Implant-associated osteomyelitis is a chronic infection that complicates orthopaedic surgeries. Once infected, 50% of patients suffer treatment failure, resulting in high healthcare costs. While various small animal models have been developed to investigate the efficacy of prophylactic and therapeutic treatments, the minute scale of murine-model bone and hardware has been prohibitive for evaluating interventions with a complete implant exchange in the setting of an infected critical defect. To address this, the aim of the present study was to develop a murine femur model in which an initial mid-diaphyseal infection was established by surgical implantation of a titanium screw contaminated with bioluminescent Staphylococcus aureus (Xen36). 7 d after the infection was established, an ostectomy was performed to remove the middle segment (3 mm flanking the infected screw hole) and a bone-cement spacer, with or without impregnated gentamicin, was secured with a plate and screws to fix the septic segmental defect. Longitudinal bioluminescent imaging revealed a significant decrease in Xen36 growth following one-stage revision, with the antibiotic-impregnated spacer treated systemically with vancomycin (p < 0.05). This result was corroborated by a significant decrease in colony forming units (CFU) recovered from spacer, bone, soft tissue and hardware 12 d post-operative (p < 0.05). However, ~ 10^5 CFU/g Xen36 still persisted within the bone despite a clinical therapeutic regimen. Therefore, the model enables the investigation of new therapeutic strategies to improve upon the current standard of care in a mouse model of implant-associated osteomyelitis that employs reconstruction of a critical defect.

Keywords: Staphylococcus aureus, osteomyelitis, one-stage revision, poly(methyl methacrylate).

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expected to exceed $1.62 billion by 2020 (Kurtz et al., 2012). Ultimately, osteomyelitis continues to be a clinical and socio-economic burden in the healthcare system despite the adherence to rigorous treatment algorithms that utilise prophylactic treatments, multiple revision surgeries and prolonged antibiotic regimens.

Staphylococcus aureus (S. aureus) is the most common pathogen in implant-associated osteomyelitis. This bacterial species accounts for 56% of all bone and joint infections (Grammatico-Guillon et al., 2012). The recalcitrant and recurring nature of chronic osteomyelitis is attributed to the ability of S. aureus to form biofilms on orthopaedic implants (Brady et al., 2006; Nishitani et al., 2015). Within the biofilm, S. aureus can resist the patient’s immune response, as well as antibiotic therapies. Thus, effective management of the infection is difficult to achieve with the standard of care, consisting of intravenous antibiotics and high-dose antibiotic therapy delivered after debridement to the infection site through vancomycin- and gentamicin-impregnated poly(methyl methacrylate) (PMMA) cement spacers (Del Pozo and Patel, 2009; Parvizi et al., 2009). Biofilm eradication requires an increased antibiotic concentration, up to 1000 times more than planktonic cells (Olson et al., 2002), and most standard-of-care antibiotics (e.g. vancomycin and gentamicin) are ineffective against bacteria in biofilms (Sanchez et al., 2015; van de Belt et al., 2000). Consequently, implant retention during staged revision surgery of established infections results in a 57% treatment failure rate (Lee et al., 2010; Marculescu et al., 2006). Implant retention is viewed as detrimental due to established biofilm on the hardware in delayed and late-onset osteomyelitis (Meteemakers et al., 2018). Thus, best-practice treatment algorithms recommend complete hardware exchange as the clinical standard when treating implant-associated chronic osteomyelitis. Yet, even with a removal strategy, a failure rate of 36% is still reported (Lee et al., 2010). Hence, alternative treatment strategies must be investigated to improve efficacy.

Small animal models of orthopaedic infection have investigated alternatives to the clinical standards in order to improve upon their shortcomings. Recently, clinically representative mouse models of one-stage revision (Yokogawa et al., 2018) have been established with bioluminescent S. aureus strains. Inzana et al. (2015a,b) created a septic defect model to investigate the local delivery of rifampin and vancomycin 3D-printed into calcium phosphate scaffolds. In this model, a mid-diaphyseal femoral defect was stabilised with plates and screws and contaminated with a methicillin-susceptible S. aureus (MSSA) collagen sheet. After 7 d, the established infection was irrigated and debrided by a 3 mm-long osteotomy, enabling insertion of antibiotic-laden spacers. However, this model retained plate stabilisation hardware during revision, which led to biofilm formation and persistence of bacteria (Yokogawa et al., 2018).

