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A new small animal model for simulating a two-stage-revision procedure in implant-related methicillin-resistant *Staphylococcus aureus* bone infection

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ABSTRACT

Background: Implant-related bone infections with *methicillin-resistant Staphylococcus aureus* (MRSA) remain a challenge for orthopedic surgeons. This devasting complication may lead to functional impairment and loss of the affected limbs. High failure rates in treatment make improvement of surgical treatment necessary. Beside an already established demanding and costly large animal model, a small animal model of a two-stage revision does not exist, yet. Thus, the purpose of this study was to establish a preclinical small animal model to simulate a two-stage revision in implant-related MRSA infection.
Materials and Methods: In twelve rabbits Steel K-wires were implanted into the intramedullary canal of the left tibia, followed by inoculation with MRSA. Two different clinical isolates of MRSA-strains were used in two different concentrations (CFUs; 10^5 and 10^7 colony forming units (CFUs). This led to four groups of three rabbits each. Eleven rabbits survived the whole study period. After four weeks the inoculated K-wires were removed and replaced with vancomycin loaded PMMA-spacers (stage 1). Twenty-eight days later new K-wire implants were placed intramedullary (stage 2). After 84 days all animals were sacrificed. Tibiae were analyzed microbiologically, radiologically and histologically.
Results: In every rabbit K-wire associated infection could be established within the first four weeks. After irrigation and debridement at revision one (stage 1), infection could be eradicated in 67% of group I, in 50% of group II and in 33% of group III and IV. Recurrence of the infection could be determined in all animals of group I and IV at day 84. X-ray analysis and histology both demonstrated clear signs of osteomyelitis after twelve weeks. Survival, clinical observations and weight assessment confirmed the ethical justifiable stress of the animals during the experiment.
Conclusion: The presented small animal model of a two-stage revision in implant-related infection is a promising preclinical set-up for assessment of new treatment strategies of implant-related infections. Both high survival as well as reinfection rates were possible by simulating the clinical gold standard of two-stage revision surgery in an MRSA implant-related infection model. Therefore, the model can be deemed suitable for further preclinical *in vivo* testing.

Introduction

Orthopedic implant-related infections such as periprosthetic joint infections (PJI) and osteosynthesis related infections are a devastating problem for both patients and surgeons. Besides being potentially life-threatening, implant related infections are a socioeconomic burden due to increasing health-care expenditures,

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functional impairment and long-lasting disabilities [1]. While *Staphylococcus aureus*, methicillin-resistant *Staphylococcus epidermidis* (MRSE), methicillin-resistant *Staphylococcus aureus* (MRSA), coagulase-negative Staphylococcus (CoNS), Streptococcus, and Enterococcus are mainly responsible for PJI, an increase of high-resistance pathogens has occurred within the last years [2]. Compared to PJI, the distribution of infect causing pathogens is similar for osteosynthesis related infections [3]. *Staphylococcus aureus* is the most common pathogen, while methicillin-resistant *Staphylococcus aureus* is the most frequent antibiotic resistant bacteria [4,5]. For MRSA-related infections a two-stage-revision is considered the gold standard in clinical routine. After an initial surgery with implant removal, debridement, irrigation and insertion of a spacer (stage 1), a second operation follows with insertion of a new implant after spacer removal (stage 2) [6].

Despite rigorous treatment, eradication rates of MRSA are worse than eradication rates of infections caused by norm-sensitive germs. Only 18% of PJIs of the knee and 48% of PJIs of the hip caused by MRSA have a positive outcome compared to 81% and 89% caused by norm-sensitive germs, respectively [7]. In MRSA infections the risk of amputation or permanent removal without reinsertion of a new implant is high. Loss of health-related quality of the patient's life is significant [7]. Furthermore, reinfection rates of up to 21% for hip prosthesis and up to 50% for MRSA-related knee prosthesis are reported in literature [8]. This demonstrates the urgent necessity of improved treatment strategies for MRSA-related infections, since approximately 112,000 implant-related infections per year with costs of 15,000–70,000$ per case are generated in the USA [9].

For preclinical testing, animal models are essential. Fintan Moriarty's group already developed a large animal model for a two-stage revision of intramedullary nail-related infection by MRSA [10]. Until today, however, there is no small animal model established that simulates a two-stage-revision procedure appropriately [11]. Hence, the aim of this study was to establish a new small animal model for simulating a two-stage-revision procedure by implant-related MRSA infections. This model should allow further *in vivo* testing to develop new strategies in the struggle against implant-related MRSA infections. For this purpose, we advanced one of our former implant-related infection models, in which implant-related osteomyelitis could reliably established within four weeks [12].

Materials and methods

Study design

All experiments were approved by the local animal welfare commission of the Freistaat Thüringen (Registration number: 14-003/13) before the start of the surgeries. For this study, twelve New Zealand white rabbits were divided into four groups depending on the strain and on the inoculation dose: Group I: Eugen Domann Culture Collection (EDCC) 5443 methicillin-resistant *Staphylococcus aureus* (MRSA) with an inoculation dose of 10^5 CFUs; Group II: MRSA EDCC 5443 with an inoculation dose of 10^7 CFUs, Group III: MRSA EDCC 5398 with an inoculation dose of 10^5 CFUs and Group IV: MRSA EDCC 5398 with an inoculation dose of 10^7 CFUs. Each group consisted of three animals. The experimental part was planned for 84 days sectioned into four parts: Initial surgery (day 0), revision 1 (day 28, stage 1), revision 2 (day 56, stage 2) and final evaluation with the euthanasia of the animals (day 84) (Fig. 1).

