Research Paper Medical Science

Identification of Key Components of The Healthy Periodontium Microbiome That Protect Against Aggressive Periodontitis by Metagenomic Sequencing.

Zorina Oxana	I.M. Sechenov First Moscow State Medical University, Moscow, Russia
Petrukhina Natalia	I.M. Sechenov First Moscow State Medical University, Moscow, Russia
Shibaeva Anna	Emanuel Institute of Biochemical Physics of Russian Academy of Sciences, Moscow, Russia
Basova Alexandra	I.M. Sechenov First Moscow State Medical University, Moscow, Russia
Shevelev Aleksey	Emanuel Institute of Biochemical Physics of Russian Academy of Sciences, Moscow, Russia

ABSTRACT

In contrast to chronic periodontitis, aggressive periodontitis is not associated with bacterial hypercolonization of the periodontium. To investigate whether AP might be caused by a shift in the composition of the periodontal microbiome, it is necessary to compare microbial present in samples from healthy individuals and patients with the condition. We report use of metagenomic sequencing to accomplish this in strictly selected cohorts of five patients and five healthy individuals. 16S rDNA libraries were generated from DNA purified from subgingival samples and analyzed using Illuminametagenomic sequencing. A complex of putative periodontoprotective species was identified through statistical analysis (*Prevotellanigrescens, Prevotellaoris, Prevotellatannerae, Veillonellaparvula, Streptococcus sanguinis, Kingellaoralis, Granulicatellaparadiacens* and species of genera Corynebacterium and Bergeyella). Hypocolonization of the periodontium with these species should be considered as an important risk factor. Reduced presence of the protective species is likely to eliminate normal mechanisms of pathogen containment. The previously identified periodontal pathogen *Porphyromonasgingivalis* was not found to be associated with the condition. *Aggregatibacteractinomycetemcomitans*, commonly acknowledged to be the periodontal pathogen with the greatest negative impact, was absent in all samples. This suggests that its clinical significance may be limited to a small proportion of severe cases. The status of Prevotella intermedia as a key periodontal pathogen was confirmed, while its closest taxonomic relative, P. nigrescens, was shown to be associated with periodontal health. Several species not previously linked to periodontal disease demonstrated a valid association with the condition (*Synergistessynergistes, Parvimonasmicra, Mycoplasma hyosynoviae* and *Filifactoralocis*). Overall, the results of this study add to our understanding periodontal microbiome shifts in aggressive periodontitis. The putative protective role of Veillonella and pathogenic roles of Synergistaceae, Filifactor, Ruminococcaceae, Mycoplasma and Parvimonas detected in our study provide rationale for their further investigation as potential therapeutic targets and/or diagnostic markers.

KEYWORDS Periodontitis, aggressive periodontitis, RT-PCR analysis, periodontium microbiome, perodontal pathogen,normoflora, periodontal health

Introduction

Periodontitis is the decay of gingival tissue, periodontal ligature and alveolar bone caused by a combination of genetic and environmental factors. Microbial infection is considered a key etiological factor in this condition [1]. While classification of clinical forms of periodontitis remains somewhat controversial and is not uniform among different countries, the terms "aggressive periodontitis" (AP) and "chronic periodontitis" (CP) are commonly acknowledged. Between these two forms of periodontitis, CP is most prevalent and is known to be primarily controlled by oral cavity hygiene. CP results from the cumulative effects of excessive bacterial colonization of the periodontium over a long period of time. Accordingly, the average age of CP patients at diagnosis is 35-40. In contrast, AP is not strongly associated with poor oral cavity hygiene. AP patients typically have a high level of oral cavity hygiene and present at younger ages than CP patients. In this form of periodontitis, changes in the qualitative composition of the periodontal microbiome has been suggested to play a more critical role than an excessive bacterial colonization. AP is a much more serious, rapidly progressing disease than CP, characterized, for example, by rapid decay of the alveolar bone [2]. Long-term clinical observations revealed a genetic predisposition to AP [3, 4], suggesting that responses of the human host to changes in the periodontal microbiome may underlie development of the condition. Despite these findings, it is clear that genetics do not fully explain AP. For example, over the last decade, the occurrence of AP in young patients (less than age 25) in the Russian population of Moscow increased ten-fold. This increase cannot be explained by genetic background which remains constant. Rather, we hypothesize that improved oral hygiene in the Russian population of Moscow over the last decade (confirmed by the reduced incidence of caries) could be responsible for the increase prevalence of AP. It is possible that a high level of oral hygiene in general, and in particular, specific anti-caries treatments, might disrupt the balance in the periodontal microbiome in a way that actually promotes AP (i.e., by exhausting the protective normoflora with or without concomitant increases in periodontopathogenic species.

