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ORIGINAL ARTICLE

Analysis of the intraimplant microflora of two-piece dental implants

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Abstract
Objective Information about the spectrum of microorganisms in the intraimplant cavities of two-piece dental implants is scarce. The purpose of this study was to assess the intraimplant microflora of two-piece dental implants by conventional biochemical testing, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and 16 s rDNA gene sequencing.
Materials and methods Ten patients (six men and four women; average age=66.7 years; age range=58–78 years) received 35 two-piece titanium implants carrying ball attachments. Biofilm sampling was performed with sterile microbrushes, and nonadherent microbial samples were obtained by injection and reuptake of predefined volumes of NaCl solution. The samples were cultured and analyzed by conventional biochemical testing, MALDI-TOF MS, and 16 s rDNA gene sequencing.
Results Of the 103 species detected, 27 and 33 were identified only in the biofilm and nonadherent microbial samples, respectively. Forty-three species were identified in both types of samples.
Conclusions Two-piece dental implants harbored a broad spectrum of gram-positive and gram-negative aerobes and anaerobes, especially rods and cocci.
Clinical relevance These findings confirm bacterial translocation from the oral cavity to intraimplant cavities. Microbiological methods as used in this study are necessary to reveal the complete vital microflora of intraimplant cavities.

Keywords Dental implants · Bacterial translocation · Intraimplant cavity · Biofilm · Microflora · Nonadherent bacteria

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Introduction

Most dental implants are two-piece systems comprising an endosteal implant body and a suprastructure-carrying abutment. This mechanical design allows the application of a submerged healing protocol and multiple prosthodontic treatment options with different types of abutments, which can be customized according to the clinical situation. The two components (i.e., implant body and abutment) are fitted at the mating zone, commonly termed the implant–abutment interface.

The occurrence of microgaps between the two components leading to hollow cavities located inside the implant body in butt-joint connections and morse taper connections have been detected by assessing the permeability of dyes, bacteria, bacterial components, and hard X-ray synchrotron radiation [1–9]. Growing clinical evidence suggests that the location of such microgaps in relation to the periimplant bony crest influences the integrity of the periimplant hard and soft tissues [10–13]. Microgaps represent interruptions in the mechanical properties of the implant and enable bacteria harbored by the periimplant system to reach intraimplant cavities [14]. Such chemotactic stimuli in intraimplant cavities and/or microgaps of two-piece implants induce and support the recruitment of inflammatory cells such as neutrophils and mononuclear cells, thus causing persistent inflammation and increased alveolar bone loss [15].

The extent to which microorganisms colonize intraimplant cavities and the composition of the biofilm on intraimplant surfaces after implant placement and during function remain unclear. At present, only few data are available regarding the

oral microorganisms colonizing the internal surfaces of two-piece dental implants, which were obtained using commercial DNA probes and sampling with sterile paper points. Further, only a small bacterial spectrum (i.e., periodontopathogenic species) was identified in these studies [14, 16, 17]. Therefore, the aim of the present study was to assess the intraimplant microflora of two-piece dental implants by conventional biochemical testing, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and 16 s rDNA gene sequencing.

Materials and methods

Patients and implants

Ten patients (six men and four women; average age=66.7 years; age range=58–78 years) participated in this study. The exclusion criteria were systemic diseases that could affect the immune response or the use of antibiotics in the 4 months before the beginning of the study. All patients had excellent oral hygiene. After approval from the University's Ethical Committee, every patient signed an informed consent form acknowledging voluntary participation and assurance of complete anonymity.

Thirty-five two-piece titanium implants (Camlog® Screw-Line, Camlog Biotechnologies, Basel, Switzerland) were inserted, with a minimum of two and maximum of four implants per patient. The implants had the following dimensions: diameter, 3.8 or 4.3 mm; length, 9.0, 11.0, or 13 mm. Three and seven patients received implants in the maxilla and mandible, respectively. The implants were inserted according to a submerged healing protocol with primary closure of the flaps using nonresorbable sutures. The sutures were removed 8–10 days after implant placement. The implants were filled with 1 % chlorhexidine gel (Chlorhexamed, GlaxoSmithKline Consumer Healthcare, Bühl, Germany) during healing cap and ball abutment placement [18]. The abutments were placed using a torque wrench according to manufacturer's recommendations.

Finally, seven and three patients received implant-supported and combined implant–tooth-supported overdentures, respectively. Ball anchors (Dalbo®-Plus, Cendrés+Metaux, Biel/Bienne, Switzerland) were used as matrices in all patients. Three, four, and three patients had complete dentures, fixed prostheses, and tooth-supported overdentures in the opposing jaws, respectively.

