Shift of microbial composition of peri-implantitis-associated oral biofilm as revealed by 16S rRNA gene cloning

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Abstract

Purpose. Micro-organisms are important triggers of peri-implant inflammation and analysing their diversity is necessary for peri-implantitis treatment. This study aimed to analyse and compare the microbiota associated with individuals with peri-implantitis, as well as clinically healthy implant sites.

Methodology. Subgingival biofilm samples were taken from 10 individuals with peri-implantitis and from at least 1 clinically healthy implant. DNA was extracted and bacterial 16S rRNA genes were amplified using universal primers. After cloning the PCR-products, amplified inserts of positive clones were digested using restriction endonucleases, and the chosen clones were sequenced. The 16S rDNA-sequences were compared to those from the public sequence databases GenBank, EMBL and DDBJ to determine the corresponding taxa.

Results. Differing distributions of taxa belonging to the phyla Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, Proteobacteria, Synergistetes, Spirochaetae and TM 7 were detected in both the healthy implant (HI) and the peri-implantitis (PI) groups. A significantly higher relative abundance of phylum Bacteroidetes, as well as of the species Fusobacterium nucleatum, were found in the PI group (P<0.05). The putative periodontal red complex (Porphyromonas gingivalis, Tannerella forsythia) was also detected at significantly higher levels in the PI group (P<0.05), whereas the yellow group, as well as the species Veillonella dispar, tended to be associated with the HI group.

Conclusion. A shift in the healthy subgingival microbiota was shown in peri-implantitis-associated biofilm. Anaerobic Gram-negative periopathogens, including P. gingivalis and T. forsythia, seem to play an important role in peri-implantitis development and should be considered in treatment and prevention strategies.

INTRODUCTION

Endosseous implants are a standard therapy in prosthetic dentistry and have been used successfully for patients with tooth loss [1–3]. Regardless of the chemical structure of the materials used in implants, microbial adhesion and biofilm accumulation can lead to peri-implant mucositis and peri-implantitis, causing bone loss and subsequent treatment failure [4–6]. Although the term peri-implantitis has been known for more than 50 years [7], the question of whether peri-implantitis should be considered to be a disease or only a marginal bone loss due to complications after having a foreign body placed in the oral cavity is a matter of controversial discussion among clinicians [8]. Recently, Albrektsson et al. summarized the discussion of the Rome meeting of 17 clinical experts in periodontology and stated that the previously depicted high prevalence of peri-implantitis is rather due to the liberal criteria that have been applied in the diagnosis of peri-implantitis [8]. The authors considered the marginal bone loss associated with oral implants to be the result of different factors that in the end lead to imuno-osteolytic reactions and subsequently to ongoing bone resorption. In this complicated cycle microorganisms and their components are still important triggers of peri-implant inflammations. Hence, the composition of the biofilm microbiota associated with peri-implantitis has

Received 30 November 2017; Accepted 10 January 2018

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Keywords: peri-implant disease; subgingival biofilm; microbiota; subgingival microbial complexes; culture-independent method.

Abbreviations: BLAST, Basic Local Alignment Search Tool; BOP, bleeding on probing; DDBJ, DNA Data Bank of Japan; dNTP, deoxyribonucleotide triphosphate; EMBL, European Molecular Biology Laboratory; HI, healthy implant; HOMD, Human Oral Microbiome Database; ISO, International Organization for Standardization; PD, probing depth; PI, Peri-implantitis; RTF, reduced transport fluid.

One supplementary table is available with the online version of this article.
been a major concern for understanding the aetiology of this disease.

Inconsistent and contradictory discussions have been going on with regard to the microbiota associated with peri-implantitis, and its similarity with the microbiota of periodontitis biofilm. As stated in several recent literature reviews, various factors have led to an unclear classification of the microbial community of peri-implantitis infections. In addition to the number of specific sample sites, one important factor is the detection method used to describe the peri-implantitis microbiota, making it difficult to compare different studies with each other [9]. In addition to classical culture and PCR techniques, checkerboard DNA-DNA hybridization, 16S rRNA gene-sequencing techniques, hybridization with specific P32 arrays directed against the DNA hybridization, and 16S rRNA gene-cloning technique.

The aim of the present cross-sectional study was to comprehensively analyse the respective microbiota associated with peri-implantitis compared to healthy implants using the culture-independent 16S rRNA gene-cloning technique.