In order to fully understand AP and how it might be best prevented and/or treated, it is necessary to identify components of the periodontal microbiome that either protect against or

promote AP. There are several papers describing analysis of the periodontal microbiome through next-generation sequencing (NGS) [5-8] and HOMIM microarray technology [9]. However, these studies only examined pathogenic bacteria, not the normoflora that might play a protective role. In addition, five commonly acknowledged periodontopathogenic species (Aggregatibacteractinomycetemcomitans and the so-called "orange complex" of Porphyromonasgingivalis, Prevotella intermedia, Treponemadenticola and Tannerella forsythia) can be routinely detected by commercial PCR kits. Although the negative impact of these and some other species (e.g.,Parvimonasmicra and Filifactoralocis) on periodontal health has been reported [6, 8], characterization of the overall state of the oral microbiome in AP patients (i.e., the ratio between pathogenic bacteria and key components of the normal periodontal microflora) has not been presented. The current study was aimed at closing this gap in our understanding of AP by established a complete picture of the microbiome of AP patients compared to normal individuals using unbiased metagenomic sequencing. This strategy has the potential to reveal mechanisms stabilizing the periodontal microbiome in healthy patients and allow evaluation of existing and new oral cavity hygiene means (e.g., tooth paste, chewing gum, antibiotics) which must not simply reduce levels of unfavorable species but optimize the ratio between favorable and unfavorable bacteria taking in account their symbiotic and antagonistic relationships. Clearly the composition of the cohorts of AP patients and healthy individuals with normal periodontium is an essential factor in this type of study. While this can be affected by the different standards of periodontal examination by dentists in different countries, we are the first to use Russian national standards for this type of study. The correct choice of OTU (operational taxonomical units) to examine is another key factor determining success of identifying bacterial groups relevant for one cohort or the other (e.g., AP versus normal). Most of the OTU chosen for evaluation in this study have a rank of genus, whereas previous studies analyzed lower order [7]or higher order [8] taxa. Overall, the results of this study showed occurrence of stable protective bacterial complexes in healthy periodintium. Degradation of these complexes should be considered as a key reason of multiplication of pathogenic or conditionally pathogenic species contributing to the condition onset.

Materials and methods

Cohorts of healthy patients (n=7; cohort 1) and patients with aggressive periodontitis (n=10; cohort 2) were established. All participating patients were from the Russian population of Moscow and had no systemic diseases. Patients signed an informed consent form describing the conditions of their involvement in the study. The study protocol was evaluated and approved by the Ethics Committee of the Central Research Institute of Stomatology and Maxilla-Facial Surgery of the Russian Ministry of Public Health. Clinical examination of patients included collection of personal complaints and life anamnesis, visual examination of the face, skin and lips, and palpation of the regional lymph nodes. Beginning at the vestibule, the oral cavity was examined, noting the state of the mucosa and gums (e.g., anatomic peculiarities, depth of the vestibule, morphology of the frenaand connective cords), the number of teeth inlays, and presence of any supporting and orthopedic constructions. The depth of the periodontal pocket of each tooth was measured with a 6 point graduated probe. Standard indexes were used to classify oral cavity hygiene level and periodontium inflammation as objectively and quantitatively as possible (Silness-Loe, [10]; Muhlemann-Cowell, [11]). Tooth movement was measured as described (by Miller in modification by Flezar). Digital orthopantograms were obtained and used as a supplementary method of visualizing the bone substrate. The following criteria were used to diagnose AP:

a) Juvenile age at the onset of disease symptoms (e.g., bleeding gums, recurrent abscesses);
b) Patient less than 35 years old at the start of the study ;
c) Family history (similar condition in close relatives);
d) Satisfactory level of oral cavity hygiene;
e) Periodontal pockets less than 5 mm deep;
f) Periodontium decay most severe near the first incisors and molars;
g) Divergence of the molar fangs demonstrated by X-ray;
h) Contradiction between the clinical and X-ray data (the last is more unfavorable).

Four samples were obtained from each patient using sterile paper points: two "A" samples from the area adjacent to molar teeth and two "B" samples from the area adjacent to incisors. To collect the samples, the points were introduced into the subgingival cavity to maximal depth and held for 5 seconds. Each point was then placed into a 1.5 ml eppendorf tube containing 0.5 ml saline with 1% bovine serum albumin. The samples were stored at -20°C for two weeks or less before use for DNA extraction and analysis.

DNA was extracted from subgingival samples using a Proba-GS kit (DNA-Technology, Russia) according to the manufacturer's instructions. Purified DNA from 100 ml of each subgingival sample was dissolved in 50 µl diluent and characterized by RT-PCR with a commercial Dentoflor kit (DNA-Technology, Russia) using 5 µl of the DNA preparation per RT-PCR reaction. This kit allowed quantification of total bacterial 16S rDNA as well as specific DNA sequences of known periodontal pathogens (A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. denticola, T. forsythia and Candida albicans) and of the human genome. The five best-quality DNA samples from each cohort were subjected to PCR with 16S-rDNA specific primers [12] The produced pair-end (2×250) libraries were then sequenced using an IlluminaMiSeq instrument with MiSeq reagent Kit v2. This yielded ~100,000 reads per sample.

The obtained 16S rDNA sequences were assigned to bacterial genera using QIIME software [13] for automated analysis and comparison of microbial communities. The statistical significance of differences in the occurrence of each bacterial genus in the "normal" cohort versus "AP" cohort was determined by Student's t-test (T-criterion) and Fisher's exact text (F-criterion) using Statistica software. P values less than or equal to 0.05 were considered statistically significant.

Results

Collection of clinical samples. While humans younger than 25 typically have healthy periodontium, individuals older than 40 frequently suffer from CP, CP and AP cannot be easily distinguished through clinical examination. Post factum identification of patients with these two conditions is possible on the basis of the clinical anamnesis, but this is often not available or is ambiguous. For this study, in order to compose a cohort of unambiguous AP cases and a cohort of normal individuals with low risk of developing AP, we aimed to thoroughly exclude CP patients, the largest segment of the population. Therefore, Cohort 1 ("normal") was comprised of the oldest persons with healthy periodontium (10 subjects, Table 1) and Cohort 2 ("AP") was composed with persons of the possibly younger age (7 subjects, Table 1).