Yokogawa et al. (2018) implanted a titanium femoral plate stabilised with two screws, one contaminated with methicillin-resistant S. aureus (MRSA). At time of revision, plate and screws were removed, screw holes reamed and new plates and screw implanted to investigate the synergy of systemic vancomycin and anti-glucosaminidase (Gmd) immunotherapy. Yet, this model lacks a cortical defect to provide local antibiotic therapy. To the authors knowledge, small animal models to perform hardware exchange in septic defects have not been developed, probably due to the technically daunting task of exchanging hardware without compromising the structural integrity of the bone (due to the minimal bone volume available for debridement). To overcome this challenge, a mouse model of implant-associated osteomyelitis that utilised a one-stage revision using complete hardware exchange combined with the insertion of an antibiotic-laden PMMA spacer was developed. This model aimed to mimic the clinical features of an implant-associated osteomyelitis and enabled the investigation of the clinically recommended treatment, including debridement, complete hardware exchange, systemic antibiotics and implantation of antibiotic spacers. Furthermore, this model could enable the investigation of new classes of antimicrobial-impregnated spacers, systemic antibiotics and additional novel therapeutics, such as antimicrobial-coated hardware. In the present validation study, one-stage revision with thorough debridement and complete hardware exchange was performed to compare two different cohorts of mice. One group of mice received only a spacer PMMA and acted as the negative control, while the other group received the clinical standard of treatment, gentamicin-laden PMMA with additional systemic antibiotics. The working hypothesis was that despite complete hardware exchange, the clinical standard treatment would fail to eradicate the bacteria on the implanted hardware, bone and soft tissue, enabling persistence of the infection.

Materials and Methods

Study design
For the development of the murine model, two groups were investigated: a negative control that received a PMMA spacer alone with no antibiotics and a clinically treated group that received both a gentamicin-laden PMMA spacer and additional daily systemic vancomycin (110 mg/kg subcutaneously twice daily) (Caston et al., 2014). Vancomycin was allometrically scaled to simulate the pharmacokinetic profile used in clinical patients, receiving 1 g of vancomycin every 12 h (Albrecht et al., 1991; Crandon et al., 2010). The systemic vancomycin was administered for 12 d spanning the remainder of the study after the revision surgery. The negative control
was used to observe the disease progression of the model with no therapeutic interventions, while the clinical control group was used to assess the efficacy of the scaled clinical standard of care used to treat the model.

During the initial infection surgery, a titanium screw contaminated with a bioluminescent strain of MSSA (Xen36) was inserted into the right femur of a 23-25-week-old female Balb/cJ mouse (Fig. 1). Prior to insertion, the screw was contaminated with Xen36 by submersion in bacterial culture for 20 min and, then, dried for 5 min, resulting in a bacterial load of ~2.5 × 10^6 CFU/mL. Next, the infection was established over the time course of 7 d during which BLI was performed at day 0, 1, 3, 5 and 7 to ensure equivalent progression of the infection. At day 7, the revision surgery was performed, in which the contaminated titanium screw was removed and the femur was fixed with a polyether ether ketone (PEEK) plate with 4 titanium screws. A 3 mm-long ostectomy was performed to debride the infected mid-diaphysis of the femur. Then, a PMMA cement spacer with or without gentamicin (1.25 wt%; Cobalt G-HV or MV; DJO Global, Vista, CA, USA) was inserted into the defect. Relapse in infection was continually monitored by BLI at day 1, 3, 5, 7 and 12 d post-revision. The mice were euthanised 12 d post-revision surgery and hardware, bone, cement spacer and surrounding soft tissue were harvested for CFU analysis (Fig. 1).

**Bioluminescent S. aureus strain**

Xen36 (PerkinElmer, Inc.) is a bioluminescent MSSA strain, which is a derivative of the clinical parental strain *S. aureus* ATCC 49525 (Wright) harbouring a stable copy of the luxABCDE operon. This strain was chosen due to its proven high bioluminescent signal *in vivo* relative to other bioluminescent *S. aureus* strains, as well as its use in other murine models of implant-associated osteomyelitis (Farnsworth et al., 2015; Inzana et al., 2015a; Pribaz et al., 2012).

