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Prevalence and microbiological diversity of Archaea in peri-implantitis subjects by 16S ribosomal RNA clonal analysis

M. Faveri[1,2], L. F. H. Gonçalves[2], M. Feres[2], L. C. Figueiredo[2], L. A. Gouveia[2], J. A. Shibli[2], M. P. A. Mayer[1]

[1]Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil and [2]Department of Periodontology, Dental Research Division, Guarulhos University, Guarulhos, São Paulo, Brazil

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Background and Objective: This study evaluated the prevalence and the molecular diversity of Archaea in the subgingival biofilm samples of subjects with peri-implantitis.

Material and Methods: Fifty subjects were assigned into two groups: Control ($n = 25$), consisting of subjects with healthy implants; and Test ($n = 25$), consisting of subjects with peri-implantitis sites, as well as a healthy implant. In the Test group, subgingival biofilm samples were taken from the deepest sites of the diseased implant. In both groups, subgingival biofilm was collected from one site with a healthy implant and from one site with a periodontally healthy tooth. DNA was extracted and the 16S ribosomal RNA gene was amplified with universal primer pairs for Archaea. Amplified genes were cloned and sequenced, and the phylotypes were identified by comparison with known 16S ribosomal RNA sequences.

Results: In the Control group, Archaea were detected in two and three sites of the implant and the tooth, respectively. In the Test group, Archaea were detected in 12, 4 and 2 sites of diseased implants, healthy implants and teeth, respectively. Diseased implants presented a significantly higher prevalence of Archaea in comparison with healthy implants and natural teeth, irrespective of group. Over 90% of the clone libraries were formed by *Methanobrevibacter oralis,* which was detected in both groups. *Methanobacterium congelense/curvum* was detected in four subjects from the Test group and in two subjects from the Control group.

Conclusion: Although *M. oralis* was the main species of Archaea associated with both healthy and diseased implant sites, the data indicated an increased prevalence of Archaea in peri-implantitis sites, and their role in pathogenesis should be further investigated.

Marcelo Faveri, Centro de Pós-Graduação e Pesquisa-CEPPE, Universidade Guarulhos, Praça Tereza Cristina, 229 Centro, 07023-070 Guarulhos, SP, Brazil
Tel: +55 11 2441 3670
Fax: +55 11 2411 3671
e-mail: mfaveri@prof.ung.br

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Peri-implantitis is described as a destructive inflammatory process that affects the soft and hard tissues around osseointegrated implants, leading to the formation of a peri-implant pocket and bone loss (1). It is well recognized that the composition of the biofilm formed around healthy implants or periodontally healthy teeth is quite similar (2).

Similarly, a peri-implant pocket seems to harbor a microbiota similar to that found in periodontitis, and including microorganisms such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Tannerella forsythia, Treponema denticola, Aggregatibacter actinomycetemcomitans* and *Staphylococcus aureus* (3–7). However, it should be highlighted that recent studies using culture-independent techniques, such as cloning and sequencing analyses, suggest that other bacterial species, such as *Filifactor alocis*, *Dialister pneumonsintes* (8) and *Selenomonas sputigena* (9), as well as Archaea (10,11), might be associated with the onset and progression of periodontitis. Therefore, the role of these microorganisms in the pathogenesis of peri-implantitis should also be investigated.

Archaea are a group of single-cell microorganisms that were classified, on the basis of the ribosomal RNA (rRNA) gene sequences, as a distinct group of organisms differing greatly from prokaryotes (bacteria) and eukaryotes (12) in terms of genetic, biochemical and structural features (13). Archaea can be found in most ecosystems and are often prevalent in supposedly uninhabitable environments (13). Several studies have shown that Archaea can be detected from subgingival biofilm samples of periodontitis patients (10,11,14,15), and an association between the severity of periodontal disease and the prevalence of Archaea (*Methanobrevibacter* spp.) has also been shown (11). As methanogens (a specific member of the Archaea that produce methane as a metabolic by-product in anoxic conditions) are potential hydrogen competitors of *Treponemes*, it may be speculated that organisms of the Archaea domain may serve as syntrophic partners with other members of the subgingival biofilm community (11). In addition, Archaea have also been detected in infected root canals (16,17), possibly interacting with bacteria such as *Synergistes* spp. (17).