DNA was prepared from four independently collected subgingival samples from each individual (two A (molar) and two B (incisor)). The yield of bacterial genomic DNA in each sample was assessed using the commercial Dentoflor quantitative RT-PCR kit with primers for total 16 s rDNA, Aggregatibacteractinomycetemcomitans, Porphyromonasgingivalis, Prevotella intermedia, Treponemadenticola, Tanerella forsythia, Candida albicans.Data for each amplified sequence was expressed as the C_t (threshold cycle) which is inversely proportional to the template DNA concentration. Samples in which the C_t exceeded 22 were discarded to avoid the risk of non-proportional losses of different bacterial species in them. Samples with C_t values lower than 22 were pooled.

At this stage, 3 patients out of the initial group of 10 (#001, 003 and 009) were excluded from Cohort 1. In contrast, all patients in Cohort 2 had sufficient DNA quantity and quality, which supports the hypothesis that periodontitis patients have

increased bacterial colonization of the gingival tissue compared to healthy individuals without periodontitis. To finalize Cohort 1, we excluded the youngest patients (#002 and 008), leaving five individuals 28-44 years old whose average C_t indicating bacterial DNA yield was 18.7-21.3 (absolute average below 6.1 times).

The final Cohort 2 was composed of 5 patients 27-35 years old with average C_t values indicating bacterial DNA yield of 16.6-21.8. Three Cohort 2 patients (#306, 308 and 310) had higher DNA yields than seen in healthy individuals (average C_t 16.6-16.9) whereas patients #300 and #303 had much lower DNA yields (comparable to what was seen in Cohort 1; average C_t 20.0-21.8).

Creation of 16S rDNA libraries and their analysis by next-generation sequencing (NGS).In order to identify and quantify bacterial species present in each subgingival sample, we generated 16S rDNA libraries via PCR amplification with specific primers as described (REF). We then sequenced the 16S rRNA gene amplicon, targeting the V4 hypervariable region corresponding to positions 515–806 in Escherichia coli. Despite being rather short, this region has been shown to enable successful discrimination between different phylotypes and provides species richness comparable to that obtained through analysis of a nearly full-length fragment [12, 13]. Furthermore, amplicons of single hypervariable regions missing conserved intermediate stretches (and short targets in general) are less prone to form chimeras, thus providing higher data quality [14, 15]. In total, 1,017,669 sequence reads, ranging from 69,566 to 139,385 per sample, were generated on the IlluminaMiSeq instrument. The sequences were deposited in NCBI BioProject Submissions system (Submission ID SUB588191; BioProject ID PRJNA256234).

Downstream sequence processing and quality filtering eliminated about 30% of the sequencing data. Clustering of denoised high quality reads at the species level (defined by 3% sequence difference) generated between 300 and 350 OTUs per specimen, most of which were equal to species or genus.

The arithmetic mean of the resulting data (% of each OTU in a sample) was calculated for each cohort (Table 2). Student's t-test was then used to calculate the T-value for each OTU (at p value >0.05), which provides an estimation of the relevance of differences in the levels of the OTU in distinguishing cohorts. A T-value above 3 indicates statistically valid prevalence of the OTU in Cohort 1 (healthy individuals) as compared to Cohort 2 (AP patients). A T-value of 2-3 means that the OTU tends to be associated with healthy phenotype. In contrast, a T-value below -3 shows statistically valid association of the OTU with AP while a T-value in the range between -2 and -3 shows a trend of OTU prevalence in AP patients. A T value between -2 and 2 indicates that occurrence of the analyzed OTU is not associated with either the healthy periodontal phenotype or the AP phenotype.

This analysis of the prevalence of bacterial genera in our cohorts (Table 2) demonstrated that the genus Veillonella is the only OTU significantly associated with high conservancy of periodontium health. Moreover, Veillonella was found to be one of the most prevalent bacterial groups (third most prevalent after Fusobacterium and Prevotella in healthy individuals). In AP patients (Cohort 2), we observed a 4-5-fold decrease in the level of Veillonella bacteria compared to the levels seen in healthy individuals (Cohort 1). In addition, the low Std.Dev. C2 parameter (Table 2) shows absence of AP cases with normal (healthy) Veillonella levels. These findings establish Veillonella bacteria as a periodontal protective species.

Five other OTUs were found to be associated with the healthy periodontium in Cohort 1, including Streptococcus, Bergeyella, Granulicatella, Kingella and Corynebacterium. However, the strength of these correlations was much lower than that of Veillonella.

A relatively larger number of OTUs (eight) was found to be significantly associated with AP in our study, including Porphyromonas, Treponema, Synergistaceae, Tannerella, Filifactor, Ruminococcaceae, Parvimonas and Mycoplasma (Table 2). Interestingly, Treponema, Synergistaceae and Filifactor showed the strongest association with the AP in our cohorts while the commonly acknowledged periodontal pathogens Porphyromonas and Tannerella were less strongly associated with this condition. Moreover, colonization of the periodontium with genera Prevotella[16] and Aggregatibacter[17] was found to be fairly independent of AP although they are often considered as the most dangerous infectious agents directly involved in periodontal tissue decay. A possible explanation for these discrepancies between our findings and previous observations may be that only certain serotypes of A. actinomycetemcomitans exhibit periodontal pathogenicity while other serotypes of this species, as well as other species of genus Aggregatibacter, are not associated with AP [17].

