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Year: 2015

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DOI: <https://doi.org/10.4161/21505594.2014.980661>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-108727>

Journal Article

Accepted Version

Originally published at:

Charalampakis, Georgios; Belibasakis, Georgios N (2015). Microbiome of peri-implant infections: lessons from conventional, molecular and metagenomic analyses. *Virulence*, 6(3):183-187.

DOI: <https://doi.org/10.4161/21505594.2014.980661>

# **Microbiome of peri-implant infections: lessons from conventional, molecular and metagenomic analyses**

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## **Abstract**

Osseointegrated dental implants are now a well-established treatment option in the armament of restorative dentistry. These technologically advanced devices are designed to functionally and esthetically replace missing teeth. Despite the revolutionary advances that implants have incurred, they have also provided the oral cavity with new artificial surfaces prone to the formation of oral biofilms, similarly to the hard tissue surfaces of natural teeth. Biofilm formation on the implant surface can trigger the inflammatory destruction of the peri-implant tissue, in what is known as peri-implantitis. The mixed microbial flora of peri-implant infections resembles that of periodontal infections, with some notable differences. These are likely to expand with the ever increasing application of metagenomics and metatranscriptomics in the analysis of oral ecology. This review presents the wealth of knowledge we have gained from microbiological methods used in the characterization of peri-implant microflora and sheds light over potential new benefits, as well as limitations, of the new sequencing technology in our understanding of peri-implant disease pathogenesis.

**Keywords:** dental implants; peri-implantitis; peri-implant mucositis; oral microbiota; molecular diagnostics; metagenomics; biofilms

## **Dental implants and peri-implant infections**

Dental implants have been in the market for more than 40 years. They are artificial devices made of titanium, introduced in order to replace one or more missing natural teeth. They are anchored to the bone, based on the concept of 'osseointegration', which ensures direct structural and functional bone to implant contact <sup>1</sup>. Nevertheless, the anchorage to the bone is different between dental implants and natural teeth. For teeth, the anchorage is established via the periodontal ligament its Sharpey's fiber extremities, anatomical elements that are absent at implants <sup>2</sup>. Despite these dissimilarities between dental implants and natural teeth, soft tissues around teeth and implants are of similar dimensions. Gingiva and peri-implant mucosa include a junctional/barrier epithelium about 2 mm long in the apico-coronal direction and one zone of supracrestal connective tissue attachment just above 1 mm. In addition, the hard non-shedding surface of the implant behaves similar to that of the tooth, colonized by microbiota in the form of a biofilm <sup>3</sup>. The colonization pattern on implants appears to be initially slower than on natural teeth <sup>4</sup>, given the pristine surfaces of the implant and the lack of the desired indigenous microbiota. However, once the biofilm is established, it acts as an orchestrated microbial challenge causing, in many respects, similar soft and hard tissue reactions around teeth and implants. Thus, long-term biological complications, biofilm-mediated, do occur around dental implants, despite the prior successful osseointegration.

More than two decades, we have witnessed the emergence of two contemporary diseases, peri-implant mucositis and peri-implantitis. Peri-implant mucositis is characterized by inflammation restricted to the peri-implant mucosa of an implant in function, presenting no distinct bone loss. Peri-implantitis is defined as a more profound inflammatory lesion, characterized by a deepened bleeding peri-implant pocket and progressive loss of supporting

bone around a functional implant. These two forms of peri-implant infections are analogous to diseases occurring on natural teeth <sup>2</sup>. Peri-implant mucositis corresponds to gingivitis and peri-implantitis to periodontitis. The prevalence of these two infections may differ according to various reports. Peri-implant mucositis affects approximately 80% of patients bearing dental implants, and 50% of the total implants installed. In the case of peri-implantitis, this prevalence can be 28%-56% and 12%-43%, respectively <sup>5</sup>. These numbers however can vary depending on the years that an implant has been in function.

The diagnosis of peri-implant infections is largely based on clinical and radiographic criteria <sup>6,7</sup>. Peri-implant mucositis may not always be revealed by mere inspection of the tissue, because of the lack of light transmission through the metal device. Bleeding of the affected site during examination by use of a periodontal probe is far more reliable. Peri-implantitis lesions in addition to bleeding on probing may present suppuration and exhibit an apical migration of the barrier epithelium as well as bone loss, which is often depicted on radiographs as a characteristic crater-shaped circumferential bony defect around the implant <sup>8</sup>. The progression of peri-implantitis may be more aggressive in some cases, and if not arrested, the developing bone loss may render the implant dysfunctional, eventually leading to complete implant loss <sup>9</sup>.

