Microbial Composition in Peri-implant Health and Disease

Authors
Malak Hamzé
Saad Fadhel

Tutor
Py Palmqvist
ABSTRACT

Although the infectious initiation of peri-implantitis is commonly accepted, differences in microbial composition in peri-implantitis and periodontitis has been the subject of some debate. The presence of periodontal pathogens at sites with peri-implantitis has been documented by many studies. However, other studies have revealed that sites with peri-implantitis harbour high levels of microorganisms which have not primarily been associated with periodontitis, such as *Candida*, *Staphylococcus* and *Enterobacteriaceae* species. These findings suggest that peri-implantitis may display a flora distinct from that of periodontitis.

The aim of the present study was to evaluate the presence of three known periodontal pathogens, *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), *Porphyromonas gingivalis* (*P. gingivalis*) and *Treponema denticola* (*T. denticola*) at healthy and diseased (peri-implantitis) sites. A second aim was to evaluate the presence of *Candida*, *Staphylococcus* and *Enterobacteriaceae*-species at the same sites. The hypothesis was that *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola*, *Candida*, *Staphylococcus* and *Enterobacteriaceae*-species would be detected at higher levels in peri-implantitis samples than healthy controls. Samples of peri-implant crevicular fluid (PICF) were obtained from peri-implant pockets in 13 subjects, of which 5 subjects had both healthy and diseased implants, 4 subjects had healthy implants only and the remaining 4 had only diseased implants. Microbial analyses were carried out using Real Time PCR and microbial culture techniques. *A. actinomycetemcomitans*, *P. gingivalis* and *T. denticola* were detected at higher levels at sites with peri-implantitis compared to healthy sites.

Furthermore, *Klebsiella oxytoca* was detected in one peri-implantitis sample whereas *Candida* and *Staphylococcus species* were not detected in any of the samples. Higher counts of periodontal pathogens were detected around implants with peri-implantitis. The suggestion that *Candida* and *Staphylococcus* may be present at higher levels in peri-implantitis was not supported by this study as these bacteria could not be detected in any of the samples.
INTRODUCTION
Dental implants as replacement for missing teeth are common today. Although highly functioning for many patient’s implants are vulnerable to infections, so called peri-implant diseases, where the two conditions peri-implant mucositis and peri-implantitis are included. Peri-implant mucositis is defined as inflammation in the surrounding mucosa of the dental implant with unaffected supporting bone, clinically seen as bleeding on probing and swelling of the mucosa. In contrast, peri-implantitis includes inflammation in the surrounding mucosa and concomitant peri-implant bone loss. Deep pathological peri-implant pockets and suppuration are common clinical findings associated with peri-implantitis (Lang and Berglundh, 2011). In 2014, 53859 dental implants were installed in Sweden. In the same year 75432 dental implants were diagnosed with peri-implantitis (Data register of the Swedish Social Insurance Agency). The mean prevalence of peri-implant mucositis and peri-implantitis is 43 % and 22 %, respectively (Jepsen et al., 2015).

Peri-implant mucositis is a reversible condition and can be treated efficiently using conservative methods while established peri-implantitis is difficult to manage, but studies have demonstrated that early diagnosis and intervention can improve the outcome of treatment (Salvi et al., 2012; Smeets et al., 2014). Studies have shown that non-surgical treatment of peri-implantitis is ineffective while surgical treatment show better results (Figuero et al., 2014; Smeets et al., 2014). However, surgical treatment demonstrates a success rate of no more than 58 % (Leonhardt et al., 2003). Altogether, this emphasizes the need for more sensitive diagnostic tools as well as adjunctive preventive and intervening treatment strategies for the disease.

