Prevalence of Antibiotic Resistance Genes in Subjects with Successful and Failing Dental Implants. A Pilot Study

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Abstract: Objectives: To investigate the prevalence of the bacterial genes encoding resistance to beta-lactams, tetracyclines and metronidazole respectively, in subjects with successful and failing dental implants and to assess the presence of Staphylococcus aureus and the meca gene encoding for Methicillin Resistant Staphylococcus aureus (MRSA) in the same samples.

Materials and Methodology: The subject sample included 20 participants with clinically healthy osseointegrated implants and 20 participants with implants exhibiting peri-implantitis. Clinical parameters were assessed with an automated probe, samples were collected from the peri-implant sulcus or pocket and analyzed with Polymerase Chain Reaction for blaTEM, tetM, tetQ and nim genes, S. aureus and MRSA using primers and conditions previously described in the literature.

Results: Findings have shown high frequencies of detection for both groups for the tetracycline resistance genes tetM (>30%), tetQ (>65%) with no statistical differences between them (z-test with Bonferroni corrections, p<0.05). The blaTEM gene, which encodes resistance to beta-lactams, was detected in <15% of the samples. The nim gene, which encodes resistance to metronidazole, S.aureus and the meca gene encoding for MRSA were not detected in any of the analyzed samples.

Conclusions: Healthy peri-implant sulci and peri-implantitis cases often harbor bacterial genes encoding for resistance to the tetracyclines and less often for beta-lactams. Thus, the antimicrobial activity of the tetracyclines and to a lower extent to beta-lactams, might be compromised for treatment of peri-implantitis. Since no metronidazole resistance genes were detected in the present study, its clinical use is supported by the current findings. S.aureus may not participate in peri-implant pathology.

Keyword: Dental implants, antibiotics, bacterial resistance genes, peri-implantitis.

INTRODUCTION

Dental implants are currently considered as an excellent alternative solution for replacing teeth.

When evaluating the performance of dental implants it should be mentioned that implant survival is no longer a reliable parameter for clinical outcome and instead it is of utmost importance that the dental implants remains free from functional and aesthetic complications, a parameter known as implant success.

A number of systematic reviews have shown that although implant survival rates can reach after 10 years 89.4% for single tooth restorations and 93.9% for prosthesis in partially edentulous subjects, the rate of complications can reach 33.6% [1, 2].

The incidence of per-implantitis as reported in clinical studies depends on definition of the disease [3, 4]. The similarity in bacterial pathogens associated with periodontitis and peri-implantitis, currently suggest a common microbial aetiology [5]. In addition to the bacterial factor “a number of other parameters such as poor integrity of the connection between the implant and the abutment” the iatrogenic introduction of cement in the peri-implant area and the practitioner’s choice and clinical management of implant design have also been shown to influence the initiation and progression of peri-implantitis [6].

The goal of treating peri-implantitis is to regenerate bone and achieve new osseointegration. Various approaches have
been applied for treating both peri-implant mucositis and peri-implantitis, based on the concept they are both infectious diseases. According to the current evidence, it is suggested that non-surgical therapy leads to unpredictable outcomes, although in some cases it has been shown that adjunctive antibiotics might assist in resolution of peri-implantitis [7-9]. Surgical treatment for treating peri-implantitis has also been applied, using various techniques with or without adjunctive antimicrobials [10]. Again, based on the current evidence, no definite clinical guidelines can be extrapolated, especially regarding the role of systemic or local antimicrobials, which could lead to predictable therapeutic outcomes.

The administration of antimicrobials is globally compromised by the growing phenomenon of antimicrobial resistance [11]. Due to the over- or misuse of antibiotic compounds worldwide, several classes of antibiotics, including the ones administered for oral infections, can now be ineffective against various bacterial species, due to dissemination of antibiotic resistance, which is frequently spread by genetic material and is favored in the oral environment due to the biofilm nature of bacterial deposits [12, 13].

Therefore, the recognition of specific genes which encode for mechanisms of resistance to various antibiotics, might have clinical significance, by guiding the clinicians in a personalized choice and prescription of specific antimicrobials.

Aim of the current pilot study was to investigate the prevalence of bacterial genes encoding for antimicrobial resistance against classes of antibiotics commonly used for treatment of peri-implantitis in healthy and diseased peri-implant sulci. The presence of S.aureus and the Methicillin Resistant Staphylococcus aureus (MRSA) was also assessed in the same samples.