**METHODS**

**Study design, individuals and clinical parameters**

Ten individuals with at least one implant with peri-implantitis, and one clinically healthy implant were involved in this study. All individuals showed good general health and were non-smokers. They had not received any antibiotic therapy in the last 2 years. The consumption of alcohol was also excluded from at least 1 week before the study commenced. The age of the individuals was between 62 and 83 years.

The following clinical examinations of the implants with peri-implantitis and the healthy implant (Table 1) were conducted and registered at six sites per tooth, as well as at six sites per implant (mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual): (1) bleeding on probing (BOP), (2) probing depth (PD), (3) recession or gingival hyperplasia and (4) discharge of pus. Intraoral radiographs were obtained and analysed for bone loss for both the peri-implantitis and the healthy implants. As shown by the clinical and radiographic data, peri-implantitis sites were selected that exhibited PD ≥ 5 mm, BOP and concomitant radiographic bone loss. Healthy implant sites showed no sign of either bone loss or increased pocket depth (PD ≤ 3 mm). All implants were made of titanium oxide with sufficient supra-constructions. The patients stated that they brushed their teeth two times a day and maintained good general oral hygiene. All patients were enrolled during their first appointment at the study center.

The study design and all study protocols were examined and approved by the Ethical Committee of the Albert-Ludwigs-University of Freiburg (502/13) and written informed consent was obtained from all patients.

**Subgingival biofilm sample collection and DNA extraction**

Subgingival plaque samples were taken from the deepest pockets of the peri-implantitis sites using three sterile paper points (ISO 45). The paper points were maintained within the pockets for 20 s and then transferred into vials containing 0.75 ml reduced transport fluid (RTF) [15] and stored at −80 °C until the DNA was extracted. Furthermore, samples from sulcus of the healthy implant sites were obtained using six paper points and stored as described above. For DNA extraction, the samples collected from each patient were centrifuged at 16,000 g for 10 min before the supernatant was discarded. The DNA extraction was performed using the DNeasy Blood and Tissue kit according to the manufacturer’s protocol for Gram-positive bacteria (Qiagen, Hilden, Germany). The DNA was eluted with 200 µl AE buffer (Qiagen) and stored at −20 °C until analysis.

**PCR amplification of 16S rRNA genes**

Bacterial 16S rRNA genes were amplified using the following universal primers, as described previously [16, 17]. The bacterial primers used were 27F-YM (5'-AGAGTTTGTATYMTGCTCAG-3') and 1492R (reverse: 5'-TACGGYTACCTGTGTTACGACTT-3'). PCR amplification was performed in a total volume of 50 µl. The reaction mixture contained 1 × PCR buffer (Qiagen), 0.2 mM each of the four deoxyribonucleoside triphosphates (dNTPs; PEQLAB, Erlangen, Germany), 0.5 µM of forward and reverse primers, 2 U Taq polymerase (Qiagen) and 5 µl of the isolated sample DNA. The PCR cycling conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 35 cycles, which included denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min, with a final extension step at 72 °C for 10 min. A positive control and a control with no DNA template were included in each set of PCR reactions. The PCR reaction products were analysed by electrophoresis in a 1.5 % agarose gel and positive reactions were used to prepare clone libraries.
Cloning of PCR products and analysis of clone libraries

The 16S rDNA amplification products were ligated into the pCR2.1-TOPO plasmid vector using the TOPO TA Cloning kit (Invitrogen, Life Technologies, Darmstadt, Germany) according to the manufacturer's protocol. All white clones from each library were picked and the presence of inserts was confirmed by PCR amplification. Hha I, Rsa I and Hinf I (New England Biolabs GmbH, Frankfurt, Germany) were used for the digestion of PCR products of recombinants that resulted from the universal bacterial PCR. Fragment length patterns were compared and grouped if they were identical. One representative clone was selected from each group and used for sequencing. The selected clones with inserts of the correct size were grown at 37°C overnight in Luria-Bertani liquid medium with kanamycin (50 mg ml⁻¹). Plasmid DNA extraction was then prepared using the PureLink Quick Miniprep kit (Invitrogen, Life Technologies, Darmstadt, Germany). Bidirectional Sequencing was performed by LGC Genomics (LGC Genomics GmbH, Berlin, Germany).

