Archaea Occurrence in the Subgingival Biofilm in Patients with Peri-implantitis and Periodontitis

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This study aimed to determine the prevalence and diversity of archaea and select bacteria in the subgingival biofilm of patients with peri-implantitis in comparison to patients with unaffected implants and patients with periodontitis. Samples of subgingival biofilm from oral sites were collected for DNA extraction (n = 139). A 16S rRNA gene–based polymerase chain reaction assay was used to determine the presence of archaea and select bacteria. Seven samples were selected for direct sequencing. Archaea were detected in 10% of samples from peri-implantitis sites, but not in samples from the unaffected dental implant. Archaea were present in 53% and 64% of samples from mild and moderate/advanced periodontitis sites, respectively. The main representative of the Archaea domain found in biofilm from periodontitis and peri-implantitis sites was Methanobrevibacter oralis*. The present results revealed that archaea are present in diseased but not healthy implants. It was also found that archaea were more abundant in periodontitis than in peri-implantitis sites. Hence, the potential role of archaea in peri-implantitis and periodontitis should be taken into consideration.*
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The human oral microbiome is comprised of over 600 prevalent taxa at the species level and has been shown to be the second most complex microbiome in the body, after the large intestine. Microorganisms present in oral sites may cause some pathologic conditions, ie, caries (tooth decay), endodontic (root canal) infections, alveolar osteitis (dry socket), and tonsillitis.[1] Additionally, peri-implantitis and periodontitis are chronic microbial-induced inflammatory conditions that include both soft tissue inflammation and progressive bone loss.[2,3] Numerous and diverse bacteria are involved in peri-implantitis and periodontitis. However, the most commonly associated with these conditions are *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, called "red complex." Although peri-implantitis is similar in symptoms to periodontitis, some differences in the composition of microbial communities are recognized.[4]

Archaea are still poorly understood organisms; they form the third domain of life more genetically similar to eukaryotes.[5,6] The most studied group of archaea in the human microbiome are methanogens, which produce energy by synthesizing methane from inorganic compounds (H_2 and CO_2) as well as organic ones.[7] Methanogenic archaea were found in the human gastrointestinal tract,

specifically in the large intestine,[8] but also in the vagina[9] and on the skin.[10] Archaea can be detected in the oral cavity. In physiologic conditions, they were identified in tongue scrapings,[11] in subgingival biofilm samples of patients with unaffected implants,[12] and in subgingival plaque of subjects with healthy periodontium.[13,14] However, it needs to be mentioned that not all studies detected those microorganisms in healthy gingival pockets.[15,16] In pathologic conditions, archaea were identified within infected dental pulp tissue,[17–22] and the present authors have previously showed archaeal presence in infected root canals.[23,24] These microorganisms were also found in subgingival plaque samples from periodontal pockets[4,13–16,25,26] and tongue biofilm obtained from patients with periodontitis.[11] However, there is limited information that archaea are present in subgingival biofilm of subjects with peri-implantitis.[4,12] Thus, this study aimed to determine the prevalence and diversity of archaea, as well as select bacteria (*Fusobacterium nucleatum*, *T denticola*, *T forsythia*, *P gingivalis*, and *Prevotella intermedia*) in samples of subgingival biofilm of patients with peri-implantitis in comparison to patients with unaffected implants and patients with periodontitis.

Materials and Methods

Study Population

A total of 139 nonsmoking patients were enrolled in this study. Before participation, the purpose and procedures were fully explained to all individuals, and written informed consent was obtained from each patient. The study protocol was approved by the Bioethics Commission.

Patients were divided into four groups. One group of 78 participants (37 men, 41 women; mean age: 54.0 ± 12.7 years) was selected randomly from the Department of Periodontology of the Medical University of Lublin. These study subjects received dental implantation. Among them, Group I consisted of 37 patients (18 men, 19 women; mean age: 52.8 ± 11.9 years) with no clinical evidence of gingival inflammation around the implant, no radiographic evidence of alveolar bone loss, and probing depth (PD) ≤ 4 mm. The remaining 41 patients (19 men, 22 women; mean age: 55.4 ± 13.2 years) had peri-implantitis and comprised Group II. Peri-implantitis was defined by PD > 4 mm, suppuration, bleeding on probing, and a noticeable three-thread loss, visible on a radiograph, of the alveolar bone to a certain extent around the implant.