![Schematic diagram](image_url)

**Fig. 1. Schematic summary of the implant-associated osteomyelitis mouse model employing a hardware exchange revision.** During the initial infection surgery, a titanium screw contaminated with a bioluminescent strain of MSSA (Xen36) was inserted into the right femur of a 23-25-week-old female Balb/cJ mouse. Prior to insertion, the screw was contaminated with Xen36 by submersion in bacterial culture for 20 min and, then, dried for 5 min, resulting in a bacterial load of ~2.5 × 10^6 CFU/mL. Next, the infection was established over the time course of 7 d during which BLI was performed at day 0, 1, 3, 5 and 7 to ensure equivalent progression of the infection. At day 7, the revision surgery was performed, in which the contaminated titanium screw was removed and the femur was fixed with a PEEK plate with 4 titanium screws. A 3 mm-long ostectomy was performed to debride the infected mid-diaphysis of the femur. CFU analysis of the removed screw and debrided bone determined bacterial counts of 1.5 × 10^6 CFU/g and 4.0 × 10^7 CFU/g, respectively. Then, a PMMA cement spacer with or without gentamicin (1.25 wt%; Cobalt G-HV or MV; DJO Global) was inserted into the defect. Relapse in infection was continually monitored through BLI at day 1, 3, 5, 7 and 12 d post-revision. Mice were euthanised 12 d post-revision surgery and hardware, bone, cement spacer and surrounding soft tissue were harvested for CFU analysis. Two groups were investigated, a standard-of-care group, that received local delivery of gentamicin through PMMA spacers and systemic antibiotics that began immediately after revision surgery (110 mg/kg vancomycin injected subcutaneously twice daily), and a negative control that received no antibiotics (both locally and systemically).
Contamination of titanium screw with bioluminescent *S. aureus*

A single colony of *Xen36* was used to prepare bacteria cultures grown at 37 °C, 225 rpm in tryptic soy broth (TSB) until the optical density (OD$_{630\text{nm}}$) of 0.4-0.5 was reached (log phase), corresponding to $\sim 1.5 \times 10^6$ CFU/mL. Subsequently, 200 µL of the bacterial cultures were transferred to a 96-well microtiter plate (Falcon; Corning Life Sciences). Then, a titanium screw (Shenzhen Qianpeng Weiye Technology Co., Ltd, Shenzen, China) was contaminated by submersion in the plate for 20 min and allowed to dry for 5 min prior to implantation. The bacterial load on each titanium screw prior to implantation was determined to be $2.475 \times 10^6 \pm 1.49 \times 10^6$ CFU/mL by sonicating adhered bacteria and plating serial dilutions on TSB agar.

Animals and inoculation surgery

All animal procedures were performed in accordance to protocols approved by the University of Rochester’s Committee on Animal Resources (UCAR protocol 2013-001). Female Balb/cJ mice, 13-15 weeks old, were purchased from Jackson Research Labs. Mice were housed until surgeries were performed, at around 23-25 weeks of age. 0.1 mg/kg buprenorphine was subcutaneously administered pre-operatively and twice daily for 3 d following each surgery. Throughout the 12-week time course of the study, no differences in body weights were observed between the two treatment groups (data not shown).

Briefly, an inoculation surgery was performed with a contaminated titanium screw to establish an implant-associated infection. Prior to surgeries, mice were anaesthetised with 12 mg/kg xylazine and 130 mg/kg ketamine through intraperitoneal injection. The right femur of the mouse was exposed by a direct lateral approach using blunt dissection to reveal the femur. A titanium screw contaminated with *Xen36* was inserted into the mid-diaphysis of the femur after drilling through the anterolateral and posterolateral cortices using a 0.31 mm drill bit (Fig. 2a,b). Then, muscle and skin were closed using 5-0 monofilament nylon sutures and the infection was allowed to establish for 7 d.

Revision surgery

After the infection was established, a revision surgery was performed, in which the contaminated screw was removed, the infected area was irrigated, the femur was fixed, the visible pathological tissue was debrided, the infected bone was ostectomised and

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**Fig. 2.** Macroscopic evidence of infection 7 d post-inoculation surgery. (a) A representative photograph of a mouse’s femur exposed using blunt dissection prior to mid-diaphysis implantation of a titanium screw contaminated with *Xen36* during the inoculation surgery is shown (day 0). Gross examination of the established infection at the time of revision (day 7 post-infection) generally showed (b) peri-implant abscesses (black arrow) and (c) subcutaneous abscesses (white arrow). At this time, the quantified viable bacteria on the screw were determined to be $\sim 7.4 \times 10^5$ CFU.

