Case Control Study to Evaluate the Role of Candida, Staphylococcus and Enterococccus Species in Peri-implant Infections in Irradiated Patients after Tumour Surgery and Non-irradiated Patients

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Abstract

Background: The purpose of the study was to evaluate the micro-flora of healthy (P-) and infected implant sites (P+) in patients with a former radiation therapy after tumour surgery (R+) in comparison with patients without history of irradiation in the head and neck region (R-), focusing on *Candida, Staphylococcus and Enterococcus* species.

Methods: Patients with healthy implant sites (n=14; group I: P-; R-); with clinical signs of peri-implant infections (n=13; group II: P+; R-); with healthy implant sites after irradiation (n=7; group III: P-; R+) and with clinical signs of peri-implant infections after irradiation (n= 6; group IV: P+; R+) took part in this case control study. An oral assessment was performed for each patient, including: plaque index, sulcular bleeding-index, pocket probing depth, and bone loss. Samples out of the peri-implant sulcus have been used to identify periodontal pathogens, *Candida*, *Staphylococcus* and *Enterococcus* species, and testing their resistance to antibiotics/antimycotics.

Results: The most periodontal pathogens, especially *Tannerella forsythia* and *Fusobacterium nucleatum/periodonticum*, were found in patients of group II (P+; R-). *Candida*, *Staphylococcus* and *Enterococcus* species were detected in all patients groups. Multi-resistant *Candida* and *Enterococcus* species were found independently of the group of patients, however no multi-resistant *Staphylococci* could be seen.

Conclusions: Peri-implant infections occurred in patients with a former radiation therapy, but the number and composition of periodontal pathogens are lower compared with patients without irradiation. Independently of clinical signs of peri-implant infections *Candida*, *Staphylococcus* and *Enterococcus* species were present in all patients groups in the peri-implant sulcus, but multi-resistance was only detected in low numbers. It puts the role of these bacteria and yeast in question, since they were found in all patients groups.

Key Words: Peri-implantitis, Healthy implant sites, Radiation therapy, Candida, Staphylococcus and Enterococcus species, Multi-resistance

Introduction

Peri-implantitis is defined as a condition of inflamed peri-implant soft tissue associated with a loss of supporting bone around an implant in function [1,2]. In patients the prevalence of peri-implantitis varies between 28% and 56% and in implant sites between 12% and 43% [3-5]. In patients with radiation therapy of the head and neck region in their past medical history similar numbers of peri-implant infections (12%) have been described in their medical history like in patients without radiation therapy [6]. A cause and effect relationship between biofilm formation on implants and peri-implant mucositis, the reversible infection of the implant surrounding soft tissue, could be demonstrated by Pontoriero *et al.* [7] and Zitzmann *et al.* [8]. The peri-implant mucositis can lead to peri-implant infections with irreversible loss of bone. The micro-flora in infected implant sites is dominated by Gram-negative obligate anaerobic rods, fusiform bacteria and spirochetes, like species of *Porphyromonas*, *Tannerella* or *Treponema* [9,10]. Thereby, healthy implant sites normally are populated by high proportions of Gram-positive coccoid bacteria [11-15]. However, several studies showed that occasionally *Candida* species, *Staphylococcus* species and *Enterococcus* species are part of the peri-implant flora in infected peri-implant sites [10,16-21]. It is not well examined, if the micro-flora of infected and healthy implant sites differs in patients with and without former radiation therapy.

However, a history of radiation therapy was detected as a factor influencing the development of clinical signs of peri-implant mucositis. But irradiation in the past failed to be a relevant factor for the detection of periodontal pathogens at the implant site [22]. *Candida, Staphylococcus and Enterococcus* colonization can be found in the oral cavity of patients after irradiation in higher amounts than in patients without radiotherapy [23]. If these micro-organisms also occur more frequently in the peri-implant sulcus in peri-implant infections in patients after radiation therapy is not known. Independent of a former radiation therapy, in patients with peri-implantitis the role of S*taphylococcus* species*, Enterococcus* species and *Candida* species remains also still unknown. No data on the susceptibility of these bacteria and yeasts of the peri-implant sulcus to antibiotics, respectively antimycotics, are available.