A group of 61 patients with periodontitis (32 men, 29 women; mean age: 53.2 ± 12.9 years) was selected from the Dental Institute at the Medical University of Lodz. Periodontitis was defined according to the current classification of the American Academy of Periodontology. Fifteen of these patients (8 men, 7 women; mean age: 49.2 ± 9.9 years) with mild periodontitis (PD ≤ 5 mm) comprised Group III, and Group IV consisted of the remaining 46 patients (24 men, 22 women; mean age: 50.5 ± 11.6 years), who had moderate/advanced periodontitis (PD > 5 mm).

Criteria for patient inclusion were as follows: *(1)* no treatment of periodontitis/peri-implantitis within the previous 6 months; *(2)* no use of antibiotics and anti-inflammatory drugs within the previous 3 months; and *(3)* no systemic diseases (eg, diabetes, osteoporosis, and immunologic disorders). Criteria for exclusion from the study were as follows: *(1)* pregnancy or breastfeeding; *(2)* cigarette smoking; *(3)* having < 20 teeth; and *(4)* undergoing orthodontic therapy.

Sampling Procedure

In patients with peri-implantitis and periodontitis, the site with the deepest PD was selected for sample-taking. If two or more sites presented similar PD values, the anterior-most site was chosen. In patients with no sign of peri-implantitis, the sample was collected from the mesial site of an implant. Before sampling, saliva and supramucosal deposits were removed, and the tooth/implant was isolated with cotton rolls. The supragingival plaque was removed, and subgingival biofilm samples were taken with individual sterile Gracey curettes and put into a sterile tube containing 1 mL of transport fluid (phosphate buffered saline [PBS]), immediately frozen and stored at –80°C until laboratory analysis.

Taking control samples was performed with a sterile Gracey curette (not used for sampling) to check for microbial contamination, using the

Table 1 PCR Primers Used for Identification of Archaea and Bacteria

Target	Sequence (5′– 3′)	Amplicon size, bp	Annealing temperature, °C
F nucleatum[a]	AGAGTTTGATCCTGGCTCAG GTCATCGTGCACACAGAATTGCTG	407	55
T denticola[a]	TAATACCGAATGTGCTCATTTACAT TCAAAGAAGCATTCCCTCTTCTTCTTA	316	57
T forsythia[a]	TACAGGGGAATAAAATGAGATACG ACGTCATCCCAACCTTCCTC	746	57
P gingivalis[a]	AGGCAGCTTGCCATACTGCG ACTGTTAGCAACTACCGATGT	405	57
P intermedia[a]	CGTGGACCAAAGATTCATCGGTGGA CCGCTTTACTCCCCAACAAA	260	57
Archaea[b]	ACKGCTCAGTAACACGT GTGCTCCCCCGCCAATTCCT	793	56

PCR = polymerase chain reaction.
[a]Detection and amplification performed as described by Vickerman et al.[18]
[b]Detection and amplification performed as described by Vianna et al.[17]

following procedure: Swabs were taken, then streaked on blood agar plates and cultivated aerobically and anaerobically. Additionally, the absence of investigated microorganisms was confirmed by polymerase chain reaction (PCR) targeting the 16S rRNA gene as described below. All procedures were performed in a way that prevents contamination, and a strict sterility regime was applied.

Genomic DNA Isolation

Samples were thawed on ice and dispersed by stirring for 3 minutes. Next, samples were discarded and centrifuged at 2,000 g for 5 minutes. After that, PBS was removed, and the pellets containing microbial cells were resuspended in 100 µL of Tris-HCl buffer (pH 8.5). To isolate microbial DNA, a Genomic Mini kit (A&A Biotechnology) was used according to the manufacturer's instructions. Cell lysis was performed using lysis buffer with proteinase K. Deoxyribonucleic acid (DNA) was adsorbed onto a silica spin-column matrix and purified from contaminants by centrifugation. The isolated microbial DNA was suspended in 100 µL of Tris-EDTA (ethylenediaminetetraacetic acid) buffer (pH 7.4). Picodrop Microliter Spectrophotometer (Picodrop Limited) was used to determine the purity (A260/A280) and the concentration (A260) of extracted DNA. DNA samples were immediately frozen at –80°C and kept until further analysis.