Determining species associated with peri-implant health and disease has been suggested as a diagnostic tool useful to improve prevention and treatment strategies (Charalampakis et al., 2011). In the oral cavity, dental implants like teeth, are exposed to biofilm formation. Pellicle is formed rapidly and the dental implants are soon colonized by multiple species. Consequently, a supra- and subgingival biofilm is established within weeks to months. In a clinical experimental study by Pontoriero et al. (Pontoriero et al., 1994), the participants were asked to refrain from all oral-hygiene practices for 3 weeks. Plaque samples were then gathered and analysed. The study demonstrated a relationship between plaque accumulation and the establishment
of peri-implant mucositis. A similar study was performed by Zitzmann et al. (Zitzmann et al., 2001). The study concluded that plaque accumulation led to an inflammatory response in the gingiva and the peri-implant mucosa. The inflammation was characterized by an increased inflammatory cell infiltrate including elevated levels of T- and B-cells. Peri-implantitis is a multifactorial disease induced and entertained by an opportunistic infection. The susceptibility for the disease differs among individuals due to many contributing factors, such as genetic disposition, the host’s immune system, presence of a virulent flora and environmental factors. Smoking, a history of periodontal disease and poor oral hygiene are known risk factors associated with implant failure and peri-implant disease. Diabetes mellitus and cardiovascular diseases, rough implant surfaces and lack of keratinized mucosa are other factors which have been proposed to increase the risk for disease (Smeets et al., 2014). Peri-implant health is achieved by an equilibrium between the flora colonizing the peri-implant pockets and the host response including the immune system. In a susceptible host, uninterrupted plaque accumulation is followed by increased depth of the peri-implant pockets. A gram-negative flora of anaerobic bacterial species with specific virulence factors increases in this environment at the expense of the less harmful gram positive aerobic flora. When the host’s immune response, being either inefficient or excessive, fails to suppress the pathogenic flora the balance maintaining peri-implant health is altered which eventually leads to destruction of the peri-implant tissues (Griffen et al., 1998).

Previous microbiological investigations have described the flora associated with peri-implant health to be very similar to that of sound periodontal sites. The flora has been demonstrated to consist of mostly gram positive cocci with low proportions of gram negative anaerobic species (Lindhe et al., 2015).

Early studies using culture techniques failed to detect species like P. gingivalis and A. actinomycetemcomitans in fully edentulous patients treated with dental implants after complete extraction of teeth due to periodontitis. Consequently, these species were believed to be eradicated when their ecological niches, i.e. the periodontal pockets, were no longer present. This led to the suggestion that periodontal pathogens do not colonize dental implants in fully edentulous patients (Danser et al., 1997). However, this idea was rejected when more sensitive detection methods such as real time PCR
and DNA-DNA hybridization became available. With these methods, periodontal pathogens such as *P. gingivalis*, *A. actinomycetemcomitans*, *T. denticola* and *T. forsythia* were detected at low levels at the sulci of newly inserted and healthy implants in fully edentulous patients with a history of severe and aggressive periodontitis (Van Assche *et al.*, 2009). Periodontal pathogens were also identified at the sulci of sound dental implants in patients with partial edentulism (Casado *et al.*, 2011). Hence, the presence of periodontal pathogens at the sulci of dental implants may not always cause disease, owing to the multifactorial nature of peri-implant disease.

The flora associated with peri-implantitis is mainly composed of gram negative anaerobic rods with high proportions of black-pigmented *Bacteroides-, Campylobacter- and Fusobacterium* species. The presence of periodontal pathogens such as *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola*, *Tannerella forsythia*, *Campylobacter gracilis* and *Campylobacter rectus* at sites with peri-implantitis has been documented in many studies (Sanz *M et al.*, 1990; Persson *et al.*, 2010). Consequently, it was concluded that peri-implantitis and periodontitis share similar microbial profiles. However, other studies have identified species not primarily associated with periodontal diseases at sites with peri-implantitis. These species include enteric rods (Leonhardt *et al.*, 2003; Renvert *et al.*, 2016), fungal organisms (Leonhardt *et al.* 1999; Schwarz *et al.* 2015), *Staphylococcus aureus* (Persson *et al.*, 2010; Renvert *et al.*, 2016) and human *Cytomegalovirus* as well as *Epstein-Barr* virus (Jankovic *et al.*, 2011), thus indicating microbiological differences between peri-implantitis and periodontitis. In a culture study by Leonhardt *et al.* (Leonhardt *et al.*, 1999) it was revealed that diseased implants harboured periodontal pathogens such as, *A. actinomycetemcomitans*, *P. gingivalis*, *Prevotella intermedia* and *Prevotella nigrescens* in 60 % of the cases. In addition, enteric rods, *Candida-* and *Staphylococci*-species were detected in 55 % of the diseased cases which led to the conclusion that peri-implantitis and periodontitis may share different microbial profiles. In a newly published systematic review 194 microbiological studies were screened of which 47 studies were included in the review. It was concluded that classic periodontal pathogens are often found in the flora of peri-implant disease, however, correlation between studies is difficult due to the use of different detection techniques. For future
investigations, the authors recommended the use of megagenomic techniques in order to avoid detection bias (Padial-Molina et al., 2016).