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**Fig. 3.** Insertion of a cement spacer was achieved by aggressive debridement of the mid-diaphysis by performing a 3-mm-long ostectomy. (a) After the contaminated screw was removed, the infection site was irrigated and a PEEK fracture fixation plate was fixed to the anterolateral surface of the femur with 4 titanium screws. (b) Radical debridement was performed ($\sim 25\%$ of the length of the femur) by attaching a cutting guide to the fixation plate and screws and using a Gigli wire to cut a 3 mm-long defect. (c) A PMMA spacer was inserted into the defect and secured in cerclage fashion with a braided suture.
an antibiotic spacer was inserted (Fig. 3a-c). Briefly, mice were anaesthetised with 12 mg/kg xylazine and 130 mg/kg ketamine injected intraperitoneally. The wound was reopened and any abscess or pathological tissue was debrided. The femur was repositioned and the contaminated titanium screw was removed and either placed in 2.5 % glutaraldehyde/4 % paraformaldehyde in 0.1 M sodium cacodylate buffer for scanning electron microscopy (SEM) analysis or frozen in 300 µL of phosphate buffered saline (PBS) at −80 °C for later CFU analysis. After removal of the screw, the femur was irrigated and, then, fixed on the anterolateral surface with a 6-hole radiolucent PEEK plate and 4 titanium screws inserted into the outermost screw holes (Shenzhen Qianpeng Weiye Technology Co., Ltd, Shenzen, China). The PEEK plate was used to enable longitudinal micro-computed tomography imaging without the production of metallic artefacts for future studies examining and quantifying bone healing. Next, a 5-0 nylon monofilament suture was tied around the plate and femur in between the proximal and distal pairs of screws, in order to prevent screw pull-out from possible septic loosening. A 3 mm-long transverse osteotomy encompassing the location of the original infected screw insertion was performed at the mid-diaphysis by using a 0.67 mm wire Gigli saw and cutting guide, allowing fixed and reproducible cuts (RSYSTEM, Davos, Switzerland). The 3 mm-long bone debrided segment was placed in 1 mL of PBS and stored at −80 °C for CFU analysis. The fixed and osteotomised femur was again irradiated. Commercial PMMA bone cement spacers, with (Cobalt G-HV, DJO Global) and without 1.25 wt % gentamicin (Cobalt G-HV, DJO Global), were prepared in accordance to the manufacturer’s guidelines and shaped into 1.8 × 3 mm cylindrical spacers using a custom negative mould machined out of aluminium to fit these dimensions. The cured PMMA spacers were inserted into the femoral defect and secured in a cerclage fashion using a 6-0 nylon braided suture (Fig. 3b,c). Finally, muscle and skin were closed and the mice underwent a treatment time of 12 d post-revision surgery. The sample size was 6 mice per group. When performing the surgical procedures, three mice died during revisions, two suffered femoral fractures and the other did not recover from anaesthesia. These outcomes were associated with the learning curve for this surgical technique. These mice were replaced to maintain sample size. Additionally, no mice died during the study; however, one mouse had to be removed from the PMMA-treated group due to a fractured femur after osteotomy.

Radiographic imaging
To qualitatively assess implant placement and bone changes, X-rays were taken at 0, 7 (before and after revision surgery) and 12 d post-revision. An X-ray cabinet (LX-60 X-ray Cabinet, Faxitron Bioptics LCC, Tucson, AZ, USA) was used to take X-rays at 26 kV, with an exposure time of 6 s. Mice were anaesthetised and placed in the supine position to capture an anteroposterior X-ray of a lateral view of the operated femur.

SEM
SEM (LEO 982 FE-SEM, Carl Zeiss SMT, Thornwood, NY, USA) of gold-sputtered titanium screws was used for qualitative assessment of S. aureus adhered to the surface of the screw after removal of the screw 7 d post-infection surgery, as previously described (Varrone et al., 2014). The surface of the hardware was carefully scanned at 2000× to identify rounded structures. S. aureus can appear as 2 dividing bacteria or in planktonic aggregates (Nishitani et al., 2015). Once a rounded structure was found, the magnification was increased to 5000× for closer morphologic assessment and verification of the structure size (1.0 to 1.25 µm).

Longitudinal BLI
BLI of the mice infected with Xen36 was performed at day 0, 1, 3, 5, 7, 8, 10, 12, 14 and 19 using a Spectrum imaging system (IVIS; PerkinElmer, Inc.). Ventral images at each time point were taken with automatic high sensitivity exposure settings. The bioluminescence was quantified using the LivingImage software 2.50.1 (PerkinElmer, Inc.) by analysing a fixed region of interest (ROI) around the infected femur of each mouse. The ROI was designated to fit around the infected femur of each mouse, encompassing the entire BLI signal. The bioluminescence was quantified as average radiant efficiency (p/s/cm²/sr).