MATERIALS AND METHODOLOGY

Subject Sample and Study Design

Differences between groups regarding the prevalence of investigated resistance genes was set as the primary outcome of the present study [14]. The experiment was set to have at least 80% power to detect differences of 25% with a significance level (a) of 0.05 (two-tailed). Therefore 20 subjects were required for each group (Statmate®, GraphPad Inc, San Diego, CA, USA).

The study was conducted according to the protocol outlined by the Research Committee, Aristotle University of Thessaloniki, Greece and approved by the Ethical Committee of the School of Dentistry (#120), in compliance with the ethical principles of the World Medical Association Declaration of Helsinki. All patients read and signed an appropriate informed consent document prior to the participation in the study.

Inclusion Criteria

To be included in the study, subjects should fulfill the following criteria:

- a) age >30 years.
- b) absence of systemic diseases or medications known to affect periodontal tissues, infectious conditions (hepatitis, HIV) or pregnancy and lactation.
- c) no periodontal treatment or antibiotic intake during the last six months.

In addition participants should fulfill one of the following conditions:

- a) subjects with periodontal health (bleeding on probing <10%, without any clinical attachment loss >3mm) and a single osseointegrated implant present for at least 5 years in the dentition with probing depth <3mm, without any clinical attachment loss on the site of the implant and no bleeding on probing.
- b) subjects at the supportive periodontal therapy phase, treated at least 5 years ago and exhibiting a single implant present for at least 5 years in the dentition with peri-implantitis (bleeding on probing/suppuration and probing depth >6mm or bone loss >2.5mm on the implant) [3].

Clinical Recordings

Clinical recordings were performed at six points of all teeth present at the dentition (mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual, distolingual). Recordings were performed by a calibrated examiner (GK) using an automated probe (Florida probe, Florida Probe Corporation, Gainesville, FL, USA) and included the following parameters:

- a) Probing Pocket Depth
- b) Recession
- c) Clinical Attachment Level
- d) Bleeding on Probing

Clinical Sampling

Samples were taken from healthy and peri-implantitis sites, with sterile paper points left in the sulcus for 30 seconds, after removing supragingival plaque with sterile Gracey curettes. All samples were immediately placed in 200 μl of TE buffer (Tris HCL 10 mM, EDTA 1 mM, pH 7.5) and stored at -20°C, until assayed.

Polymerase Chain Reaction (PCR)

Analysis of samples was performed blindly (coded samples). All experiments were performed in the Department of Microbiology, School of Biology, Aristotle University of Thessaloniki, Greece.

The main methodology applied for analysis of samples was Polymerase Chain Reaction (PCR). First of all, PCR was performed for the detection of the 16S ribosomal RNA gene, in order to verify that the clinical samples contained identifiable bacterial DNA [15]. Samples were further analyzed by PCR for the presence of the tetM, tetQ, blaTEM, nim, nuc and mecA genes. All experiments were run with PCR.
Table 1. Polymerase Chain Reaction (PCR) primers used in the study.

<table>
<thead>
<tr>
<th>PCR Primers</th>
<th>16S Primer Sequence</th>
<th>tetM Primer Sequence</th>
<th>tetQ Primer Sequence</th>
<th>blatTEM Primer Sequence</th>
<th>nim Primer Sequence</th>
<th>nuc Primer Sequence</th>
<th>mecA Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S</td>
<td>5'-CAG GAT TAG ATA CCC TGG TAG TCC ACG C-3' and 5'- GAC GGG CGG TGT GTA CAA GGC CCG GGA ACG-3' [15]</td>
<td>5'-GAC ACG CCA GGA CAT ATG G-3' and 5'-TGC TTT CCT CTT GTT CGA G-3' [16]</td>
<td>5'-GGC TTC ATC GAC ATC TA- 3' and 5'-CAT CAA CAT TTA TCT CTC TG-3' [17]</td>
<td>5'-AGATCAGTTGGGTGACGAG -3' and 5'-AACGATTGTGACACGATAGCC-3' [21]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Polymerase Chain Reaction (PCR) conditions.

