CLINICAL ORAL IMPLANTS RESEARCH

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Assessment of periodontal and opportunistic flora in patients with peri-implantitis

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Abstract

Aim: To assess the presence of periodontal and opportunistic organisms in patients with peri-implantitis.

Material and methods: Thirty-three partially edentulous subjects (22 women, 11 men), aged 32–90 years, who had one or more implants with peri-implantitis were included. Peri-implantitis was defined as: (i) the presence of bleeding on probing and/or suppuration and (ii) radiographic images showed marginal bone loss >1.8 mm after 1 year in function. Criteria for inclusion were: (i) partially edentulous patients having at least one implant diagnosed with peri-implantitis; (ii) no antibiotic therapy for 6 months prior to clinical examination. Following this definition, a total of 48 implants were diagnosed with peri-implantitis. Subgingival bacterial samples were obtained with sterile paper points from infected implants and selected teeth of each individual. Periodontopathogens (*Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia* and *Treponema denticola*) were detected by multiplex PCR targeting 16S rDNA. Samples were placed in reduced transport medium and cultured for opportunistic pathogens (*Staphylococcus aureus, enteric bacteria, Pseudomonas and yeasts*).

Results: Twenty-two patients yielded positive results for *P. gingivalis*, 25 for *T. forsythia,* eight for *P. intermedia* and 13 for *T. denticola*. None of the patients yielded a positive result for *A. actinomycetemcomitans*. Non-periodontal species were found in five patients (15% of total). *P. aeruginosa* was found in four (12%) patients, and *C. albicans* (3%) and *S. aureus* in one patient (3%) each. In two cases of peri-implantitis, none of the periodontal or opportunistic microorganisms studied were detected in either implant or tooth samples. When results of the periodontopathic bacteria from the implant and tooth samples of the same patient were compared, 18 patients (54%) showed the same results for both samples and 15 (45%) patients different results.

Conclusions: The implant surface may be colonized with pathogens different from periodontal bacteria. Opportunistic pathogens such as *P. aeruginosa, S. aureus* and *C. albicans* may be associated with implant failure.

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The inflammatory lesions that develop in the tissues surrounding implants are collectively recognized as peri-implant diseases and include two disease entities: mucositis and peri-implantitis. Peri-implantitis is defined as an inflammatory lesion of bacterial aetiology, characterized by the loss of supporting bone, as well as inflammation of the mucosa (Albrektsson & Isidor 2004; Zitzmann & Berglundh 2008). The prevalence of peri-implantitis depends on the clinical threshold used to define it, recent results varied between 6.6% and 47% (AAP Board of Trustees 2013).Poor oral hygiene, a history of periodontitis and cigarette smoking have all been identified as risk indicators for peri-implant disease.

A history of periodontitis or current periodontitis in the remaining natural teeth may have a significant impact on peri-implant

microbiota. Bacterial colonization in implants is similar to that in teeth and may be due to the transmission of periodontal pathogens from the residual dentition to the implant (Apse et al. 1989; Kohavi et al. 1994; Mombelli et al. 1995). Various analytical methods have found that the microbiota associated with peri-implant disease is mixed, somewhat variable and, in most cases, dominated by diverse Gram-negative anaerobic bacteria, as is the case with chronic periodontal disease (Mombelli et al. 2012). *Tannerella forsythia* (Tf), *Porphyromonas gingivalis* (Pg), *Treponema denticola* (Td), *Prevotella nigrescens, Prevotella intermedia* (Pi), *Fusobacterium nucleatum, Campylobacter* spp, *Parvimonas micra and Aggregatibacter actinomycetemcomitans* (Aa) have all been isolated around failing implants both in patients who are completely edentulous (Adell et al. 1986; Mombelli et al. 1988; Bower et al. 1989; Hultin et al. 2002; Quirynen et al. 2005; Devides & Franco 2006) and partially edentulous (Mombelli et al. 1987; Leonhardt et al. 1999; Botero et al. 2005; Shibli et al. 2008; Tabanella et al. 2009).