Quantifying bacterial colonisation of harvested tissue and implants
At the end of the time course, mice were sacrificed by carbon dioxide inhalation followed by cervical dislocation. Hardware, spacer, bone and soft tissue (quadricep and hamstring muscle blocks) were harvested to quantify the viable bacteria load for each sample. The PMMA cement spacer, fixation hardware and screw harvested at revision surgery were sonicated in 1 mL of sterile PBS for 3 min at 35 kHz (VWR Symphony Ultrasonic Cleaner VWR Intl., Radnor, PA, USA) and vortexed to release any adhered bacteria. Bone, soft tissue and ostectomised bone at revision surgery were homogenised in 3 mL of sterile PBS using a T10 basic ULTRA-TURRAX® disperser (IKA Works, Inc., Wilmington, NC, USA). Then, the homogenised and sonicated solutions were plated on TSB agar in 10-fold serial dilutions and incubated overnight at 37 °C to count for CFU the next day. CFU counts were normalised to the mass of each collected sample and transformed using log2(1 + X) before statistical analysis.

Statistical analysis
Longitudinal body weight and bioluminescence differences were assessed by performing a multiplicity adjusted two-way ANOVA. A log
transformation of the bioluminescence data was performed prior to performing the two-way ANOVA, which was followed by Sidak’s test for multiple comparisons. The means of the area under the curve of the bioluminescence data was compared by performing an unpaired student t-test. Bacterial CFU quantification was tested for Gaussian distribution by performing a Kolmogorov-Smirnov test with Dallas-Wilkinson-Lillie for corrected p-value. The failure in the normality test resulted in data being analysed by Mann-Whitney nonparametric t-test. In addition, CFU data were binarised and Fisher’s exact test was performed on the 2 × 2 contingency table. All differences were considered significant for p < 0.05. All data were represented as mean, except for bacterial CFU quantification, which was represented as median due to the bimodal distribution. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

Results

Similar levels of infection in the two groups were achieved from the inoculation surgery as assessed at day 7 by four independent outcomes. First, occurrences of subcutaneous and peri-implant abscesses were observed in infected mice during the revision surgery (Fig. 2b,c). Also, no difference in CFU were found on the inoculation screw [1.22 × 10³ ± 4.60 × 10² CFU for PMMA and 1.85 × 10³ ± 1.22 × 10³ CFU for gentamicin-PMMA + SC Vanco (systemic vancomycin); p = 0.3054 by Mann-Whitney U test] and in the ostectomy (2.63 × 10⁶ ± 8.69 × 10⁵ CFU/g for PMMA and 5.14 × 10⁶ ± 6.47 × 10⁶ for gentamicin-PMMA + SC Vanco; p = 0.9697 by Mann-Whitney U test) harvested during revision surgery at day 7. Furthermore, SEM showed similar levels of bacterial colonisation on the recovered screws, observing the presence of S. aureus cells located on the screw as well as embedded within a biofilm (Fig. 4a-c).

BLI of the infected mice (Fig. 5i) confirmed the lack of statistically significant differences in the levels of bacterial presence in both groups after the inoculation surgery (p > 0.05 by 2-way ANOVA). Consistent with similar murine models (Inzana et al., 2015a; Inzana et al., 2015b; Yokogawa et al., 2018), BLI peaked at day 3 (~ 3.5 × 10⁴ photons/s/cm²/sr) and declined to ~ 9.3 × 10³ photons/s/cm²/sr prior to revision surgery at day 7. Longitudinal BLI also detected relapse of the infection in the PMMA-only group, which peak between day 1 and 3 of the revision surgery (day 8-10 post inoculation surgery). In contrast, gentamicin-PMMA + SC Vanco significantly reduced the BLI levels to background levels following revision surgery (p < 0.001 by 2-way ANOVA).

Radiographic imaging revealed no osteolysis in any mice prior to revision surgery. However, at the end of the experiment, radiographic evidence of peri-implant osteolysis was present in all the mice in the PMMA-only group. In contrast, there was no radiographic evidence of osteolysis in any of the mice in the gentamicin-PMMA + SC Vanco group (Fig. 6a-h).