<table>
<thead>
<tr>
<th>PCR Conditions</th>
<th>16S</th>
<th>tetM</th>
<th>tetQ</th>
<th>blatTEM</th>
<th>nim</th>
<th>nuc</th>
<th>mecA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C for 5min</td>
<td>95°C for 5min</td>
<td>95°C for 5min</td>
<td>94°C for 10min</td>
<td>94°C for 10min</td>
<td>94°C for 10min</td>
<td>94°C for 10min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C for 1min</td>
<td>94°C for 30sec</td>
<td>94°C for 30 sec</td>
<td>94°C for 1min</td>
<td>94°C for 30s</td>
<td>94°C for 30s</td>
<td>94°C for 30s</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C for 1min</td>
<td>55°C for 1min</td>
<td>50°C for 1min</td>
<td>62°C for 1min</td>
<td>62°C for 1min</td>
<td>58°C for 1min</td>
<td>55°C for 1min</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C for 34s</td>
<td>72°C for 90 s</td>
<td>72°C for 160 s</td>
<td>72°C for 1min</td>
<td>72°C for 1min</td>
<td>72°C for 1min</td>
<td>72°C for 1min</td>
</tr>
<tr>
<td>Step 2 to 4</td>
<td>34 cycles</td>
<td>37 cycles</td>
<td>37 cycles</td>
<td>35 cycles</td>
<td>35 cycles</td>
<td>35 cycles</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C for 3min</td>
<td>72°C for 10min</td>
<td>72°C for 10min</td>
<td>72°C for 10min</td>
<td>72°C for 10min</td>
<td>72°C for 10min</td>
<td>72°C for 10min</td>
</tr>
<tr>
<td>DNA template</td>
<td>5 μl</td>
<td>5 μl</td>
<td>5 μl</td>
<td>5 μl</td>
<td>5 μl</td>
<td>5 μl</td>
<td>5 μl</td>
</tr>
<tr>
<td>dNTPs mix, 10mM</td>
<td>1 μl (200μM)</td>
<td>1 μl (200μM)</td>
<td>1 μl (200μM)</td>
<td>2 μl (400μM)</td>
<td>1 μl (200μM)</td>
<td>2 μl (400μM)</td>
<td>2 μl (400μM)</td>
</tr>
<tr>
<td>Reaction Buffer 5x</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
</tr>
<tr>
<td>MgCl₂ Solution, 25mM</td>
<td>3 μl (1.5mM)</td>
<td>3 μl (1.5mM)</td>
<td>3 μl (1.5mM)</td>
<td>4 μl (2mM)</td>
<td>3 μl (1.5mM)</td>
<td>4 μl (1.5mM)</td>
<td>3 μl (1.5mM)</td>
</tr>
<tr>
<td>Upstream Primer, 10μM</td>
<td>2.5 μl (0.5μM)</td>
<td>2.5 μl (0.5μM)</td>
<td>2.5 μl (0.5μM)</td>
<td>5 μl (1μM)</td>
<td>5 μl (1μM)</td>
<td>6 μl (1μM)</td>
<td>5 μl (1μM)</td>
</tr>
<tr>
<td>Downstream Primer, 10μM</td>
<td>2.5 μl (0.5μM)</td>
<td>2.5 μl (0.5μM)</td>
<td>2.5 μl (0.5μM)</td>
<td>5 μl (1μM)</td>
<td>5 μl (1μM)</td>
<td>6 μl (1μM)</td>
<td>5 μl (1μM)</td>
</tr>
<tr>
<td>Taq polymerase, 5U/μl</td>
<td>0.25μl (1.25U)</td>
<td>0.25μl (1.25U)</td>
<td>0.25μl (1.25U)</td>
<td>0.25μl (1.25U)</td>
<td>0.25μl (1.25U)</td>
<td>0.25μl (1.25U)</td>
<td>0.25μl (1.25U)</td>
</tr>
<tr>
<td>Water for injection</td>
<td>25.75μl</td>
<td>25.75μl</td>
<td>25.75μl</td>
<td>18.75μl</td>
<td>20.75μl</td>
<td>19.75μl</td>
<td>19.75μl</td>
</tr>
</tbody>
</table>

Conditions and primers previously reported to the literature and are described in Tables 1 and 2 [15-21].

The tetM and tetQ genes confer resistance to the tetracyclines, the blatTEM gene to beta-lactams, and the nim genes to metronidazole. The nuc gene encodes a thermonuclease exclusive for S.aureus, while the mecA gene is characteristic for MRSA.

The final volume of the reaction mixture for each PCR assay was 50 μl. A Peltier Thermal Cycler (PTC-100, Peltier Thermal Cycler, MJ Research) was used for experiments.

For each set of samples analyzed by PCR a negative and a positive control were used. Sterile water for injection (Demo S.A. Pharmaceutical Industry, Athens, Greece), was used as negative control (replacing DNA template into the PCR reactions). Positive controls included bacterial species positive for the above mentioned bacterial genes [22].