Several studies have indicated the possibility that some cases may harbour microorganisms that are not frequently found among oral flora, such as *Staphylococcus aureus*, Enterobacteriaceae, *Candida albicans*, *Pseudomonas aeruginosa* (Alcoforado et al. 1991; Leonhardt et al. 1999; Renvert et al. 2008; Salvi et al. 2008). All of these associated only very rarely with periodontal disease but are able to successfully attach themselves to titanium surfaces (Truong et al. 2010; do Nascimento et al. 2013). In addition, these pathogens have been found to cause orthopaedic device-related infections, such as early mandibular osteomyelitis after implant surgery (Rokadiya & Malden 2008). Current information about the prevalence of opportunistic pathogens is limited because most studies focus on anaerobic gram-negative bacteria. The aim of this study was to assess the presence of periodontal and opportunistic organisms in patients with peri-implantitis and to compare the microbiological findings of implants and natural teeth.

Material and methods

Patient selection

Patients were recruited over a 2-year period (May 2010 till May 2012). Thirty-three partially edentulous subjects (22 women, 11 men) with 225 titanium implants, aged between 32 and 90 years old, were included in the study. Patients were recruited from the Department of Periodontics at the University of Seville Dental School and from a private practice in Barcelona, both Spain. Peri-implantitis was defined as: (i) the presence of bleeding on probing and/or suppuration; and (ii) a radiographic image that showed marginal bone loss of more than 1.8 mm (corresponding to three threads of implant with 0.6 mm thread pitch) after 1 year in function. The criteria for inclusion were: (i) partially edentulous patients having at least one implant with a diagnosis of periimplantitis; (ii) no antibiotic therapy for 6 months prior to the clinical examination. Case was defined as a subject with untreated periimplantitis.

A total of 48 implants were selected for the study, all screw-shaped implants with a rough surface. The Ethics Committee at the University of Seville approved the trial and all patients gave written informed consent before the study commenced.

Clinical procedures

Information regarding medical history and smoking status was collected. Clinical parameters, such as the presence of bleeding on probing (BOP) and pocket probing depth (PPD) were recorded. PPD measurements, obtained using a North Carolina periodontal probe (PCPNU-15, Hu-Friedy, Chicago, IL, USA) at six sites (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) of every implant and tooth, were recorded to the nearest millimetre. Machtei criteria (CAL $\geq$ 6 mm in two or more teeth; one or more sites with PPD $\geq$ 5 mm) were used to diagnose periodontitis (Machtei et al. 1992). The deepest PPD for each implant was recorded.

Microbiological procedures

Supragingival plaque was removed using sterile cotton pellets. Sampling sites were isolated using cotton rolls and each one gently dried using an air syringe. Subgingival samples were obtained by inserting a sterile paper point into the deepest part of the periodontal pocket and kept in place for 60 s. For every patient, two paper point samples were taken from two teeth with the deepest pockets and placed in the same vial for PCR detection. Two additional paper point samples were taken from every peri-implant site and placed in individual vials with selective media for culture and PCR. In the case of a patient with more than one implant with peri-implantitis, samples were taken for each implant and processed individually. Although results were counted as a sample pool. The samples were sent within 48 h to the Department of Microbiology, School of Medicine, University of Seville.

Periodontopathogen bacteria were considered *A. actinomycetemcomitans*, *P. gingivalis, P. intermedia, T. forsythia* and *T. denticola* according to Consensus report of the 5th European Workshop in Periodontology (Sanz & Quirynen 2005). Our detection method has a threshold of 10^4 CFU/ml, according to manufacture' s recommendations, so a positive PCR reaction in our study agrees with a high level of periodontopathogens, avoiding considering as aetiological agents those yielded at low amount. Opportunistic bacteria were considered potential pathogens that are not traditionally accepted to be important in periodontal diseases, but are important in extraoral infections such as gramnegative non-fermentative rods, enteric bacteria, staphylococci and yeasts.