Animals

Twelve New Zealand white rabbits were used as laboratory animals. This species was chosen because of good results in prior studies [11,12]. The twelve animals were obtained from Dipl. Ing. Agr. Ronald Krieg (Niederwünsch, Germany). They aged 6–8 months. Average weight was 5.8 ± 0.24 kg. Each rabbit was kept in an air-conditioned stud in single boxes.

Bacteria

Two clinical isolates of *methicillin-resistant Staphylococcus aureus*, termed EDCC 5443 and EDCC 5398 (Eugen Domann Culture Collection, Institute of Medical Microbiology, Giessen, Germany) were used. The isolates were identified by API biochemical characteristic testing (bioMerieux, Marcy L'Etoile, France), by sequencing the 16S rDNA gene, and by specific polymerase chain reaction (PCR) to detect Enterotoxin G und I and MecA genes. These strains exhibited strong hemolytic activity and biofilm formation capacity.

The isolates were cultivated in BHI broth at 37 °C under vigorous shaking for 16 h. The cultures were diluted 1:50 and cultivated again as for four hours. The cultures were diluted 1:10 in

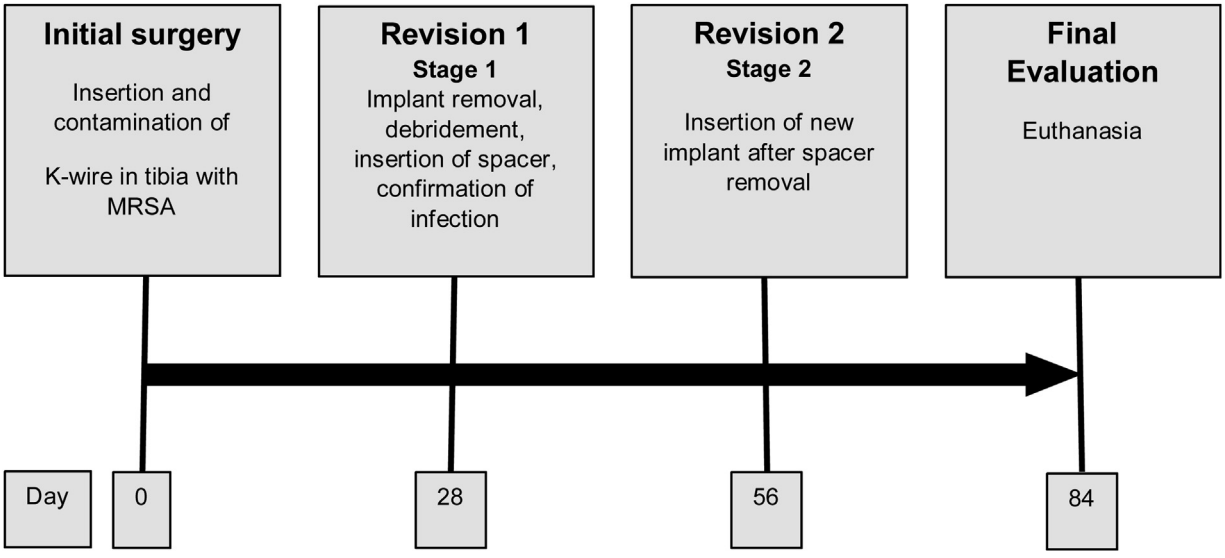

Fig. 1. Time schedule for two-stage-revision rabbit model.

phosphate-buffered saline (PBS) and plated on BHI agar plates using a spiral plater for the enumeration of *Staphylococcus aureus* in CFUs per ml. Suspensions were generated with a final volume of 200 µl for inoculations with a concentration of 10^5 and 10^7 CFUs per 20 µl in BHI/20% glycerol and stored at −80 °C until usage.

Spacer

For treatment of the K-wire related infection after the first four weeks, vancomycin loaded cement spacers (Fa. aap Biomaterials GmbH, Dieburg, Germany) were used and placed intramedullary at revision 1. 2 g of vancomycin was used per 40 g of PMMA These spacers were 60 mm long with a diameter of 2 mm and had a local vancomycin release rate of 1.569 mg vancomycin over four days (data not shown).

Surgical procedure

Surgical technique

After official approval of the local animal care committee, surgeries were carried out in general anesthesia using ketamine (35 mg/kg bodyweight) and medetomidin (025 mg/kg bodyweight) under aseptic conditions. After shaving and disinfection of the left lower limb, the knee region was draped in sterile manner.

