Microbial Diversity of Peri-Implant Biofilms on Implant Fixed Bar and Telescopic Double Crown Attachments

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One of the principal problems in oral implantation is inflammation of peri-implant hard and soft tissues caused by bacterial biofilms. The purpose of the present study was to evaluate the microbial diversity of peri-implant biofilms on 2 different implant-anchored attachment types in vivo. Samples of peri-implant sulcus fluid were collected from 8 patients with implant-supported bar attachments and 8 patients with implant-anchored telescopic double crown attachments. Samples of sulcus fluid of the adjacent teeth were also collected from the partially edentulous patients with implant fixed telescopic double crowns. The mixed amplicons of 16S rRNA fragments of different bacterial origins were separated by use of single-strand conformation polymorphism analysis to identify the predominant bacterial genera. With 3.5 ± 2.1 different predominant bacterial genera in the sulcus fluid surrounding implant-supported bar attachments and 6.3 ± 3.1 different predominant genera in the sulcular fluid of implant-anchored double crown attachments, the differences were not statistically significant (P = .11). The microbial diversity in the sulcus fluid surrounding the remaining dentition was similar to that of the implant fixed telescopic attachments (6.3 ± 2.1). Aside from host response and other individual factors, the microbial diversity of peri-implant biofilms seems to be impaired by cofactors such as the possibility of cleaning the implant-supported supraconstructions and the different plaque-retaining sites. Nevertheless, these differences do not lead to statistically significant differences in the microbial diversity of peri-implant plaques.

Key Words: bacterial biofilms, dental implants, microbial diversity, single strand conformation polymorphism

INTRODUCTION

Biofilm formation on oral implants can cause bacterial inflammation of peri-implant hard and soft tissues, which endangers the long-term success of osseointegrated implants. It has been reported in several studies that implants revealing signs of peri-implantitis, such as radiographic bone loss, increased bleeding on probing, or probing pocket depth, contain microbiota similar to those of natural teeth with signs of periodontitis. However, there is a lack of evidence in the literature regarding which putative pathogens are mainly responsible for the inflammation of peri-implant hard and soft tissues. These pathogens are often organized in 3-dimensional matrices of extracellular polysaccharides (EPSs). Provided by its protective matrix, the growing biofilm has a high tolerance against antibiotic therapies or shear forces of oral fluids and movement of tongue and jaw. After the

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development of the EPS matrix, the mature biofilm grows until parts of its cells detach, adhere to other regions of the oral cavity, and elicit other bacterial colonies. Because of these sophisticated survival strategies and the optimal environments for such microsystems in the plaque-retentive sites of implant attachments or implant-anchored supraconstructions, bacterial biofilms are currently one of the principal problems in the long-term stability of oral implants.

The results of several studies have shown that the microbial composition of peri-implant biofilms shifts toward a higher proportion of periodontal pathogen species, such as *Aggregatibacter* or *Porphyromonas*, during peri-implant biofilm formation.7–10 This shift, as well as the general process of biofilm formation, are affected by host response and individual ecological cofactors, such as tongue activity, salivary composition, or nutrition.11–14 Furthermore, it can be assumed that differences in the possibility of cleaning the implant-supported supraconstructions or the different plaque-retaining sites at the implant attachments or supraconstructions may lead to different peri-implant bacterial communities.

Therefore, the object of the present study was to evaluate the microbial diversity in the peri-implant crevicular fluid at implant-supported bar attachments as well as in the crevicular fluid at implant-fixed telescopic double crown attachments, using the molecular biological single-strand conformation polymorphism method and subsequent sequence analysis. In addition, samples of sulcus fluid from the remaining dentition were analyzed from the partially edentulous patients with implant-anchored double crowns in order to evaluate cross-infections between implants and teeth.

**Material and Methods**

**Patients**

The study protocol was approved by the ethics committee of Hannover Medical School (No. 3791). The examination was performed with the understanding and written consent of each subject.

Qualitative and quantitative analyses were based on 8 patients (fully edentulous, 6 women and 2 men, aged between 52 and 80 years [mean 66 ± 10 years]) who received an implant-supported bar-attached supraconstruction and 8 patients (4 partially edentulous, 4 fully edentulous, 7 women and 1 man, aged between 54 and 81 years [mean 63 ± 10 years]) with implant-anchored telescopic double crown attachments. All patients were treated within the period of 2005 to 2006 with at least 4 oral 2-piece implants made of titanium per jaw. Furthermore, the following exclusion criteria were defined: systemic illness, smoking, pregnancy, active periodontitis or peri-implantitis (probing pocket depth or radiographic bone loss ≥3 mm), pharmacologic treatment or antibiotic therapy during or up to 4 months before the study.