The purpose of the present study was to evaluate the micro-flora of patients with healthy and peri-implant infected implant sites with and without a former radiation therapy in the head and neck region after tumour surgery. The main focus has been the detection of *Candida*, *Staphylococcus* and *Enterococcus* species in the peri-implant sulcus to clarify the role of these pathogens. In addition these species were tested for their

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the QIA quick PCR Purification Kit (Qiagen) according to the manufacturer's instructions, and the DNA concentrations were determined using a NanoVue system (GE Healthcare). The copy number was calculated and serial 10- fold dilutions were made in the range of 1×10^1 to 1×10^7 copies [29].

After identification the isolates as well as the test bacteria were mixed with top agar and poured onto agar plates to determine possible resistance of the strains against specific antibiotics and antimycotics. The susceptibility of the cultures was tested against amoxicillin, amoxicillin/clavulanic acid, ampicillin, ampicillin + sulbactam (2:1), penicillin, azithromycin, linezolid, and minocyclin in the concentration of 0.016-256 µg/ ml and moxifloxacin in the concentration of 0.002-32 µg/ml using the Etest®® (AB BIODISK, Dalvägen, Solnam Sweden). The yeasts were tested against amphotericin B, ketoconazol and voriconazol in a concentration of 0.016-32 µg/ml and fluconazole in a concentration of 0.016-256 µg/ml. The minimum inhibitory concentration (MIC) was measured after 24-48 hours of incubation according to the manufacturers' instructions. The MIC values have been divided into susceptible, intermediate susceptible or resistant using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines as reference.

Statistical Analysis

Data collection, data management and data analysis were performed with the statistical software package SPSS® Version 21. For the qualitative variables, absolute and relative frequencies were calculated. Values were given as mean and standard deviation. The Kruskal Wallis test and Mann-Whitney U test was used to test for possible statistical significance. A p value <0.05 was considered as statistically significant.

Results

Patient collective

Out of the study sample of 40 patients, 14 patients (35%) belonged to group I (P-; R-), and 13 patients (33%) to group II (P+; R-). Seven patients (18%) have been assigned to group III (P-; R) and six patients (15%) to group IV (P+; R+). Looking at the age and the gender of the patients, there was no difference of the patients collective comparing the four groups. The average length after the prosthetic reconstruction differed between the groups with and without radiation therapy. In patients with a former radiation therapy (group III, and group IV) the restoring time was much shorter than in the other groups (p = 0.002). Most smokers belonged to group II without a statistical significance *(Table 1)*.

Description of peri-implant pathogens present at the implant site in the different patient groups

Periodontal pathogens were found in 7/14 (50%) implant sites of group I (P-; R-), and in 12/13 (92%) implant sites of group II (P+; R-). Whereas in patients with a former radiation therapy in the past in 2/7 (29%) implant sites (group III: P-; R+), respectively in 4/6 (67%) implant sites (group IV: P+; R+) periodontal pathogens could be detected *(Table 2)*.

Table 1. *Patients collective.*

	Group P^-R^- (n = 14)	**Group P^+R^- (n = 13)**	**Group P^-R^+(n = 7)**	**Group P^+R^+(n = 6)**
Age	68 ± 4.61; (63-81)	68 ± 7.25; (56-79)	65 ± 13.50 (54-75)	68 ± 12.29 (53-81)
Gender (male/female)	6/8	4/9	6/1	3/3
Average length after prosthetic reconstruction (years)	14 [11-15]	14 [11-16]	2 [2-10]	4 [2-9]
Radiation dosage (Gy)	0	0	60 [60-60]	60 [60-60]
Smoking (number of patients)	3	7	2	1

Table 2*. Clinical signs of peri-implant inflammation at the implant site, where the microbiologic samples were obtained.*