PCR Amplification of the 16S rRNA Gene

Detection, as well as amplification, of archaeal and bacterial DNA was performed with 2720 Thermal Cycler (Life Technologies, Fisher Scientific) under standardized conditions, as described by Vianna et al[17] and Vickerman et al.[18] For detection of select bacteria (*F nucleatum*, *T denticola*, *T forsythia*, *P gingivalis*, and *P intermedia*) and archaea, oligonucleotide-specific primers (presented in Table 1) targeting the 16S rRNA gene were used. The Primer-BLAST tool[27] was used for selecting the PCR primer in particular, to avoid primer-dimer interference. The reactions were performed using previously described methods.[24] Electrophoresis in a 2% agarose gel in TBE buffer (90 mM Tris-borate; 2 mM EDTA) was performed to separate the PCR products. Next, gel was stained with ethidium bromide and visualized under ultraviolet light. The negative controls consisted of *(1)* ultra-pure water instead of template DNA, and *(2)* DNA extracted from a subgingival plaque sample of periodontally healthy patients who earlier tested negative for presence of archaea and select bacteria. The positive contained: *(1)* DNA extracted from a subgingival plaque sample that earlier tested positive for presence of archaea and chosen bacteria, and *(2)* reference DNA isolated from

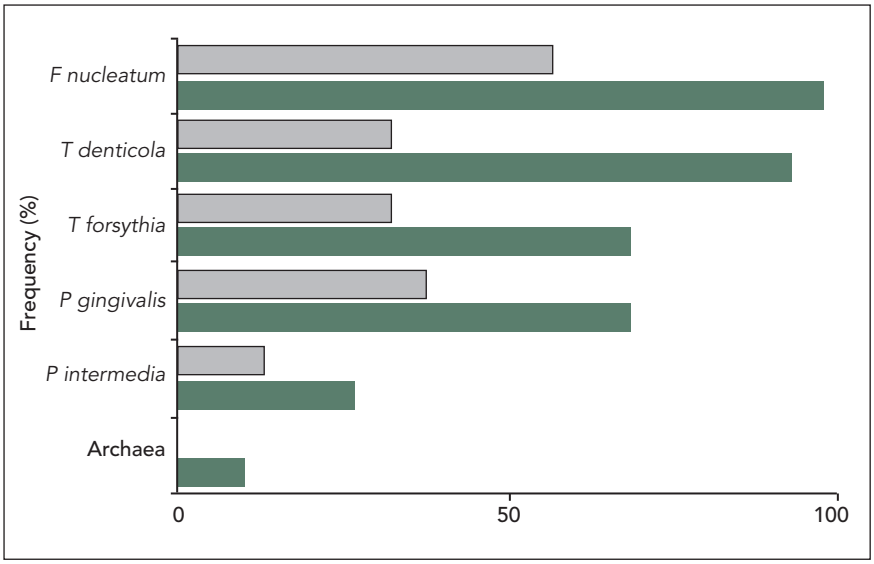

Fig 1 *Frequency of archaea and select bacteria in samples from peri-implantitis sites* (green) *and from healthy implants* (gray).

Methanobrevibacter oralis DSM 7256.[28] All experiments were performed in duplicate.

Gene Sequencing

Identification of archaea was based on a sequence of the 16S rRNA gene. Three archaea-positive samples from patients with peri-implantitis and four archaea-positive samples of patients with periodontitis were randomly selected for direct sequencing using previously described methods.[24]

Statistical Analysis

Statistical analysis was made using the Statistica 13.1 program (TIBCO Software). The association between the presence of archaea and bacteria in samples from peri-implantitis and periodontitis sites was evaluated using Pearson chi-square test. The significance level was $P < .05$.

Results

First, the presence of select bacterial species and archaea in samples from peri-implantitis sites (n = 41) and healthy implant sites (n = 37) was evaluated (Fig 1). Twelve percent of the samples from diseased implants and 8% of samples from healthy implant sites were positive for all tested bacteria. The most prevalent bacterial species in peri-implantitis probes were *F nucleatum* (98%) and *T denticola* (93%). In samples from healthy implants, their frequencies were 57% and 32%, respectively. *T forsythia* was detected in 68% of samples from diseased sites and only in 32% of samples from healthy sites. Similarly, *P gingivalis* was found in 68% of samples from peri-implantitis sites and 38% of samples from healthy sites. *P intermedia* was the least-prevalent bacterial species, found only in 27% of samples from peri-implantitis sites and 14% from healthy implants. The statistical analysis (chi-square test) showed no association in coexistence between any of bacterial species ($P > .05$). In all samples taken from healthy implant sites, no archaeal DNA was detected. However, archaeal DNA was found in 10% of samples from peri-implantitis sites. Only one sample from an implant with peri-implantitis was positive for all bacterial species and archaea simultaneously.