Bacterial samples were taken at 4 sites for each implant and tooth. The sampling area was isolated from saliva and gently dried by air. Four paper points were inserted for 10 seconds into the peri-implant or gingival sulcus (mesiobuccal, distobuccal, mesiopalatal/lingual, distopalatal/lingual) and pooled for every implant or tooth (Figure 1). All samples were stored in Eppendorf tubes (Eppendorf, Hamburg, Germany) at −80°C before processing.

**Nucleic acid extraction**

The genomic DNA was isolated using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Preparation was according to the manufacturer’s protocol for bacteria. In addition, a mechanical disruption step was performed. For this purpose, samples were treated with 20 mg/mL hen egg white lysozyme (Fluka, Buchs, Switzerland) for 30 minutes at 37°C in lysis buffer (20 mM TrisHCl, 2 mM ethylenediaminetetraacetic acid [EDTA], 1.2 % Triton X-100, pH 8.00), followed by proteinase K digestion. The cell suspension was then homogenized (6500 rpm, 3 × 20 seconds with a 15-second break) with a Precellys 24 bead mill (Bertin Technologies, Montigny-le-Bretonneux, France) using 0.5-mm glass beads (Roth, Karlsruhe, Germany).

**Amplification of the 16S ribosomal DNA and exonuclease digestion**

An approximately 500-bp fragment of the 16S ribosomal DNA (rDNA) was amplified using the universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′; MWG Biotech, Ebersberg, Germany) and 5′-prime phosphorylated 521revP (5′-ACCGCGCTGCTGGCAC-3′; MWG Biotech). These primers target conserved regions flanking the V1 and V3 hypervariable regions within the 16S rRNA
A polymerase chain reaction (PCR) was performed on a TProfessional thermocycler (Bio- metra, Göttingen, Germany). The PCR mix contained 50 ng of template DNA, 200 nM of each primer, 1× PCR buffer (including 1.5 mM magnesium chloride; Qiagen), 1.5 U HotStar Taq polymerase (Qiagen), 200 mM of each deoxyribonucleotide triphosphate (dNTP; Roth), and PCR-grade water (Roche, Penzberg, Germany) in a total reaction volume of 50 µL. The PCR conditions were as follows: initial denaturation at 95°C for 15 minutes; 30 amplification cycles consisting of denaturation at 94°C for 1 minute, annealing at 52°C for 40 seconds, elongation at 72°C for 1 minute, and final extension at 72°C for 10 minutes. A total volume of 5 µL of each amplification reaction was analyzed by agarose gel electrophoresis (Agarose MP, AppliChem, Darmstadt, Germany). The PCR products were purified using the QIAquick PCR Purification kit (Qiagen). Single-stranded DNA (ssDNA) was generated by enzymatic cleavage. For this purpose, 1.5 µg of each amplification product was digested with 10 U lambda exonuclease (New England Biolabs, Frankfurt am Main, Germany) in 1× exonuclease buffer (New England Biolabs) for 1 hour at 37°C in a total volume of 55 µL. Enzymatic reaction products were purified using the QIAquick PCR Purification kit (Qiagen). Samples were dried overnight in a thermal shaker (40°C, 800 rpm; Thermomixer comfort, Eppendorf).

**Sequence-dependent separation of 16S rDNA fragments**

Single-stranded conformation polymorphism (SSCP) analyses were performed on a DCode Universal Mutation Detection System (Bio-Rad, Hercules, Calif). For this purpose, ssDNA fragments were resuspended in 5 µL 1× SSCP buffer (Bio-Rad), heated for 5 minutes to 95°C and kept on ice for 3 minutes before electrophoresis. Subsequently, samples were loaded on a 0.625× mutation detection enhancement (MDE) gel (Lonza, Rockland, Me). Electrophoresis was performed at constant voltage and temperature (300 V, 20°C) for 24 hours in 0.7 tris(hydroxymethyl)aminomethane/boric acid/ethylenediaminetetraacetic acid (TBE) buffer (Bio-Rad). The DNA bands were visualized by silver-staining according to the manufacturer’s instructions (SilverStain kit, Bio-Rad) (Figure 2).