For the first surgery with introduction and bacterial contamination of the K-wire a similar technique was used as previously described by our group [12]. In brief, the tibial tuberosity was approached *via* a 1 cm incision followed by splitting of the patellar tendon. The tibial tuberosity was perforated by a 2 mm hand brace and the K-wire was subsequently introduced into the intramedullary canal. The K-wire was driven to the distal part of the tibial canal and its overlapping part was cut. This was followed by inoculating the different bacteria with different inoculation doses into the medullary canal according to the study protocol after randomization of the animals. For revision 1 (day 28, stage 1), the K-wires were explanted and the bone marrow canal was irrigated with 20 ml of 0.9% saline solution and debrided followed by the insertion of a vancomycin-loaded polymethacrylate (PMMA) spacer (Fig. 2). The explanted K-wires were rolled out on Brain heart infusion (BHI) plates to determine the bacterial load on the implant. For revision 2 (day 56, stage 2), the spacer was removed and also rolled out on BHI plates. Another irrigation and

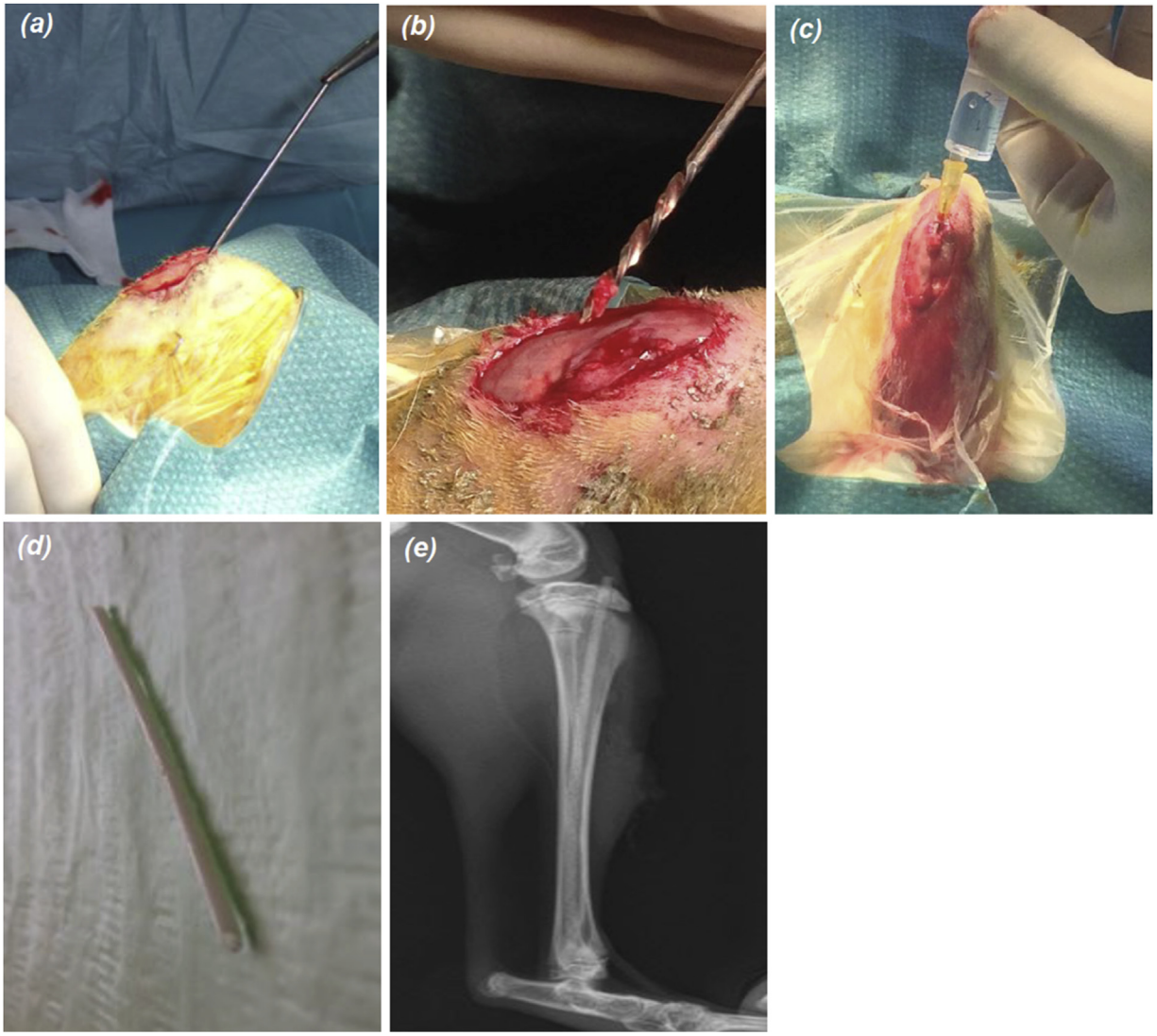

Fig. 2. Surgical technique of revision procedure#1.
Removal of the intramedullary implant after transpatellar approach **(a)**, debridement of the intramedullary canal with a drill **(b)**, followed by saline irrigation **(c)**, introduction of a vancomycin-loaded PMMA spacer **(d)** into the intramedullary tibial canal **(e)**.

debridement procedure were conducted as for revision 1. For final evaluation (day 84), rabbits were euthanized and clinical assessment of the wounds was performed. The K-wires were removed and processed for sonication and CFUs assessment. The tibia was split into two halves for histological analysis and microbiological assessment of CFUs of MRSA in the bone marrow tissue samples after curettage.

Postoperative follow-up

A daily postoperative follow-up on all animals was performed by a veterinarian. It based on a score sheet with criteria such as behavior, general condition, weight bearing of the operated limb, wound healing, animals´ weight and temperature. Further, any animals displaying persisting secretion of pus, rejection of nutriment, a massive loss of weight (20% or more within a few days) or a complete loss of function of the left limb were considered for early euthanasia.

