A Novel Rat Model of Polymicrobial Peri-implantitis: A Preliminary Study

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Background: Peri-implantitis is a complex polymicrobial biofilm-induced inflammatory osteolytic gingival infection that results in orofacial implant failures. There are no preclinical in vivo studies in implant dentistry that have investigated the inflammatory response to known microbial biofilms observed in humans. The aim is to develop a novel peri-implant rat model using an established model of polymicrobial periodontitis.

Methods: Wistar rats were used for the study of experimental peri-implantitis. One month following extraction of maxillary first molars, a mini-titanium implant was inserted. Two months following implant healing, implants were uncovered, and abutment fixing was done using cyanoacrylate in order to prevent abutment loosening. Rats were separated into two groups (Group A: polymicrobial–infected, Group B: sham-infected). One week following healing of abutment, rats were infected with Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia for 12 weeks. Bacterial colonization, bone resorption, and implant inflammation were evaluated by PCR, microCT, and histology, respectively.

Results: Three rats with 4 implants in the infection group and 2 rats with 3 implants in the sham-infection group were analyzed. PCR analysis revealed presence of bacterial genomic DNA and infection elicited significant IgG and IgM antibody responses, indicating bacterial colonization/infection around implants. Infection-induced enhanced mean distance from the implant platform to the first bone to implant contact, extensive peri-implantitis with advanced bone resorption, and extensive inflammation with granulation tissue and PMNs.

Conclusions: This is the first study to develop a novel rat model of polymicrobial peri-implantitis. With modifications to improve implant retention it could offer significant advantages for studies of initiation and progression of peri-implantitis.

KEY WORDS:

Peri-implantitis; Dental implants; Porphyromonas gingivalis; Treponema denticola; Tannerella forsythia; polymicrobial infection.

Over the last few decades, dental implants is a common treatment alternative for missing teeth replacement, improving the quality of life of millions of patients and preventing disuse atrophy of the alveolar bone.1 However, biological complications such as peri-implant diseases commonly occur.2 Peri-implant disease is a collective phrase for inflammatory reactions, which are infectious in nature, in the tissues around an implant.3 Peri-implant mucositis is a common
term to describe the existence of inflammation in the mucosa at an implant with no signs of loss of supporting bone in addition to inflammation in the mucosa. While peri-implant mucositis represents the host response of the peri-implant tissues against a bacterial challenge, gingivitis, is a sum of responses of the host to a bacterial challenge in the gingiva. Peri-implantitis may also differ from periodontitis, both in the extent of lesion and composition of cells in the lesion as well as the rate of progression.

Using a ligature-induced defect model the association between bacterial biofilm formation and the pathogenesis of peri-implant diseases was demonstrated in animal studies. In this experimental model, mucositis and peri-implantitis lesions were induced by terminating the plaque control regimen and the placement and exchange of ligatures around the implant neck in a submucosal position. At peri-implantitis sites, this active breakdown period is usually terminated by a ligature removal, which was associated with spontaneous disease progression in a majority of sites (i.e. progression period). It is critical to note that the ligature-induced breakdown is an experimentally induced invasive procedure in order to achieve a certain degree of breakdown so that the final result can mimic a natural peri-implantitis lesion. Thus, it fails to serve as an ideal model to study progression as the investigator controls the ligature-induced breakdown process artificially. Further, the type of ligature, coronal–apical position of the ligature, and how often the ligature is replaced during the plaque formation interval determines the amount and rate of breakdown. Therefore, ligature-induced tissue destruction model fails to recapitulate in vivo peri-implant pathogenesis.

Rodent models serve as important tools for exploring molecular mechanisms behind the pathogenesis of infection-induced inflammatory diseases. In relation to the process of periodontal disease, rodent models have revealed that periodontal pathogens play a primordial role in initiating gingival inflammation and alveolar bone resorption, and humoral immune response to bacteria significantly influences periodontal tissue destruction.