### **Microbial etiology of peri-implant infections**

Given the infectious nature of peri-implantitis, by the accumulation of a complex biofilm community along the implant surface, it makes sense to try to map out the microbial profile of this disease. However, up to date any hopes to associate specific microorganisms with peri-implantitis in a way that would imply causation have been shattered. This has happened far earlier for periodontitis, and a lot of common lessons apply for both pathological entities. Thus,

the fundamental principle for periodontitis applies equally well for peri-implantitis, i.e. it is an endogenous, polymicrobial, opportunistic infection <sup>10</sup>. This implies that species associated with the destructive disease are part of the normal oral microbiota but under certain ecological shifts, become pathogens, never alone but always acting in concert. It is not the mere presence of specific bacteria instigating the disease rather than the interplay of divergent abundant bacteria from different phyla.

Over the years different methods have been applied to characterize the peri-implant microbiota. Culture-dependent methods have traditionally been the gold standard, but molecular methods have also been introduced to avoid conventional time-consuming laboratory work. As technology improves, sequencing methods, including the latest next-generation sequencing, have emerged to reveal in more detail differences between healthy and diseased oral microbiomes.

This review aims to present the wealth of knowledge we have gained from microbiological methods to characterize peri-implant microbiology and to shed light over potential new benefits as well as limitations of the new sequencing technology in our understanding of peri-implant disease pathogenesis.

### **Lessons from conventional methods**

The development of agar-containing solid culture media for the growth of bacteria more than a century ago was the first and invaluable source of information for elucidating the bacterial etiology of infectious diseases. Regarding peri-implant infections, the earliest studies to reveal the healthy and diseased microbiome around dental implants used culture techniques and phase-contrast microscopy <sup>6, 11-15</sup>. The peri-implant microflora in health consists mainly of Gram-

positive cocci and non-motile bacilli, and a limited number Gram-negative anaerobic species<sup>13</sup>.<sup>16</sup>. Microbial composition of biofilms on healthy implants may be similar to that on the surfaces of periodontally healthy teeth in the adolescent. Early reports favor this aspect, i.e. microbiota at well-maintained implants resembles the microbiota associated with healthy conditions at teeth<sup>14</sup>,<sup>17-20</sup>. The switch to peri-implant mucositis is associated with increased presence of cocci, motile bacilli and spirochetes, a trend which is comparable to gingivitis<sup>21</sup>. The transition to peri-implantitis is the result of a deepened peri-implant pocket and this change of habitat with low oxygen conditions does not favor the growth of aerobic bacteria any more. Culture-based techniques demonstrated the emergence of Gram-negative, black-pigmented, motile, and anaerobic species that are also commonly found in deepened periodontal pockets<sup>13, 22</sup>. Phase-contrast microscopy revealed the presence of spirochetes and motile rods<sup>11, 13, 23, 24</sup>. In another study<sup>25</sup>, by use of latex agglutination test, up to 39% of the peri-implantitis sites were shown to host black-pigmented species whereas up to 17% of the sites hosted *Aggregatibacter actinomycetemcomitans*. This facultative anaerobic species was also detected by culture at peri-implantitis sites<sup>26</sup>. Other studies detected aerobic Gram negative bacilli (AGNB) at peri-implantitis sites<sup>19, 27, 28</sup>, as well as *Candida* spp.<sup>19, 24, 27</sup> and staphylococci<sup>19, 28</sup>.

### **Lessons from molecular methods**

Molecular methods have been adopted around 20-30 years ago in an attempt to speed up microbiological analyses and avoid time-consuming biochemical and physiological tests, as well as the culture of fastidious microbiota.

Polymerase chain reaction (PCR) was the first molecular technique developed in the mid-80s<sup>29</sup> enabling the enzymatic replication of DNA without the use of a living organism. PCR

analysis offers great sensitivity as it allows a small amount of DNA of the microbial sample to be amplified many times in an exponential manner. It has been used for the analysis of microbial samples from dental implants for the detection of *A. actinomycetemcomitans*<sup>30</sup>, *Porphyromonas gingivalis*<sup>30,31</sup>, *Prevotella intermedia*<sup>30,32</sup>, *Fusobacterium* spp<sup>32</sup>.