**Band extraction, reamplification, and sequencing**

Bands were excised from the gel, homogenized and resuspended in 100 µL elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulfate, pH 8.0). The DNA was eluted overnight on a thermal shaker (50°C, 800 rpm; Thermomixer comfort, Eppendorf). Samples were concentrated by ethanol precipitation and resuspended in 10 µL double distilled water. The complete DNA solution was used as template for PCR reamplification with the primer.
pair 27f/521revP. The PCR conditions were the same as described earlier. However, the cycle number was increased to 33, and the annealing temperature was raised to 54°C. Afterward, PCR products were purified using the QIAquick MinElute kit (Qiagen) and subsequently sequenced (Seqlab, Göttingen, Germany). Obtained sequences were checked using the BioEdit software package (version 7.0.9, Ibis Biosciences, Carlsbad, Calif) and compared with the 16S rDNA database from the National Center for Biotechnology Information. For identification of the closest match, both the BLAST Basic Local Alignment Search Tool and the SEQMATCH Tool from the Ribosome Database Project were used.15,16

Counting the SSCP profiles and statistical analysis

Using G-Power (G-Power 3.2.1, Franz Faul, University of Kiel, Kiel, Germany), power and sample sizes were calculated. Power calculation revealed that a sample size of 8 would have a power of 80% to detect difference in means of 2.8 counts (eg, a first condition mean [μ1] of 6.3 and a second condition mean [μ2] of 3.5), assuming that the standard deviation was 2.1.

The 16S rDNA banding pattern of each sample was analyzed using the Quantity One 1D-Analysis Software package (version 4.6.5). The total number of bands was determined after background subtraction (rolling circle correction; disc size: 30) with preset values for sensitivity of 5.1 and a minimal band intensity ≥2%.17,18 Documentation and evaluation of the data were performed with data processing program SPSS/PC version 17.0 for Windows (SPSS Inc, Chicago, Ill). Comparison of microbial diversity was performed 2-tailed with the Mann-Whitney U test for unpaired non-normally distributed data, with a significance level of P ≤ .05.

RESULTS

Amplified bacterial 16S rDNA was separated, sequence dependent, by use of the SSCP method. Figure 3 summarizes the number of predominant bands in relation to the implant-supported superstructures. With 3.5 ± 2.1 different predominant bacterial genera in the peri-implant liquid plaque surrounding implant-supported bar attachments and 6.3 ± 3.1 different predominant genera in the peri-implant liquid plaque of implant-anchored double crown attachments, the differences were not statistically significant (P = .11). The microbial diversity of the biofilms surrounding the remaining dentition (6.3 ± 2.1) was almost similar to those of the implant-fixed telescopic attachments for the partially edentulous patients (Figure 4). For identification of the most abundant bacterial genera in the crevicular fluid of the different implant attachments, the predominant bands of the SSCP fingerprints were excised and the DNA sequences of the fragments were determined. Figure 5 presents the bacterial genera in the crevicular fluid of implant-anchored bar attachments and implant-fixed telescopic double crowns in relation to the number of patients.

DISCUSSION

The long-term success of osseointegrated oral implants is endangered by chronic inflammation of peri-implant hard and soft tissues caused by bacterial biofilms. The early process of oral biofilm formation on all solid surfaces is characterized by the generation of an acquired pellicle from salivary biopolymers or enzymes and the subsequent adherence of initial microorganisms, such as Streptococcus or Actinomyces spp, which are known to create the preconditions for the accumulation of gram-negative, anaerobic, late-colonizing microorganisms, such as Fusobacterium or Prevotella spp.19–22 The appearance of such periodontal pathogens is influenced by oral hygiene and other interindividual cofactors, including nutrition, tongue activity, or salivary composition. In particular, the number of sites retaining biofilm at dental prostheses or orthodontic appliances can influence the microbial diversity of oral biofilms and the corresponding clinical indices.12

This knowledge and the fact that the risk of peri-implantitis of osseointegrated implants not only depends on the quantity of the biofilm but also on the species in the biofilm,23–26 demonstrate the high clinical relevance of the present study. However, it has to be considered that the risk of periodontitis or peri-implantitis with the loss of hard and soft tissues also depends on differences in individual immune response and host susceptibility.14,27

