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Assessment of *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* Counts around Healthy Implants, Diseased Implants and Sound Teeth: A Preliminary Study

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Abstract

Background. This study aimed to assess *Aggregatibacter actinomycetemcomitans* (Aa) and *Prevotella intermedia* (PI) counts in gingival crevicular fluid (GCF) around healthy implants, diseased implants and sound teeth.

Methods. Eight patients (four males and four females), who had healthy implants, implants with peri-implantitis and sound teeth, were selected. Samples (GCF) were analyzed using real-time polymerase chain reaction (RT-PCR). The above-mentioned bacteria were detected and counted. Data analysis in RT-PCR was carried out based on the standard curve using Prism software to compare Pi and Aa counts between the three areas (GCF around sound teeth, healthy implants and implants with peri-implantitis).

Results. Pi counts were significantly higher in GCF around implants with peri-implantitis (8 implants) than around healthy implants (8 implants) ($P<0.001$) and sound teeth (8) ($P=0.012$). No significant differences were found in Pi counts in GCF around healthy implants and sound teeth ($P=0.063$). Aa counts in GCF around implants with peri-implantitis were significantly higher than those around healthy implants ($P=0.002$) and sound teeth ($P=0.024$). No significant differences were noted in Aa counts in GCF around healthy implants and sound teeth ($P=0.57$).

Conclusion. Aa and Pi counts in GCF around diseased implants were higher than around healthy implants and sound teeth. Also, Aa counts were significantly higher than Pi counts.

Key words: **Dental implant, peri-implantitis, tooth, gingival crevicular fluid, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, bone loss.**

Introduction

The recent increase in demand for dental implant treatment is attributed to several factors, including but not limited to the increased life expectancy in developing and developed countries, consequences of the failure of fixed partial dentures, poor performance of removable partial dentures and enhanced public knowledge.[1]

Dental implant treatment has a high rate of success; however, it suffers some limitations as well and there is always a risk of bacterial infection of the implant surrounding tissues and subsequent inflammatory reactions.[1] Inflammation of the gingival margins around implants is similar to gingivitis and periodontitis. The pattern of dental plaque accumulation also resembles that around natural teeth. Bacterial colonization of the implant surface occurs a few minutes following its exposure to the oral cavity.[2] In case of accumulation of high load of microbial plaque on the implant surface, neutrophils and the epithelial barrier are no longer adequate for infection control and consequently, inflammation develops in the peri-implant tissue; this condition is clinically referred to as peri-implant mucositis.[3] Pathogenic microorganisms migrate from natural tooth surfaces to other parts of the oral cavity particularly to implant surfaces, forming a microbial biofilm. Microbial agents in the oral cavity play a significant role in the formation of bacterial biofilm on the surface of freshly placed implants. Patients with periodontal diseases are at higher risk for developing peri-implantitis compared to other patients because pathogenic microorganisms migrate from the infected areas (periodontitis) to peri-implant areas.[4] Moreover, the tongue is a major source of bacterial transfer to dental surfaces and formation of dental biofilms.[5]

An in vivo study demonstrated that bacterial colonization of implant surfaces occurs within the first 30 minutes following the placement of implants.[6] Another study showed that following the placement of dental implants in the oral cavity, *Streptococci* became the dominant bacterial strain after four hours and anaerobic bacteria significantly increased within the first 48 hours. Bacterial colonization of implant surfaces is the main cause of peri-implantitis, leading to treatment failure. Since the implant surfaces are located subgingivally, the patient and the clinician have limited access for infection control at the site. Thus, the implant design affects the penetration of microorganisms into the implant surfaces and its internal structure.[6]

Peri-implantitis occurs due to the activity of complex microorganisms, which include the common periodontal pathogens such as Aa, *Porphyromonas gingivalis* (Pg), *Tannerella forsythia* (Tf), *Peptostreptococcus micros* (Pm), *Enterobacteriaceae*, *Candida albicans* (Ca) and *Staphylococci* found in abundance around implants.[7] High counts of *Staphylococcus aureus* and *Streptococcus epidermidis* have also been reported on the surfaces of intraoral dental implants.[8] Relative resistance of these microorganisms to conventionally used antibiotics further confirms that their presence may indicate their opportunistic colonization following systemic antibiotic therapy.[9]