Later studies has been demonstrated that P. gingivalis, T. denticola, and T. forsythia can colonize the rat and mouse gingival cavity, leading to gingival inflammation with induction of enhanced IgG immune response, and significant alveolar bone loss, typical of polymicrobial periodontitis. At present there are only limited rodent models available for evaluating biofilm-mediated pathogenesis of peri-implant diseases. Thus, this study is aimed to evaluate a rat model for polybacterial infection in the progression of peri-implantitis.

**MATERIALS AND METHODS**

**Rats**

The protocol of the study was approved by the Institutional Animal Care and Use Committee of the University of Florida (Protocol #201408568). Twelve 5-week-old male Wistar rats (weighing 150 grams) were used. Rats were kept in groups, housed under microisolator conditions, fed standard powdered chow, and given H2O ad libitum. The design of the study is described in Fig. 1A. The rats were separated into two experimental groups. Rats in Group A (n=6) were exposed to polymicrobial infection and rats in Group B (n=6) served as sham-infected controls.
Experimental Procedure and Scheduling

During all surgical procedures, rats were kept under general anesthesia by intramuscular injection of 1 mg/kg of a solution containing 100 mg/ml ketamine and 20 mg/ml xylazine. Extraction of maxillary first molars was performed (Fig. 2A and 2G). One month after healing (period 1), mucoperiosteal flaps were elevated at the edentulous maxillary molar region and osteotomies were prepared with a 1.7 mm diameter reamer. Custom-made, pure titanium, machined-surfaced implants with 1.5 mm diameter and 2 mm lengths (Fig. 2E) were installed* (Fig. 2B). The alveolar mucosa was sutured back with absorbable sutures and implants submerged. Two months (healing period 2) following implant installation implants were uncovered and 1 mm custom abutments (Fig. 2F) were fixed using cyanoacrylate in order to prevent abutment loosening (Fig. 2C, D). One week following healing (Period 3), polybacterial oral infection was initiated for rats in Group A and rats in Group B were sham-infected with the vehicle.

Bacterial Strains and Polybacterial Inocula

P. gingivalis ATCC 53977, T. denticola ATCC 35404, and T. forsythia ATCC 43037 were cultured anaerobically at 37°C in anaerobic chamber and maintained for gingival infection as described previously.15, 19, 21 Bacterial amounts were determined quantitatively and the organisms were suspended in equal proportions in reduced transport fluid at 10^10 bacteria per ml. To initiate gingival infection, P. gingivalis (3.3 x 10^9) cells were mixed with an equal amount of T. denticola (3.3 x 10^9) cells and permitted to interact for 5 min. Then, T. forsythia (3.3 x 10^9) cells were added to the tube gently vortexed for 1-2 min and permitted to interact for 5 min. Finally, an equal amount of sterile 4% low-viscosity carboxymethylcellulose† was added, vortexed thoroughly, and 0.5 ml was administered by gingival lavage using isoflurane inhalation anesthesia.

Peri-Implantitis Induction With P. gingivalis, T. denticola, and T. forsythia

All rats were administered kanamycin (500μg/ml) in the drinking water for 4 consecutive days.15, 22 The gingival surfaces were swabbed with chlorhexidine gluconate‡ mouth rinse15, 22 to inhibit the rat oral microflora and enhance subsequent colonization of P. gingivalis/T. denticola/T. forsythia.21 Following a 3-day antibiotic washout period, polymicrobial inocula were rendered by gingival lavage for 4 consecutive days per week on 6 alternate weeks for a total of 24 inoculations over infection period of 12 weeks of the experimental Group A. Sham-infected rats (Group B) received vehicle (sterile 4% CMC) only.

Gingival Plaque Sampling and Bacterial Colonization

Gingival plaque samples from around mini-implants were gathered using sterile veterinary cotton swab rubbing over the abutment surfaces after infection.15 For the sake of monitoring the colonization/infection with minimal disruption, a total of two post-infection microbial samples (fourth and fifth infections) were collected following the 8th and 10th weeks from all polybacterial-infected rats. At the end of the experiment the rats were euthanized. Gingival plaque samples were gathered 72h post-infection from the implants both infected and sham-
infected rats. Subsequently, PCR was done using 16S rRNA bacterial species-specific oligonucleotide primers in a thermal cycler as described previously.\textsuperscript{15, 21, 22} PCR products were then separated by agarose (1.5\%) gel electrophoresis and ethidium bromide staining. The known genomic DNA of \textit{P. gingivalis}, \textit{T. denticola}, and \textit{T. forsythia} was used as positive controls and no template DNA was used as a negative control.