Sequence analysis

The sequence data were proofread visually and trimmed using Ridom TraceEdit software (Ridom GmbH Münster, Germany). Trimmed sequences were assembled using BioEdit [18]. The partial and almost full-length 16S rDNA sequences were compared to those from the public sequence databases, GenBank, EMBL and DDBJ, using the BLAST program. The program was run through the server hosted by the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [19, 20]. Sequences that showed 98 % similarity or less with public database sequences were checked for chimeras with the Pintail program, version 1.0 [21]. Chimeric sequences were excluded from further analysis. Sequences with a >98 % match to a database sequence were considered to be of the same species as the one with the highest similarity and score bits. Additionally, all 16S rDNA sequences were compared with the database sequences of the Ribosomal Database Project (https://rdp.cme.msu.edu/) [22] and the Human Oral Microbiome Database (HOMD, http://www.homd.org/) [23] to confirm the results of the BLAST search and to obtain further information. Sequences that could not be assigned to any database sequence were considered to be a novel phylotype if they were less than 98 % similar to the closest GenBank entry. The taxa revealed from all the sequences are depicted in Table S1 (available in the online version of this article), including the accession numbers.

Statistical analysis

First, a descriptive analysis of the data was performed. A McNemar test and a Wilcoxon signed rank test were used to check for group differences. The statistical analysis was performed with STATA 14.

RESULTS

In a cross-sectional split-mouth clinical study with 10 individuals, subgingival biofilm samples from 10 healthy implants and 10 implants with peri-implantitis were analysed using the culture-independent cloning technique. On average, 18 clones were analysed per sample. In the implant

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Site</th>
<th>Tooth number</th>
<th>BOP</th>
<th>PD (mm)</th>
<th>Pus</th>
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<td>36</td>
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<td>7</td>
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<td>48</td>
<td>Negative</td>
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</table>
group with peri-implantitis (PI) and in the group of healthy implants (HI), 94 and 73 clones were sequenced, respectively. As shown in Fig. 1(b), the revealed taxa belonged to eight phyla with differing distribution within each group. In the PI group, the most abundant phylum was Bacteroidetes, with 40.3% of all sequenced clones, whereas for the HI group it was Firmicutes, with 51.9% of the clones. The second most frequent phylum in the PI group was Firmicutes (30.6%) followed by Fusobacteria (13.9%). In the HI group the second most frequent phylum was Bacteroidetes (18.5%) followed by Fusobacteria (11.1%). All other sequenced clones were distributed among the phyla Actinobacteria, Proteobacteria, Spirochaetae and TM 7. Only the difference between the percentages of Bacteroidetes was found to be significant (P<0.05). Members of this phylum were found in the peri-implantitis implants of all individuals, but were only found in the healthy implants of six individuals (Fig. 1a). All other differences regarding the distributions among the other phyla were not significant (P>0.5), although different relative abundance values of the phyla can be seen in Fig. 1(a). For example, taxa of Fusobacteria were detected in the samples from the healthy implants of five individuals and in the peri-implantitis implant samples of seven individuals.

Fig. 2(a) summarizes the distributions and relative abundance values of the bacterial genera (including different species) and single species detected for the PI and HI groups for all individuals. The distribution of some genera is different between the two groups. As shown in Fig. 2(b), Streptococcus spp. were detected at a relative abundance of 24.1 and 16.4% in the HI and PI groups, respectively. The relative abundance of Prevotella spp. was 19.2% in the PI group and 9.3% in the control group (HI). Fusobacterium spp. were also found at a high abundance of 13.7% in the PI group compared to 9.3% in the HI group. Differences have also been shown for other genera and species, among which some were only detected in one of the groups, although only at a low abundance.

To further analyse the detected taxa (especially those belonging to the phylum Bacteroidetes), we depicted the results in Fig. 3 presenting the known periodontitis...
subgingival bacterial complexes that were suggested by Socransky [24], in addition to *F. nucleatum*, *P. gingivalis* and *V. dispar*. Members of the red complex (*P. gingivalis*, *T. forsythia*) were present in significantly higher abundance in the PI group (*P*<0.05). None of the other complexes showed statistically significant differences (*P*>0.05), although members of the orange complex tended to be more abundant in the PI group (*P*=0.09), and the abundance of the yellow complex showed a tendency to be higher in the HI group (*P*=0.08). When considering the comparison of single taxa, with the exception of *F. nucleatum*, which was detected at significantly higher frequency in the PI group (*P*<0.05), no statistically significant differences could be determined among the HI and PI groups. Nevertheless, *P. gingivalis* showed a tendency towards a higher abundance in the PI group (*P*=0.08) and *V. dispar* showed a tendency towards higher abundance in the HI group (*P*=0.08). The three aforementioned taxa were therefore depicted in Fig. 3 next to the other complexes. Moreover, as can be seen in Fig. 2, the distributions of the different taxa among the HI and PI groups and the individuals are different. The species names, as well as the corresponding accession numbers, are depicted in Table S1.