The presence of *A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythia and T. denticola* was determined by PCR. DNA from the samples was extracted using the DNeasy Spin Column kit (QIAGEN, Düsseldorf, Germany), in accordance with the manufacturer's instructions. 16S rDNA amplification was carried out using multiplex PCR (microIDENT®, Hain Lifescience, Hehbren, Germany). Reverse hybridization was performed in accordance with microDent® kit instructions.

The presence of *Staphylococcus aureus,* enteric Gramnegative bacteria*, Pseudomonas spp,* and *Candida albicans* was evaluated by traditional microbiological culture. Samples were placed in liquid dental transport (AS-916 Anaerobe Systems®, Morgan Hill, CA, USA) with reducing agents designed as a holding medium for maintaining the viability of the microorganisms. In the laboratory, the liquid transport was inoculated into various media (Chromagar, Mannitol salt agar, McConkey agar and 5% sheep blood Mueller Hinton agar) to detect growth of *C. albicans, S. aureus,* Enterobacteriaceae and *Pseudomonas* spp. Plates were incubated at 35–37°C for 48 h.

Subsequently, biochemical tests were carried out for identification purposes: catalase test, coagulase tube test, and agglutination with Staphaurex Plus (Remel Europe Ltd., Kent, UK) for staphylococci; catalase test, esculine test and API Strept (BioMérieux, Mercy l'Etoile, France) for streptococci; catalase test, oxidase test and API 20NE (BioMérieux) for enteric gramnegative nonfermentative bacteria; and API ID32C for yeasts

(Versalovic et al. 2011). Susceptibility studies were carried out by disc diffusion test, following CLSI guidelines.

Results

A total of 33 cases with 48 affected implants with peri-implantitis were recorded; 11 (33.3%) were males and 22 females (66.6%). The mean age was 67.1 years (range 20–85 years). All patients (100%) were diagnosed with untreated periodontitis, and 10 (30.3%) of the subjects were smokers. The implants studied had been in function for a mean of 4.5 years (range 2–9.5 years). There were similar numbers of implants with peri-implantitis in mandibles (22/48) and maxillae (26/48,) with more in the posterior sextants (43/48) compared to the anterior (5/48). Regarding the clinical parameters, mean PPD at implant sites was 6.58 mm (range 4–10 mm). 76.9% of teeth and 97.9% of implant sites showed BOP. Suppuration was present at 54.1% of implant sites and 7.69% of tooth sites.

Nineteen (57.5%) patients, with a total of 124 implants, had one implant with peri-implantitis, and 14 (42.5%) patients, with a total of 106 implants had two or more with periimplantitis. Twenty-two (67%) patients yielded a positive result for *P. gingivalis;* 25 (75%) for *T. forsythia;* 8 (24%) for *P. intermedia;* and 13 (39%) for *T. denticola* (Table 1). None of the patients gave a positive result for *A. actinomycetemcomitans*. The two most prevalent associations found were *P. gingivalis* + *T. forsythia* and *T. forsythia* + *T. denticola*, which were recovered in 11 (33%) and 8 (24%) patients, respectively.

When the results of the implant and teeth samples were compared, no significant differences were found between implants and teeth for the prevalence of the periodontopathic bacteria, *P. gingivalis, T. forsythia*, *P. intermedia* or *T. denticola*, whether considered individually (Table 1) or in different combinations.

Staphylococcus aureus, P. aeruginosa and *C. albicans* were only studied in implants and they were found in five (15%) patients. *P. aeruginosa* was found in four (12%) patients, and both *C. albicans* (3%) and *S. aureus* (3%) in one patient each. These pathogens were detected along with the periodontal bacteria in four out of the total of five patients; in one case, *P. aeruginosa* was the only bacterial pathogen recovered from an implant that had no periodontal bacteria. In two cases of peri-implantitis, none of the periodontal or opportunistic microorganisms studied were detected in the samples taken from either implants or teeth.

All *P. aeruginosa* isolates were shown to be susceptible to ciprofloxacin, ceftazidime and aminoglycosides, and the *S. aureus* isolate was also susceptible to methicillin.