**Serum IgG and IgM antibody analysis.** After euthanasia, blood was collected from each rat by cardiac puncture and sera were removed and used for analyzing IgG and IgM antibody concentrations with a standard ELISA protocol.\textsuperscript{19, 23-26} Mean bacteria-specific antibody titers of infected rat were divided by sham-infected mean antibody titers, and the result represented the level change in mean bacteria-specific antibody titer as a result of polybacterial infection. Graphs demonstrate mean fold-change in specific antibody titer of infected rats.

**Morphometric Analysis of Alveolar Bone Resorption (ABR)**

The horizontal alveolar bone resorption (ABR) area was evaluated by histomorphometry as described previously.\textsuperscript{24, 25} The rat mandibles were autoclaved, defleshed and later were suspended in 3\% (vol/vol) hydrogen peroxide for 3 hours. Digital images of the root surfaces of three molar teeth were acquired under a 10× stereo dissecting microscope.\textsuperscript{5} Line tool was used to evaluate the ABR area from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC). The surface perimeters of CEJ and ABC were traced with the help of the calibrated line tool.

**Analysis of Microcomputed Tomography (microCT) Peri-Implant Bone Levels**

Peri-implant bone levels were evaluated by microCT analysis. Rat maxillae were scanned by microCT\textsuperscript{**} with 18 × 18 × 18 μm\textsuperscript{3} voxel size. Peri-implant bone volume was evaluated in central transaxial and sagittal sections of the implants as the distance from the implant platform (IP) to the first bone to implant contact (BIP) using ImageJ\textsuperscript{††} image processing software (NIH).

**Histological Evaluation of Inflammation**

The maxillae of the control and infected rats were fixed in buffered formalin (10\%) and then decalcified in phosphate buffered saline containing 0.4 M EDTA and 2\% formaldehyde. The solution was replaced every other day for six weeks. Implants with healing abutments were then gently removed by unscrewing with pliers. The specimens were then embedded in paraffin and sectioning was done using routine protocols. The specimens were sectioned (7 μm) in the mesiodistal direction. From each block of section every fifth slide was stained with hematoxylin and eosin. Stained sections were used to assess the migration of the peri-implant epithelium, connectives tissue, bone contact and the level of inflammation by light microscopy. Images of stained sections were captured using a digital camera and transferred to image editing software Adobe Photoshop\textsuperscript{‡‡}.

**Statistical Analyses**

Serum IgG and IgM antibody responses were analyzed using unpaired two-tailed Student’s \textit{t} test, and expressed as mean fold change between infected and sham-infected rats. MicroCT data of peri-implant bone levels between infected and sham-infected rats were studied d using the Mann-
Whitney U-test. Mandibular horizontal ABR levels between infected and sham-infected rats were analyzed using unpaired two-tailed Student’s $t$ test with statistic software GraphPad Prism 5.0 software. A P value of <0.05 was considered statistically significant.

RESULTS

**Clinical Results of Titanium Implants**

One rat in Group A and two rats in Group B were euthanized due to corneal infection following the mini-implant installation surgical procedure. The development of corneal infection was not related to the implant placement procedure, but was the result of a self-injury. In addition, two other rats in Group B died due to aspiration of blood during recovery time after the implant installation procedure. At the time of implant recovery, two rats in Group A and one rat in Group B had one implant that failed to integrate. At the end of the experiment, two more rats in Group A had both implants fail. Thus, at the end of the experiment 3 rats in Group A with 4 implants and 2 rats in Group B with 3 implants were available for microCT and histologic evaluation (Fig. 3A-D). For two out of four implants in the Group A and one out of three implants in the Group B the healing abutment was not present at the end of the experiment.