The products of the DNA amplification were electrophoresed through a 2% agarose gel, stained with ethidium bromide, exposed under UV light and photographed. A 100bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was also...
loaded on agarose gel as a molecular weight standard. The amplified fragment sizes were 625bp for 16S rRNA, 754bp for tetQ, 397bp for tetM, 458bp for nim, 618bp for blaTEM, 255bp for nuc, and 527bp for mecA. The electrophoresis for each PCR product was carried out twice in order to test the reproducibility of the method.

Statistical Analyses

The statistical analysis of the data was carried out with the statistical package SPSS 19.0 version (SPSS Inc., Chicago, IL, USA).

Differences regarding the distribution of participants, according to gender and smoking were sought by applying the z-test for proportions adjusted with Bonferroni corrections and differences between the two groups regarding mean age were sought by applying the Kruskal-Wallis test.

For clinical parameters, indicators of Descriptive Statistics were used, such as mean and standard deviation for each group, with the patient as the observational unit. Differences in clinical parameters for full-mouth periodontal recordings and investigated implants respectively, were sought by applying the Kruskal-Wallis test.

Differences in the prevalence of investigated genes were also sought between the group with healthy implants and peri-implantitis, by applying the z-test for proportions adjusted with Bonferroni corrections.

Statistical significance was set for all comparisons at the 0.05 level.

RESULTS

Demographic data for participants are presented in Table 3. Participants were age and smoking status matched (Kruskal-Wallis and z-test with Bonferroni corrections, p>0.05).

Clinical parameters for the two groups and for investigated implants are presented in Tables 4 and 5 respectively. Groups were statistically different regarding clinical parameters of full mouth periodontal conditions (Kruskal-Wallis and z-test with Bonferroni corrections-tests, p<0.05) as depicted in Table 4. Clinical parameters of investigated implants also displayed significant statistical differences between the two groups as presented in Table 5 (Kruskal-Wallis and z-test with Bonferroni corrections-tests, p<0.05) with significant peri implant disease (mean probing depth, clinical attachment loss and bleeding on probing) in the periimplantitis group.

The distribution of investigated genes is displayed in Table 6. According to the findings of the present study, no differences were observed between the healthy implants vs peri-implantitis groups regarding the prevalence of three out of six investigated antimicrobial resistance genes. Findings have shown high frequencies of detection for both groups for the tetracycline resistance genes tetM (>30%), tetQ (>65%) with no statistical differences between them (z-test with Bonferroni corrections, p<0.05). The blaTEM gene, which encodes resistance to beta-lactams, was detected in <15% of the samples. The nim gene, which encodes resistance to metronidazole, S.aureus and the mecA gene encoding for MRSA were not detected in any of the analyzed samples (Table 6).
DISCUSSION

Peri-implant diseases and their effective treatment pose an increasing clinical challenge. Studies regarding the incidence of peri-implant mucositis have reported that up to 48\% of implants in a follow-up period of up to 14 years can be affected by this condition which can be both preventable and reversible once early diagnosed [23].

In contrast, the reported prevalence of peri-implantitis presents distinct differences, attributed mainly to the variety of clinical and radiographic thresholds set for the disease.

Regarding the etiology and pathogenetic mechanisms, it is currently accepted, that peri-implant diseases are infectious in nature, similar to periodontal diseases and occur as the result of an imbalance between the bacterial challenge and host response. At the present time, Gram-negative anaerobic species known consensus pathogens for periodontitis, have also been associated with peri-implantitis. Thus, members of the “red complex” as described by Socransky et al.1998, including Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia, along with Gram-negative anaerobes such as Prevotella and Fusobacterium spp, have been shown to be present in peri-implantitis lesions [24]. It should be noticed that species not known to clearly be involved in periodontal pathology such as Staphylococcus aureus, have been associated to correlate with the initiation of peri-implantitis [23, 25, 26].

The implication of S. aureus and MRSA in peri-implant pathology is not supported by findings of the present study, albeit the limited subject sample since neither was detected in any of the investigated samples. Nevertheless it should be kept in mind that the aetiological significance of species such as Candida albicans, Gram-negative enteric rods and Staphylococci spp remains unclear and more studies are required [25].

Previous studies, displaying the presence of Staphylococcus spp and S. aureus in peri-implant lesions, by applying cultural techniques, reported that participants had previously used antibiotics [27]. Therefore, findings of the present study are not comparable with the above mentioned data, since according to criteria of inclusion of the study, subjects have not been administered antibiotics for at least 6 months prior to enrollment. The findings are also in agreement with previous studies, which reported that when Staphylococci spp have been detected in peri-implantitis lesions, they belonged mainly to the species S. epidermidis and not to S. aureus [26,28].