Sampling and processing

Two different types of intraimplant samples were collected: nonadherent microbial samples and biofilm samples. The intraimplant volume was determined by filling one implant of each diameter–length combination with NaCl solution up to 1 mm beneath the margin of the implant shoulder under a light microscope (×20 magnification).

Before sampling, the implant site was isolated with sterile gauze pads to prevent contamination by saliva. The ball abutment and surrounding tissues were decontaminated using sterile cotton balls soaked with 0.2 % chlorhexidine solution. For nonadherent microbial samples, a 1-ml syringe was filled with 100 μl sterile NaCl solution. After removing the abutment, the implant-specific volume was injected into the intraimplant cavity and collected into the syringe. Biofilm samples were collected using sterile microbrushes. Each microbrush was inserted into the deepest part of the implant and the inner threads were brushed twice in counterclockwise direction. Thereafter, the microbrushes were placed into sterile tubes filled with 1.95-ml prereduced culture medium (thioglycolate broth enriched with vitamin K_1 and hemin; BBL™, Becton, Dickinson and Company, Sparks, MD, USA) such that only minimal air volume remained. Both types of samples were microbiologically processed within 15 min after collection.

Cultivation

The nonadherent microbial samples were plated in serial dilutions onto Columbia blood agar (Oxoid Ltd., Cambridge, UK) and Brucella agar (Becton, Dickinson and Company). The thioglycolate broth tubes, containing the microbrushes, were vortexed and then stirred by rotation for 20 min. Aliquots of the microbial suspensions were plated onto Columbia blood agar, chocolate agar (bioMérieux, Marcy l'Etoile, France), Brucella agar, KV agar and Karmali agar (Oxoid Ltd.), and Actinomyces agar (Difco, Becton, Dickinson and Company). Blood and chocolate agar plates were incubated in air enriched with 10 % CO_2, whereas Brucella, KV, Karmali, and Actinomyces agar plates were placed in anaerobic jars for up to 7 days at 37 °C. Representative colonies were isolated according to growth morphology, pigmentation, and hemolysis. All isolates were subjected to various microbiological methods for identification.

Conventional biochemical testing

Commercial microbial identification assays, such as VITEK® 2 system and API® strips (bioMérieux) and RAPID™ system (Remel Products, Thermo Fisher Scientific, Lenexa, KS, USA), were used according to the manufacturers' instructions.

MALDI-TOF MS

MALDI-TOF MS fingerprint analysis was performed using a Bruker Microflex LT instrument equipped with MALDI Biotyper 2.0 software (Bruker Daltonics, Billerica, MA,

USA). A test colony was suspended in deionized water, and proteins were extracted using formic acid and acetonitrile according to the manufacturer's instructions. One microliter of the extract was spotted on a polished steel target and air dried. Then, the spot was overlaid with 1 μl matrix solution (saturated solution of alpha-cyano-4-hydroxycinnamic acid) in organic solvent (50 % acetonitrile and 2.5 % trifluoroacetic acid) and air dried again. Measurements were performed with the MALDI-TOF spectrometer. Mass spectral profiles (mass range=2–20 kDa) were analyzed and interpreted to the species level by the MALDI Biotyper software.

16 s rDNA gene sequencing

DNA was extracted from a single colony using a QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). For 16 s rDNA gene amplification, universal primers fD1 (5′-AGAGTTT GATCCTGGCTCAG) and rP2 (5′-ACGGCTACCTTGT TACGACTT) were used. Sequencing reactions were carried out using a BigDye™ Terminator cycle sequencing kit (Applied Biosystems, Life Technologies, Foster City, CA, USA) and primers fD1, rP2, and PL06rev (5′-GCGCTCG TTGCGGGACTTAACC) and were determined in an ABI Prism® 310 genetic analyzer (Applied Biosystems). Electropherograms were exported to Vector NTI software, and the sequences were searched against the databases of BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Results

Seventy species were isolated from the biofilm, and 76 species from the nonadherent microbial samples. Most isolates were differentiated adequately (93); 6.1 % were not completely differentiated, and identification of 0.9 % was questionable (without doubt only at the genus level). In the nonadherent microbial samples, 4.15×10^5 CFU/ml (1.0×10^2 to 3.6×10^6 CFU/ml) were detected on average.

Of the 103 species detected, 27 and 33 were identified only in the biofilm and nonadherent microbial samples, respectively, and 43 species were detected in both types of samples (Table 1). The incidence of the predominant bacterial species isolated in this study is shown in Table 2. The detected microbial species are characterized in Tables 3 and 4.