Analysis of bacterial species (in contrast to genera) revealed additional microorganisms prevalent in healthy periodontium samples (Table 3). Interestingly, three species of genus Prevotella (P. nigrescens, P. orisand P. tannerae) showed trends toward association with periodontal health (average share of these species in cohorts 1 and 2 comprises 2.1 and 0.6% for P. nigrescens, 3.1 and 1% for P. orisand1.1 and 0.3% for P. tannerae), while P. intermedia, which is commonly acknowledged as a periodontal pathogen, was confirmed to have a negative clinical impact in our study (average share in cohorts 1 and 2 comprises 0.4 and 1.8%). Other relatively massive species (Veillonellaparvula, Streptococcus sanguinis, KingellaoralisandGranulicatellaparadiacens) were also found to be associated with the healthy periodontium, with their levels decreased in samples from AP patients (average share of these species in cohorts 1 and 2 comprises 1.9 and 0.4%; 3.9 and 0.9%; 0.8 and 0.2%; 1.0 and 0.1% respectively).

Validation of 16S rDNA NGS sequencing results by real-time PCR. Quantitative RT-PCR (using a Dentoflor kit) was used as an independent method to validate the results obtained by NGS sequencing. Confirmation of the accuracy of our DNA purification procedure in this way was particularly important since our findings for several OTUs (e.g., Filifactor, Prevotella and Aggregatibacter) were not in a good agreement with previously reported data [18]. In addition, comparison of the percentage of each OTU in clinical samples as determined by metagenomic sequencing versus RT-PCR was necessary for establishment of RT -PCR as a routine method allowing high through-put assay of waste panels of samples.

RT-PCR analysis demonstrated complete absence of Aggregatibacteractinomycetemcomitans in the periodontal microbiome of all individuals in the study, both healthy and AP patients (Table 4). This suggests that A. actinomycetemcomitans was not an essential factor contributing to AP in the analyzed Russian population. Interestingly, however, the RT-PCR data for genus Aggregatibacter shown in Table 4 apparently contradicts those obtained by metagenomic sequencing (Table 2). Comparison of the two right columns of Table 4 suggests that this discrepancy may be explained by investigation of species within genus Aggregatibacter used as OTU for metagenomic analysis. This kind of analysis (Table 5) demonstrated that Aggregatibactersegnisis highly prevalent in the periodontal microbiome of all patients, both normal and AP. A. haemophilus similae and A. aphrophilus were found to be less prevalent; however, their levels also exceeded that of Aggregatibacteractinomycetemcomitans. Taken together, these results call into question consideration of genus Aggregaticabter as a causative agent of AP. It should be noted that the PCR-based Dentoflor kit uses species-specific molecular markers and thus provides results corresponding to metagenomic sequencing.

Similar comparison of RT-PCR and metagenomic sequencing data obtained for our cohorts was performed for acknowledged periodontal pathogens Porphyromonasgingivalis, Tannerella forsythia and Treponemadenticola (Table 6). This

demonstrated fairly good agreement between the two types of data. As expected, RT-PCR analysis accurately quantified the levels of a given microorganism in a particular individual relative to other individuals. However, due to differences in the sensitivity of different primer pairs, RT-PCR analysis was not useful for accurate comparison of the extent of periodontal colonization by different species. This is clearly illustrated by comparing the data for P. gingivalisand T. denticola, for which the relative C_t parameters in both cohorts are higher for the first species (indicating lower presence) while actual colonization of the periodontium with P. gingivaliswas 2-3-fold greater than with T. denticola.

Discussion

Our data demonstrate that Veillonella is the only OTU (of genus or higher rank taxa) strongly associated with the healthy periodontium. This finding is in agreement with data from Heller et al. [19] and Silva-Boghossian et al. [20] who reported that Veillonella was associated with CP rather than with AP. Heinrich-Weltzien et al. [21] reported that a high level of Veillonella colonization is associated with caries in deciduous teeth. Streptococcus is another OTU known to contribute to development of caries [21]; however, its high prevalence in the cohort of healthy individuals in our study demonstrates association with low AP susceptibility. These findings support a hypothesis that hygienic means aimed at prevention of caries may induce onset of AP due to disruption of the balance between normal microbiome components. It is possible that Veillonella in particular may contribute to maintenance of periodontal health through consumption of succinate and lactate which are converted to acetate and propionate [22]. It should be noted, however, that while Veillonella may act as a periodontoprotective agent and a positive prognostic marker of AP, it may cause other serious conditions such as endocarditis [23], meningitis and shunt [24], septic shock [25], pyelonephritis and secondary bacteremia [26].