The GCF, a serum transudate or inflammatory exudate, contains a wide spectrum of biochemical factors with the potential of use for the assessment of periodontal status. It also contains parts of the connective tissue, epithelial inflammatory cells, serum and microbial flora of the gingival sulcus or periodontal pocket.[10]

In two-piece implants, a microgap exists between the two components at the fixture–abutment interface (FAI) when the prosthetic abutment is placed over the fixture. This microgap is not cleanable and microorganisms can colonize the FAI.[11]

This study aimed to assess Aa and Pi counts in GCF around healthy implants, diseased implants and sound teeth.

Methods

Type of study and the population studied

Eight patients (four males and four females), with healthy implants, implants with peri-implantitis and sound teeth, with a mean age of 53±1.2 years, who met the inclusion and exclusion criteria were evaluated.

The study population comprised of patients who presented to the Dental Implant Department, Dental School of Tehran University of Medical Sciences and received at least two dental implants. The following inclusion and exclusion criteria were applied as well.

The inclusion criteria

1. Presence of at least one sound tooth and two implants in the dental arch
2. A minimum of one year after the loading of implants
3. Presence of at least one healthy implant and one implant with peri-implantitis
4. No history of periodontal disease

The criteria for selection of a sound tooth: No periodontal pocket (<3 mm), pale pink color of the gin-

giva, presence of scalloping, adequate consistency, firmness and resilience.[12]

Peri-implantitis was defined as extension of inflammation from the marginal gingiva to the implant supporting tissues causing bone destruction and attachment loss.[3] Peri-implant probing depth of at least one site had to be≥5 mm with or without suppur ation/bleeding on probing.

To differentiate healthy implants from implants with peri-implantitis, previous radiographs of the areas are required in order to make a comparison and use them as reference for assessment of bone loss in recent radiographs. In case of unavailability of previous radiographs, the vertical distance threshold from the respective reference point might be considered as 2 mm for bone margin.[7]

The exclusion criteria

1. Smoking
2. Pregnancy
3. History of a systemic disease (cardiovascular diseases, renal disease, diabetes mellitus, liver disease, etc.) affecting the tooth and implant-supporting structures
4. History of antibiotic therapy in the past one month
5. Uncooperative patients

Sample size

Considering α=0.05, β=0.2, significant difference of 30 units and standard deviation (SD) of 21 units, the minimum sample size required for each group was calculated to be eight using the Minitab software.

Extraction of GCF around the teeth and dental implants: The proper site for sampling of GCF is the deepest point of the pocket around the teeth and dental implants because the deepest part of the pocket contains the highest count of microorganisms.[13] GCF was collected using the following steps:

1. Isolation of teeth and respective implants by cotton rolls
2. Drying the area using air spray (low pressure)
3. Mechanical removal of supragingival plaque by a curette
4. Insertion of a paper point into the gingival sulcus

In this study, paper points were used for collection of GCF as described by Griffiths et al.[13] Each paper point was maintained in the gingival crevice for 30 seconds and guided downward until resistance was felt.[13] The paper points were replaced when blood or saliva contamination occurred. After collection, the samples were immediately placed in Eppendorf tubes containing 50 γ of phosphate-buffered saline (PBS). Immediately after sampling and immersion in PBS, the samples were frozen at -20°C and transferred to a microbiology laboratory in a cold box.