Discussion

In this study, the intraimplant microflora of two-piece dental implants with a long implant–abutment interface was assessed

Table 1 Species detected in biofilm and nonadherent microbial samples

Species	Biofilm samples only	Nonadherent microbial samples only	Both samples
Actinomyces gerencseriae	−	+	−
Actinomyces graevenitzii	−	−	+
Actinomyces israelii	+	−	−
Actinomyces meyeri	−	+	
Actinomyces naeslundii	−	−	+
Actinomyces odontolyticus	−	−	+
Actinomyces turicensis	−	+	−
Actinomyces viscosus	−	+	−
Aeromonas salmonicida	−	−	+
Anaeroglobus geminatus	−	+	−
Atopobium parvulum	−	−	+
Atopobium rimae	−	−	+
Bacteroides caccae	+	−	−
Bacteroides distasonis	+	−	−
Bifidobacterium dentium	+	−	−
Bifidobacterium scardovii	−	+	−
Bulleidia extructa	−	+	−
Campylobacter concisus	−	−	+
Campylobacter gracilis	−	−	+
Campylobacter rectus	+	−	−
Campylobacter showae	−	−	+
Candida albicans	−	−	+
Candida glabrata	+	−	−
Capnocytophaga gingivalis	−	−	+
Capnocytophaga granulosa	+	−	−
Capnocytophaga ochracea	−	−	+
Clostridium clostridioforme	−	+	−
Clostridium difficile	+	−	−
Clostridium innocuum	+	−	−
Corynebacterium jeikeium	−	+	−
Enterococcus faecalis	−	+	−
Exiguobacterium profundum	−	+	−
Fusobacterium naviforme	+	−	−
Fusobacterium nucleatum	−	+	−
Gemella haemolysans	−	−	+
Gemella morbillorum	−	−	+
Haemophilus parainfluenzae	+	−	−
Lactobacillus acidophilus	−	+	−
Lactobacillus crispatus	−	−	+
Lactobacillus gasseri	+	−	−
Lactobacillus paracasei	+	−	−
Lactobacillus reuteri	−	+	−
Lactobacillus rhamnosus	−	−	+
Lactobacillus salivarius	−	+	−
Lactobacillus vaginalis	+	−	−
Lautropia sp.	−	+	−

Table 1 (continued)

Species	Biofilm samples only	Nonadherent microbial samples only	Both samples
Leptotrichia trevisanii	−	+	−
Leptotrichia wadei	+	−	−
Megasphera micronuciformis	+	−	−
Micrococcus luteus	−	−	+
Neisseria flava	−	−	+
Neisseria flavescens	−	+	−
Neisseria macacae	−	+	−
Neisseria perflava	−	+	−
Olsenella profusa	−	−	+
Olsenella uli	−	+	−
Paenibacillus amylolyticus	+	−	−
Parascardovia denticolens	−	+	−
Parvimonas micra	−	+	−
Peptostreptococcus micros	−	+	−
Prevotella histicola	−	+	−
Prevotella intermedia	−	−	+
Prevotella maclosa	+	−	−
Prevotella melaninogenica	−	−	+
Prevotella nigrescens	−	−	+
Prevotella oralis	−	−	+
Prevotella pallens	+	−	−
Prevotella veroralis	+	−	−
Propionibacterium acnes	−	−	+
Propionibacterium freudenreichii	+	−	−
Propionibacterium granulosum	−	+	−
Rothia aeria	−	−	+
Rothia dentocariosa	−	−	+
Rothia mucilaginosa	−	−	+
Scardovia inopinata	+	−	−
Selenomonas artemidis	−	+	−
Selenomonas dianae	−	+	−
Selenomonas infelix	−	−	+
Selenomonas sputigena	+	−	−
Slackia exigua	+	−	−
Staphylococcus epidermidis	−	−	+
Streptococcus anginosus	−	−	+
Staphylococcus capitis	−	+	−
Streptococcus downei	+	−	−
Streptococcus constellatus	−	+	−
Streptococcus gordonii	−	−	+
Staphylococcus hominis	−	+	−
Streptococcus infantis	+	−	−
Streptococcus intermedius	−	−	+
Streptococcus mitis	−	−	+
Streptococcus mutans	−	−	+
Streptococcus oralis	−	−	+
Streptococcus oralis/mitis	−	+	−
Streptococcus parasanguis	−	−	+
Streptococcus pseudopneumoniae	−	−	+
Streptococcus salivarius	−	−	+
Streptococcus sanguinis	−	−	+
Streptococcus vestibularis	+	−	−
Tissierella praeacuta	−	+	−
Veillonella atypica	−	−	+
Veillonella dispar	−	−	+
Veillonella parvula	−	−	+
Veillonella ratti	−	−	+

by biochemical testing, MALDI-TOF MS, and 16 s rDNA gene sequencing of biofilm and nonadherent microbial samples collected from intraimplant cavities. The implants harbored a broad spectrum of gram-positive and gram-negative aerobes and anaerobes, especially rods and cocci.