In view of the fact that Archaea have been detected in periodontal pockets and in infected root canals, the purpose of the present study was to analyze the prevalence and the molecular diversity of Archaea in the subgingival biofilm samples of subjects with peri-implantitis and to compare the findings with the data collected from healthy implants and healthy subgingival sites of natural teeth.

Material and methods

Subject population

Fifty systemically healthy subjects were selected from the population referred to the Oral Implantology Clinic of Guarulhos University (Guarulhos, SP, Brazil). Their medical and dental histories were obtained, and a full-mouth periodontal and implant examination was performed. Based on these data, the diagnosis of peri-implantitis 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 previously approved by the Clinical Research Ethics Committee of Guarulhos University.

Inclusion criteria

The subjects were assigned to two groups.

Control group (n = 25) – Subjects with at least one healthy dental implant (probing depth ≤ 4 mm and no bleeding on probing) and at least 10 periodontally healthy teeth (probing depth ≤ 3 mm and no bleeding on probing).

Test group (n = 25)– Subjects with at least one implant with peri-implantitis, one healthy implant and at least 10 periodontally healthy teeth. peri-implantitis was characterized by saucer-shaped osseous defects of > 3 mm, a probing depth of ≥ 5 mm and an inflamed peri-implant mucosa exhibiting bleeding on probing and/or suppuration (1,6).

Exclusion criteria

Subjects were excluded if they were completely edentulous, had an implant with a coated surface, had moderate to severe chronic periodontitis (i.e. suppuration and/or bleeding on probing in more than 30% of the subgingival sites or in any site with a probing depth of ≥ 4 mm), had taken antibiotics or anti-inflammatory drugs within 6 mo before the clinical examination, had received periodontal or peri-implant therapy within 6 mo before the start of the study, had a chronic medical disease or condition, or were smokers.

Clinical and radiographic examination

Visible plaque (0/1), 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 implant (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual). Probing depth and clinical attachment level measurements were recorded to the nearest millimeter using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA). The clinical examination was performed by one trained and calibrated examiner.

Investigator calibration

The examiner participated in a calibration exercise that was performed in 10 nonstudy subjects with periodontitis. The examiner measured one quadrant per subject. The quadrant chosen had at least six teeth. If a quadrant presented fewer than six teeth, a second quadrant was chosen. For better standardization, quadrant 1 was the first choice, followed by quadrants 2, 3 and 4, respectively. Initially, the examiner measured probing depth and clinical attachment level in a given quadrant and 60 min later the protocol was repeated. Therefore, all 10 subjects were probed twice in the same visit by the examiner. Upon completion of all measurements, the intra-examiner variability for probing depth and clinical attachment level measurements was assessed. Calibration was conducted according to the protocol developed by Araujo *et al.* (18) and the standard error of measurement was calculated. Intra-examiner variability was 0.17 mm for probing depth and 0.20 mm for clinical attachment level.

Microbiological examination

Sample collection— In the Test group the peri-implant site with the deepest probing depth was selected for sampling. If two or more sites presented similar probing depth values, the more anterior site was chosen. Another sample was collected from the mesial site of a healthy dental implant and of the crevicular sulcus of a tooth in a condition of periodontal health in the same subject. Samples from the mesial surface of a healthy dental implant and a periodontally healthy tooth were collected from each subject of the Control group. After the clinical parameters had been recorded, the supragingival plaque was removed and subgingival biofilm samples were taken with individual sterile Gracey curettes and immediately placed in separate polypropylene tubes containing 50 μL of TE buffer (10 mm Tris–HCl, 1 mm EDTA, pH 7.6).

Bacterial lysis— For cell lysis, subgingival biofilm samples were directly suspended in 50 μL of TE buffer containing 0.5% Tween-20. Proteinase K (200 mg/mL) (Roche Applied Science, Indianapolis, IN, USA) was added to the mixture. The samples were then incubated at 55°C for 2 h and proteinase K was inactivated by heating at 95°C for 5 min.