Other bacterial species have been identified as putative markers of low AP susceptibility in previous reports. Bergeyella prevalence was described as a positive periodontitis and plaque predictive marker in dogs [27]. Both Granulicatella and Veillonella were described as components of the normal oral cavity microbiome [28] although these species may be responsible for endodontic infection and may cause fetal injury in pregnant women. Shaddox et al. [29] studied metagenomes of healthy and diseased sites in localized AP in Afro-American children in the US. The most prevalent species in healthy sites were Selenomonasspp, Veillonellaspp, Streptococcus spp, Bergeyellasp, and Kingellaoralis. Overall, Streptococcus spp, Campylobacter gracilis, Capnocytophagagranulosa, Haemophilus parainfluenzae, and Lautropia mirabilis were most abundant in healthy children, while A. actinomycetecomitans, Filifactoralocis, Tannerellasp, Solobacteriummoorei, Parvimonasmicra, and Capnocytophagasp were most abundant in those with AP.

Comparison of changes in the subgingival microbiota of individuals with "refractory" periodontitis versus treatable periodontitis before and after periodontal therapy using Human Oral Microbe Identification Microarray (HOMIM) analysis were reported by Colombo et al. [30] and yielded conclusions similar to ours. Presence of Capnocytophagasputigena, Cardiobacteriumhominis, Gemellahaemolysans, Haemophilus parainfluenzae, Kingellaoralis, Lautropia mirabilis, Neisseria elongata, Rothiadentocariosa, Streptococcus australis, and Veillonella spp. was found to be associated with therapeutic success.

Corynebacterium has not been formerly associated with periodontitis. Its role in AP prognosis may correlate with the fact that this genus belongs to aerobic bacteria in contrast to all abovementioned OTUs.

Our finding of significant association of Synergistaceae, Filifactor, Ruminococcaceae, Mycoplasma and Parvimonas representatives with AP suggests that these microorganisms should be considered as putative negative markers of periodontal health beyond those traditionally used (Aggregatibacteractinomycetemcomitans and the so-called "orange complex" of Porphyromonasgingivalis, Prevotella intermedia, Treponemadenticola and Tannerella forsythia).

Finally, Fusobacter was identified in our study as the most abundant OTU in the periodontium that shows no positive or negative association with AP. Therefore, its specific chromosomal markers may be considered as additional useful targets for normalizing RT-PCR data concerning the prevalence of periodontopathogenic or periodontoprotective species.Overall, the results of this study add to our understanding the healthy periodontal microbiome and how it shifts in AP. In particular, the putative protective role of Veillonella and pathogenic roles of Synergistes, Filifactor,Ruminococcus, Mycoplasma and Parvimonas detected in our study provide rationale for their further investigation as potential therapeutic targets and/or diagnostic or prognostic markers in periodontal disease.

Acknowledgments

We acknowledge Patricia Baker for proof-reading of the English spelling, grammar and style.

Table 1. Cohorts of patients with healthy periodontium (Cohort 1) and aggressive periodontitis (AP) involved in the study. DNA yield was determined via real-time PCR with 16S-rDNA specific primers from the Dentoflor kit. C_t values for the final pooled samples are shown. Samples included in the final cohort are bolded.

Cohort 1. Healthy periodontium				
Personal Code	Year of birth	Gender	Clinical findings	DNA yield, C_t
001	1987	F	No complaints, no pathological changes	25.1
002	1983	M	No complaints, no pathological changes	19.9
003	1977	F	No complaints, no pathological changes	25.2
004	1985	F	No complaints, no pathological changes	18.7
005	1964	F	No complaints, no pathological changes	19.6
006	1974	M	No complaints, no pathological changes	21.3
007	1969	M	No complaints, no pathological changes	18.4
008	1980	F	No complaints, no pathological changes	18.5
009	1963	F	No complaints, no pathological changes	23.3
010	1985	F	No complaints, no pathological changes	20.0
Cohort 2. Aggressive periodontitis				
0300	1986	F	Periodontal pocket 8 mm, Sl=-2,1; Muhl =-2,5; no worn tubera, negative maternal inheritance, annual exacerbation, 1-2 stage mobility	20.0
0303	1982	F	Periodontal pocket 5mm, Sl=-1,6; Muhl=1,8; no worn tubera, negative maternal inheritance, 2 times per year exacerbation, 1-2 stage mobility	21.8
0304	1983	M	Periodontal pocket 6мм, Sl=-1,9; Muhl=-2,1; no worn tubera, negative paternal inheritance, annual exacerbation, 1 stage mobility	21.4
0306	1983	M	Periodontal pocket 10мм, Sl=-2,9; Muhl=-2,9; no worn tubera, negative maternal inheritance, even 3-4 months exacerbation, 3 stage mobility	16.8
0308	1978	M	Periodontal pocket 8мм, S-l=-1,6; Muhl=-1,9; no worn tubera, negative maternal inheritance, 2 times per year exacerbation, 2 stage mobility	16.9
0309	1971	M	Periodontal pocket 7мм, S-l=-1,8; Muhl=-2,3; no worn tubera, negative multiple indirect inheritance, 2 times per year exacerbation, 2 stage mobility	20.1
0310	1980	F	Periodontal pocket 10мм, Sl=-2,9; Muhl=-1,9; no worn tubera, negative maternal inheritance, annual exacerbation, 2-3 stage mobility	16.6

Table 2. Prevalence of the most massive bacterial OTUs in the periodontium of healthy individuals (cohort 1 = C1) and patients with AP (cohort 2 = C2). "Mean C1" and "mean C2" columns indicate the percentage of the OTU within the total number of high-quality sequences in the cohort. T-value indicates the statistical difference between the cohorts. OTUs associated or tending to association with resistance to AP are highlighted with grey. OTUs associated or tending to association with susceptibility to aggressive periodontitis are shown in boxes, marked with * symbol and bolded.