To further validate this observation, bone, soft tissue, cement spacer and hardware were harvested at the end time point and either homogenised or sonicated and serially diluted for bacterial enumeration and CFU plating. At the time of harvest, all cement spacers remained intact in the defect (Fig. 6). CFU counts demonstrated a significant reduction in bacterial colonisation for each of the 4 harvest samples between PMMA and gentamicin-PMMA + SC Vanco groups, resulting in 0.9-, 1.7-, 0.9- and 3.1-log reductions for bone, soft tissue, hardware and cement spacer, respectively (Fig. 7a-d). This finding corroborated the longitudinal bioluminescent measurements.

Fig. 4. Bacterial colonisation of the titanium screw 7 d after infection surgery. Representative SEM images of an explanted screw 7 d post-inoculation surgery obtained initially at nominal instrument magnifications of (a) ×25, (b) ×15,000 and (c) ×5,000. (a) S. aureus cells were located throughout the infected screw. (b) S. aureus cells located at the head of the screw (white arrows), as well as the tip of the screw (c; white arrows) embedded within biofilm. (c) Cluster of cocci (arrows), which was indicative of S. aureus proliferation within the biofilm on the implant.
Discussion

Implant-associated osteomyelitis is a clinical challenge due to its recurring and unpredictable nature. Complications and failure rates remain high, despite well-established surgical principles (Crockarell et al., 1998; Schoifet and Morrey, 1990; Teeny et al., 1990). To better understand clinical outcomes and potential therapeutic avenues, a clinically representative mouse model of established implant-associated osteomyelitis was developed, modelling the first step in a two-stage revision. The present work expanded upon several established murine models designed to evaluate interventions for established implant-related osteomyelitis (Reizner et al., 2014).

The most common mouse models are periprosthetic models of infection, which are employed to investigate the efficacy of different treatment modalities in a post-total knee joint arthroplasty infection (Bernthal et al., 2010; Berretta et al., 2017; Kaur et al., 2016; Niska et al., 2012; Pribaz et al., 2012; Stavrakis et al., 2016; Thompson et al., 2017; Young et al., 2015). In these models, the inoculation strategy generally consists of directly injecting $10^5$ to $10^6$ CFU of *S. aureus* into the joint space after that an orthopaedic hardware, typically a k-wire, has been implanted in a retrograde fashion. Investigated treatment strategies for this model generally consist of systemic antibiotics (Thompson et al., 2017), antimicrobial-coated hardware (Bernthal et al., 2010; Stavrakis et al., 2016), antibiotic-laden pastes (Berretta et al., 2017) or prophylactic treatments.
Mouse models of implant-associated osteomyelitis have also been developed using internal fixation. Traditionally, internal fixation has been performed in mouse models with either a transcortical pin or an intramedullary implant that has been previously infected (Li et al., 2008; Matsumoto et al., 2006; Shandley et al., 2012; Varoga et al., 2008). However, these models lack the ability to perform a revision surgery in order to debride the infected bone and insert an antibiotic-laden spacer, which is a common practice for trauma-related cases. In order to circumvent this, a model utilising a locking fracture fixation plate was developed to study the efficacy of bone debridement and the utilisation of antibiotic-laden cement spacers (Inzana et al., 2015a). In this model, an infection is established in a mid-diaphyseal femoral ostectomy through implantation of a contaminated sponge. Then, a revision surgery is performed 7 d post-infection, in which debridement of the infected bone is accomplished by widening the ostectomy to 3 mm, a partial exchange of the hardware is performed and an antibiotic-laden spacer is inserted to assess infection management. Although this model has some relevance, such as displaying the bone infection hallmarks of chronic osteomyelitis, the retention of the hardware is inconsistent with most revision surgeries.

A similar mouse osteomyelitis model established an infection by using a contaminated screw for fixation of a femoral plate to investigate the synergy between systemic vancomycin treatment with anti-glucosaminidase monoclonal antibodies (anti-Gmd) immunotherapy (Yokogawa et al., 2018). This model replicates common clinical practices in which the hardware is exchanged during the one-stage revision surgery. However, this model does not contain a cortical defect to allow for structural bone healing outcomes. Additionally, while the study demonstrated synergistic effects between antibiotic and immunotherapy, resulting in reduced bacterial burden and osseointegration of the new implants, ~ 10^5 CFU/mL were still recovered on the newly exchanged hardware. This indicated the need for more radical debridement and local delivery of antibiotics in addition to systemic antibiotics in order to better manage the infection.