Microbiological analysis

The samples were sent to a microbiology laboratory for RT-PCR relying upon the detection and quantitation of a fluorescent reporter. The fluorescent signal of reporter increases in direct proportion to DNA amplification and the amount of PCR product in a reaction. Circle threshold (CT) is defined as the first circle with the intensity of fluorescence above the baseline. CT value estimates the amount of primary mRNA. In other words, in the primary exponential phase, the florescence increases until it reaches a plateau, which is to a certain amount, higher than the background level. This cycle is defined as CT, also known as the crossing point. This phase is the onset of transcription of the pattern. Real-time PCR has four phases of the baseline region, the exponential phase, the linear phase and the plateau region.[14]

Study procedure

DNA extraction is the first step to measure the concentration of each of the bacteria, done with boiling and proteinase K application. The paper points were immersed in 50 µL of distilled water for spread of bacteria in water. Next, 5 µL of proteinase K (20 µL/mL) was added to break down bacterial cell walls. The mixture was incubated at 56°C for 15 minutes for protein denaturation and extraction of bacterial DNA. Extracted DNA was stored at -20°C.

To calculate the amount (number of amplified bands/mL) of the unknown samples, a standard curve was generated for each of the bacteria under study. The CT value of each standard bacterial sample is inversely correlated with its concentration.

Quantitative RT-PCR of 16 unspecified samples was carried out with specific primers using SLAN®-96S Real-Time PCR System.

Statistical analysis

Data analysis in RT-PCR was carried out based on the standard curve using Prism software and data were transferred to Excel software. To compare the Pi and Aa counts in the three areas (GCF around sound teeth, healthy implants and implants with peri-implantitis), repeated-measures ANOVA was applied. Bonferroni adjustment was used for pairwise comparisons of the areas. Data were analyzed using SPSS 20 (Microsoft, IL, USA).

Results

Prevotella intermedia: The mean counts of Pi in GCF of the three sampled areas are shown in Table 1. Based on repeated-measures ANOVA, the differences in 2 of the 3 comparisons were statistically significant. The Pi counts in GCF around implants with peri-implantitis (8) were significant higher than those around healthy implants (8) (P<0.001). The Pi counts in GCF around implants with peri-implantitis were significantly higher than those around sound teeth (8) (P=0.012). No significant differences were found in this respect between sound teeth and healthy implants (P=0.063).

Aggregatibacter actinomycetemcomitans: The mean counts of Aa in the three sampled areas are shown in Table 2. Significant differences existed in this regard in 2 out of 3 comparisons using repeated-measures ANOVA. The Aa counts in GCF around implants with peri-implantitis were significantly higher than those around healthy implants (P=0.002). The Aa counts in GCF around implants with peri-implantitis were significantly higher than those around sound teeth (P=0.024). However, no significant differences were found between sound teeth and healthy implants in this respect (P=0.57; Tables 3 & 4).

Discussion

In the current study on 16 dental implants, Pi counts in GCF around implants with peri-implantitis were significantly higher than those around healthy implants and sound teeth. However, the difference in this regard between sound teeth and healthy implants was not significant. Similar results were obtained for Aa.

Transmucosal abutment of implants provides a suitable surface for colonization of microbial biofilms. Similar to the gingival crevice around natural teeth, the peri-implant mucosa covering the alveolar bone closely adapts to the implant surface.[15] In partially edentulous patients, the microorganisms around implants highly resemble the microbial flora around natural teeth.[16] In fact, opportunistic microorganisms and periodontal pathogens migrate from the pockets around the remaining natural teeth to implant surfaces. This phenomenon, known as "intraoral translocation", plays an important role in the composition of subgingival microbial biofilms.

Ebadian et al[17] demonstrated that in chronic periodontitis (CP) and peri-implantitis, the microbial flora around natural teeth and implants were highly similar, comprising mainly of the red and orange complex bacteria. They explained that these two diseases have the same etiology and bacterial involvement triggers the host inflammatory response (due to the release of virulence factors). Consequently, the pocket depth increases, and bone loss occurs leading to eventual tooth loss or implant failure.