**Peri-Implantitis Induction With Periodontal Bacteria**

Gingival plaque specimens were collected from rats with titanium-implant and abutment infected with a polybacterial inoculum 3 days after each infection to detect colonization/infection by three periodontal bacteria. Using bacterium-specific primers revealed all rats were positive for *P. gingivalis*, *T. denticola*, and *T. forsythia* genomic DNA indicating bacterial adherence to the gingival surfaces around the implants and demonstrating the ability of these periodontal bacteria to colonize in rat gingiva after infection (Table 1). None of the sham-infected rats contained genomic DNA for the three bacteria.

**Polybacterial Infection Elicits Humoral Antibody Response**

To evaluate the induction of specific humoral antibody response against the three periodontal bacteria, serum levels of IgG and IgM antibodies in infected and sham-infected rats were measured. Infection induced significant serum IgG (Fig. 1Bi) and IgM antibodies (Fig. 1Bii) ($P <0.05$) against all three bacteria compared to sham-infected rats. These antibody data corroborated the bacterial adherence to and colonization/infection of implanted rat gingival surface.

**Polybacterial Infection Elicits Mandibular Alveolar Bone Resorption**

Mandibular (left and right lingual) horizontal ABR area was measured following polybacterial infection by morphometry. Infected rat mandible had significant ($P <0.05$) ABR compared to sham-infected rats (Fig. 1C and Di, ii). These results clearly indicate infection-induced ABR in rats with polymicrobial infection. In addition, ABR correlates with bacterial colonization of gingival surfaces and induction of bacterial-specific immune responses following infection.
**MicroCT Imaging Analysis of Peri-Implant Bone**

The peri-implant maxillae bone resorption was measured in infected and sham-infected rats (Fig. 4A-B). Rats with mini-implants and polymicrobial infection showed greater mean distance from the implant platform (IP) to the first bone to implant contact (BIC) (Fig. 4Ci, ii) compared to implant plus sham-infected rats (Fig. 4 Di, ii) (0.80±0.37 mm vs 0.48 ± 0.13 mm). However, there was no significant difference in bone resorption between infected and sham-infected rats ($P >0.05$).

**Peri-Implant Histology**

In the control group, three implants were retained in two rats for histological assessment. Peri-implant epithelium (PIE) was composed of extended keratinized oral epithelium with no resemblance to typical tooth lining junctional epithelium (Fig. 5A and 5B). The connective tissue (CT) immediately underneath the epithelium was practically free of inflammation. The connective tissue above the bone crest (BC) and below the PIE that leans against the implant abutment had a mild inflammatory infiltrate with capillaries and many PMN cells. Implant-bone contact was well preserved in all control specimens (Fig. 5C). Four implants were recovered in three rats in the infected group (40%). The histological view of two implants in one rat in this group was similar to the control implants (not shown). However, the other two infected implants showed extensive peri-implantitis with advanced bone resorption and extensive inflammation (Fig. 5D-F). Interestingly, the location of the PIE in these specimens was comparable to the sham-infected specimens. There were no signs of formation of pocket epithelium in the infected samples. Bone-contact areas were often minimal and replaced by the inflammatory granulation tissue (Fig. 5E). Intriguingly, inflammatory cells facing the implant threads were predominantly PMNs (Fig. 5F).