PCR amplification of the Archaea 16S rRNA gene— The *16S rRNA* gene was amplified under standardized conditions using a universal primer pair for *Euryarchaea* (forward primer: 300fEyAr, 5′-AGCRRGAGCCCGGAGATGG3′; and reverse primer: 954rEyAr, 5′-CGGCGTTGARTCCAATTAAAC-3′), as described by Kulik *et al.* (10). The primers were commercially synthesized (Invitrogen Life Technologies, Carlsbad, CA, USA). PCR was performed in thin-walled tubes with Peltier Thermal Cycles PTC-200 (MJ Research, Inc., Watertown, MA, USA). DNA-extracted samples from subgingival biofilm samples (1 μL) were used as template DNA. The reaction in a total volume of 50 μL contained 2 mM $MgCl_2$, 25 pmol of each primer, 0.2 mM deoxynucleoside triphosphates and 2U Platinum® Taq DNA Polymerase (Invitrogen). The negative control consisted of the same reaction mix but with no added template DNA, and the positive control consisted of the same reaction mix plus 1 μL of a pool of methanogenic Archaea DNA (*Methanosaeta concilli*, *Methanosaeta lacustris*, *Methanobrevibacter arboriphilus* and *Methanobrevibacter smithii*). The samples were preheated at 94°C for 4 min, amplified for 35 cycles under the conditions of denaturation at 94°C for 15 s, annealing at 64°C for 30 s and elongation at 72°C for 15 s, then a final elongation step at 72°C for 7 min was performed. The PCR products (0.5–0.7 kb) were separated by electrophoresis in a 1.5% agarose gel (Invitrogen) in TAE buffer (40 mM Tris acetate, pH 8.5, 2 mM EDTA), then stained with ethidium bromide (Sigma- Aldrich, St. Louis, MO, EUA) and visualized under short-wavelength ultraviolet light (UV20; Pharmacia Biotech, San Francisco, CA, USA). The PCR reaction was performed in triplicate.

Diversity of Archaea

Cloning procedures— The amplicons obtained in the archaeal *16S rRNA* amplifications were cloned using the TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instructions. Twenty five clones per sample were selected. The sizes of the inserts were determined by PCR, using the M13(-20) forward primer and the M13- reverse primer (Invitrogen), and the PCR-amplified *16S rRNA* gene fragments were purified and concentrated according to Paster *et al.* (19).

16S rRNA sequencing— Sequencing was performed with ABI Prism fluorescent bases (BigDye Terminator Cycle Sequencing kit with AmpliTaq DNA Polymerase FS; Perkin-Elmer, Foster City, CA, USA) using 3.2 μM reverse primer (13) and purified PCR product in a final volume of 20 μL. Cycle sequencing was performed with a GeneAmp PCR system 2400 (ABI), with 35 cycles of denaturation at 96°C (45 s), annealing at 64°C (30 s) and elongation at 60°C (4 min). The sequencing reactions were run on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

16S rRNA sequencing and data analysis of unrecognized inserts— A sequence of approximately 500 bases was obtained to determine identity or approximate phylogenetic position. For identification of the closest relatives, the sequences of unrecognized inserts were compared with the *16S rRNA* sequences of over 10,000 microorganisms in our database and over 100,000 sequences in the Ribosomal Data Project and GenBank. A level of 98.5% sequence identity was used as the cut-off point for identification of a specific taxon. Phylogenetic trees were constructed using the Unweighted Pair Group Method with Arithmetic Mean method using the software package BioNumerics (Applied Maths Inc., Austin, TE, USA). Chimeric sequences were identified using the Chimera Check program in the Ribosomal Database Project, by treeing analysis and by base signature analysis.