Koyanagi et al[18] evaluated the bacteria forming biofilms on implants with peri-implantitis and stated that the source and origin of bacteria on the implant surfaces were the remaining natural teeth or the saliva. This finding has also been reported in some other studies.[19] Haririan et al[20] showed that detection of periopathogenic bacteria via saliva sampling wound be similar or even more accurate than sampling from the pockets around natural teeth. Implants with peri-implantitis often have a more complex microbial composition compared to healthy implants and teeth with periodontitis, and gram-negative anaerobic bacteria more commonly colonize these areas. In addition to gram-negative anaerobic rods with dark stains, other microbial strains might also cause peri-implantitis as well such as Tf, *Fusobacterium nucleatum* (Fn), *C. rectus* (Cr), Pm, Pi, Pg, Td and Aa, particularly the b serotype. Less common microorganisms such as *S. aureus*, enteric bacilli, and Ca have been isolated in 55% of cases of peri-implantitis.[20,21] Pg, Pi and Aa have been frequently detected around implants with peri-implantitis.[6] Borgerello et al[4] reported that *Stomatococcus*, *Peptostreptococcus*, Pi, Fn and Aa were the dominant microorganisms around implants. Advanced form of periodontitis is among the main causes of tooth loss in adult populations and a high percentage of patients receiving dental implants have a history of pe-

Table 1. Comparison of Pi counts between the three groups

	Mean	SD	Minimum	Maximum
Pi around implants with peri-implantitis	8.45	9.03	8.69	8.69
Pi around healthy implants	8.25	8.73	8.48	8.48
Pi around sound teeth	8.41	8.82	8.57	8.57

Table 2. Pairwise comparisons of groups in terms of Pi counts

Variable	Variable	P-value
Pi around implants with peri-implantitis	**Pi around healthy implants**	0.001
Pi around healthy implants	**Pi around sound teeth**	0.063
Pi around sound teeth	**Pi around implants with peri-implantitis**	0.012

Table 3. Comparisons of Aa counts between the three groups

	Mean	SD	Minimum	Maximum
Aa around implants with peri-implantitis	2.98	9.48	13.04	4.02
Aa around healthy implants	2.5558	6.78	10.19	3.03
Aa around sound teeth	2.5556	7.21	9.54	3.58

Table 4. Pairwise comparisons of groups in terms of Aa counts

Variable	Variable	P-value
Aa around implants with peri-implantitis	**Aa around healthy implants**	0.002
Aa around healthy implants	**Aa around sound teeth**	0.57
Aa around sound teeth	**Aa around implants with peri-implantitis**	0.024

riodontal disease. Papaioannou et al[23] evaluated the prevalence of periodontal pathogens in partially edentulous and fully edentulous patients with positive history of periodontal disease using phase-contrast microscopy and DNA probe. In partially edentulous patients, the microbiological profile was the same around teeth and implants with equal depth of pockets, which indicates that pockets around teeth serve as a reservoir for microbial transfer to peri-implant areas. In completely edentulous patients, the prevalence of Aa and Pg was not correlated to peri-implant infections as it was in partially edentulous patients.

Shape, type, design and surface characteristics of implants (roughness and wettability) are important factors for bacterial colonization and progression of peri-implantitis. Quirynen et al,[11] in an in vitro study demonstrated that bacterial invasion into the micro-gap at the FAI in two-stage implants occurred when the abutment was placed over the implant and the complex was immersed in a liquid containing oral microorganisms. Also, an in vivo study demonstrated that specific amounts of microorganisms were found in the internal areas of all the implant abutment screws. Some studies have recommended disinfecting the internal parts of two-stage implants by applying 1% chlorhexidine gel into the internal parts of the fixture before placement of abutment and screw tightening in order to decrease bacterial colonization in a 6-month period.[23]

Callan et al[24] found moderate to high amounts of eight periodontopathogenic microorganisms such as Aa and Pg at the FAI using DNA probe analysis and paper point sampling. Adell et al compared the bacterial strains in the gingival crevice around one and two-stage implants and in contrast to the current study, they did not find Aa in any group. Pg was found around one-stage implants. Pi in small amounts and Fn in large amounts were detected in both groups. Also, in a study by Shahabouee et al,[24] all the implants were one-stage and Aa, Tf or Fn were not detected in any group; while Pg and Pi were both isolated. Pg is an exogenous bacterium capable of firmly attaching to periodontal tissue. It can compete with other microorganisms and attenuate the humoral immunity system of the host.[26]