OTU	Mean C1	Mean C2	T-value	df	p	Std.Dev.	Std.Dev.	F-ratio	p
Prevotella	11,65825	7,92996	0,91368	8	0,383123	8,096486	4,098364	3,90	0,215672
Fusobacterium	13,05302	13,94555	-	8	0,693050	6,621698	4,257331	2,43	0,411364
Selenomonas	6,47897	4,55765	1,33382	8	0,219612	3,129343	1,704155	3,37	0,266054
Veillonella	10,44074	2,21493	4,91707	8	0,001168	3,731150	0,267648	194,34	0,000157
Streptococcus	6,61704	2,28866	2,22603	8	0,056802	4,233962	0,977388	18,83	0,014738
Porphyromonas *	5,25431	14,75697	-	8	0,617368	1,988579	6,822742	11,77	0,038865
Dialister	2,00807	1,77627	0,28814	8	0,780566	1,157305	1,376980	1,42	0,744617
Treponema *	3,28366	17,82401	-	8	0,007413	0,782318	5,941260	57,68	0,001722
Synergistaceae *	3,13117	10,18970	-	8	0,016804	0,922154	5,158220	31,29	0,005636
Campylobacter	3,45338	2,53127	1,49724	8	0,172703	1,218197	0,648626	3,53	0,249624
Oribacterium	0,70363	0,15223	1,34214	8	0,216093	0,913191	0,062571	213,00	0,000131
Lautropia	2,38803	0,56250	1,20390	8	0,263068	4,118750	0,141817	843,83	0,000008
Neisseria	6,52359	1,94308	1,85281	8	0,101152	5,098794	1,533794	10,70	0,031336
Tannerella *	1,49621	3,95056	-	8	0,055309	0,624582	2,367577	14,37	0,024259
Capnocytophaga	4,75307	1,26772	1,80756	8	0,092880	4,041040	0,632723	40,79	0,003331
Bergeyella	0,39906	0,14003	2,12279	8	0,066626	0,474033	0,093714	26,71	0,007624
Leptotrichia	3,18794	1,56863	1,69841	8	0,127860	2,357909	0,423058	31,36	0,005611
Paludibacter	1,24076	1,13819	0,20212	8	0,844870	1,065644	0,389833	7,47	0,077066
Granulicatella	1,00149	0,14826	2,54026	8	0,03469[illegible]	0,750589	0,026365	810,48	0,000009
Johnsonella	0,57620	0,15887	1,92231	8	0,090652	0,473309	0,106758	19,66	0,015609
Actinomyces	0,76488	0,50139	1,39981	8	0,188620	0,172663	0,321712	3,41	0,259329
Filifactor *	0,53883	2,45718	-	8	0,009987	0,050166	1,277068	1792,23	0,000062
Abiotrophia	0,47957	0,05665	1,22155	8	0,256650	0,784992	0,016228	5890,97	0,000060
Peptostreptococcaceae	0,18007	0,41615	-	8	0,262828	0,131001	0,495624	14,31	0,024471
Rothia	0,30651	0,29517	0,16044	8	0,876614	0,071382	0,186758	6,84	0,089217
Eubacterium	0,20154	0,65522	-	8	0,217722	0,077901	0,756889	92,51	0,000676
Aggregatibacter	1,51733	0,45994	0,95025	8	0,369804	2,473020	0,138559	318,55	0,000059
Ruminococcaceae	0,25794	0,71619	-	8	0,024458	0,051892	0,166618	46,47	0,002571
Parvimonas *	0,25149	0,71188	-	8	0,017776	0,086666	0,327545	14,28	0,024565
Dysgonomonas	0,26611	1,32021	-	8	0,198759	0,058008	1,676465	835,24	0,000009
Kingella	0,78830	0,18056	2,62609	8	0,030548	0,512344	0,078168	42,96	0,003058
Mycoplasma *	0,23286	1,27028	-	8	0,063295	0,041669	1,072709	666,43	0,000013
Megasphaera	0,25741	0,19358	0,88805	8	0,400426	0,141814	0,077761	3,31	0,252063
Streptobacillus	0,20936	0,82977	-	8	0,348861	0,012398	1,393704	1167684	0,000000
Corynebacterium	0,60677	0,10096	2,05302	8	0,076498	0,345781	0,026658	470,03	0,000014
Paracoccus	0,17366	0,16479	0,36129	8	0,389970	0,017438	0,023063	1,48	0,723226

Table 3.Analysis of bacterial species prevalence in the periodontium. Percentage of a species is shown for each individual patient. Except for pathogenic Prevotella intermedia, only putatively protective species are shown.

