EFFICACY OF A NEW COATING OF IMPLANT-ABUTMENT CONNECTIONS IN REDUCING BACTERIAL LOADING: AN *IN VITRO* STUDY

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SUMMARY
Background. Titanium is the gold standard for dental implants, since it has demonstrated excellent biocompatibility and osseointegration properties. The rate of osseointegration is however affected by the surface morphology and peri-implant infections may affect fixture and the long-term osseointegration outcome. Thus chemical composition of a coating at the implant-abutment junction (IAJ) surface is expected to play a key role in preventing bacterial infection.
Purpose. In the present study a new antimicrobial polysiloxane coating functionalized with chlorexidine digluconate (PXT) has been tested in an *in vitro* model.
Materials and methods. Twenty implants were coated in the internal chamber with PXT and twenty were used as controls.
Results and conclusions. Ten of the coated implants, preliminarily tested against Gram positive and negative bacteria and fungi, showed a complete inactivation of the microbial species after a 15 min contact. On the remaining ten treated implants a series of microbiological tests and PCR analysis, after contamination of the implant external medium, in which the implant have been immersed, with genetic modified *Tannerella forsythia* (TF) and *Porphyromonas Gingivalis* (PG), leads to the conclusion that the coating is capable of inactivating the microbial species penetrating the internal of the implant through the implant abutment junction.

Key words: implant-abutment connection, antimicrobial siloxane coating, peri-implantitis.

Introduction

Titanium is the gold standard for the manufacture of dental implants since it has demonstrated excellent biocompatibility and osseointegration properties (1-46).
Peri-implant mucositis, a reversible inflammation of peri-implant soft tissues, occurs in approximately 80% of subjects (47). The most severe forms of peri-implantitis occur in about 28-56% of subjects and, as well as soft tissue inflammation, results in loss of the supporting bone (48). Treatment of peri-implantitis requests a perfect control of the plaque surrounding the implant. Upon exposure to the oral cavity, implants, like all materials, are rapidly coated with a microbiota pellicle called biofilm (49) which can be responsible of periodontal diseases and peri-implantitis (50, 51). The inflammatory content may increase as a consequence of the adhesion and proliferation of bacteria on the biofilm around implant-abutment connection (IAC), during soft tissue manipulation for prosthetic component installation. The presence of a cavity inside dental implant and close to the bone may influence the development of peri-implant inflammation and bone resorption. In fact, bacterial

species present inside the implant chamber can be responsible of an inflammatory process in the peri-implant tissues at the alveolar bone crest level, and bacterial infection can interfere with osseo-integration.
Similarly to implant surface, implant-abutment junction (IAJ) is affected by the type of coating. Coated IAJ (CIAJ) surfaces show a decreased susceptibility to bacterial adhesion, at least *in vitro*, and this has been attributed to increased protection from bacterial leakage (52). Translating the *in vitro* data, we can infer that the CIAJs show a lower propensity for occurrence of peri-implantitis *in vivo* (53). Peri-implantitis is a chronic and multifactorial disease caused by pathogen bacteria, aggregated in biofilm along IAJ. Periodontopathogenic bacteria and in particular the species of "red complex" [*Porphyromonas Gingivalis* (PG), *Tanerella Forsythia* (TF) and *Treponema Denticola* (TD)] cause peri-implant tissues inflammation, which may lead to the destruction of the peri-implant bone, resulting in implant loss (53). The treatment of peri-implantitis has unpredictable results, while the control of bacterial plaque is crucial (54-57).
Aim of the present *in vitro* study is to evaluate the effectiveness of a new polysiloxane coating containing chlorexidine (PXT) in reducing bacterial loading inside and outside IAJ.

Materials and methods

Implant preparation

Forty Edierre Implants System SpA (Genoa, Italy) were used in two different sets of measurements. Twenty of them were coated in the internal chamber with an antimicrobial polysiloxane coating functionalized with chlorexidine digluconate (abbreviated as PXT). The corresponding abutments and screws were also treated with PXT according to the procedure described below. The remaining twenty untreated implants abutments and screws were used as controls.
The PXT product, which was provided by Edierre, is composed by an alcoholic solution containg polysiloxane-titanate oligomers and chlorexidine digluconate at 1%. The product has the ability of binding the titanium surface via interaction of the OH functionalities present on the polysiloxane chains and at the titanium surface (antimicrobial coating composition for dental implants"PCT/IT2015/000142). The role of the alkyl chains present on the siloxane units is that of trapping through Van der Waals interactions chlorhexidine molecules, allowing their subsequent slow release when in contact with an aqueous medium.
Coating of the internal chamber of the implants was realized by filling with the PXT solution, which was left in contact with the surface for at least 10 min, followed by draining and implant heating at 60°C for 45 min. Coating of the abutments and screws was realized by immersion in the PXT solution for 10 min followed by centrifugation on a sintered glass filter and subsequent heating at 60°C for 45 min.