Methods which are used for microbial detection involve culture techniques, DNA hybridization, polymerase chain reaction (PCR), immunofluorescence and 16S RNA sequencing. The different detection methods all have advantages and disadvantages and analytical results vary depending on the technique used. Hence, comparison of results generated by different techniques in studies may be difficult to make. (Rylev and Kilian., 2008).

**Hypothesis**

The hypothesis of the study was that *A. actinomycetemcomitans*, *P. gingivalis* and *T. denticola* would be detected at higher levels around implants with peri-implantitis compared to healthy controls. Secondarily, we hypothesized that *Candida-, Enterobacteriaceae- and Staphylococcus- species* would also be detected at higher levels around implants with peri-implantitis compared to healthy controls.

**Aim**

The aim of the present study was to evaluate the presence and detection levels of three known periodontal pathogens, *A. actinomycetemcomitans*, *P. gingivalis* and *T. denticola* at healthy and diseased (peri-implantitis) sites and secondly to evaluate the presence of *Candida-, Staphylococcus- and Enterobacteriaceae-species* at these sites by cultivation and quantitative polymerase chain reaction (qPCR).

**MATERIAL AND METHODS**

**Ethical considerations**

The ethical committee at the institution of Odontology at Umeå University approved this study. The subjects were informed verbally about the content and aim of this study and written consent was given from all the enrolled patients. Patients were given the chance to decline to participate at any time.

All patient data was handled with confidentiality and all operators were bound to secrecy. Patient data was coded by the supervisor of the study, senior
consultant in periodontology Py Palmqvist. Data was handled in a coded manner by the dental students Malak Hamzé and Saad Fadhel and presented in a decoded manner in the report. Only the supervisor had access to the passkey which was store locked at the Department of Periodontology.

**Literature search**

Literature search was performed using the electronic PubMed database using a combination of specific mesh-terms such as “peri-implantitis”, “microbiology”, “microbiota” and “dental implants”. Additionally, hand search was made in the textbook Clinical Periodontology and Implant Dentistry (Lindhe et al., 2008).

**Statistical Analysis**

Descriptive data analysis was performed the Microsoft® Excel program (Microsoft Corporation, USA Production).

**Patient sample**

A total of 13 consecutive adult patients who were transferred to the specialist clinics for Periodontology and Prosthodontics, respectively, in County Council of Västerbotten, Umeå, for treatment of either peri-implantitis or prosthodontic treatment between years 2016 and 2017 were enrolled in the study. The clinical examinations were carried out by three periodontists and one prosthodontist. Only our supervisor PhD Py Palmqvist and the clinicians who treated the patients had access to the uncoded patient data.

**Questionnaire**

A questionnaire was delivered to the involved clinicians. The questionnaire was used to ensure that samples of peri-implant crevicular fluid were collected and obtained only from patients with implant sites meeting the inclusion criteria. The questionnaire included clinical data from the patients’ dental records including radiographic data from the clinical periodontal registration, i.e. peri-implant diagnosis, probing pocket depth (PPD) and bleeding on probing/suppuration (BOP/SUP). Furthermore, the questionnaire contained questions about smoking.
habits, history of periodontitis and presence of systemic disease and medication. However, these anamnestic data were not presented or analysed in this study.

Inclusion criteria
Patients with at least two implants of which one was diagnosed with peri-implantitis and one was healthy were included in the study. However, patients not meeting these inclusion criteria, by demonstrating either diseased or healthy implants but not both, were also included in the study. These patients were included to increase the number of analyzed samples as patients fulfilling these inclusion criteria were lacking in sufficient numbers at the clinic at the time of the study.

Implants were considered healthy when demonstrating no BOP/suppuration at any site, a PPD $\leq$ 4 mm in all 4 sites and intact marginal bone level which was determined using intraoral radiographs. The diagnostic criteria for peri-implantitis were presence of radiographic marginal bone loss around implants when compared to baseline radiographs one year after implant placement in combination with presence of PPD $\geq$ 4 mm and BOP/SUP. (Lindhe and Meyle, 2008).