The investigation of the oral microflora was not part of the present investigation. Therefore, a direct comparison between the intra- and extraimplant microflora was not performed. But given the fact that the investigated implants were noncontaminated at the time of insertion, the observed bacterial colonization might be a consequence of bacterial translocation during

Table 2 Incidence of the predominant bacterial species

Bacterial species	Number
Biofilm samples only	
Actinomyces naeslundii	5/10
Streptococcus anginosus	5/10
Streptococcus oralis	5/10
Streptococcus salivarius	5/10
Veillonella parvula	5/10
Nonadherent microbial samples only	
Actinomyces naeslundii	7/10
Streptococcus mitis	6/10
Veillonella parvula	6/10
Streptococcus anginosus	5/10
Streptococcus mutans	5/10
Streptococcus salivarius	5/10
Both samples	
Streptococcus mitis	5/10
Streptococcus salivarius	4/10
Veillonella parvula	4/10
Streptococcus anginosus	4/10
Actinomyces naeslundii	3/10
Actinomyces odontolyticus	3/10
Streptococcus mutans	3/10

Table 3 Characterization of the species detected only in biofilm samples

Species	Gram stain	Morphology	Growth requirement
Bacteroides caccae	Gram negative	Rod	Anaerobe
Bacteroides distasonis			
Fusobacterium naviforme			
Prevotella maclosa			
Prevotella pallens			
Prevotella veroralis			
Selenomonas sputigena			
Leptotrichia wadei			Facultative anaerobe
Haemophilus parainfluenzae			
Campylobacter rectus			Microaerophile
Capnocytophaga granulosa		Coccus	Anaerobe
Megasphera micronuciformis	Gram positive	Rod	Anaerobe
Actinomyces israelii			
Bifidobacterium dentium			
Clostridium difficile			
Clostridium innocuum			
Paenibacillus amylolyticus			
Propionibacterium acnes			
Propionibacterium freudenreichii			
Slackia exigua			Facultative anaerobe
Lactobacillus gasseri			
Lactobacillus paracasei			
Lactobacillus vaginalis			Aerobe
Scardovia inopinata		Coccus	Facultative anaerobe
Streptococcus downei			
Streptococcus infantis			
Streptococcus vestibularis		Fungus	
Candida glabrata			

the replacement of prosthetic components or due to the prevalence of microgaps between the implant and its abutment.

Microgaps at butt–joint connections reportedly range from <10 to >100 μm [5, 19, 20]. Rack et al. visualized microgaps even at conical implant–abutment connections under dynamic loading using monochromatic hard X-ray synchrotron radiation. Several in vitro studies have demonstrated microleakage of fluids, dyes, bacteria, and bacterial components (e.g., lipopolysaccharide (LPS)) through the implant–abutment interface [1–7, 21]. Therefore, microgaps at this interface enable bacterial colonization and biofilm formation. Most studies of microleakage phenomena were conducted under static load conditions. Occlusal and extra-axial loading of implants may enlarge existing microgaps at the implant–abutment interface and thereby increase the potential for bacterial penetration and fluid percolation with transport of molecules such as LPS. LPS is a component of the outer cell wall of gram-negative bacteria and one of various microbe-associated molecular patterns recognized by host cells and known to elicit cytokine responses [22–24]. In addition, LPS modulates bone resorption by inducing osteoclastogenesis [25, 26]. Therefore, the presence of microgaps at the epicrestal level may increase the risk of periimplant bone loss because of bacterial colonization of the internal surfaces of implants.

Modifications made to the implant–abutment interface design with an extension of the horizontal implant shoulder (platform switching) seem to positively influence the preservation of the periimplant bone level [27–30]. To what extent the larger distance between the microgap of the implant and its abutment and the periimplant bone is responsible for the observed effects remains still unclear. However, the data of our study suggest that a vital intraimplant microflora and, therefore, a possible origin point for a chemotactic stimulus leading to bone resorbing processes exist.