Statistical analysis

The chi-square test was used to analyze whether the prevalence of Archaea differed between Test and Control groups. The significance of differences within subjects in each group was sought using McNemar's chi-square test. The mean percentage of sites with visible plaque, gingival bleeding, bleeding on probing and suppuration, as well as mean full-mouth probing depth and clinical attachment level, and the mean probing depth and clinical attachment level of the sample sites, were computed for each subject and then averaged across subjects in both groups. The significance of differences between the two groups for age and the clinical parameters was sought using the Mann–Whitney *U*-test. The chi-square test was employed to compare the differences in the frequency of gender. The level of significance was set at 5%.

Results

Demographic and clinical parameters

The demographic characteristics and clinical parameters of the study

population are presented in Tables 1 and 2. A total of 50 subjects with healthy ($n = 25$) and diseased ($n = 25$) implants participated in this investigation. In the full-mouth data, no statistically significant differences were observed between groups for any clinical parameter, except for the clinical attachment level, which was significantly higher in the peri-implantitis group (Table 1). The mean probing depth and clinical attachment level measurements and the percentage of sites exhibiting plaque, gingival bleeding, bleeding on probing and suppuration, were higher in the diseased implants of the peri-implantitis group in comparison with healthy teeth and healthy implants in both groups (Table 2).

Prevalence of Archaea in subgingival biofilm samples

A total of 50 subjects ($n = 25$/group) participated in the present study. Of the 125 samples collected, two were lost during the DNA extraction. Therefore, a total of 123 subgingival biofilm samples (73 from the Test group and 50 from the Control group) were analyzed. The prevalence of samples testing positive for Archaea in the two groups is presented in Fig. 1. Dental implants with peri-implantitis showed a higher frequency of sites testing positive for Archaea than healthy implants or subgingival sites of healthy natural teeth from the Control and Test groups ($p < 0.05$). No significant differences were observed in the prevalence of Archaea in healthy teeth or healthy implants between test and control groups ($p > 0.05$). No significant differences were found between healthy implants and healthy teeth within each group.

Diversity of Archaea

Diversity was determined in all samples testing positive for Archaea in both groups (five samples from the Control group and 18 samples from the Test group). A total of 575 clones yielded a *16S rRNA* insert of approximately 614 bp [125 clones from the control group samples (25 per sample) and 450 clones from the test group (25 per sample)], were sequenced. The number of *16S rRNA* clones available for identification ranged from 20 to 25 per sample in both groups.

The phylogenetic identity of the 545 available clones (30 clones could not be identified due to technical reasons) was determined by sequencing 400–600 bp of the amplified 16S rRNA product. A level of 98.5% sequence identity was used as the cut-off for identification of a specific taxon. Overall, two different genera of methanogenic Archaea were identified in both clinical groups (*Methanobrevibacter* and *Methanobacterium*), as shown in Figs 2 and 3.

Methanobrevibacter oralis was the most prevalent phylotype and was detected in all Archaeal positive samples, representing 92% of the clones identified in the Control group, and 95.3% in the Test group. *Methanobacterium congolense/curvum* was detected in two samples from the Control group (one from a healthy implant and the other from a healthy tooth), while in the Test group this species was found in five samples (three from diseased implants, one from a healthy implant and one from a healthy tooth). *M. oralis* represented over 10% of the Archaea detected in all samples, whereas when *M. congolense/curvum* was detected, its proportion represented < 10% of the Archaea DNA detected.

Discussion

Archaea are usually present in different environments, and may be part of the

Table 1. Demographic characteristics and full-mouth clinical parameters of the subjects in both groups

Clinical variables	Healthy subjects ($n = 25$)	Peri-implantitis subjects ($n = 25$)
Age (years)	46.5 ± 11.0	49.4 ± 12.2
Gender (M : F)	10:15	12:13
Probing depth (mm)	2.1 ± 0.8	3.1 ± 1.5
Clinical attachment level (mm)*	0.8 ± 0.4	2.6 ± 1.8
Percentage of sites with		
Plaque	30.7 ± 7.8	37.5 ± 10.6
Gingival bleeding	7.1 ± 2.8	11.1 ± 3.2
Bleeding on probing	8.1 ± 1.8	12.5 ± 4.6
Suppuration	0 ± 0	0.09 ± 0.01

Data are given as mean ± standard deviation, unless indicated otherwise.
The significance of differences between groups was assessed using the Mann–Whitney *U*-test (*$p < 0.05$).
F, female; M, male.