Weight assessment

Daily weight controls were performed over the whole period of twelve weeks. Weight changes were analyzed by students *t*-test with a p value < 0.05 considered significant.

X-Ray imaging

After every surgery, X-rays in two planes of the left limb were taken to confirm the correct position of the implanted K-wire, spacer or its complete removal. Criteria for osteomyelitis were lytic lesions and periosteal scalloping.

Clinical assessment after euthanasia

For all animals the calf, the adjacent knee and the ankle joint were evaluated for any clinical signs of infection. such as swelling, redness, fistula, soft-tissue defects and pus drainage. Both soft tissue and the intramedullary canal of the tibia were assessed for pus, abscess formation, cortical lysis and for joint effusion of the adjacent knee joint.

Microbiological evaluation

Standard agar plating

After sterile removal of the K-wires (day 28 and day 84) and spacers (day 56), they were rolled out on BHI agar plates. Positive culture growth after an incubation time of 18 h at 37 °C indicated colonization of the implant and was interpreted as infection.

Sonication

K-wires were aseptically removed and immersed in 10 ml sterile PBS in Greiner tubes. The tubes were vortexed for 1 min and then subjected to sonication (Sonomatic 300 PC Apparatebau Clemens Bous, Cologne, Germany) for 5 min, followed by 1 min of vortexing. Suspensions were 1:10 and 1:100 diluted. 50 μl of non-diluted as well as diluted suspensions were streaked out on BHI plates. The plates were incubated for 18 h in 37 °C. Afterwards, bacterial colonies were counted.

Agar plating of bone marrow content

After splitting the tibial bone longitudinally, the bone marrow was aseptically isolated, weighed, and added to 5 ml of sterile 1x PBS in Greiner tubes. The tubes were vortexed for 1 min. and suspensions were 1:10 and 1:100 diluted. 50 μl from non-diluted as well as diluted suspensions were streaked out on BHI plates. The plates were incubated for 18 h in 37 °C. Infection was defined as positive culture growth on the agar plates. The number of CFU's was counted for each agar plate. The number of CFU's/g bone marrow for each dilution was determined by division of the number of CFU's by the initially total weight of the bone marrow samples. The average of the two dilutions was calculated to obtain the number of CFU's/g bone marrow for each animal.

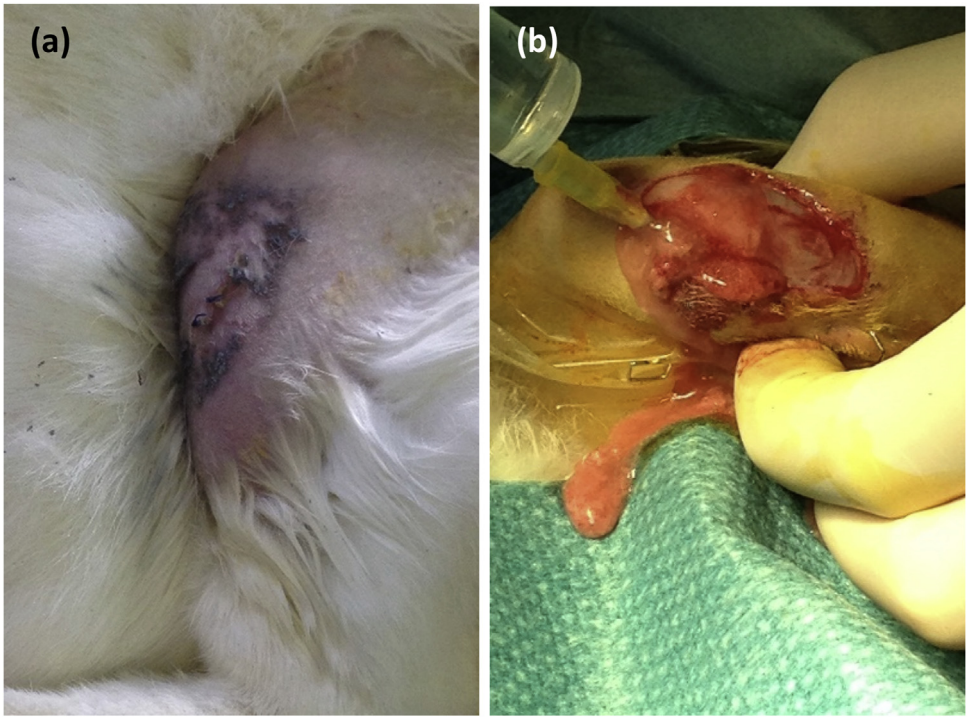

Fig. 3. Clinical observation at revision 1 (day 28). **(a)** Swelling and redness of the knee and tibia region. **(b)** Irrigation of the intramedullary canal with drain of pus.