According to findings of the present study, the genes encoding for resistance to the tetracyclines were detected in high frequencies in the subject sample irrelevant of peri-implant conditions (> 30\% for tetM and >65\% for tetQ). These genes have been shown to correlate with Gram-positive non pathogenic species and Gram-negative

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**Table 5. Clinical parameters of evaluated implants.**

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Probing Depth (mm) (mean ± SD)</th>
<th>Reseccion (mean ± SD)</th>
<th>Clinical Attachment Level (mm) (mean ± SD)</th>
<th>Bleeding on Probing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinically healthy osseointegrated implants</td>
<td>20</td>
<td>2.1±0.5 (a)</td>
<td>0.1±0.2 (b)</td>
<td>2.1±0.5 (b)</td>
<td>0 (a)</td>
</tr>
<tr>
<td>Peri-implantitis</td>
<td>20</td>
<td>5.9±1 (a)</td>
<td>-0.5±1.3 (b)</td>
<td>5.4±1.7 (b)</td>
<td>88±20% (a)</td>
</tr>
</tbody>
</table>

Statistically significant differences between groups are indicated by the same letter (Kruskal-Wallis test and z-test with Bonferroni corrections, p<0.05)

**Table 6. Frequency of detection of tetM, tetQ, blaTEM, nim, Staphylococcus aureus and mecA in evaluated implants.**

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>tetM % (positive/total)</th>
<th>tetQ % (positive/total)</th>
<th>blaTEM % (positive/total)</th>
<th>nim % (positive/total)</th>
<th>S.aureus % (positive/total)</th>
<th>mecA % (positive/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants with clinically healthy osseointegrated implants</td>
<td>20</td>
<td>30% (6/20)</td>
<td>65% (13/20)</td>
<td>15% (3/20)</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Participants with peri-implantitis</td>
<td>20</td>
<td>40% (8/20)</td>
<td>75% (15/20)</td>
<td>5% (1/20)</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>35%</td>
<td>70%</td>
<td>10%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

No differences were observed between groups (z-test for proportions with Bonferroni corrections, p>0.05)
periodontal pathogens respectively [16, 17]. These high frequencies of detection might suggest that this class of antibiotics is not suitable for treating peri-implant infection. Previous studies in the literature have generally positive effects in clinical parameters of peri-implant lesions after local administration of tetracycline’s [29-32], but the outcome is generally unpredictable for all patients and further clinical trials are required and no data exist in the literature regarding the effect of antimicrobial resistance in the outcome of these treatments.

The bla<sub>TEM</sub> gene, which encodes for resistance to the beta-lactams, was detected, according to present findings, in much lower frequencies compared to tet<sub>M</sub> and tet<sub>Q</sub> in investigated samples (15% from healthy implants and 5% for peri-implantitis cases respectively). These findings support the use of this class of antibiotics for treatment of peri-implant infections. In addition, the fact that the nim gene, which encodes for resistance to metronidazole, was not detected in any of investigated samples supports the administration of this antibiotic alone or in combination with beta-lactams for resolution of peri-implantitis.

Limitations of the current pilot study include the confined subject sample and the fact that cultural techniques such as antibiotic sensitivity were not applied and therefore the bacterial source of investigated antibiotic resistance genes cannot be identified. However it is known that the biofilm structure of bacterial accumulations in the oral cavity allows for the dissemination of genetic material between different microbial species and even genera [12, 13, 33].

Thus, taking the above mentioned findings into account, the administration of tetracyclines for treatment of peri-implant diseases might be compromised due to the high prevalence of detection of genes encoding resistance to these antibiotics. In contrast, data regarding the bla<sub>TEM</sub> gene suggest that beta-lactams could be effective in treating peri-implantitis as an infection. The same is suggested for metronidazole, as an antimicrobial for treating Gram-negative infections or the combination of the two antibiotics.

CONCLUSION

Healthy peri-implant sulci and peri-implantitis cases often harbor bacterial genes encoding for resistance to the tetracyclines and less often for beta-lactams. Thus, the antimicrobial activity of the tetracyclines and to a lower extent of beta-lactams, might be compromised for treatment of peri-implantitis. Since no metronidazole resistance genes were detected in the present study, its clinical use is supported by the current findings. <i>S. aureus</i> may not participate in peri-implant pathology since it was not detected in any of the investigated samples.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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