Table 2. Clinical parameters of the sample sites of subjects in both groups

	Healthy		Peri-implantitis		
	HI	HT	HI	HT	DI
Probing depth (mm)*	2.3 ± 1.1a	2.6 ± 0.8a	2.4 ± 0.9a	2.4 ± 0.9a	6.1 ± 2.2b
Clinical attachment level (mm)*	0.9 ± 0.7a	1.2 ± 0.9a	1.1 ± 1.0a	1.1 ± 1.2a	5.9 ± 2.9b
Percentage of samples with					
Plaque	24	32	24	28	88
Gingival bleeding	0	0	0	0	100
Bleeding on probing	0	0	0	0	100
Suppuration	0	0	0	0	52

Data are given as mean ± standard deviation, or as a percentage.
The significance of differences between groups was assessed using the Mann–Whitney *U*-test (*$p < 0.05$; different lower case letters indicate the differences among sample sites).
DI, diseased implant; HI, healthy implant; HT, healthy teeth.

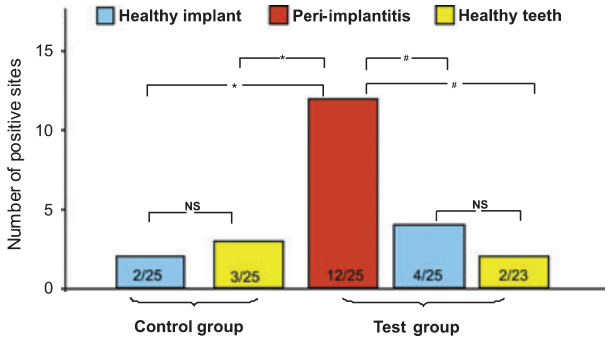

Fig. 1. Number of sites testing positive for Archaea spp. in subjects from Control and Test groups. The significance of differences between Control and Test groups was assessed using the chi-square test (*$p < 0.05$). The significance of differences within subjects of each group was assessed using McNemar's test (#$p < 0.05$). NS, non significant.

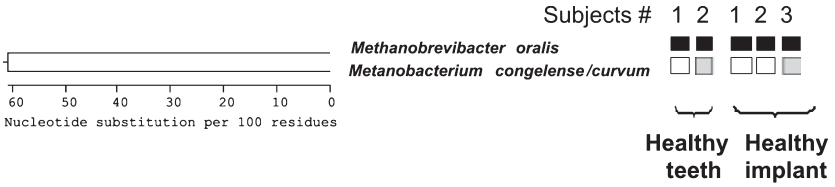

Fig. 2. Phylogenetic tree of Archaea phylotypes detected in the Control group. The distribution and levels of Archaea species among three subjects are shown by the columns of boxes to the right of the tree. Grey-shaded boxes indicate the presence of species detected at < 10% of the total number of clones analyzed. Black-shaded boxes indicate the presence of species detected at > 10% of the total number of clones analyzed. Clear boxes indicate that species were not detected (below the limit of detection). The 10% cut-off was chosen arbitrarily.

Fig. 3. Phylogenetic tree of Archaea phylotypes detected in the Test group. The distribution and levels of Archaea species among 12 subjects are shown by the columns of boxes to the right of the tree. Grey-shaded boxes indicate the presence of species detected at < 10% of the total number of clones analyzed. Black-shaded boxes indicate the presence of species detected at > 10% of the total number of clones analyzed. White boxes indicate that the species were not detected (i.e. were below the limit of detection). The 10% cut-off was chosen arbitrarily.

microbiota of the oral cavity and other sites in humans such as the vagina and intestine (20). Other observations associating the presence of Archaea with pathological conditions such as periodontitis (10,11), colon cancer and diverticulosis (20,21), raised the question about their possible role in pathogenesis. Therefore, the aim of this study was to establish the prevalence and diversity of these organisms in subjects with peri-implant diseases and to compare the findings with data obtained from samples of healthy implants and healthy natural teeth. Overall, 19% of the samples evaluated in the present study contained Archaea. These findings are in agreement with, and extend data from, previous investigations which suggested that Archaea colonize eukaryotic hosts (10,11,14,22).