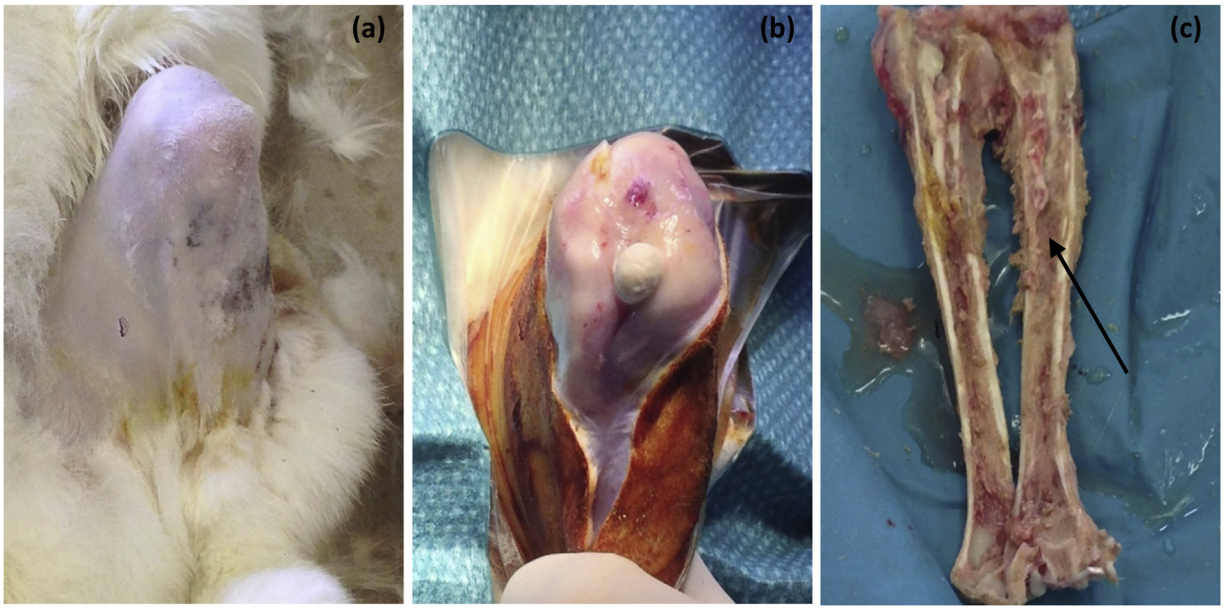

Fig. 4. Clinical observation at time of euthanasia (day 84). Pretibial swelling at the tibial tuberosity **(a)** with pus drainage at the subcutaneous soft tissue layer **(b)** and purulent appearance of the intramedullary canal after sagittal splitting of the tibia (**(c)**, arrow).

Histological evaluation

The medial half of the tibia was brought into 4% paraformaldehyde solution for 4d and subsequently 5 μm longitudinal sections were cut using a rotary microtome (Fa. Leica Biosystems Nussloch GmbH, Germany). These sections were stained with hematoxylin-eosin (HE), toluidine-blue (TB) and Gram staining. To assess a manifest bone infection after 84 days the following criteria were used: abscess formation in the intramedullary canal or the cortical bone, periosteal scalloping, any bone necrosis or bone sequesters as well as bacterial settlement in the histological section [13,14].

Results

Clinical assessment

Eleven of 12 animals (91.7%) survived the entire observation period. One rabbit had to be euthanized on day ten due to a back-hand palsy, which was not related to the bacterial infection. In general, signs of bacterial infection of the lower limb developed within the first 4 weeks with swelling of the knee and tibia region (Fig. 3). Furthermore, weight loss could be evidenced (see next paragraph). During debridement of the intramedullary canal at revision 1 (day 28) drainage of pus out of the intramedullary canal could be determined as sign of MRSA induced infection (Fig. 3). After revision 1, clinical infection signs improved with reduction of swelling and most animals regained weight. At revision 2 (day 56), no acute signs of infection could be detected. However, reimplantation of the K-wire was leading to recurrence of infection signs in all remaining 11 animals with purulent appearance of the bone marrow on day 84 (Fig. 4).

Weight assessment

Initial weight on day 0 of all animals was 5.8 ± 0.24 kg. After initial surgery, a considerable weight loss was observed in all rabbits within the first 4 weeks (Fig. 5). The mean weight loss was 14% ($p < 0.001$). After revision 1, most animals (8 of 11) regained weight again. There was a mean weight increase of 4.2%, which was

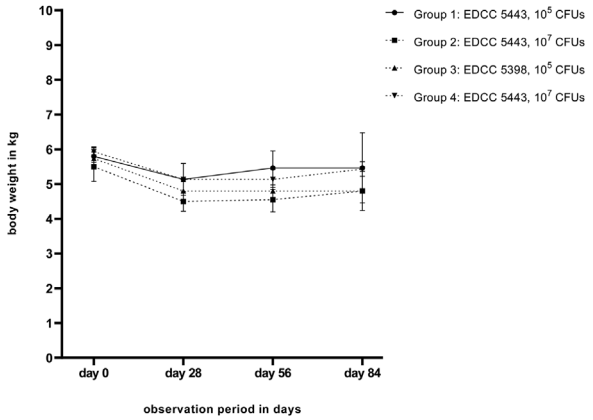

Fig. 5. Weight analysis of the rabbits of the different groups over the entire observation time period of 84 days.

not statistically significant ($p = 0.0531$). One rabbit of group 1 continued to lose weight. The body weight of the other two animals of group 3 stabilized during the last eight weeks.

Microbiological results

For all groups, reliable induction of K-wire associated infection could be established with infection rates of 100% in the first 4 weeks (stage 1) determined by positive bacterial culture growth of the K-wire roll-outs. Microbiological analysis of spacers rolled out on BHI plates after revision 2, could evidence eradication rates of 67% in group I, 50% in group II and 33% in group III and IV due to irrigation and debridement at stage 1.