Prevalence of archaea and select bacteria in healthy and peri-implantitis sites was compared with the occurrence of those microorganisms in samples obtained from periodontal pockets (mild [PD ≤ 5 mm] and moderate/advanced periodontitis [PD > 5 mm]; Fig 2). Similar to samples from peri-implantitis sites, *F nucleatum* was the most prevalent bacterial species in periodontitis sites, with a 93% prevalence in plaque samples from mild periodontitis sites and 98% in samples from moderate/advanced sites. *T denticola* was found in 53% and 60% of samples from mild and moderate/advanced periodontitis sites, respectively; hence, its prevalence was lower than in peri-implantitis sites. *T forsythia* was present in 53% (mild periodontitis) and 70% (moderate/advanced periodontitis) of samples, which was similar to its occurrence in peri-implantitis subgingival plaque. *P gingivalis* was present in 87% and 72% of samples from mild and

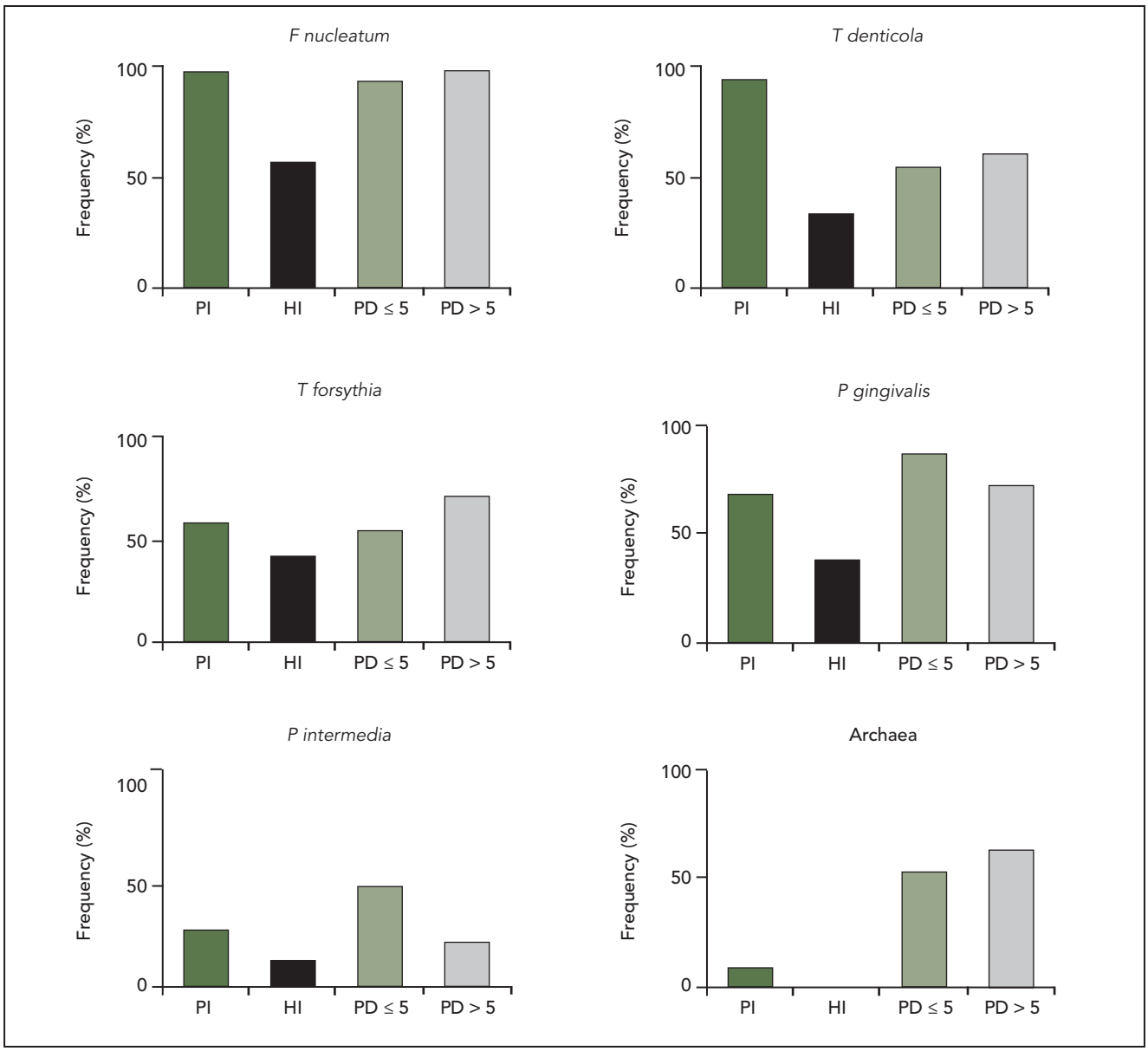

Fig 2 *Frequency of archaea and select bacteria in samples from peri-implantitis (PI), healthy implant (HI), and periodontitis sites with probing depth (PD) ≤ 5 mm and PD > 5 mm.*

moderate/advanced sites, respectively. Similar to samples from peri-implantitis, *P intermedia* was the least-prevalent bacterial species; its occurrence was 47% in samples from mild sites and 21% in samples from moderate/advanced sites. Presence of archaea was more pronounced in samples from periodontitis. Archaeal DNA was found in 53% of samples from mild periodontitis sites and 64% of samples from moderate/advanced periodontitis sites.