When the periodontopathic bacteria from the implant and tooth samples of the same patient were compared, 18 patients (54%) showed the same results in both sets of samples, and 15 (45%) showed different results; thirteen (39%) patients showed a positive result for periodontal pathogens in the implant samples but a negative one for tooth samples. On the other hand, in just two patients (6%), the sample taken from the teeth was positive, whereas the implant sample was negative (Table 2).

Bleeding on probing was positive in all implants except in one of them. Concerning PPD, very similar mean values were obtained when positive or negative periodontal flora were recorded (Table 3).

A third of patients (10 cases) were smokers. *P. gingivalis* and *T. denticola* were more prevalent in patients who smoked, in 90% and 60% of samples, respectively, than in those who were non-smokers, in 69% and 34% of samples, respectively. On the other hand, *T. forsythia* and *P. Intermedia* were more frequently recovered from non-smokers, 69% and 47%, respectively, than from smokers, 60% and 30%, respectively. With respect to opportunistic microorganisms, *P. aeruginosa* was more prevalent in smokers than in non-smokers, 20%, vs. 9%, respectively. *S. aureus* was found only in one smoker (10%), whereas *C. albicans* was found only in one non-smoker (4%). These differences were not statistically significant.

Table 1. **Comparative analysis of periodontal flora found in implant and tooth samples (33 patients)**

Microbiological findings	Implant samples No. of cases (%)	Tooth samples No. of cases (%)
Occurrence of each pathogen		
Pg	22 (66)	16 (48)
Tf	25 (75)	20 (60)
Pi	8 (24)	9 (27)
Td	13 (39)	9 (27)
Occurrence of combinations		
Pg alone	5 (15)	3 (9)
Tf alone	0	2 (6)
Pg+Tf (with or without Pi)	11 (33)	9 (27)
Pg+Tf+Td (with or without Pi)	6 (18)	3 (9)
Tf+Td (with or without Pi)	8 (24)	5 (15)
Td+Pi	0	1 (3)

Pg: *Porphyromona gingivalis,* Tf: *Tannerella forsythia*, Pi: *Prevotella intermedia*, Td: *Treponema denticola*.

Discussion

We evaluated the possibility that the peri-implant pockets might be colonized by pathogens other than anaerobic periodontal flora. *In vitro* studies have identified the high affinity of *P. aeruginosa* (Truong et al. 2010), *S. aureus* (Truong et al. 2010) and yeast (do Nascimento et al. 2013) for titanium surfaces. Some of these pathogens can cause early post-surgical infections (Cobo et al. 2011; D'Ovidio et al. 2011). The present study showed a significant prevalence (five cases) of the opportunistic flora targeted; indeed, in one of these five cases, they were the only microbiological finding. It should also be highlighted that in another two cases, no periodontal or opportunistic flora was isolated at all. Whilst the majority of studies have recovered anaerobic periodontal microbiota from around implants and teeth (Renvert et al. 2008), there is very little available data about the prevalence of species such as gram-negative enteric rods, *P. aeruginosa, S. aureus* or *Candida* sp. Early transmission of periodontal pathogens from periodontal to implant sites has been demonstrated months after implant placement (van Winkelhoff et al. 2000; De Boever & De Boever 2006). Consequently, although some studies have indicated the possibility that some peri-implantitis cases may harbour microorganisms that are rarely found in oral flora, such as *S. aureus, Enterobacteriaceae, C. albicans, P. aeruginosa*, most have focused on periodontal flora using both molecular and culture-based methods.

Pseudomonas aeruginosa was the most prevalent of the opportunistic bacteria found in our study. A case of *S. aureus* was also detected, although we did not recover species such as *S. epidermidis* or enteric gram-negative rods. Other studies, in contrast, have

Table 2. **Discordant findings between implant and tooth samples taken from the same patient**

Microbiological findings	Positive implant samples/Negative tooth samples No. of cases (%)	Negative implant samples/Positive tooth samples No. of cases (%)
Pg alone	3 (20)	0
Pg+Tf (with or without Pi)	3 (20)	0
Tf+Td (with or without Pi)	6 (40)	1 (7)
Pg+Tf+Td (with or without Pi)	1 (7)	0
Tf (with or without Pi)	0 (0)	1 (7)

Pg: *Porphyromona gingivalis,* Tf: *Tannerella forsythia*, Pi: *Prevotella intermedia*, Td: *Treponema denticola*.