**DISCUSSION**

Peri-implantitis has become a global clinical problem and affects 10-20% of implants to varying degrees. The peri-implantitis microbiome differs from periodontitis but still harbors many periodontal pathogens, including *P. gingivalis, T. denticola,* and *T. forsythia* used in this study. The pathogenesis of peri-implant lesions remains poorly understood due to a nonavailability inexpensive animal models for peri-implantitis. To date, most animal studies have been performed with dogs. Limitations of the dog model include the cost of required number of dogs as well as the emotional aversion to using family pets for research purposes. Use of a small number of dogs does not allow dissection of longitudinal cellular and molecular events that lead to establishment of peri-implant lesions. In addition, peri-implant lesions need to be initiated with ligatures around the implants that may not truly mimic initiation of peri-implantitis. Animal studies and cross-sectional human histological data collectively suggest that peri-implant lesions differ from periodontitis lesions in at least three aspects. First, the formation of barrier pocket epithelium in peri-implant lesions is limited at the apical aspect of the lesion and thus it does not “insulate” the biofilm from the inflammatory infiltrate. This means that bacteria are in direct contact with inflammatory cells. In our rat model, peri-implant epithelium (PIE) showed practically no migration to form a true pocket epithelium in the infected peri-implantitis.
specimens, thus supporting the previous findings. Second, there are more PMN cells in the peri-implant lesions compared to those with periodontitis. This finding was also corroborated in our rat model as PMN cells were found lining the implant surface. It is not surprising, therefore, that peri-implantitis lesions are frequently associated with pus discharge (dead PMN and other leukocytes and bacteria). Third, inflammation extends to the alveolar crest in peri-implantitis while inflammation is rarely seen within 1 mm of bone in periodontitis. This finding was also supported in our rat model. In summary, the rat model of multi-species infection replicates well the histological observations made previously with the dog models, as well as the human peri-implant histology data.

The disadvantage of the rat model used in this study was substantial loss of implants or healing abutments during the course of 24 weeks. Abutments were finger torqued and secured with cyanoacrylate to prevent loosening. The abutment loosening can be reduced/prevented by using torque device, and/or making the healing abutments even thinner (1 mm to 0.5 mm) and possibly reduce the coronal dimension so as to decrease the amount of abutment surface area subjected to functional forces. There were a few more implants lost in the infected group than sham-infected group which could possibly be construed due to the loosening of the implant due to peri-implantitis. However, the difference was not significant so it is more likely that in the rodent model more implants are lost due to function and trauma. Softer diet might offer better implant survival in rodents. The fact that peri-implantitis was advanced in the infected group in 24 weeks indicates that a shorter experimental timeframe should be used, which may lead to preservation of more implants with initial peri-implantitis lesions. It is conceivable that a shorter infection time frame will be sufficient for introduction of initial to moderate peri-implant lesions in suspected rats. Understanding the development of initial to moderate lesions is likely more important for understanding the pathological process than further advancement of moderate lesions. Increasing the number of rats is relatively easy and less expensive than using dogs. In addition, a clear advantage is the natural process of disease without mechanical trauma from ligatures, thus better mimicking natural peri-implantitis in humans.

In the current study, the peri-implant tissue reaction of implants exposed to polymicrobial infection was analyzed. It was observed that the amount of bone resorption present at the end of the study period was greater for implants exposed to polymicrobial infection than control implants. The histological analysis showed that implants subjected to polymicrobial infection had peri-implant bone replaced by granulation tissue with PMN lining the surface that was used previously in contact with bone. This suggests that microbial infection with P. gingivalis/T. denticola/T. forsythia induced an inflammatory reaction to peri-implant tissues and subsequent ABR in rats.