Patient (sample)	*P. nigrescens*	*P. oris*	*P. tannerae*	*P. intermedia*	*Veillonella parvula*	*Streptococcus sanguinis*	*Kingella oralis*	*Granulicatella adiacens*
Cohort 1. Healthy periodontium								
4-A	3,1	4,3	3,1	**0,5**	1,8	1,9	0,2	0,7
5-A	2,7	4,1	0,5	**0,4**	3,3	1,3	0,9	0,4
6-A	0,4	0,4	0,1	**0,3**	1,8	9,0	1,4	2,3
7-A	1,3	3,0	1,3	**0,4**	0,6	4,1	1,1	0,6
10-A	0,8	3,7	0,2	**0,3**	2,0	2,9	0,3	1,0
Mean	**2,1**	**3,1**	**1,1**	0,4	**1,9**	**3,9**	**0,8**	**1,0**
Cohort 2. Aggressive periodontitis								
300-A	0,4	0,7	0,2	**0,5**	0,4	0,7	0,1	0,1
303-A	0,4	0,5	0,2	**1,4**	0,3	0,7	0,3	0,2
306-A	0,6	0,7	0,5	**5,1**	0,5	1,6	0,2	0,2
308-A	0,9	2,6	0,4	**0,2**	0,4	1,1	0,1	0,1
310-A	0,5	0,6	0,2	**1,9**	0,3	0,6	0,1	0,1
Mean	**0,6**	**1,0**	**0,3**	**1,5**	**0,4**	**0,9**	**0,2**	**0,1**

Table 4.Comparison of species of Aggregatibacter genus prevalence in the microbiome of patients with healthy periodontium and AP. The C_t values in the "RT-PCR" column for each species are normalized to the 16S rDNA signal of the same sample.

Patient (sample)	RTPCR	MS genus-specific	MS species-specific
Cohort 1. Healthy periodontium			
4-A	no	0,33	no
5-A	no	0,55	no
6-A	no	0,34	no
7-A	no	0,41	no
10-A	no	5,93	no
Mean	no	1,51	no
Cohort 2. Aggressive periodontitis			
300-A	no	0,32	no
303-A	no	0,38	no
306-A	no	0,60	no
308-A	no	0,62	no
310-A	no	0,38	no
Mean	no	0,46	no

Table 5. Prevalence of different species of genus Aggregatibacter in the microbiome of individual patients determined bmetagenomic sequencing.

Patient (sample)	Genus	Species		
	Aggregatibacter	A. segnis	A. haemophilus similae	A. aphrophilus
4-A	0,33	0,31	0,0000	0,013
5-A	0,55	0,52	0,0015	0,025
6-A	0,34	0,31	0,0062	0,027
7-A	0,41	0,33	0,0042	0,073
10-A	5,93	5,89	0,0045	0,042
300-A	0,32	0,30	0,0000	0,028
303-A	0,38	0,30	0,0126	0,064
306-A	0,60	0,56	0,0014	0,038
308-A	0,62	0,54	0,0016	0,075
310-A	0,38	0,37	0,0000	0,015

Table 6.Comparison of the prevalence of acknowledged periodontal pathogens in the periodontal microbiome by RT-PCR and metagenomic sequencing analysis. C_t parameters in columns "RT-PCR" for each species are normalized to the 16S rDNA signal in the same sample.

	Porphyromonasgingivalis			*Tannerellaforsythia*			*Treponemadenticola*		
	RT-PCR (Ct)	MS genus-specific	MS species-specific	RT-PCR (Ct)	MS genus-specific	MS species-specific	RT-PCR (Ct)	MS genus-specific	MS species-specific
4-A	18,1	4,06	2,89	13,9	1,26	no	17,4	2,95	0,89
5-A	no	4,55	3,00	16,9	1,24	no	14,9	3,44	0,95
6-A	17,9	3,39	1,85	12,5	0,89	no	15,6	2,68	0,81
7-A	27,5	8,43	2,28	4,2	2,53	no	8,2	4,58	1,72
10-A	No	5,85	2,89	no	1,56	no	0	2,77	0,94
Mean	**12,7**	**5,26**	**2,58**	**11,875**	**1,50**	**no**	**11,22**	**3,28**	**1,06**
300-A	No	20,01	16,69	3,6	8,14	no	5,3	5,21	0,86
303-A	No	12,75	2,12	0,1	2,44	no	0,1	20,86	3,10
306-A	0,7	3,64	2,35	3,6	2,75	no	6,6	10,52	5,73
308-A	0,3	18,88	16,32	3,4	3,23	no	3,1	11,35	5,52
310-A	0,7	18,50	15,81	1,8	3,19	no	0	16,19	6,34
Mean	**0,34**	**14,75**	**10,66**	**2,5**	**3,95**	**no**	**3,02**	**12,82**	**4,31**