After reinsertion of the K-wire at revision 2, 9 of the 11 animals showed recurrence or persistence of the infection at the final timepoint of 84 days. All six animals of group I and IV showed positive culture group after K-wire roll-out. One animal of group II and one animal of group III were free of positive culture after K-wire roll-out. Sonication confirmed the previous results in all but two animals, in which culture of the sonication fluid remained

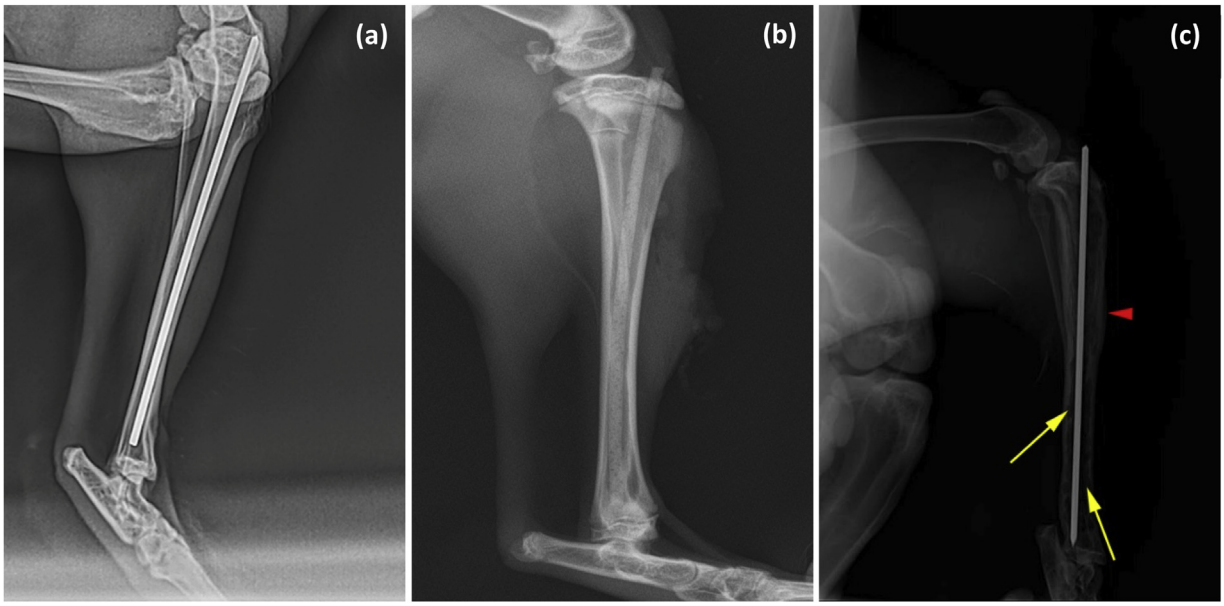

Fig. 6. X-rays of the left tibia after initial surgery (day 0) **(a)**, revision one (day 28) **(b)** and final evaluation (day 84). **(a)** and **(b)** intramedullary position monitoring of the implanted K-wire respectively of the spacer directly after operation. **(c)** X-ray analysis with typical signs of osteomyelitis such as periosteal scalloping (red arrowheads) and osteolysis (yellow arrows) at day 84. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

negative (one animal of group I and one animal of group IV). Values of 1×10^5 CFUs/g of bone marrow and 5×10^5 CFUs/g of bone marrow for two animals of group I revealed a stable chronic infection in this group at the time of reinfection (day 84). Data of the remaining four animals at the time of reinfection evidenced a change from acute to chronic infection.

X-ray assessment

X-rays on day 84 showed clear radiological signs of osteomyelitis (Fig. 6). Lytic lesions and new periosteal bone formations could be found in 10 of 11 animals. One animal of group I showed no specific signs of osteomyelitis on the left tibia. The radiological signs of stable bone inflammation, could be underlined in most cases by clinical and histological assessment.

Histology evaluation

Histological results showed chronic infection-related bone alterations characterized by pronounced, reactive periosteal scalloping in the tibia cortex sample (Fig. 7). In some cases, this could be detected over the whole length of the tibia. Furthermore, bone sequesters with empty lacunae as sign of cortical bone necrosis and large abscess formations and diverse bacterial colonies could be detected in the bone marrow in combination with bone marrow necrosis (Fig. 8).

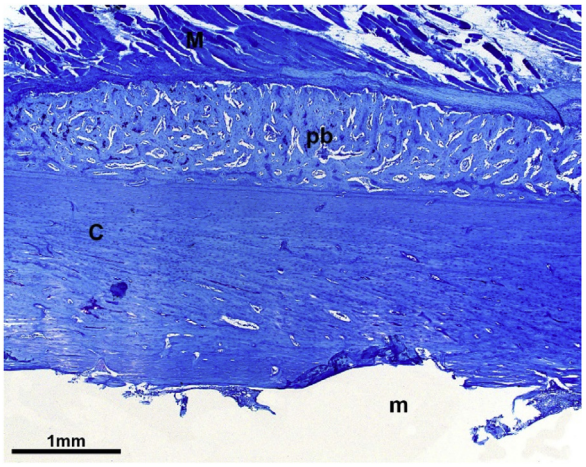

Fig. 7. Toluidine blue staining with reactive pathological new bone formation (pb) as a clear sign of osteomyelitis at the outer side of the cortical bone (C) (M: muscle; m: medullary space) (magnification: 2,5×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Discussion