Poor oral hygiene, history of periodontitis and cigarette smoking are among the important risk factors for development and progression of peri-implantitis. For peri-implant mucositis, mechanical removal of plaque and use of antimicrobial mouthwashes seem to be effective. However, for peri-implantitis, surgical treatment in conjunction with local or systemic antibiotic therapy is often required. Thus, it is clear that periodontopathogenic bacteria are the main cause of peri-implantitis and conduction of a test to isolate bacteria and determine the bacterial load can be clinically valuable.[27] Progression of peri-implantitis depends on the amount and composition of microorganisms in pockets around implants and also high occlusal load.[28]

PCR is the most sensitive and fastest method for detection of microbial pathogens in clinical samples. Its diagnostic value is particularly important when culture of some pathogenic microorganisms is difficult in the laboratory setting or requires a long time (i.e. anaerobes involved in periodontal disease). RT-PCR is the advanced form of PCR that enables counting the target DNA using fluorogenic probes. Moreover, it is a closed system and the reaction tube is not opened after amplification and consequently, laboratory cross-contamination and false positive results are prevented. Also, presence of probe along with 2 PCR primers increases the specificity of this method. RT-PCR enables isolation and counting of main pathogens involved in periodontitis and peri-implantitis. This test provides information about the microbiological status of tissues around teeth and implants to improve oral health care or assess the efficacy of treatment protocols.[27]

Shibi et al compared the composition of subgingival and supragingival microbial plaque and found that the mean counts of Tf, Pg, Td, Fn and Pi around diseased implants were higher than those around

healthy implants. All these bacteria have pathogenic potential and can cause periodontal destruction (key periodontal pathogens).[29] This finding is in line with the current study results. Another study using DNA probe and checkerboard assay found no significant difference in the counts of the above-mentioned bacteria in subgingival areas between healthy and diseased implants.[10] This result is in contrast to our findings. Such differences in the results might be attributed to differences in the methods of sampling and processes of bacterial storage. In our study, sampling was carried out using a paper point and isolation and counting of Aa and Pi were carried out using RT-PCR. The highest counts of Aa and Pi were found around implants with peri-implantitis, around healthy teeth and around healthy implants, in a descending order of frequency. Based on the results of Shibli et al, implants with peri-implantitis and supragingival areas harbored higher bacterial loads compared to areas around healthy implants and subgingival areas. They demonstrated that the ratio of non-pathogenic to pathogenic microorganisms was very low around implants with peri-implantitis and also showed that periodontal pathogens moderately increased around implants with peri-implantitis in both subgingival and supragingival areas. Another study demonstrated that in partially edentulous patients, the longer the exposure of implants to the oral cavity, the higher the amount of motile microorganisms and spirochetes around implants with peri-implantitis. This explains the results obtained in the current study. Higher bacterial load around natural teeth compared to healthy implants is probably due to the longer presence of natural teeth in the oral cavity compared to implants. A previous study indicated that in fully edentulous patients who immediately received implant-retained fixed partial dentures of the mandible, the longer the exposure of implants to the oral cavity, the higher the prevalence of Aa, Pg and Pi in gingival crevice around implants.[28] One month after the placement of dental implants, periodontal pathogens are detectable around them and can be isolated. This explains the high percentage of positive results for periodontal pathogens in both healthy and diseased areas. Colonization of bacteria on the implant surfaces starts approximately 30 minutes following the placement of dental implants.

Ebadian et al[17] in their study collected the samples using paper points and found high loads (>50%) of red-complex bacteria and some other anaerobic strains such as Pi, Fn and Cr in deep periodontal pockets and also in peri-implantitis areas in Iranian patients, which is in line with the current findings. However, in contrast to the current study results, Aa counts in their study were very low. In other communities, Aa has been abundantly found in these areas. This indicates that the bacterial composition may not be the same in different communities.