Direct sequencing of the archaeal 16S rRNA gene was used for identification of archaea. Obtained sequences were compared with the GenBank database (using BLAST) and between each other. In samples from periodontitis and peri-implantitis sites, the chief representative of archaea domain was *M oralis*, as established based on the profile of obtained sequencing profiles. The similarity of all analyzed fragments compared to the reference sequence was between 98% and 100%.

Discussion

The composition of microbial biofilms from peri-implantitis and periodontitis is similar at the phylum level, and "red complex" bacteria are abundant in both inflammatory conditions.[4,11,25,29,30] However, the incidence of some genera differs significantly; in comparison to periodontitis, higher levels of the genera *Olsenella*, *Sphingomonas*, and *Peptostreptococcus* and lower levels of the genus *Desulfomicrobium* were documented in peri-implantitis.[4] At the species level, differences in the presence of bacteria were also noted; for example, *Prevotella nigrescens* and *Prevotella oris* were more abundant in peri-implantitis samples, whereas *Desulfomicrobium orale* were more frequently seen in periodontitis sites.[4,29,30] In the present study, the most prevalent species in both peri-implantitis and periodontitis sites was *F nucleatum*. However, it was noticed that *T denticola* was more frequently found in samples from peri-implantitis sites than from periodontitis ones. In turn, *P gingivalis* was less frequent in samples from peri-implantitis than periodontitis. Hence, the present data seems to be consistent with the view that there are some differences between the composition of bacterial flora between peri-implantitis and periodontitis.

Data indicate that biofilm associated with peri-implantitis or periodontitis can be composed of not only bacteria but also archaea. There is information that archaea are present in gingival plaque from periodontitis sites.[4,13–16,25,26] However, there are only two studies about archaea in peri-implantitis sites. Faveri et al[12] showed that archaea are present in biofilm from peri-implantitis sites. Moreover, these authors showed a higher number of sites positive for archaea from peri-implantitis (12/25) than in sites from healthy implants (2/25). Thus, the present authors' observations are consistent with data obtained by Faveri et al.[12] Maruyama et al[4] also found archaea in biofilm samples from peri-implantitis sites and noticed that archaea were more abundant in peri-implantitis than in periodontitis probes. In the current study, archaea were present in both periodontitis and peri-implantitis sites, but its prevalence was lower in peri-implantitis than periodontitis ones. It should be stressed that the surface of an implant is different from the tooth's tissue. Thus, microorganisms with various adhesive properties may be included in those inflammatory conditions.

Available data show that *M oralis* and *M oralis*–like phyloptype is the most frequently detected archaea in samples from oral sites. *M oralis* was found in biofilm from periodontitis[9,10,20–23] and peri-implantitis[12] sites. *M oralis* was detected in infected root canals, as well.[23,24] Besides, *Methanobacterium curvum/congelese* was detected in samples from peri-implantits[12] and periodontitis[13] sites. Few studies found other archaeal species, like *Methanobrevibacter smithii*[14] and *Methanosarcina mazeii*.[13] In the present study, only *M oralis* was found in peri-implantitis and periodontitis samples.

Presence of microorganisms in healthy tissue around a tooth as well as the dental implant is quite apparent. Numerous oral bacteria species may promote inflammation and/or tissue degradation. Additionally, the architecture of such microbial communities and, essentially, the coexistence of bacterial and archaeal species and a network of mutual interdependencies may be significant. For example, by eliminating hydrogen from the environment, methanogenic archaea can promote the growth of bacteria able to oxidize volatile fatty acids.[31] Methanogenic archaea may compete with acetogenic bacteria for H_2 using some representatives of treponemes, for example.[15,32] The discussion about potential pathogenic properties of archaea is still ongoing, and there are no well-supported data that archaea could be pathogens.[26]

Conclusions

The present results revealed that archaea are present in diseased but not healthy implants. It was also found that archaea were more abundant in periodontitis sites than in peri-implantitis ones. Hence, the potential archaeal role in peri-implantitis as well as in periodontitis should be taken into consideration. However, future studies are needed to provide more insight into the archaeal role in both inflammatory conditions.

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