Exclusion criteria
The participants had to be non-smokers and free from systemic diseases. In addition, patients treated with antibiotics for the last 3 months were excluded. However, one unmatched diseased sample did not meet the exclusion criteria for smoking. This sample was included to increase the number of samples.

Clinical Investigation
The clinical examinations and assessments were carried out by the same four specialists who collected the samples. Peri-implant bone level was evaluated using intraoral radiographs. PPD was measured using a manual periodontal probe (Hu-Friedy PCP-11, Hu-Friedy Mfg. Co., Chicago, USA) at four sites per implant; mesial, distal, buccal and lingual. Values $\geq$ 3 mm were registered. BOP/SUP from the bottom of the peri-implant pocket was registered 30 seconds after probing with an estimated force of 0.25 N (Schwarz et al., 2011). Non-
bleeding sites were registered as zero (0) and bleeding sites were registered as one (1).

**Collection of peri-implant fluid**

Samples of peri-implant crevicular fluid (PICF) were collected with paper points from healthy and diseased sites in 13 subjects, of which 5 subjects fulfilled the inclusions criteria and had both healthy and diseased implants in the same mouth (matched samples). The remaining 8 subjects were unmatched demonstrating either healthy or diseased implants, of which 4 were healthy and 4 were diseased.

The dental implants were polished to remove supra-mucosal plaque and the sites were isolated with cotton rolls. Sterile absorbent endodontic paper points were inserted deep into each peri-implant pocket for 30 seconds. The deepest site of each implant was chosen for sampling at implants with peri-implantitis. Two samples were collected from the same site, one for cultivation and one for PCR-analysis.

The paper points were inserted into VGMAIII, an anaerobic transport medium (Viable medium of Department of Bacteriology, University of Gothenburg, Anaerobic medium) for cultivation and Eppendorf tubes with 1 ml TE-buffer (Tris EDTA buffer, 10 µM/1µM; pH 8.0) for qPCR analysis.

The samples were transported to the Clinical laboratory of Department of Odontology, Medical Faculty, Umeå University for microbiological analyzes.

**Microbiological Analysis**

Detection of *Candida* -, *Enterobacteriaceae*- and *Staphylococcus species* by cultivation

The samples transported in VGMAIII tubes were incubated at 37 degrees for 2-3 minutes and subsequently diluted 100 times in a salt buffer (Johansson et al., 2014). They were then gently shaken on a Vortex mixer for 1 min. Aliquots of 50 µl of undiluted as well as 100 times diluted samples were spread on *Candida*- (ChromAgar, Mast Diagnostics, U.K), *Enterobacteriaceae*- (Violet Red Bile
Agar; Difco Laboratories; Detroit, Michigan, USA), and *Staphylococcus* – (Staphylococcus Medium 110, Becton, Dickinson and Company, Sparks, MD USA) specific plates. The selective plates were incubated for 2-3 days at 37˚C before they were examined.

**Detection of A. actinomycetemcomitans, P. gingivalis and T. denticola and quantification of total number of bacteria by qPCR.**

Bacterial DNA in the samples which were transported in the TE-buffer were purified by a DNA preparation robot (Diasorin). DNA from standard solutions (10^1-10^8 bacterial cells/ml) of *A. actinomycetemcomitans* and *P. gingivalis* were purified in the same way.

Due to the absence of standard solutions for *T. denticola*, the samples were studied in a semi-quantitative manner. When total number of bacteria in the samples was determined the standard suspension for *P. gingivalis* was used.

The PCR mixture of in total 10µl contained 5µl of Kapa Cyber Green (KK 4601, Kapa Biosystems, Boston USA), 4 µl of template and 1µl of species specific primers (5 µM each). The primers for *A. actinomycetemcomitans*, *P. gingivalis* and *T. denticola*, total number of bacteria, and the PCR programs used are shown in the Appendix. The quantifications were performed by q-PCR Corbett research Rotor-Gene 6000 Rotary Analyze (QIAGEN, Valencia, CA, USA).

**RESULTS**

In 5 of the 13 subjects, samples from both healthy and diseased sites could be collected from the same individual, i.e. samples were matched. In 4 of the subjects, only diseased unmatched samples were collected and the remaining 4 were healthy unmatched samples.