Forty eight per cent of the diseased implants were colonized by Archaea, a significantly higher prevalence than found in healthy implants and natural healthy teeth. These data reinforce the association of Archaea with disease, as suggested for chronic periodontitis (11,14,15), aggressive periodontitis (10,15) and infected root canals (22,23). Although a significant association was observed between the presence of Archaea and peri-implantitis in this study, and periodontitis in previous investigations (11,15), or endodontic infection (20,22), it has been suggested that the pathogenic mechanism by which Archaea would collaborate with the pathogenesis may differ from that of classic oral pathogens, such as tissue invasion and toxin release (20). Archaea seems to enhance the growth of other, nonarchaeal, organisms that are either pathogens themselves or promote the pathogenicity of other pathogens. Recently, Vianna *et al.* (25) suggested a positive association between methanogens and *Synergites* spp., which are considered to be putative pathogens of humans (24,25).

It is interesting to note that the prevalence of Archaea was very similar in healthy implants and adjacent teeth from subjects of the Control and Test groups ($p > 0.05$). Indeed, the colonization of Archaea in healthy sites observed in the present study was rather unexpected. This should further investigated, given that these data contradict previous studies in which Archaea was not detectable in periodontally healthy sites of subjects from the USA (24), Japan (14) and China (15). It could be speculated that differences in geographical regions may result in differences of the oral microbiota, as previously shown for some periodontopathogenic organisms (26). Likewise, there is evidence that the levels of methane could differ among populations (27). Morii *et al.* (27) reported that the proportion of methane producers among adult subjects in the USA or Great Britain was higher

than it was in Japan. It should also be borne in mind that Lepp *et al*. (11) and Yamabe (15) used a different pair of primers from the one used in this study, although this fact, by itself, should not lead to differences in the study results (14).

The analysis of diversity of oral Archaea revealed the dominance of *M. oralis*, which was found in all positive samples irrespective of the condition of the implant, and even in healthy subgingival sites of natural teeth. *M. oralis*, as well as *M. congolense*/*curvum* (the other phylotype detected) are methanogenens (i.e. produce methane gas from various substrates, such as H_2 and CO_2, acetate and methylamines) (20). Both H_2 and CO_2 are required for its growth. Previous studies have reported that *M. oralis* was the dominant methanogen isolated from different oral environments, such as subgingival biofilm samples (10,11,15) or root canals (22,23), and these data indicated that this species should be considered as part of the human oral microbiota.

M. congolense/*curvum* is a nonmotile, mesophilic, hydrogenotrophic species that was first isolated from an anaerobic digester (28) in the Congo and had not been previously detected in the oral cavity. Despite the fact that these organisms were found in both Control and Test group samples, they were found to occur at a low proportion among the Archaea, suggesting that they may not be part of the resident microbiota of the oral cavity, but may be the result of ingestion of contaminated food (29).

The increased prevalence of Archaea in diseased implant sites may not indicate their involvement with tissue destruction or induction of inflammation, but it is indicative of an altered ecosystem providing a more anaerobic environment which stimulates the growth of strict anaerobes represented not only by methanogens but also by members of the red complex such as *T. forsythia*, *T. denticola* and *P. gingivalis*, and the decrease in beneficial facultative anaerobic microorganisms compatible with a healthy periodontal condition.

Conclusion

In conclusion, Archaea were detected at a higher prevalence in peri-implantitis sites than in clinically healthy implants and natural teeth. *Methanobrevibacter oralis* was the most prevalent species in the subgingival biofilm samples among the Archaea domain in sites with healthy or diseased implants and their role in the pathogenesis of peri-implantitis should be further investigated.

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