further investigation.

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