Microbial species

Staphilococcus aureus, ATCC 6538; *Escherichia coli*, ATCC 10536; *Pseudomonas aeruginosa*, ATCC 15442; *Enterococcus hirae*, ATCC 10541 and *Candida albicans*, ATCC 10231 were purchased from Liofilchem. Genetic modified *Tannerella Forsythia* (TF) and *Porphyromonas Gingivalis* (PG), purchased from Eurofins Genomics (MWG Operon, Ebersberg, Germany), both contain synthetic DNA target sequences in their plasmid, which guarantee antibiotic resistance.
Bacteria were cultured in lysogeny broath (LB) containing both Kanamycin and Ampicillin (50ug/ml both) at 37°C for 24h in a shacking incubator. Ten microliters of this culture were used to "contaminate" fresh LB with antibiotics contained in a microcentrifuge tube together with

the implant. Tubes were then left at 37°C for 48h in a thermal bath, in order to allow bacterial growth and their hypothetical passage within the implant.

Procedures

A first preliminary microbiological test on ten implants coated with PXT was performed by contaminating the internal implant chambers with 20µl of a microbial pool constituted by: *Staphilococcus aureus*, ATCC 6538; *Escherichia coli*, ATCC 10536; *Pseudomonas aeruginosa*, ATCC 15442; *Enterococcus hirae*, ATCC 10541 and *Candida albicans*, ATCC 10231, at the concentration level of 1-5 x 10^6 ufc/ml. After 15 minutes of contact, development in LBA (Luria-Bertani Agar) and incubation at 37°C for 24h, the developed colonies were counted.
On the remaining ten implants treated with PXT, twenty microliters of sterile culture medium with antibiotics (kanamycin and ampicillin, each at 50ug/ml) were placed in the internal chamber of each implant, the abutments were screwed at 35 newton and fixtures placed in plastic tubes. Ten microliter of medium infected by genetic modified *Tannerella forsythia* (TF) and *Porphyromonas Gingivalis* (PG) were placed in 700µl of the external medium of each tube, an amount which was sufficient to cover perfectly the IAJ of the immersed implants. The tubes containing the implants in the contaminated medium were finally incubated at 37°C for 48h. After this time bacteria sampling was performed inside and outside implants with two paper tips capable of absorbing 10 µl each of the liquid medium. Twenty of these tips, ten for the internal sampling and ten for the external culture medium of the PXT treated implants, were object of quantitative PCR analysis which was performed to detect the amount of bacterial loading. Sampling of the external was performed after tubes agitation to homogenize the bacterial suspension. Analogous experiments were performed for the untreated implants. Finally ten paper tips, sampling the internal of the treated implants, and ten additional paper tips sampling the external culture medium were inserted in LBA Petri plates and incubated at 37°C for 24h. The antimicrobial activity was evaluated by the number of developed colonies. Also these experiments were repeated in parallel for the untreated control implants.

DNA extraction

Once collected, paper probe were put on a new microcentrifuge tube and processed for bacterial DNA extraction, by using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St., St. Louis, MO, USA), following the manufacturing procedures. Briefly, samples were incubated with lysozyme and, subsequently with proteinase K to isolate DNA. Once extracted, DNA was purified by spin-column method.

Real-time polymerase chain reaction

Bacterial quantification was performed by Real-Time Polymerase Chain Reaction using the absolute quantification with the standard curve method.
Primers and probes oligonucleotides for TF and PG were designed basing on 16S rRNA gene sequences of the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq Version 10.1).
For the quantitative analysis, plasmid (Eurofin MWG Operon, Ebersberg Germany) containing the specific DNA target sequence was employed as standard.
All reactions were performed in duplex, in 20ul final volumes; with 2X TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and 50nM concentration of each primers and 200nM of the probes. Amplifications were carry out by using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

SPSS program was used for statistical purposes and paired samples T-test done.

Results

Microbiological experiments

Ten of the coated implants were rinsed in 95% ethanol and water, dried and the internal part was then filled with 20µl of a suspension of a microbial pool constituted by: *Staphilococcus aureus*, ATCC 6538; *Escherichia coli*, ATCC 10536; *Pseudomonas aeruginosa*, ATCC 15442; *Enterococcus hirae*, ATCC 10541 and *Candida albicans*, ATCC 10231, at the concentration level of 1-5 x 10^6 ufc/ml. After 15 minutes of contact the implants were put in Petri dishes, LBA was added, and they were then incubated at 37°C for 24h. After this time the capsule was examined evaluating the colonies proliferation. Figure 1 shows the complete absence of colonies in the treated sample as observed for all coated implants, with respect to the control plates in which a 20µl amount of the same microbial pool were seeded in the Petri dishes.