The current study presented a novel murine model of implant-associated osteomyelitis, which utilised a one-stage revision surgery for removal of the infected hardware, debridement of the infected ROI and introduction of a new sterile hardware for femoral fixation of a critical defect with local antibiotic delivery. In this model, a bioluminescent strain of MSSA, Xen36, was chosen in order to longitudinally monitor the progression of the infection in vivo. The bioluminescent signal peaked 3 d post-infection surgery, which was similar both in timing and magnitude to other models of implant-associated osteomyelitis that utilise bioluminescent MSSA (Inzana et al., 2015a; Inzana et al., 2015b; Li et al., 2008; Yokogawa et al., 2018). The revision surgery resulted in a reduction in bioluminescence, that quickly rebounded after 24 h for the untreated control PMMA group. The infection was established with an inserted titanium screw that had a diameter of ~ 0.3 mm, yet despite a radical 3 mm ostectomy (~ 25 % of the femur), the infection still quickly rebounded. This highlighted the ability of the deep infection to spread to adjacent tissues and prevail in seemingly healthy tissue despite debridement and irrigation of the identified devitalised and infected tissues. However, the clinical standard of gentamicin-impregnated PMMA spacer with systemic antibiotics displayed no such rebound due to the administration of local and systemic antibiotics. The observed decline in signal after day 3 prior to the revision surgery, which was also seen in the group of mice that received PMMA after revision, was attributed to the transition from planktonic bacterial growth (high metabolic

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**Fig. 6.** Local and systemic antibiotic therapy prevented peri-implant osteolysis following revision surgery.

(a,d) A contaminated titanium screw with Xen36 was inserted into the mid-diaphysis of a mouse’s femur.

(b,f) The infection was allowed to establish for 7 d, in which a revision surgery was performed. No osteolysis was observed prior to revision surgery. For revision, the contaminated screw was removed and a new hardware was inserted.

(c,g) Debridement of the mid-diaphysis was performed resulting in a 3 mm-long defect in which a bone cement spacer with or without gentamicin was inserted.

(d) Radiographic evidence of osteolysis was apparent by day 19 in the PMMA-only group (white arrows).

(e) No osteolysis was observed in the clinical treatment group; PMMA spacer with gentamicin and SC vancomycin. Representative X-rays for each treatment group are all from the same mouse.
rate) to colonised bacteria growth (low metabolic rate) (Li et al., 2008), biofilm formation (Inzana et al., 2015), and the ability of planktonic S. aureus cells to become localised within both macrophages and osteoblasts, while still surviving (Hamza and Li, 2014; Josse et al., 2015), and to propagate into the osteocytic-canalicular networks (de Mesy Bentley et al., 2017; de Mesy Bentley et al., 2018).

Treating the established infection with the clinical standard of care enabled the bacterial burden to be significantly reduced after the revision surgery in comparison to the PMMA-only-treated mice (Fig. 5i,j). The clinically treated mice displayed a bioluminescent signal that drifted just above the sterile control post-revision (Fig. 5i), no osteolysis in longitudinal radiographs (Fig. 6) and a benign skin suture surface. Thus, qualitative signs of infection were not apparent in the clinically treated group. However, recovered bacteria in both groups indicated the inability to sufficiently clear the infection even with the antibiotic treatment. Although the clinical treatment significantly reduced the bacterial count in hardware, spacer, bone and soft tissue, only minimal log reductions were observed and none were completely sterile (Fig. 7a-d). Furthermore, Fisher’s exact test did not yield significance when calculating the ability to eradicate the infection for the recovered hardware, spacer, bone and soft tissue. This indicated that even when managed with a standard clinical treatment, the infection was not eradicated, which is analogous to the persistence of infection observed clinically. Thus, the new described model provided the capacity to investigate novel therapeutics that could be delivered locally to improve upon current standards.

The ability of the bacteria to persist highlights the elusive nature of chronic osteomyelitis, especially with the absence of clinical signs (Mouzopoulos et al., 2011; Panteli and Giannoudis, 2016). Explanations as to why the reinfection rates of osteomyelitis are as high as 20-30 % (Conterno and Turchi, 2013), even with debridement beyond the margins, is still poorly understood. However, one explanation is the ability of S. aureus to propagate into the bone canaluli to evade therapeutic regimens and host immune responses and, thus, emerging after revision surgery with debridement and insertion of a new hardware (de Mesy Bentley et al., 2017). Hence, the presented model provided a platform to investigate experimental treatments consisting of new classes of spacers and antimicrobials that could improve upon therapeutic outcomes even with thorough debridement.