Today, two-stage revision is the favored surgical therapy for chronic implant related infection [6,15]. Therefore, we transferred the actual clinical gold standard of staged exchange of orthopedic implants in an animal model by enhancing a former rabbit model of implant-related infection [12]. This new model describes for the first time a model of two-stage revision failure of an implant-related infection in a small animal model. Various studies and models for implant-related osteomyelitis in different species have been described in literature. Reizner et al. have given an extensive overview about animal models for *Staphylococcus aureus* osteomyelitis [11]. Focusing on rabbit models of MRSA bone infection several animal models exist. Nevertheless, none of them mimics a two-stage revision procedure in implant related infection. Both Belmatoug et al. [16] and Craig et al. [17] only made an initial surgery for implant insertion and inoculation with MRSA to establish PJI. While Craig and coworkers sacrificed animals after seven days Belmatoug et al. euthanized animals 8 weeks after infection. Furthermore, the inoculation method and the type of implant is different from our study. Giavaresi et al. [18], El-Kamel et al. [19] and Ismael et al. [20], performed a one-stage procedure to test new antibiotic therapy strategies, like a gentamicin-vancomycin impregnated PMMA coating nail, a biodegradable implantable gatifloxacin delivery system or teicoplanin cement. Nonetheless, a two-stage-revision procedure was not been used. The longest time period for development of infection was four weeks [18].

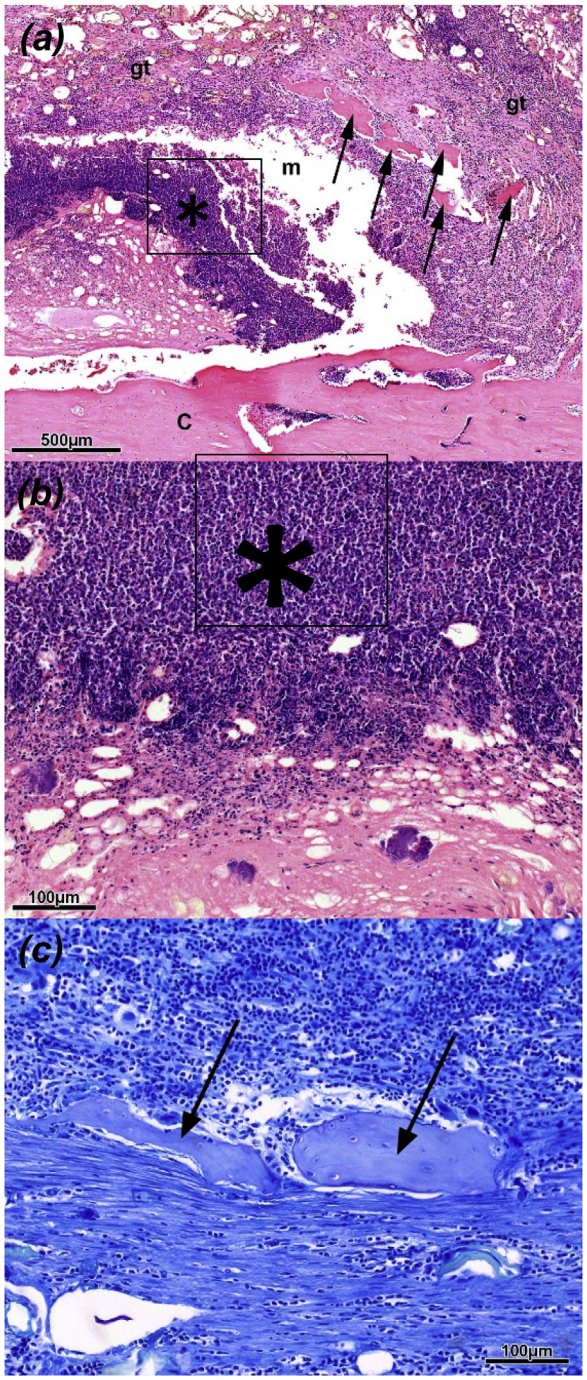

Fig. 8. Histological analysis of the tibia at day 84. ***(a)*** and ***(b)*** HE staining with diverse spectrum of osteomyelitis signs, such as granulation tissue (gt), massive abscess formation (*) and bone sequesters (arrows). ***(c)*** Toluidine blue staining with bone sequesters (arrows) and surrounding abcess formation. c: cortical bone, m: medullary space. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Other MRSA osteomyelitis models, not reviewed by Reizner et al., published by Helbig et al. [21] and Gaudin et al. [22], did not use a two-stage study design either. To the best of our knowledge, a two-stage-revision procedure for MRSA implant-related infections in a rabbit or any another small animal model has not been described in literature yet.

Since failure rate in two-stage revision procedures is still considerably high [23,24], our goal was to establish a model of failed two-stage revision for chronic implant related infection. Chronic MRSA-related osteomyelitis could reliably be replicated using not only two different MRSA strains but also two different inoculation dosages although implant removal, debridement and irrigation and debridement have been performed according to clinical practice in a two-stage manner. Therapy concepts could be assessed in the presented small animal model prior to evaluate new therapeutic approaches in a more costly and demanding large animal model of failed two-stage implant-related infection in sheep described by Moriarty et al. [10].