The DNA-DNA hybridization ('checkerboard') technique belongs to molecular techniques, developed by Socransky and co-workers<sup>33</sup>. It allows for the assessment of large amounts of plaque samples and multitude of species by hybridizing DNA samples against whole genomic DNA probes on a single support membrane. The sensitivity of the 'checkerboard' DNA-DNA hybridization assay is usually set to 10<sup>4</sup> cells of a bacterial species by adjusting the concentration of each DNA probe in the hybridization buffer. Whole-genomic probes can display cross-reactivity with even heterologous species, which may compromise the validity of the results due to false-positive outcomes (low specificity). Hence, probe quality and stringency of the hybridization conditions are critical for the successful diagnostic performance of the method. The 'checkerboard' format has been widely used for peri-implantitis cases, as it is ideal for the analysis of a large number of samples. Various species have been targeted in the relevant studies; from just three<sup>34</sup>, five<sup>35</sup>, eight<sup>36</sup> and 12<sup>28,37</sup> up to 23<sup>38</sup>, 36<sup>39</sup>, 40<sup>4,40-42</sup> and 78<sup>43</sup>.

Notably, the wealth of knowledge on the microbiological profile of peri-implantitis derives from periodontitis, given the fact that the target microbiota for peri-implantitis 'checkerboard' studies were based on the traditional cultural studies performed at periodontitis sites. The majority of the latter studies were able to confirm that the peri-implant pocket shares commonalities in its microbial profile with the periodontal pocket. The cluster of the so-called 'red complex' (*P. gingivalis*, *Tannerella forsythia*, *Treponema denticola*) was found at higher counts at peri-implantitis sites compared to healthy ones<sup>37,39</sup>. The only distinct microbiological

difference between dental implants and teeth emerging from ‘checkerboard’ analysis is the higher count of *S. aureus* at implants than at teeth <sup>41</sup>. In addition, *S. aureus* was found at higher counts at implants with peri-implantitis than at healthy implants <sup>43</sup>.

Importantly, the obvious disadvantage of the DNA-based method is the need to preselect DNA probes for the specific bacterial taxa investigated. This pre-selection inevitably creates a form of bias but more importantly does not leave any chance to investigate the ‘unexpected’ microbiota. In addition, low specificity of the whole-genomic probes, often used in the checkerboard format cannot be neglected due to unavoidable cross-reactivity with uncultivable phylotypes.

### **Lessons from metagenomics**

Metagenomics, mainly including 16S rRNA and shotgun sequencing, is a revolutionary approach to characterize the microbiome as it overcomes serious limitations of conventional and previous molecular DNA-based methods. The time-consuming and costly process for isolation and lab culture of individual species is essentially bypassed <sup>44</sup>. The whole field has its roots in the culture-independent retrieval of 16S rRNA genes, pioneered by Pace and co-workers around twenty years ago <sup>45</sup>. The basic approach was to identify microbes in a complex community by exploiting universal and conserved targets, and in this respect, the bacterial 16S rRNA gene was proven to conceal a wealth of phylogenetic information. Sanger sequencing of 16S rRNA has, nevertheless, the limitation of low-depth coverage. Although able to detect the predominant members of the microbial community, it may not detect the rare members with divergent target sequences. It is highly likely that the distinction between microbiomes in health and disease lies primarily at the strain level, while genes encoding for virulence factors may only be found in a

subset of strains. The relevance of species-level identification is therefore questionable and 16S rRNA sequence has essentially no power at a deeper taxonomic level. Such disadvantages of single gene-based amplicon sequencing can be supplanted by whole-genome shotgun sequencing, offering comprehensive coverage by high throughput, parallel DNA-sequencing platforms. Two studies <sup>46, 47</sup> have applied metagenomic sequencing at a small number of subgingival samples in order to characterize the microbiome in periodontitis. Compared to a 16S-based study <sup>48</sup>, the deep sequencing approach identified, as expected, additional low-abundance genera associated with periodontitis, including *Alistipes*, *Bulleidia*, *Butyrivibrio* and *Parabacteroides*. In addition, screening for functional genes potentially associated with periodontal health and disease was also feasible to perform. A recent systematic review compiling data from culture-independent diagnostic methods concluded that there is literature evidence to support the association of periodontal disease with 17 species or phylotypes from the phyla *Bacteroidetes*, *Candidatus Saccharibacteria*, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, *Synergistetes*, as well as the *Archaea* domain <sup>49</sup>.