REFERENCES

1. Aas JA, Paster BJ, Stokes LN, Olsen I and Dewhirst FE (2005) Defining the normal bacterial flora of the oral cavity. Journal of clinical microbiology 43: 5721-5732. | 2. Armitage GC (1999) Development of a classification system for periodontal diseases and conditions. Annals of periodontology / the American Academy of Periodontology 4: 1-6. | 3. Belstrom D, Fiehn NE, Nielsen CH, Kirkby N, Twetman S, et al. (2014) Differences in bacterial saliva profile between periodontitis patients and a control cohort. Journal of clinical periodontology 41: 104-112. | 4. Brigido JA, da Silveira VR, Rego RO and Nogueira NA (2014) Serotypes of Aggregatibacteractinomycetemcomitans in relation to periodontal status and geographic origin of individuals-a review of the literature. Medicina oral, patologia oral y cirugiabucal 19: e184-191. | 5. Brook I (2002) Meningitis and shunt infection caused by anaerobic bacteria in children. Pediatric neurology 26: 99-105. | 6. Bahrani-Mougeot FK, Paster BJ, Coleman S, Ashar J, Barbuto S, et al. (2008) Diverse and novel oral bacterial species in blood following dental procedures. Journal of clinical microbiology 46: 2129-2132. | 7. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Gonzalez Pena A, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J and Knight R (2010); Nature Methods 7(5): 335-336. | 8. Chahbouni H, Maltouf AF and Ennibi O (2013) [Aggregatibacteractinomycetemcomitans and Porphyromonasgingivalis in aggressive periodontitis in Morocco -- preliminary study]. Odonto-stomatologietropicale = Tropical dental journal 36: 5-10. | 9. Colombo AP, Bennet S, Cotton SL, Goodson JM, Kent R, et al. (2012) Impact of periodontal therapy on the subgingival microbiota of severe periodontitis: comparison between good responders and individuals with refractory periodontitis using the human oral microbe identification microarray. Journal of periodontology 83: 1279-1287. | 10. Davis IJ, Wallis C, Deusch O, Colyer A, Milella L, et al. (2013) A cross-sectional survey of bacterial species in plaque from client owned dogs with healthy gingiva, gingivitis or mild periodontitis. PloS one 8: e83158. | 11. Heinrich-Weltzien R, Bartsch B and Eick S (2014) Dental Caries and Microbiota in Children with Black Stain and Non-discoloured Dental Plaque. Caries research 48: 118-125. | 12. Hou YY, Zhen YH, Wang D, Zhu J, Sun DX, et al. (2014) Protective effect of an egg yolk-derived immunoglobulin (IgY) against Prevotella intermedia-mediated gingivitis. Journal of applied microbiology 116: 1020-1027. | 13. Heller D, Silva-Boghossian CM, do Souto RM and Colombo AP (2012) Subgingival microbial profiles of generalized aggressive and chronic periodontal diseases. Archives of oral biology 57: 973-980. | 14. Huse SM, Dethlefsen L, Huber JA, Mark Welch D, Relman DA, et al. (2008) Exploring microbial diversity and taxonomy using SSU rRNAhypervariable tag sequencing. PLoS genetics 4: e1000255. | 15. Junemann S, Prior K, Szczepanowski R, Harks I, Ehmke B, et al. (2012) Bacterial community shift in treated periodontitis patients revealed by ion torrent 16S rRNA gene amplicon sequencing. PloS one 7: e41606. | 16. Keijser BJ, Zaura E, Huse SM, van der Vossen JM, Schuren FH, et al. (2008) Pyrosequencing analysis of the oral microflora of healthy adults. Journal of dental research 87: 1016-1020. | 17. Lazarevic V, Whiteson K, Huse S, Hernandez D, Farinelli L, et al. (2009) Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. Journal of microbiological methods 79: 266-271. | 18. Ling Z, Liu X, Wang Y, Li L and Xiang C (2013) Pyrosequencing analysis of the salivary microbiota of healthy Chinese children and adults. Microbial ecology 65: 487-495. | 19. Loesche WJ and Grossman NS (2001) Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment. Clinical microbiology reviews 14: 727-752. | 20. Liu Z, Lozupone C, Hamady M, Bushman FD and Knight R (2007) Short pyrosequencing reads suffice for accurate microbial community analysis. Nucleic acids research 35: e120. | 21. Michalowicz BS, Aeppli D, Virag JG, Klump DG, Hinrichs JE, et al. (1991) Periodontal findings in adult twins. Journal of periodontology 62: 293-299. | 22. Michalowicz BS, Diehl SR, Gunsolley JC, Sparks BS, Brooks CN, et al. (2000) Evidence of a substantial genetic basis for risk of adult periodontitis. Journal of periodontology 71: 1699-1707. | 23. Muhlemann HR and Son S (1971) Gingival sulcus bleeding--a leading symptom in initial gingivitis. Helvetica odontologicaacta 15: 107-113. | 24. Ng SK and Hamilton IR (1971) Lactate metabolism by Veillonellaparvula. Journal of bacteriology 105: 999-1005 | 25. Prpic-Mehicic G, Marsan T, Miletic I and Buntak-Kobler D (1998) Infective endocarditis caused by Veillonella of dental origin. Collegium antropologicum 22 Suppl: 39-43. | 26. Silness J and Loe H (1964) Periodontal Disease in Pregnancy. Ii. Correlation between Oral Hygiene and Periodontal Condtion. ActaodontologicaScandinavica 22: 121-135. | 27. Silva-Boghossian CM, Neves AB, Resende FA and Colombo AP (2013) Suppuration-associated bacteria in patients with chronic and aggressive periodontitis. Journal of periodontology 84: e9-e16. | 28. Sogin ML, Morrison HG, Huber JA, Mark Welch D, Huse SM, et al. (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". Proceedings of the National Academy of Sciences of the United States of America 103: 12115-12120. | 29. Shaddox LM, Huang H, Lin T, Hou W, Harrison PL, et al. (2012) Microbiological characterization in children with aggressive periodontitis. Journal of dental research 91: 927-933. | 30. Yagihashi Y and Arakaki Y (2012) Acute pyelonephritis and secondary bacteraemia caused by Veillonella during pregnancy. BMJ case reports 2012. | 31. Youssef N, Sheik CS, Krumholz LR, Najar FZ, Roe BA, et al. (2009) Comparison of species richness estimates obtained using nearly complete fragments and simulated pyrosequencing-generated fragments in 16S rRNA gene-based environmental surveys. Applied and environmental microbiology 75: 5227-5236. |