It should be noted that in sampling by use of a paper point, volume of samples is limited and some strains might be missed and not sampled during the procedure of sample collection. Moreover, although this technique is suitable for sampling from deep untreated pockets, it may not be adequate for sampling of GCF around implants with peri-implantitis with exposed screw due to the interference of the screw and preventing the paper point from reaching the pocket depth; this may explain many of the negative results obtained in different studies. Another point to remember is that storage of samples in dry tubes after sampling must not take too long because it may change the load composition and ratio of some bacteria. In our study, all the specimens were immediately frozen after sampling and sent to the laboratory in a cold box.

An ideal method of sampling has yet to be found. In our study, similar to that of Casado et al,[28] peri-implant crevicular fluid (PICF) was used as a reliable source of sampling for conduction of RT-PCR and detection of Aa and Pi. It should be noted that the supragingival plaque must be necessarily removed by gauze prior to sampling and the area must be isolated with cotton rolls. PICF is an inflammatory exudate and its flow rate, composition and profile changes by alterations in the status of peri-implant tissues. PICF analysis can be very helpful for early diagnosis of metabolic and biochemical conditions that have yet to be clinically detectable. Also, monitoring of the osseointegration process and bone response to occlusal loads improves the long-term success of dental implants.

Nowzari et al[29] determined the composition of subgingival microbial plaque and the GCF around teeth and implants using paper point sampling and laboratory cultures. In contrast to our study, they did not isolate Aa in any group but the Pi load around sound teeth was higher than around healthy implants, which is consistent with our results. Moreover, the number of periodontal bacteria around teeth was higher than that around implants (even twice that in some cases), which confirms the current study results. In areas with positive bacterial culture, the level of cytokines had increased as well.

In a study by Kocar et al, oral *Streptococci* were dominant around all the healthy implants evaluated.

In fully edentulous patients, Pi was only detected around one of the healthy implants evaluated. Aa, Pg, Tf and Td were detected neither in the gingival crevice around implants nor in the alveolar mucosa. But, in partially edentulous patients, Tg, Tf and Td were found in 40–45% (almost half) of the cases around implants.[30,31] Aa was found in 15% and Pi in only one case, which is contrary to our results. Also, in each patient, the prevalence of the respective bacteria around natural teeth and implants was almost the same. Daser et al[32] reported that after extracting all the teeth in patients with severe periodontitis, Aa and Pg were no longer detectable in the mucosa or saliva, but Pi was detected in half of the patients. Also, after implant placement in these patients, Aa and Pg were not detected in peri-implant pockets. Therefore, although the number of periodontal pathogens significantly decreases following the extraction of teeth or periodontal therapy, oral mucosa can still serve as a source of re-infection around dental implants.

It is noteworthy that studies might yield variable results, depending on the method of sampling, time of analysis, laboratory methods and differences in clinical conditions. Monitoring of the periodontal status and peri-implant areas using microbiological parameters might significantly improve the long-term success rate of osseointegrated implants.

Conclusion

The results of the current study demonstrated that:

- Pi counts in GCF around diseased implants were significantly higher than those around sound teeth and healthy implants. Although the difference in this regard between healthy implants and sound teeth was not significant, it was demonstrated that the bacterial counts around sound teeth were higher than those around healthy implants.
- Similar results were obtained for Aa. Aa counts in GCF around diseased implants were significantly higher than those around sound teeth and healthy implants. However, the range of distribution of the results for Aa was significantly wider than that for Pi; in other words, the results for Pi had a narrower range of distribution (diseased implants>teeth>healthy implants).
- Aa loads around dental implants with peri-implantitis were significantly higher than those in the other two areas. Based on this finding, Aa plays a more important role in peri-implantitis.

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Conflict of Interests

The authors have declared no conflict of interests.

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