**Matched samples (Subjects 1-5)**

*A. actinomycetemcomitans* was detected in 4 out of 5 subjects (2 out of 5 sound implants and 4 out of 5 diseased implants). The diseased implants showed higher detection levels of *A. actinomycetemcomitans* except in one subject where the opposite was noted.

*P. gingivalis* was detected in 2 out of 5 subjects. In these two subjects, *P. gingivalis* was detected in samples obtained from both healthy and diseased implants.
T. denticola was detected in 3 of out 5 subjects. The diseased implants showed higher detection levels of T. denticola compared to healthy implants (Table 1a).

**Healthy unmatched samples (Subjects 6-9)**

A. actinomycetemcomitans and P. gingivalis were not detected in any of the 4 subjects. On the other hand, T. denticola were detected in all 4 subjects. It was detected at low levels in all but one of the samples, subject number 6, which revealed a high detection level for T. denticola (Table 1b).

**Diseased unmatched samples (Subjects 10-13)**

A. actinomycetemcomitans was detected in 2 out of 4 subjects. P. gingivalis was detected in 1 out of 4 subjects. However, one sample needed to be re-analyzed for P. gingivalis. Unfortunately, re-analysis could not be carried out due to lack of time. Thus, the presence of P. gingivalis could not be evaluated in one of the subjects. T. denticola was detected in 3 out of 4 subjects (Table 1c).

**Cultivation**

K. oxytoca, a member of the Enterobacteriaceae family, was detected in one peri-implantitis sample, obtained from subject number 3, whereas Candida- and Staphylococcus-species were not detected in any of the samples.

**DISCUSSION**

In the present study, A. actinomycetemcomitans, P. ginigivalis and T. denticola were detected in healthy as well as diseased (peri-implantitis) samples. However, the diseased samples demonstrated higher detection levels of these bacteria. These results are in line with Hultin et al. (Hultin et al., 2002). Hultin et al. used DNA-DNA hybridization for microbial detection and demonstrated that periodontal pathogens such as A. actinomycetemcomitans, P. gingivalis and T. denticola could be detected around both sound and diseased implants. In line with our results, these bacteria were detected more frequently and at higher levels around diseased implants. Since putative pathogens could be found around both healthy and diseased implants, the microbiological composition has not been demonstrated to have a predictive value for peri-implantitis (Casado et al., 2011; Persson and Renvert, 2014). The complex nature of peri-implantitis and the involvement of host and environmental factors makes it hard to predict the long-term health of dental implants solely based on microbial composition.
In a culture study by Leonhardt et al. (Leonhardt et al., 1999), enteric rods, Candida- and Staphylococcus-species were detected in 55% of the diseased sites. In our study, Candida- and Staphylococcus-species were not detected in either diseased or healthy sites. Hence, the findings of Leonhardt and coworkers could not be substantiated by our study. However, in line with their results is the detection, in one peri-implantitis sample, of K. oxytoca, which is a member of the Enterobacterceae family. It is possible that we failed to detect Candida and Staphylococcus-species due to the low number of participants in the present study. Furthermore, C. albicans, S. aureus and Enterobacteriaceae species are rarely found in the oral cavity and their presence have been associated with opportunistic infections in immunocompromised patients. A study evaluating their prevalence was carried out by Öhman et al. (Öhman et al., 1995) in a group of 79-year-olds in Gothenburg, Sweden. 100 subjects were included in the study and the majority of the subjects suffered from systemic diseases. The species were detected in few subjects and their presence was correlated to the use of dentures. This might be another possible explanation to why these microbes were not detected in the present study since none of our participants wore dentures.