In the microbiological evaluation, no living bacteria were detected in the internal part of the treated implants as well as in the external culture medium, as shown by the complete absence of colony forming units (UFC) (Table 1). For untreated implants the number of UFC detected in the internal part was on average 90% lower than that detected in the external culture medium (Table 1).

PCR analysis

PCR analysis on coated *vs* uncoated implants indicated a remarkable decrease of the bacterial count both in the internal part and in the external medium. A comparison between the bacterial count obtained in the internal chamber is reported in Figure 2 and in Figure 3 that obtained for the external medium. In all cases there was a statistical count reduction (see Figure 3 *vs* 2) and the sensitivity of the two genetically modified PG and TF to the antimicrobial coating was comparable. In fact for treated implants, on average, the total amount of bacteria, without any discrimination between living or inactivated species, detected in the internal part of the implants with respect to those present outside was 0.31% for PG and 0.32% for TF.

Table 1 - Bacterial count in the internal implant chamber and in the external culture medium observed for treated with PXT and untreated implants.

Untreated	**N1**	**N2**	**N3**	**N4**	**N5**	**N6**	**N7**	**N8**	**N9**	**N10**
External	C	G_{21}	G_{120}	G_2	G_{82}	G_{50}	G_{41}	C	G_9	G_{68}
Internal	G_{25}	G_2	G_{28}	NG	G_{19}	NG	G_{14}	G_{21}	G_1	NG
Treated	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**
Esternal	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
Internal	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG

Abbreviations: N=untreated implants; T=Treated implants; C=bacterial colonies at confluence; Gn=number of ufc/Petri; NG=no bacterial growth

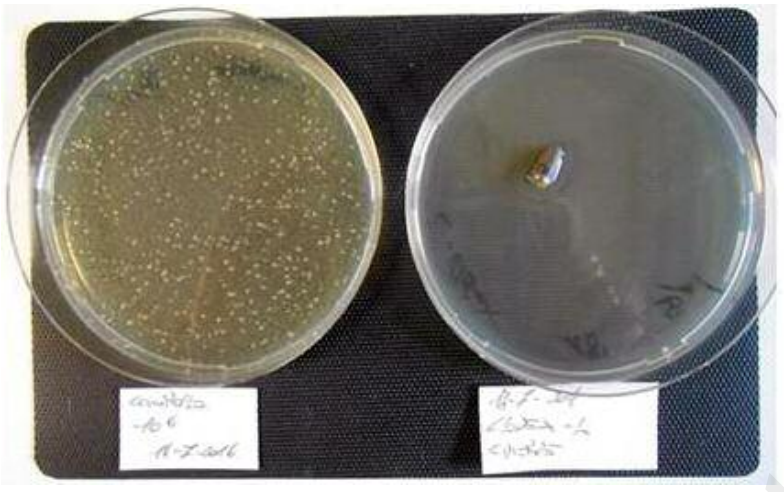

Figure 1
Left, control plate seeded with 20µl of the microbial pool after incubation. Right, Petri with the coated implant after contamination and incubation showing absence of colony forming units.

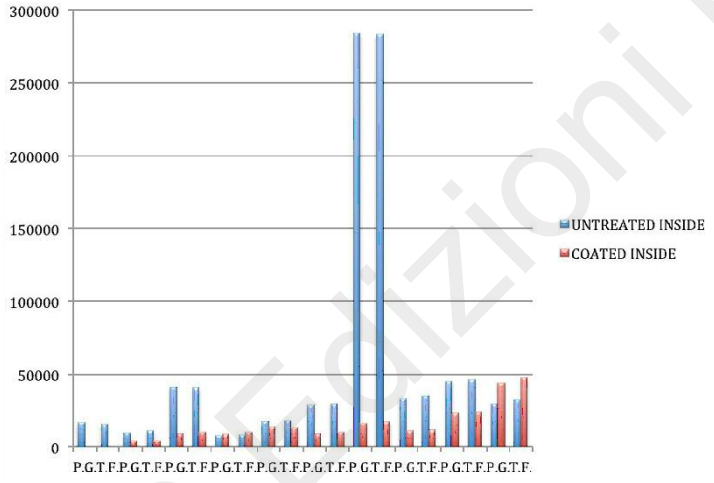

Figure 2
Bacterial count from PCR analysis in the internal implant chamber. P.G.-*Porphyromonas Gingivalis*; T.F. -*Tannerella forsythia.*