An animal survival rate of 91.7% could be achieved in the current model although surgical procedure was complex, and observation time of 84 days was long. In addition, every rabbit had to undergo three surgeries. Considering weight assessment as well as the survival rate, the stress for the animals in this demanding infection model can be deemed ethically justifiable. Weight loss within the first 4 weeks (initial surgery till revision 1) was probably due to severe acute infection. This could be locally evidenced by clinical observation at revision 1, where swelling of the knee and the tibia region in all animals as a sign of a purulent infection could be detected (Fig. 2(a)). However, most animals regained weight after revision 1, which indicated infect eradication or at least suppression of infection after revision 1. Microbiological results with positive bacterial culture growth of the K-wire roll-outs revealed an overall reinfection rate at day 84 of 81.8% (9 of 11 animals). In group I and IV reinfection rates were 100%. Microbiological analysis confirmed a reliably failure of revision of the two-stage procedure, which was the primary goal of this work. Radiological signs of chronic osteomyelitis in 10 of 11 animals after 84 days underline the intended successful induction of chronic osteomyelitis. Bacterial load of bone marrow content (1×10^5 CFU/g - 5×10^7 CFU/g) and 1×10^4 CFU/wire) and of the sonication fluid of the K-wires (1×10^4 CFU/wire - 1×10^7 CFU/wire) at day 84 suggest, that a change from acute to chronic infection has been appeared in the course of the surgical treatment.

Secondary aim of this study was both identification of the optimal MRSA strain and inoculation dose for achieving a reliable induction of reinfection at the time of euthanasia (day 84). For this purpose, we used two different MRSA strains (EDCC 5443 and EDCC 5398) with two different inoculation doses (10^5 and 10^7 CFUs). Although numbers of animals in the group were small, microbiological results indicated that group I with strain EDCC 5443 in a dose of 10^5 CFUs was best. Eradication of the infection in 67% could be achieved by irrigation, debridement and local antibiotic treatment by 2 g vancomycin loaded PMMA-spacers. In this group reinfection occurred in 100% after reinsertion of the K-wire. In 2 of 3 cases, histological analysis could evidence chronic osteomyelitis.

Our study has several limitations. The small number of animals and the missing of a control group have to be mentioned. This compromise was made to minimize the burden of experimental animals. With the presented data, use of a larger number of animals is reasonable in future studies to achieve more reliable results and better correlation between different assessment methods. Furthermore, the question which inoculation dose and which MRSA strain are best to achieve reinfection in the presented model with the least possible burden for the experimental animals still has to be elucidated.

Besides surgical therapy and local antibiotic application of vancomycin the authors did not administer systemic antibiotics for experimental treatment. Some previous experimental studies of bone infection in rabbits applied a systemic antibiotic treatment [20,25]. We have refrained doing so, since we did not want to risk complete eradication of infection in the small animal model of two-stage revision surgery first described in literature. Virulence of MRSA strains in this rabbit experiment was unforeseeable and

reinfection characteristics were not known with systemic antibiotic therapy. Since we dedicated ourselves to the 3R principles, reduction of animal numbers could be achieved by evaluating the two-stage infection model without further groups with additional systemic antibiotic treatment. The second aim of the study was to identify an optimal MRSA strain and dose for failed two-stage revision. Small groups of 3 each were used since it was not clear if any reinfection could be achieved at all. Hence, additional systemic antibiotic treatment at this early experimental stage would have been counterproductive.

To identify the best MRSA strain and bacterial load for failure of two-stage revision, microbiological assessment of bone marrow content for each revision surgery would be desirable. To determine bacterial load of the bone marrow during treatment would be a further improvement to answer the important question of microbial growth during surgical, local as well as systemic antibiotic treatment. Revealing growth patterns which not only depend on surgical manner and timing but also on additional local or systemic medical treatment, could be of invaluable benefit for future therapeutic optimization.

The model itself has shortcomings, which have been accepted due to animal welfare. The induced intramedullary infection does not present a fracture-related infection. The inflammatory reaction and biomechanical changes in fracture healing [26] are regarded as key factors for establishment, treatment and outcome of patients in fracture related infections [10]. Since an additional fracture was deemed too stressful for the animals, the authors regarded a non-fracture model best for initial model establishing. For the same reasons the authors did not use a PJI model. Carli et al. postulated criteria of clinically representative experimental PJI models [27]. Animals chosen should have immune and musculoskeletal characteristics similar to humans. Bacteria should be clinically representative and capable of creating biofilms. Further, the model should include quantitative measurements of bacteria and immune response of the host. Implants used should consist out of clinically relevant materials and should allow weight bearing to reproduce the periprosthetic environment. The last was not achieved by our study. Due to animal welfare and practical reasons, a simpler infection model was developed rather than infection models with additional fracture or prosthesis application.

Conclusion

The presented rabbit model is the first model of a two-stage revision procedure for MRSA induced implant-related infection. A high rate of reinfections could be evidenced by histological, radiological and microbiological analysis. Although refinement of the model remains necessary in the future, it can be deemed suitable for preclinical testing of new therapeutic strategies in implant-related bone infections based on the underlying two-sage revision principle.

Declaration of Competing Interest

All authors state that they have no conflict of interest regarding the content of this article.

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