	Group P^-R^- (n = 14)	**Group P^+R^-(n = 13)**	**Group P^-R^+(n = 7)**	**Group P^+R^+(n = 6)**
PI positive	11 (79%)	13(100%)	3 (43%)	6 (100%)
BOP positive	3 (21%)	13 (100%)	2 (29%)	6 (100%)
PD (mm, mean, SD)	2.6 (± 0.9)	5.5 (± 1.5)	2.6 (± 1.1)	5.2 (± 0.4)
BL (mm, mean, SD)	0.94 (± 1.37)	2.31 (± 1.57)	0.55 (± 0.60)	1.18 (± 1.04)

Table 3. Number of patients with positive micro-organisms on the implant sites and range of the concentration of the micro-organisms.

	Group P^-R^-(n = 14)	**Group P^+R^-(n = 13)**	**Group P^-R^+(n = 7)**	**Group P^+R^+(n = 6)**
Aggregatibacter actinomycetemcomitans	0	1 (= 10^4)	0	0
Porphyromonas gingivalis.	2 (< 10^5 - < 10^6)	4 (= 10^4 - < 10^6)	0	1 (= 10^4)
Tannerella forsythia	0	4 (= 10^4 - < 10^6)	0	0
Treponema denticola	0	3 (= 10^4 - < 10^6)	0	0
Prevotella intermedia	0	3(= 10^4 - > 10^7)	0	0
Micromonas micros	3 (< 10^5)	7 (= 10^4 - < 10^6)	0	1(= 10^4)
Fusobacterium nucleatum	6 (= 10^4 - < 10^6)	12 (< 10^5 - > 10^7)	2 (< 10^5 - < 10^6)	4 (= 10^4-< 10^5)
Campylobacter rectus	1 (< 10^6)	2 (< 10^6)	0	0
Eubacterium nodatum	0	1 (= 10^4)	0	0
Eikenella corrodens	5 (= 10^4 - < 10^6)	6 (= 10^4 - < 10^6)	0	2 (= 10^4)
Capnocythophaga spp.	4 (= 10^4 - > 10^7)	4 (= 10^4 - < 10^6)	1 (< 10^6)	0

Table 4. *Numbers of bacteria and yeast with a positive growth on the selective culture medium.*

	Group P^-R^-(n = 14)	**Group P^+R^-(n = 13)**	**Group P^-R^+(n = 7)**	**Group P^+R^+(n = 6)**
Candida species	2 (14%)	3 (23%)	1 (14%)	3 (50%)
Staphylococcus species	2 (14%)	1 (8%)	1 (14%)	1 (17%)
Enterococcus species	11 (71%)	10 (69%)	6 (71%)	5 (67%)

Table 5. *Specific primer and samples and optimized temperature conditions for PCR.*

PCR assay (amplicon size, annealing temp)	Oligonucleotide Sequence (5'-3')	Reference
Staphylococcus aureus (279 bp, 64°C)	Forw: 5'-GATTGATGGTGATACGGTT-3' Rev: 5'-AGCCAAGCCTTGACGAACTAAAG-3'	PMID: 1629319 [35]
Staphylococcus epidermidis (125 bp, 60°C).	Forw:5'-ATCAAAAAGTTGGCGAACCTTTTCA-'3 Rev: 5'-CAAAAGAGCGTGGAGAAAAGTA-'3	PMID: 1629319 [35]
Candida glabrata (127 bp, 60°C)	Forw: 5'-AAGAAGGCTGCCTGTTGTAATG'-3 Rev: 5'-AACCAAGTATGCAGGGTCTGTT-'3 Forw: 5'-	PMID: 11526177 [36]
Candida albicans (273 bp, 58°C)	TTTATCAACTTGTCACACCAGA-'3 Rev: 5'-ATCCCGCCTTACCACTACCG-'3 Forw: 5'-	PMID:15713607 [37]
Enterococcus faecalis (357 bp, 60°C)	AACCTACCCATCAGAGGG-'3 Rev: 5'-GACGTTCAGTTACTAACG-'3 Forw: 5'-	PMID: 15184159 [38]
Enterococcus faecium (75 bp, 62°C)	TTCTTTGCTTTATCCGATGT-'3 Rev: 5'-CGGTTTTCTGCTTTTGTAAT-'3	PMID: 14742209 [39]
Enterococcus spp. (144 bp, 68°C)	Forw:5'-CCCTTATTGTTAGTTGCCATCATT-'3 Rev: 5'-ACTCGTTGTACTTCCCATTGT-'3	PMID: 15546407 [40]
Universal U16S: (170 bp, 60°C)	Forw: 5'-TTAAACTCAAAGGAATTGACGG-'3 Rev: 5'-CTCACGRCACGAGCTGACGAC'-3	PMID: 15848151 [41]