**DISCUSSION**

It is well understood that oral biofilm can form on dental implants, affecting the surrounding tissue and leading to inflammation of the peri-implant mucosa (mucositis) and then progressing to bone destruction (peri-implantitis) [14]. Irrespective of the prevalence rate and the exact definition of peri-implantitis, the diverse microbiota associated with this disease are still a subject of great interest for oral microbiologists and clinicians. However, due to the diverse microbiological methods reported in the literature, as well as the diverse study designs implemented therein to reveal the aetiology of peri-implantitis, heterogeneous findings and statements have been reported regarding the associated microbiota to date [9, 10]. Padial-Molina *et al.* [9], in their recent review on microbial profiles of peri-implant disease, emphasized that correlation between studies is difficult, particularly because of the use of different methods. Hence, a sufficient number of studies using the same microbiological technique is required to form a reliable picture of the peri-implantitis microbiota compared to that associated with healthy implants. The cloning technique has only been used in only two studies to date to characterize the microbiota of peri-implantitis and healthy implants in the same oral cavity.

**Fig. 2.** Abundances (%) of detected genera and species in each individual (a) and average abundances over all individuals (b). The species names and the corresponding accession numbers are depicted in Table S1.
in three and six individuals, respectively [12, 13]. Due to individual differences in the resident microbiota among patients, the shift in microbiota associated with a healthy state to that associated with peri-implantitis should be studied in the same individuals. With this background, the present study used the cloning technique with the aim of comparing peri-implantitis microbiota and the microbiota associated with healthy implants sites in the same oral cavities of 10 German patients.

We showed that members of the phylum *Bacteroidetes* increased significantly in the peri-implantitis implant group (PI) as compared to the healthy implant group (HI). Furthermore, the abundance of the red complex (*P. gingivalis, T. forsythia*) as well as *F. nucleatum* showed significantly higher values in the PI group than in the HI group. It should be stressed that regardless of these statistically significant results, the composition of the microbiota showed additional high tendencies for different patterns, which indicates a shift of the microbiota from a healthy status to a diseased status.

In their first study using the cloning technique, Koynagi et al. [12] also reported a shift in the peri-implantitis microbiota and stressed the role of the phylum *Synergistetes* in this disease. The authors only found members of this phylum in the peri-implantitis group. In a follow-up study by the same authors published 3 years later, the role of the phylum *Synergistetes* was revised, since members of this phylum were also found in the healthy implant group of six patients [13]. In our study, we only detected two members of this phylum (*Fretibacterium fastidiosum, Synergistales bacteria*) in one patient of the PI group, which agrees with the aforementioned study by the authors. In a recent study that focused on the prevalence of *Synergistetes*, no clear
correlation of this phylum with the occurrence of peri-implantitis was found [25]. In their recent review describing peri-implantitis microbiota, Faveri et al. [10] stated that the similarities of peri-implantitis and periodontitis microbiota, as suggested in the literature and widely accepted at the end of the 2000s, was caused by the detection methods used at the time, such as culture technique, DNA–DNA-checkerboard hybridization and other molecular methods. The authors focused on the new results gained by microbiome analysis using 16S rDNA-sequencing and high-throughput sequencing methods. These results suggest that the microbiota associated with peri-implantitis differs more from the periodontitis microbiota than has been reported to date. For example, Kumar et al. [26] showed that the microbial profiles of peri-implantitis differ from the microbiota in periodontitis. On the other hand, a recent comprehensive study that analysed samples from healthy implants, peri-implant mucositis and peri-implantitis revealed that periodontal pathogens such as P. gingivalis, T. forsythia and Prevotella intermedia clustered together in peri-implant mucositis sites, suggesting that periodontal pathogens may play an important role in the pathogenesis of peri-implant diseases [27]. This is in agreement with the findings of the present study, which showed a correlation between the periodontal red complex members (P. gingivalis, T. forsythia) and the PI group. Furthermore, the significant association of F. nucleatum (as a primary member of the periodontal orange complex) with the PI group in the present study is in line with the aforementioned statement and the findings of other authors. These authors reported a high prevalence of this species and other anaerobe Gram-negative periopathogens in association with peri-implantitis [28–32]. It should be emphasized that in this study members of the orange complex also tended to have a higher relative abundance in the PI group. Similar results were shown by de Silva et al. [29], who at the same time reported an association of V. dispar with healthy implants, as was revealed in our study. Using 16S rRNA gene cloning and sequencing, Kumar et al. also associated a Veillonella species (Veillonella sp. oral clone X042) with healthy individuals in comparison to periodontitis patients [33]. Again, the contradictory results listed above concerning the similarity of the microbiota of peri-implantitis and periodontitis, as revealed by the high-throughput sequencing methods, are reminiscent of the controversial discussion about the similarities of peri-implantitis microbiota and periodontal microbiota as shown by culture, DNA–DNA-hybridization and other culture-independent techniques [9, 10, 14, 34]. As mentioned above, variations in the composition of the peri-implantitis microbiota, and the degree of its shift from the healthy microbiota found in the peri-implant sulcus, are dependent on the different microbiological detection methods used in the various studies. In addition to inter-individual differences in the oral microbiota, the study design (longitudinal or cross-sectional, number of individuals) and the sampling technique should also be taken into consideration when discussing the similarities of peri-implantitis microbiota to periodontal microbiota [35]. This point was highlighted by some