The SSCP method used in this study, in combination with subsequent sequence analysis, allows the detection and identification of all
important bacteria in the peri-implant crevicular fluid of implant-fixed bar attachments and implant-anchored telescopic double crown attachments. Although it is known that the bacterial pathogenicity differs between the planktonic and biofilm forms, the microbial diversity of the crevicular fluid indicates that the spectrum of supra- and subgingival biofilms is fairly typical.\textsuperscript{28,29} Thus, this approach permits an analysis of the microbial diversity of peri-implant circumfluent biofilms without the principal disadvantage of conventional PCR and DNA hybridization methods, which is that only the expected bacteria can be detected, as specific primers are used.\textsuperscript{30} Moreover, the detection and identification of oral bacteria on the basis of 16S rDNA fingerprints avoids time-consuming and unreliable cell culture.\textsuperscript{31}

Groessner-Schreiber et al\textsuperscript{18,32} have analyzed bacterial adhesion on different modified implant surfaces in the oral cavity using the SSCP method. In these studies, the microbial community structure was analyzed in biofilms formed on differently modified implant surfaces fixed on acrylic splints during intraoral exposure of 24 hours. This procedure permits a realistic in vivo analysis of early biofilm formation, but not analysis of the microbial diversity of peri-implant biofilms in the long term with different implant-fixed supraconstructions.

A search in the Medline database using the key words “bacteria” and “telescopic double crown” showed that the results of the present study provide the first information about the microbial diversity of peri-implant biofilms on implant-anchored telescopic double crown attachments. Heckmann et al\textsuperscript{33} described the clinical and microbiological results of implant therapy with telescopic attachments for a 71-year-old patient after liver transplantation. However, in this study, only 8 periodontal marker organisms were analyzed using a DNA probe technology. Heckmann et al\textsuperscript{33} found low levels of \textit{Porphyromonas gingivalis}, \textit{Fusobacterium nucleatum}, and \textit{Eikenella corrodens}, but the data must be compared extremely cautiously because of the different study designs.

The higher numbers of predominant bacterial genera in the peri-implant crevicular fluid of the implant-anchored telescopic double crown attachments compared with those of the implant-fixed bar attachments could be explained by cross-infections between implants and adjacent teeth.\textsuperscript{34,35} This explanation is supported by the similar numbers of predominant bands in the remaining dentition and the telescopic implant attachments (Figure 3). These results are in accordance with the results of Leonhardt et al,\textsuperscript{36} who
found similar numbers of periodontopathic bacteria around teeth as with implants in clinically healthy peri-implant and periodontal sites. However, an analysis of the microbiota at implant-fixed telescopic attachments in fully edentulous patients only would be unrealistic, because in contrast to bar attachments, telescopic attachments are mainly used in partially edentulous patients because of the possibility of combining implants and teeth in one jaw as attachments for overdentures.37

Quirynen et al38 have described the microbial diversity of implant-supported bar attachments in relation to magnet or ball attachments. In this study, a checkerboard DNA–DNA hybridization method was used, and the detected microbial communities were grouped into microbial complexes, as described by Socransky et al.39 However, an accurate comparison of the findings was not possible, as these were not given in detail. In contrast to the present study, Quirynen et al38 found Aggregatibacter actinomycetemcomitans in nearly all the patients. This discrepancy could be explained by the different observation period. The patients in their study were rehabilitated with implants for 10 years, whereas the patients in the present study received their implants 2 to 3 years before evaluation. The present study is the first to show that Veillonella frequently occurs in the crevicular fluid of implant-fixed bar attachments. The reason for this must be addressed in future studies.

CONCLUSION

The microbial diversity of peri-implant biofilms seems to be influenced by the different types of prosthetic supraconstructions. Furthermore, the influence of the remaining dentition on the microbial diversity of peri-implant biofilms appears to be very high. Nevertheless, further research has to be performed to clarify the relationships between the periodontal and peri-implant microsystems.

ABBREVIATIONS

dNTP: deoxyribonucleotide triphosphate
EDTA: ethylenediaminetetraacetic acid
EPS: extracellular polysaccharides
MDE: mutation detection enhancement
PCR: polymerase chain reaction
rRNA: ribosomal RNA
SSCP: single-stranded conformation polymorphism
ssDNA: single-stranded DNA
TBE: tris(hydroxymethyl)aminomethane/boric acid/ethylenediaminetetraacetic acid

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REFERENCES