The present study addressed the feasibility of inducing peri-implantitis in rats utilizing a polymicrobial infection model. The majority of the information regarding peri-implant infections is derived from larger animals utilizing a ligature-induced peri-implantitis model. However, ligature-induced breakdown is a an experimental invasive procedure to achieve a certain degree of breakdown with the investigator controlling the process. The type of ligature, the coronal–apical position of the ligature, and how frequently the ligature is replaced during the formation of plaque determine the rate and amount of breakdown. Thus, ligature-induced tissue destruction fails to precisely recapitulate in vivo peri-implant pathogenesis.
Rodents have been utilized in order to evaluate peri-implant bone reactions under local conditions such as excessive loading\textsuperscript{32, 33} and systemic conditions such as osteoporosis\textsuperscript{34} and diabetes.\textsuperscript{35} There is limited information for evaluation of peri-implant soft and hard tissue responses to bacterial infection in rodents. Freire et al.\textsuperscript{20} utilized implants containing an established biofilm contaminated with \textit{A. actinomycetemcomitans} in rats. They demonstrated that after 6 weeks implants containing the \textit{A. actinomycetemcomitans} biofilm expressed greater inflammation assessed by clinical means and greater bone volume loss assessed by microCT. In the current study, we have utilized a multi-species infection model with \textit{P. gingivalis/T. denticola/T. forsythia} that has been extensively used in induction of periodontitis in rats\textsuperscript{15-17} and mice.\textsuperscript{18, 19, 24, 36} This consortium of bacteria has been recognized as the sign of the pathogenic bacterial biofilm in deep periodontal pockets in chronic periodontitis\textsuperscript{37} and stimulates maxillary and mandibular ABR in rats.\textsuperscript{15} We reported for the first time that this polymicrobial consortium has the potential to induce peri-implant bone resorption and inflammation in rats. Although it has reported earlier\textsuperscript{38} that supposed periodontal pathogens such as \textit{P. gingivalis, P. intermedia,} and \textit{A. actinomycetemcomitans} to be present in majority of peri-implant lesions examined, more current studies\textsuperscript{27, 28} have reported distinct differences in microbiological profiles of periodontal and peri-implant lesions. In peri-implant areas \textit{Staphylococci, enterococci,} and yeasts were found almost as frequently as periopathogens indicating differences regarding the microbiota around affected teeth. The currently used polymicrobial infection model may facilitate better knowing of the role of different bacteria on the peri-implantitis pathogenesis.

In the present study, implants with machined surface were used. It has been reported that implant surface characteristics can influence the advancement of peri-implantitis, with modified implant surfaces demonstrating greater rate of ligature-induced peri-implantitis progression compared to machined surfaces.\textsuperscript{31, 39, 40} However, the influence of implant surface characteristics on peri-implantitis progression not yet fully understood in physiological infection-induced models or in clinical observations. Thus, a model for physiological induction of peri-implantitis such as a polymicrobial infection model is highly relevant for further understanding the effects of implant surface characteristics on the pathogenesis and progression of peri-implantitis.

The amount of bone resorption observed for implants subjected to polymicrobial infection was greater compared to control implants, but the difference not attained statistical significance. This may be due to the small sample size of this study. The experiment initiated with 6 rats per group having 12 implants total. At the end of the experiment, only 3 rats with 4 implants in the test group and 2 rats with 3 implants in the control group were available for analysis. It is also critical to note that the most extensive bone loss was observed in two out of four implants in the test group. As this is a major limitation of the study, the current findings need to be confirmed with further studies. However, the histological observations confirm the inflammatory changes in the peri-implant area and validate the findings despite the small sample size.

**CONCLUSION:**

In summary, our preliminary studies using a rat model for peri-implantitis with polymicrobial infection with human pathogens suggest that it could offer significant advantages for studies on initiation and progression of peri-implantitis and the roles of implant, host and microbes on the peri-implantitis disease process.
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COMPETING INTERESTS:
The authors have declared that no competing interests exist.

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Figure 1.

(A) Schematic diagram of the experimental design illustrating rat teeth extractions, implant and abutment placement, polymicrobial infection, gingival plaque sampling, PCR, and euthanasia. Six polymicrobial infections (Infection I - VI) are indicated at 1, 3, 5, 7, 9, and 11 weeks of age. Gingival plaque samplings are indicated by PCR at week 8 and 10 following the initial infection. **B** Polymbacterial-infection induced bacteria-specific IgG (B i) and IgM (B ii) humoral immune responses in infected rats that is significantly greater than sham-infected rats (*P* <0.05). **C** Polymbacterial-infection induced total (left and right lingual mandible) ABR in infected rats that is significantly greater than sham-infected rats (*P* <0.05). **D** Representative left mandibular lingual view of infected (D i) and sham-infected (D ii) rats with area of ABR outlined from alveolar bone crest (ABC) to cementoenamel junction (CEJ). Pg/Td/Tf-polymbacterial infection Pg–P. gingivalis, Td–T. denticola, Tf–T. forsythia. *P* <0.05.