Fig. 3. Abundance of the subgingival bacterial complexes suggested by Socransky, as well as F. nucleatum, P. gingivalis and V. dispar. The P-values of complexes and species that showed differences or tendencies towards a higher relative abundance are depicted for both the peri-implantitis implants (PI) and the healthy implants (HI).
authors who questioned the reliability of using putative periodontal pathogens as diagnostic markers for periodontitis [36]. The authors reported that Capnocytophaga ochracea was associated with healthy individuals, regardless of the probing depth. On the other hand, the authors found that Treponema lecithinolyticus was the only species that was coincident with periodontitis regardless of the probing depth, whereas most putative periodontal pathogens were associated with the probing depth and not with the disease itself. It should be mentioned that the authors used PCR of 16S rRNA gene fragments and subsequent dot blot hybridization, using specific oligonucleotide probes to analyse the microbiota of healthy and periodontitis patients. The detection of Helicobacter pylori by molecular methods is an interesting example of the influence that the experimental method used can have on the oral microbiota that are then detected. Although there is no study demonstrating the cultivation of H. pylori from the oral cavity independently of its occurrence in the stomach [37, 38], it has been reported to belong to the microbiota associated with peri-implantitis after its detection using culture-independent methods [10, 39]. The 16S rRNA primer pair used in our study has been shown to perform well as a universal primer system to detect a broad range of bacterial taxa [17, 40]. However, different parameters of 16S rDNA analysis can introduce a bias (e.g. DNA extraction, 16S rRNA gene copy number variation) and certain taxa may have escaped detection due to less effective primer binding or differential amplification.

More reliable results regarding the microbiota associated with peri-implantitis would be gained if different microbiological detection methods were used simultaneously to analyse samples from the same healthy and peri-implantitis patient groups, or from the same individuals, by using a cross-sectional study design. This was emphasized in a review written by a biofilm expert group after a workshop during the 5th American Society for Microbiology (ASM) Biofilm Conference in 2009. In this review Hall-Stoodley et al. [41] requested a new diagnostic algorithm combining research criteria for biofilms and clinical criteria for infection to clarify the aetiology of biofilm-associated infections such as peri-implantitis and periodontitis.

In conclusion, the present cross-sectional split-mouth study showed a shift in peri-implantitis biofilm diversity to anaerobic Gram-negative periopathogens, including putative periodontal bacteria of the red complex (P. gingivalis, T. forsythia). Studies including the use of diverse microbiological methods and the same design are strongly recommended to reveal the aetiology of peri-implantitis and the homology of its microbiota with periodontitis-associated biofilm to compensate for the contradictory results reported in the literature, and to develop specific antimicrobial, immunological treatment and prevention strategies.

Acknowledgements
The authors thank Bettina Spitzmüller and Kristina Kollmar for excellent technical assistance.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The study design and all study protocols were examined and approved by the Ethical Committee of the Albert-Ludwigs-University of Freiburg (502/13) and written informed consent was obtained from all subjects.

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