Table 3. **Mean PPD value in each implant and the presence of periodontal flora**

	Pg	Pi	Tf	Td	Pa	
Mean PPD value (mm)	6.0	6.32	6.43	6.20	6.51	**Negative**
	6.70	6.93	6.48	6.88	6.20	**Positive**

Pg: *Porphyromona gingivalis,* Pi: *Prevotella intermedia,* Tf: *Tannerella forsythia*, Td: *Treponema denticola, Pa: Pseudomonas aeruginosa.*

found a high prevalence of enteric rods such as *Enterobacter* sp. or *Klebsiella* sp. (Leonhardt et al. 1999; Botero et al. 2005) or coagulase-negative staphylococci and *Candida* spp. (Rosenberg et al. 1991). In a recent comparative analysis of 166 individuals with peri-implantitis and 47 individuals with healthy dental implant conditions, both *P. aeruginosa* and *S. aureus* were found more frequently in peri-implantitis samples, alongside classic periodontopathogens such as *T. forsythia* and *A. actinomycetemcomitans* (Persson & Renvert 2013). *P. aeruginosa* is a highly successful opportunistic pathogen that displays intrinsic multidrug resistance and has a tremendous capacity for acquiring extra resistance mechanisms (Kiska & Gilligan 2003). According to the literature (Lang et al. 2004), amoxicillin/clavulanate and metronidazole are recommended for the treatment of peri-implantitis. Clinical trials have shown a good efficacy of amoxicillin plus metronidazol combination in peri-implant infections therapy. Those antibiotics are not active against *P. aeruginosa* or *Candida* spp. Therefore, further studies are needed to assess the role of potential pathogens that are not considered to be important in periodontal diseases, but are important in extra-oral titanium-implants associated infections.

In addition, chlorhexidine, which is in general use for treating peri-implantitis, may present some problems, because it is not effective and can enhance the growth of *P. aeruginosa* at some low concentrations (Morales-Fernández et al. 2014). A microbiological analysis should be considered when designing peri-implantitis therapy and further studies are needed to assess the role of opportunistic flora in these infections.

In the majority of cases (93%), periodontal flora was found colonizing implants. We observed that *T. forsythia* was the most prevalent, followed by *P. gingivalis* and *T. denticola*, which is consistent with recent periodontal models. Laine, using a decision tree as a potential modelling tool for periodontitis, showed that the simultaneous detection of these pathogens is associated more with periodontitis than a single bacterial species (Laine et al. 2013). It is very interesting that, in some patients (39%), periodontal bacteria were detected only in implants. The tissues around implants react poorly to oral colonization by pathogens (Heitz-Mayfield 2008), so that the bacterial count would be expected to be higher in implant samples than in teeth, as our study demonstrates. The limit of detection of the PCR method used in our study was 10^4 CFU/ml, following manufacturer's recommendations, and the bacterial load from tooth samples was below this threshold.

The limitations of this study include the absence of information about the colonization of teeth by non-oral pathogens. Our aim was to assess the presence of microorganisms other than periodontal flora in implant samples, and the purpose of the samples taken from teeth was simply to evaluate the periodontal status of the patients. The strength of our study is that it included the use of a culture-based method to recover opportunistic bacteria, unlike most previous studies, which used detection methods based on DNA-DNA hybridization.

Conclusions

The surface of implants could be colonized with pathogens different from periodontal bacteria. Further clinical studies are needed to assess the role of opportunistic pathogens such as *P. aeruginosa, S. aureus* and *C. albicans* in failing implants.

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Conflict of interest

The authors have no conflict of interest to declare.

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