The model of implant-associated osteomyelitis with one-stage hardware exchange was not without limitations. First, to perform the ostectomy of the mid-diaphysis after implant removal and irrigation, the femur had to be fixed prior to debridement. In a clinical setting, complete debridement and irrigation would be carried out prior to the implantation of a new hardware. However, because of the limitations imposed on surgical procedures by the small size of the murine bone, the femur was first fixed and then the ostectomy was performed. This was required for the surgical procedure because a cutting guide was attached to the fixation hardware in order to perform consistent ostotomies mouse to mouse. Secondly, the infection surgery did not represent a truly realistic scenario when a single titanium screw would be implanted. Similar models have utilised screws, pins and k-wires to establish an infection (Choe et al., 2015; Li et al., 2008; Pribaz et al., 2012). However, this limitation was abated by the clinically relevant revision surgery used to treat the established infection. Lastly, the limitations of using a small...
animal model must also be considered in the context of assessing local drug delivery. This impacted the effective drug diffusion distance needed for effective treatment, which could bias the clinical interpretation of the strength of delivered drugs.

Conclusion

A mouse model of a one-stage revision surgery for implant-associated osteomyelitis was developed, demonstrating the limitations of antimicrobial PMMA spacers. This model could serve as a platform to examine novel therapeutics for osteomyelitis in future studies.

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Inzana JA, Schwarz EM, Kates SL, Awad HA (2015a) A novel murine model of established Staphylococcal bone infection in the presence of a...


Discussion with Reviewers

Reviewer 2: In clinical practice, patients are often taken off antibiotics for a few days to reduce the risk of false negatives from cultured biopsies. In the present study, animals received antibiotic therapy up to the day of euthanasia. Could this influence the results of the study?

Authors: We understand the merit of having a washout period and how in an ideal scenario this would enable no presence of antibiotics to confound the resulting bacterial counts. However, we believe this is not an issue in the present study because all mice, except for one, still had bacterial colonisation in bone, soft tissue and hardware samples. In the presence of consistent culture negative samples, this would warrant a washout period to ensure antibiotics were not present during CFU plating.

Yet, it is important to consider that a washout period in a preclinical model would also enable any surviving persister cells or biofilm to rebound, causing an observed increase in virulence as well as increased CFU that no longer would correspond to the true quantified measure at time of halting the therapeutic treatment. This would additionally skew any bacterial counts higher.

The fact that CFU were observed and counted in all but one sample for bone, soft tissue and hardware confirmed the recalcitrant nature of implant-associated osteomyelitis, which was the study original hypothesis.

Reviewer 2: How do you interpret the failed biofilm formation. Acta

Authors: Vancomycin may be used with MSSA (with possible exception of patients with penicillin allergy). Knowing that vancomycin is a weakly bactericidal, could alternative, first choice antibiotic regimens improve treatment outcome?

Authors: Vancomycin may be used with MSSA infections for patients who have penicillin allergies. Also, other antibiotics may be used as well. But herein lies an issue with the treatment of osteomyelitis, where there is no consensus amongst literature or clinicians on a doctrine of antibiotics to be used in certain scenarios with various broad-spectrum antibiotics, various strains of Staphylococcus aureus and the increasing rise of antibiotic resistance.

Reviewer 2: Why PEEK plates were used instead of titanium plates, as done for the screws?

Authors: PEEK plates were used over titanium plates to eliminate the metallic artefacts that titanium plates produce during micro-computed tomography imaging. This was necessary to accurately quantify bone healing within the osteotomised defect.
**Willemijn Boot:** Why was vancomycin chosen in this model for treatment of a MSSA infection?

**Authors:** Vancomycin is commonly employed as a systemic antibiotic for MSSA infections, especially for those patients who have a penicillin allergy. Furthermore, previous mouse models and treatments have administered systemic vancomycin for MSSA infections (Inzana et al., 2015b; Wang et al., 2006, additional reference). Additionally, no appreciable differences in minimum inhibitory concentration (MIC) are observed between MRSA and MSSA for vancomycin.

**Scot Guelcher:** Why was a group with PMMA + subcutaneous Vanco (no gentamicin) not included?

**Authors:** A PMMA + SC Vanco group has similar measured outcomes (BLI and recovered CFU) to that of a PMMA-antibiotics (i.e. Vanco) + SC antibiotic group (Inzana et al., 2015a; Inzana et al., 2015b). Additionally, in aseptic defect models representing trauma cases, antibiotic-laden bone cement will be inserted, while PMMA absent of antibiotics will be not. Therefore, the inclusion of PMMA + SC Vanco only in the current study was waived.

**Additional Reference**


**Editor’s note:** The Scientific Editor responsible for this paper was Fintan Moriarty.