Figure 2.

(A, B) A representative image depicting the maxillary bilateral implant placement into the palatal process in rats. **C** A representative right maxillary image of a mini-implant (inset) with abutment into the palatal process. **D** Abutments measured 1.0 mm in height. **E** Representative image of mini-titanium implant used in the present study and rat maxillary extracted tooth (M1). Implants measured 1.5 mm in width and 2.0 mm in height.

Figure 3.

(A) Representative clinical photographic images of rat maxillary implants with abutments were taken after 12 weeks of polymicrobial infection of implants **A** (n=5) and **B** sham-infection of implants (n=2). Red arrow indicates implants and abutments clinically visible **A iv and B ii** and submerged **A i, A iii, and B i** under overgrown palatal tissues but visible by microCT.

Figure 4.

Representative sagittal **A** and frontal **B** views of a maxillary left implant. Marginal bone height at implants was measured using ImageJ analysis software. **IP** indicates implant platform, **BIC** indicates the first (most coronal)
bone-to-implant contact, and AP indicates the most apical part of the implant. Sagittal view of implant and infected maxillary right (C i) and left (C ii) implants. Sagittal view of implant and sham-infected maxillary right (D i) and left (D ii) implants. M2, M3 are remaining molars. Implants were placed into original M1 area.

**Figure 5.**

Histological staining of the peri-implant tissues in the control A-C and infected D-F rats showing bone resorption and inflammation. A) Overview of a control implant shows normal peri-implant epithelium (PIE, magnified in panel B), mild inflamed connective tissue (CT) and normal bone contact with threads clearly visible (BC, magnified in panel C). D) Overview of the infected implant showing blunt and thickened PIE (magnified in panel E) with little apical migration. The connective tissues (CT) and bone to implant contact (BC) areas have been replaced by granulation tissue (GT) (D,E). Some thread sites that previously had bone contact have been replaced by inflammatory tissue with polymorphonuclear leukocytes lining the surface that used to have implant contact F). Arrows in panels B and E point to the termination of the PIE.

**Table 1.**

<table>
<thead>
<tr>
<th>Polymicrobial Infection</th>
<th>Rats</th>
<th>No. of gingival microbial samples positive for PCR</th>
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<tr>
<td></td>
<td></td>
<td>P. gingivalis 8 week</td>
</tr>
<tr>
<td>Pg+Td+Tf</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>0</td>
</tr>
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</table>

Gingival plaque samples were collected from polybacterial-infected and sham-infected rats at the 8th and 10th weeks and examined for *P. gingivalis* (*Pg*), *T. denticola* (*Td*), and *T. forsythia* (*Tf*) using bacteria-specific primers.

* JMR, Niigata, Japan
† CMC, Sigma, St Louis, MO
‡ PerioGard, Colgate-Palmolive Co, New York, NY
§ SteReo Discovery V8; Carl Zeiss Microimaging Inc, Thornwood, NY
** AxioVision LE 29A software version 4.6.3
†† Image J, NIH, Bethesda, MD
‡‡ Adobe Photoshop, Seattle, WA
§§ GraphPad Prism, La Jolla, CA
Figure 1

A

Fold increase over control

1 m 2 m 1 wk 3 d 1 3 5 7 9 11 13 weeks

Antibiotics

PCR PCR

B i

IgG fold change

Fold increase over control

Pg Td Tf

* *

B ii

IgM fold change

Fold increase over control

Pg Td Tf

*

C

Σ bone loss (mm²)

Infected Ctrl

*

D

i

M3 M2 M1 3.2 mm²

CEJ

ii

M3 M2 M1 2.35 mm²

ABC

13.
Figure 2
Figure 4

A

B

C i

C ii

D i

D ii

IP

BIC

AP

Distal

Mesial

Buccal

Lingual

Right implant Sagittal

Left implant Sagittal

Right implant Sagittal

Left implant Sagittal

M2

M3

M2

M3

M2

M3

M2

M3

M2

M3