It is of interest that culture-independent methods have been recently used to characterize the subgingival microbiota of the “classical” cohort of Sri Lankan tea workers naïve to oral hygiene practices, which has been monitored for over 40 years for their periodontal health status <sup>50</sup>. This study identified that the subgingival microbiota of this population was dominated by *Firmicutes*, *Proteobacteria*, and *Fusobacteria*. Distinctive differences in the overall microbial composition were identified between shallow and deep sites. However, there was no obvious microbial clustering of the subjects according to their respective clinical progression groups, confirming the important role of the individual host response in the progression of the disease.

There are relatively few studies that have employed culture-free techniques to study peri-implantitis-associated flora <sup>51-54</sup>. These have all applied 16S-based methodology and to date no deep metagenomic sequencing analyses of peri-implantitis samples are available. However, emerging evidence from the above-mentioned studies suggests the peri-implant microbiome could be distinct from the periodontal microbiome. It may sound logical that implants and neighboring teeth share similar microbiota since they share a similar ecological niche i.e. interdental space. However, the distinct topography and immunological characteristics of the peri-implant tissues may explain why tooth- or implant-associated biofilms may harbor diverse bacterial lineages.

### **Further projections on metagenomics**

The science of metagenomics is currently in its pioneering stages of development as a field, and current tools and technologies are undergoing rapid evolution. We appreciate the breadth of microbial diversity in oral biofilms, as demonstrated by this technology. Next generation sequencing has revealed an unexpectedly high diversity of the human oral microbiome, reaching up to 19,000 different phylotypes <sup>55-57</sup>. This order of magnitude is much higher than the previously reported 700 to 1000 oral microbial phylotypes, as identified by cultivation or traditional cloning and sequencing <sup>58, 59</sup>. A new era is open for uncultivable bacteria, as for example members of the TM7 phylum, which were previously unknown and their role in the disease process is yet to be understood. Such powerful culture-independent molecular analysis is expected to lengthen the list of bacterial species associated with peri-implantitis and tackle phylogenomic diversity of highly complex microbial populations. Nevertheless, taxonomic

enrichment with different microbial consortia neither necessarily relates to the disease process, nor does it explain the etiopathogenesis of peri-implantitis in a better way.

Beyond the taxonomic details, metagenomics offer functional characterization of bacterial genes at different levels of resolution, identifying a major systemic change between healthy and diseased microbiomes<sup>46</sup>. Disease samples harbor a more diverse microbial community, but they are more similar to each other compared to health samples. Thus, although disease-associated microbiomes are more complex in terms of community structure, this structure is quite similar across different patients. In contrast, the health-associated microbiome exhibits lower taxonomic diversity, but its exact composition varies significantly across patients. This knowledge refers to periodontitis, but it could similarly apply to peri-implantitis. An important technical issue that we still cannot accurately define, is how much plaque sample is needed to obtain a sufficient amount of DNA for sequence coverage and depth. A recommendation is that pooled plaque samples from each individual would be required, especially from patients with periodontal/peri-implant health.

Another great future challenge is the problem of analyzing large amounts of data, stemming from these high-throughput methods. A descriptive presentation of the data at gene level may be informative for other bacterial communities in the soil and other environments but not for the oral cavity. From a phylogenetic aspect, closely related species might have completely different relationships in oral health and disease (i.e. *T. forsythia* associated with disease and the uncultivable *Tannerella* BU063 associated with health) and presentation of such results at genus level would seem obscure. Sufficient computational power and computing infrastructure involving bioinformaticians would be needed in the near future to accommodate the large volumes of data acquired, and explore them at species or even strain level.

An additional consideration in gene finding with metagenomics is that we do not expect all genes to be found in all members of the species. This implies that genes encoding virulence factors may be associated with specific strains of the species. Therefore, for investigations of virulence, cultivable organisms are required. The results of metagenomics need the function verification based on the culture. Metagenomics may provide the target, but the culture-based methods still provide the proof.

## **Conclusions**

The complex microbial composition of implant-associated biofilms is endogenous, part of the resident oral microbiota, including the neighboring teeth. Bacterial culture has been the most studied conventional laboratory method for microbial sample analysis and demonstrated that healthy dental implants are populated by aerobic Gram positive cocci, whereas the transition to disease is associated with Gram negative anaerobic rods. Molecular methods based on PCR and 'checkerboard' facilitated the analysis of large number of samples in a cost-effective way, confirming the results derived from conventional methods. Metagenomics revealed that peri-implant microbiota is far more diverse than previously thought and uncultivated species have also been associated with the disease. In addition, we are now aware of the fact that the number of oral commensal taxa hosted on healthy dental implants is expected to further increase in the future. Large efforts are still needed to understand the role of microbial communities in health and disease by use of these contemporary microbial methods.

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