Persson and Renvert (Persson and Renvert, 2014) used DNA-DNA hybridization and demonstrated higher detection levels of a cluster of 7 bacterial species, i.e. T. forsythia, P. gingivalis, Streptococcus mitis, Streptococcus intermedius, S. aureus, Staphylococcus anaerobius and Treponema socranskii, around implants with peri-implantitis compared to healthy controls. The authors suggested a possible association between this cluster of bacteria and the development of peri-implantitis and they highlighted the role of S. aureus and T. forsythia as potential key pathogens since T. forsythia was found in 45% of the diseased sites compared to 15% of the healthy sites. S. aureus is often found on infected medical devices and is a microbe which is believed to be associated with infections around metallic biomaterials. Hence, its involvement in the development of peri-implantitis has been addressed in studies (Leonhardt et al., 1999). Interestingly, S. aureus, like other Staphylococci, has been shown to have high affinity to titanium surfaces (Harris et al., 2006) and subjects with failing implants have been shown to have less efficient antibodies against S. aureus as well as T. forsythia (Kronström et al., 2001). The present study failed to detect Staphylococcus-species in any of the samples, thus, their possible contribution to implant failure could not be confirmed.
The discrepancy in the microbial profile of peri-implantitis between studies is in part due to the use of different detection methods. Culture analysis has long been the golden standard for microbial analysis and it has many advantages. However, non-cultivable and dead species are not detected by this method which increases the risk for false negative results. It requires sufficient sample volumes as well as adequate transportation conditions. In contrast, molecular detection methods such as qPCR and DNA-DNA hybridization are less time consuming and more sensitive, allowing the detection of species present at low levels. These methods can detect uncultivable as well as non-vital bacteria. Consequently, microbial data obtained from studies using different detection methods may be very difficult to compare (Padial-Molina et al., 2016).

The present study had several limitations and weaknesses. According to the inclusion criteria only matched subjects would be included in the study. However, few patients demonstrated both sound and diseased implants in the same mouth at the time when the study was conducted. Consequently, unmatched subjects with either sound or diseased implants were also included in the study in order to increase the number of participants. Hence, the unmatched samples did not meet the intended inclusion criteria of this study. Anamnestic data such as smoking habits, history of periodontal disease and presence of systemic diseases were not analysed since this study included a small number of participants, which made conclusions difficult to draw. Furthermore, one of the collected unmatched samples did not meet the exclusion criteria regarding smoking habits. This was probably due to inattention to the study protocol by one specialist at the specialist clinic of prosthodontics. This sample was included and analysed because we did not want to further reduce the number of participants. Additionally, clinical assessments may have differed between clinicians and a deviation from the sampling protocol may have occurred. Inter-individual calibration of clinical assessment between the clinicians involved was not performed or evaluated. Other weaknesses were the facts that two samples were lost during the DNA-preparation process and 4 samples were not tested due to lack of time. Finally, collection of samples from only one site per implant may be a weakness as testing multiple sites per implant could contribute to more representative microbial data.
Conclusion
The results of this study confirm the findings in several previous studies showing that the periodontal pathogens *A. actinomycetemcomitans*, *P. gingivalis* and *T. denticola* are present at higher levels in diseased peri-implant pockets compared to healthy controls. These pathogens were also detected in clinically healthy peri-implant sites. Furthermore, the study failed to corroborate the findings of previous studies demonstrating presence of *Candida-* and *Staphylococcus-species* in peri-implantitis. For future studies, a higher number of participants is recommended and the promotion of megagenomic detection methods in order to avoid detection bias.

ACKNOWLEDGMENTS
We would like to thank our supervisor, PhD Py Palmqvist for her support and guidance throughout the course of the study and for collecting clinical data and microbial samples together with Martin Ågren, Uday Najim and Peder Willhelmsson at the department of Periodontology and Prosthodontics at Umeå University. We are very grateful to Rolf Claesson for the microbiogical laboratory work at the Clinical Laboratory of Dental School in Umeå. And finally, we would like to express our gratitude to Anders Esberg for helping us with the statistics and making of the graphs.
REFERENCES


Tables

Table 1a. Proportion of periodontal pathogens in matched implants with peri-implantitis compared to healthy implants.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Peri-implant status</th>
<th>Total viable counts per ml $10^3$</th>
<th>A.a TVC/ml (%)</th>
<th>P.g TVC/ml (%)</th>
<th>T.d TVC/ml (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>28 21000</td>
<td>0.5 (1.8 %)</td>
<td>Nd</td>
<td>Nd</td>
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<tr>
<td></td>
<td>1</td>
<td></td>
<td>0.2 (0.001 %)</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>42 290</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>54 (18.6 %)</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>20 2600</td>
<td>Nd</td>
<td>9.83 (46.5 %)</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>Nd</td>
<td>859 (33.0 %)</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>410 1200</td>
<td>8.2 (2.0 %)</td>
<td>Nd</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>77 (6.4 %)</td>
<td>Nd</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>430 5600</td>
<td>Nd</td>
<td>282 (65.6 %)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>410 (7.3 %)</td>
<td>4900 (87.5 %)</td>
<td>+++</td>
</tr>
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</table>