Forw = sense primer; Rev = anti-sense primer; PMID = PupMed identifier

The highest amount of periodontal pathogens could be found in patients with peri-implantitis without radiation therapy (P+; R-; p = 0.007; *Table 3*). The periodontal pathogens *Tannerella forsythia* (*T.f.*) (p=0.029) and *Fusobacterium nucleatum/periodonticum* (*F.n.*) (p=0.018) dominated.

Description of Candida, Staphylococcus and Enterococcus species

Candida species were found in nine samples (*Candida albicans* n=8; *Candida glabrata* n=1). *Staphylococcus* species were found in five samples *(Staphylococcus epidermidis* n=2; *Staphylococcus aureus* n=3). *Enterococcus* species were found in 29 samples (*Enterococcus faecium* n=7; *Enterococcus faecalis* n=12; *Enterococcus species* n=10) taken from the peri-implant sulci. *Candida, Staphylococcus* and *Enterococcus* species were detected in all four of the patients groups, in 8% -71% of the patients (Table 4).

Susceptibility of the Candida, Staphylococcus and Enterococcus species

Eight *Candida* species 8/9 (89%) were resistant against FL, VO and KE (256 µg/ml). Out of these eight strains, one strain was susceptible to AP (*Candida albicans*), four strains showed intermediate susceptibility and three have been resistant to AP (two *Candida ablicans*; one *Candida glabrata*). Therefore, these three strains showed multi-resistance against the tested antifungal agents. The test strain *Candida albicans* was susceptible against the tested antimycotics and *Candida glabrata* was susceptible against AP, VO and intermediate susceptible against FL, and KE.

Two *Staphylococcus epidermidis* and one *Staphylococcus aureus* were resistant (256 µg/ml) to azithromycin. The test strains S*taphylococcus aureus* and *Staphylococcus epidermidis* were susceptible to the nine tested antibiotics.

4/32 (13%) *Enterococcus* isolates showed a resistance against the tested antibiotics (two *Enterococcus faecalis,* two *Enterococcus* species). They were identified and treated as multi-resistance strains. The two test strains (*Enterococcus faecalis*, *Enterococcus faecium*) have been susceptible to the tested antibiotics.

No difference of susceptibility of the tested *Candida, Staphylococcus* and *Enterococcus* species against the antibiotics, respectively antimycotics was present in the four groups of patients *(Table 5).*

Discussion

The peri-implant sulcus of patients with a peri-implant infection harbored more periodontal pathogens with higher total numbers of bacteria in contrast to patients with healthy implant sites. Thereby in patients with a past radiation therapy periodontal pathogens were detected in lower levels in the peri-implant sulcus, independent of clinical signs of peri-implant infections on the implant site. *Candida*, *Staphylococcus* and *Enterococcus* species were found in the peri-implant sulcus in all four of the patients groups. In low numbers multi-resistant *Candida* and *Enterococcus* species occurred in the peri-implant sulcus, independent of clinical signs of a peri-implant infection or irradiation.

The finding of high proportions of periodontal pathogens at the implant site in patients with peri-implantitis without irradiation is comparable to the study of da Silva *et al.* (2013) and others [9,11,12]. Da Silva *et al.* (2013) used PCR amplification of universal 16S rRNA to identify bacteria from healthy and infected implant sites out of the supragingival

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