Discussion

Dental implants are an excellent treatment option for restoring areas that are missing one or more teeth. The majority of dental implants have two components: the implant placed in the bone during the surgical procedure, and the abutment, screwed into the implant to support the prosthetic rehabilitation. These two-piece implant systems present slots and cavities between the implant and the abutment that can act as a trap for the bacteria causing the accumulation of pathogens and the onset of peri-implantitis (58, 59). Peri-implantitis can happen with high frequencies in bone regeneration (60-63) also after cancer resection (64-68). In some pediatric conditions can be useful to have a low bacterial loading (69-77). During the prosthetic rehabilitation, bacterial dissemination is unavoidable,

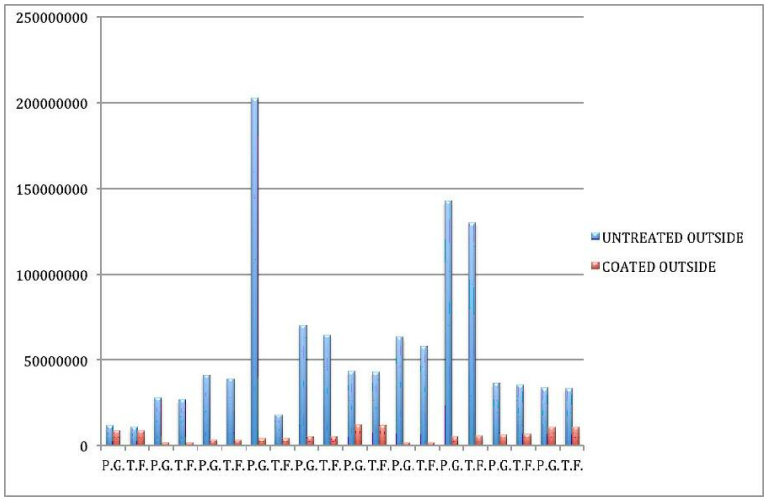

Figure 3
Bacterial count from PCR analysis in the external implant medium. P.G.-*Porphyromonas Gingivalis*; T.F.-*Tannerella forsythia.*

and when the IAJ is located at the level of the bone crest, the formation of biofilms in this area causes bone resorption, observed in the early stages of prosthetic load. Bacterial loading is an important factor in peri-implantitis, occurring during soft tissue manipulation of prosthetic rehabilitation (78, 79). It is clear that peri-implantitis occurs due to the presence of pathogenic microorganisms colonizing the surrounding implant area and the suppression or eradication of these microbes prevents peri-implantitis. The main cause of peri-implantitis consists in the passage of pathogenic bacteria in the abutment-implant gap. The inner spaces are easily colonized, and bacteria may leak out from these spaces through the IAJ into the peri-implant area. Peri-implantitis is usually associated with gram-negative bacteria similar to those that cause periodontal disease (80-84). Peri-implantitis, such as periodontal disease, is the result of the bacterial insult and the subsequent host response. Many studies have shown that bacterial species of periodontal disease are very similar to those that cause peri-implantitis (85-87). Covering IAJ surfaces with coatings, by adding biomimetic bioactive substances to improve its biological characteristics, has also been recently investigated (87). Modifications of IAJ using various modalities aim to improve prevention of bacterial infection and promote faster healing times. These aspects are of paramount importance in modern dentistry, since immediate or early loading has become a predictable treatment protocol (87). Coating IAJ surfaces with cell-adhesive proteins, improves cells attachment, proliferation, and activity (87). IAC coating has been tested in previous studies (86, 87), even if the interactions between coating and biomaterial surfaces are not fully understood.

The results of the present study demonstrate that the applications of the PXT antimicrobial coating to the IAC surface prevents the bacterial growth inside the implant chamber, eliminating thus one of the possible cause of infection, with differences between treated and untreated samples which are statistically significant. The microbiological experiments clearly show that in the internal part of the implants no bacterial growth is observed. The fact that also the external culture medium behave similarly can be consequence of the contact between the culture medium and the abutment treated with the antimicrobial PXT which can release during the 48h of immersion the active species chlorhexidine.

Conclusions

Aim of this study was to investigate the antimicrobial activity of a polymeric coating which has been designed to release a chlorhexidine component trapped in the polysiloxane matrix. The coating was applied to the internal chamber of the implants and to abutment surface. PCR analysis shows that in 48 h only 0.3% ca of the bacterial contaminating the external of the implant-abutment junction penetrates the internal implant chamber and is then completely inactivated, as clearly demonstrated by microbiological tests. The report demonstrated therefore, that the new coating determines a remarkable reduction of bacterial loading inside and outside IAJ thus becoming a very useful medical device in preventing peri-implantitis which are the main cause of failure in implantology.

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