Table 1b. Proportion of periodontal pathogens in unmatched healthy implants.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Total viable counts per ml $10^3$</th>
<th>A.a TVC/ml (%)</th>
<th>P.g TVC/ml (%)</th>
<th>T.d TVC/ml (%)</th>
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<tbody>
<tr>
<td>6</td>
<td>160</td>
<td>Nd</td>
<td>Nd</td>
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<td>7</td>
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<td>9</td>
<td>-</td>
<td>Nd</td>
<td>Nd</td>
<td>+</td>
</tr>
</tbody>
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Table 1c. Proportion of periodontal pathogens in unmatched implants with peri-implantitis.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total viable counts per ml $10^3$</th>
<th>A.a TVC/ml (%)</th>
<th>P.g TVC/ml (%)</th>
<th>T.d TVC/ml (%)</th>
</tr>
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<tr>
<td>10</td>
<td>3000</td>
<td>Nd</td>
<td>Nd</td>
<td>++</td>
</tr>
<tr>
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<td>54</td>
<td>Nd</td>
<td>-</td>
<td>Nd</td>
</tr>
<tr>
<td>12</td>
<td>1300</td>
<td>2.1 (0.2 %)</td>
<td>Nd</td>
<td>+++</td>
</tr>
<tr>
<td>13</td>
<td>10 200</td>
<td>850 (8.3 %)</td>
<td>9350 (91.7 %)</td>
<td>+++</td>
</tr>
</tbody>
</table>

0 = Healthy implant
1 = Implant with peri-implantitis
TVC/ml = Total viable counts per ml
Nd = Not detected
Nt = Not tested
- = Lost sample
Figures

**Figure 1a.** Proportion of *A. actinomycetemcomitans* in matched subjects 1-5.

**Figure 1b.** Proportion of *P. gingivalis* in matched subjects 1-5.
Figure 1c. Proportions of A. actinomyctemcomitans (left) and P. gingivalis (right) in healthy implants (Subjects 6-9) compared to implants with peri-implantitis (subjects 10-13).
APPENDIX

16S rDNA primer pairs for detecting the total number of bacteria and A. actinomycetemcomitans, P. gingivalis and T. denticola (Kirakodu et al., 2008).

<table>
<thead>
<tr>
<th>Universal</th>
<th>A.a</th>
<th>P.g</th>
<th>T.d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse</td>
<td>GATTAGATAACCTGG TAGTCCAC</td>
<td>CTAGGTATTGGCCGAACAT TTG</td>
<td>AGGCAGCTTGCCATACCT GCG</td>
</tr>
<tr>
<td>Forward</td>
<td>TACCTTGTTACGAGC TT</td>
<td>CGTGAATTAAGCTGTA ATC</td>
<td>ACTGTTAGCAACTACCG ATCT</td>
</tr>
</tbody>
</table>

Cycling setting and annealing temperatures for quantification of bacteria using Corbette Research Rotor – Gene 6000 Rotary Analyze (QIAGEN, Valencia, CA, USA)

<table>
<thead>
<tr>
<th>Prime</th>
<th>Universal</th>
<th>A.a</th>
<th>P.g</th>
<th>T.d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold/time</td>
<td>95°/600 sec</td>
<td>95°/600 sec</td>
<td>95°/600 sec</td>
<td>95°/600 sec</td>
</tr>
<tr>
<td>Cycling/time</td>
<td>95°/10 sec</td>
<td>95°/10 sec</td>
<td>95°/10 sec</td>
<td>95°/10 sec</td>
</tr>
<tr>
<td>Cycling/time</td>
<td>52°/5 sec</td>
<td>55°/5 sec</td>
<td>60°/5 sec</td>
<td>59°/5 sec</td>
</tr>
<tr>
<td>Cycling/time</td>
<td>72°/30 sec</td>
<td>72°/10 sec</td>
<td>72°/16 sec</td>
<td>72°/13 sec</td>
</tr>
</tbody>
</table>