Bacterial identification in the oral environment is challenging, and currently, no gold standard methodology exists. The limitations of the microbiological methods used in the present study include the need to preserve microbial viability during sampling and transportation, inability to detect low levels of microorganisms (the detection limit averages 10^3–10^4 bacterial cells), labor intensiveness, need for experienced personnel, and considerable time delay before

Table 4 Characterization of the species detected only in nonadherent microbial samples

Species	Gram stain	Morphology	Growth requirement
Fusobacterium nucleatum	Gram negative	Rod	Anaerobe
Leptotrichia trevisanii			
Prevotella histicola			
Selenomonas artemidis			
Selenomonas dianae			
Tissierella praeacuta		Coccus	
Anaeroglobus geminatus			
Lautropia sp.			Aerobe
Neisseria macacae			
Neisseria flavescens			
Neisseria macacae			
Neisseria perflava			Microaerophile
Peptostreptococcus micros	Gram positive	Rod	Anaerobe
Actinomyces gerencseriae			
Actinomyces meyeri			
Actinomyces turicensis			
Actinomyces viscosus			
Bifidobacterium scardovii			
Clostridium clostridioforme			
Exiguobacterium profundum			
Propionibacterium acnes			
Propionibacterium granulosum			Facultative anaerobe
Lactobacillus acidophilus			
Lactobacillus reuteri			
Lactobacillus salivarius			Aerobe
Corynebacterium jeikeium			
Enterococcus faecalis			Anaerobe/aerobe
Bulleidia extructa		Coccus	Anaerobe
Olsenella uli			
Parascardovia denticolens			
Parvimonas micra			Facultative anaerobe
Staphylococcus capitis			
Staphylococcus hominis			
Streptococcus constellatus			
Streptococcus oralis/mitis			

results are obtained [31, 32]. However, the advantages are the ability to detect multiple species coincidentally and identify unexpected bacteria, which is possible only by cell culture. Cell culture is widely used in studies to characterize the composition of the subgingival microflora and is still considered the reference for determining the feasibility of new microbial identification methods [33]. In addition, the use of MALDI-TOF MS and 16 s rDNA gene sequencing may close the diagnostic gaps in the detection of some putative pathogens that are difficult to culture [33, 34].

In recent published investigations, the intraimplant microflora was assessed by sampling with sterile paper points and using DNA probes with specific primers against the following putative periodontal pathogens: *Actinobacillus actinomycetemcomitans*, *Tannerella forsythensis*, *Campylobacter rectus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Treponema denticola* [16, 35]. Callan et al. [16] found moderate-to-high levels of these periodontopathogenic bacteria at the implant–abutment interface. However, in the present study, none of these periodontal pathogens were found in intraimplant cavities. This discrepancy might be explained by the absence of any sign of periimplantitis in the present subjects. *Actinomyces* species were the most abundant group of gram-positive rods, and *Neisseria* species predominated among the gram-negative cocci. *Actinomyces* species also dominate supragingival and subgingival areas in subjects with healthy periodontium and those with periodontitis [36].

By DNA extraction and polymerase chain reaction (PCR) amplification, Paolantonio et al. [35] sporadically detected bacterial species in implants, similar to the present findings; they used specific DNA probes for detecting the aforementioned periodontopathogenic bacteria. However, by using DNA checkerboard hybridization, Cosyn et al. [14] showed extensive bacterial contamination in intraimplant cavities of screw-retained fixed prostheses, also in accordance with the present findings.

An inherent problem of sampling with paper points is that it does not ensure the collection of all surface-adhering microbes. On the other hand, the use of sterile microbrushes ensures sampling of most adherent bacteria in intraimplant cavities. Further, PCR methods are difficult to apply for assessing small quantities of DNA because the ingredients necessary for PCR (oligonucleotide primers, dNTPs, and Taq polymerase) may be exhausted before a sufficient target is produced. A major limitation of PCR is the susceptibility of the process to contamination, particularly in experiments to detect rare DNA sequences [37, 38]. Furthermore, it does not differentiate between DNA fragments from vital intraimplant bacteria and those from cell remnants that penetrated through microgaps at the implant–abutment interface. In contrast to the previous studies, the microbiological methods of the current study have revealed the complete vital microflora in intraimplant cavities.

Conclusions

Within the limitations of this study, the following conclusions can be drawn:

1. The findings confirm bacterial translocation from the oral cavity to intraimplant cavities.
2. Biofilm and nonadherent (free-floating) bacterial species have different characteristics.
3. A combination of microbiological methods, as used in this study, is necessary to reveal the complete intraimplant microflora.

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Conflict of interest None.

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