

Research Article

# Development and Validation of a Preclinical Canine Model for Delayed Onset Fracture-Related Infections

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## Abstract

**Background:** Fracture-Related Infections (FRIs) are a challenging complication in orthopaedics. FRI incidence is considerably high, particularly in open fractures. FRI management typically involves multiple surgical interventions and prolonged antibiotic therapies. This regimen is often ineffective at infection eradication, resulting in poor outcomes and inefficient use of healthcare resources such that improved preventative and therapeutic interventions are needed. To effectively address these gaps, valid preclinical animal models for FRIs are needed. The purpose of this study was to develop and validate a canine ulna model for delayed FRIs that accurately mimics the clinical course noted in patients.

**Methods:** In this model, a distal ulnar osteotomy was established, then internally stabilized with plates and screws that were pre-incubated with methicillin-resistant *Staphylococcus aureus*. After a 3-week period, all animals underwent irrigation and debridement of the fracture site followed by clinical, radiographic, bacteriologic, and histologic assessments over the subsequent 8 weeks.

**Results:** This preclinical canine model established a valid representation of delayed FRI in patients based on clinical, radiographic, bacteriologic, and histologic features. Bilateral distal ulnar osteotomies stabilized with MRSA-incubated implants were consistently associated with clinical signs of local infection, radiographic evidence for delayed union with osteomyelitis and implant failure, and implant-associated biofilm formation 3 weeks after “fracture” creation.

**Conclusion:** The translational rigor of the model allows for efficient and effective testing of novel preventive and therapeutic interventions aimed at improving outcomes for FRI patients.

**Keywords:** Fracture-Related Infection; Animal Model; Canine Research; Ulna; Methicillin-Resistant *Staphylococcus aureus*

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## Introduction

Fracture-Related Infections (FRIs) are among the most challenging complications in orthopaedics. FRIs are difficult to treat, manage, and resolve; they are associated with significant physical and financial burdens for patients and are costly for the healthcare system. Reported FRI incidence in the United States ranges from 1% in closed fractures to up to 30% in complex open fractures with lower extremity FRIs being most common [1-3]. Standard-of-care treatment for FRIs typically involves additional surgeries for Irrigation and Debridement (I&D) of the fracture site and/or implant exchange, as well as prolonged antibiotic therapy. Unfortunately, this regimen is often ineffective at resolution, resulting in a reported treatment failure rate of up to 38% [4]. FRI treatment failure can involve delayed union, nonunion, or malunion; permanent loss of limb function; and/or need for amputation, which can have catastrophic impacts on affected patients' quality of life. Financially, FRIs have drastic economic consequences with median treatment costs of ~\$108,000 per FRI patient compared to ~\$57,000 per uninfected patient [5]. These economic costs are greatly exacerbated by the indirect costs associated with lost productivity and wages.

In clinically characterizing FRIs, onset of infection is classified as early (<2 weeks), delayed (2-10 weeks), or late (>10 weeks) [6,7]. Diagnostic criteria have been categorized as confirmatory or suggestive by an international FRI consensus group (Table 1) [7]. The difficulty in effectively resolving FRIs lies largely within the unique microbial pathology of the infection. *Staphylococcus aureus* (*S. aureus*) is the most common pathogen isolated from FRIs, with reported involvement of Methicillin-Resistant *S. aureus* (MRSA) as high as 25% in monomicrobial and polymicrobial infections [8]. These pathogens characteristically form a biofilm on fracture-fixation implants that acts as a bacterial reservoir enclosed by a dense matrix of exopolysaccharides, rendering the causative organisms up to 1,000 times more resistant to antibiotic agents compared to their planktonic form [9,10]. Together, antibiotic-resistant pathogens and their associated biofilms are defining features of FRIs that make eradication of the infection extremely difficult such that high treatment failure rates persist. This is exacerbated by the fact that stabilized FRIs often heal in the face of infection such that related clinical signs and symptoms often diminish for months to years until irreversible pathology manifests [3,7,11,12].

Given the profound challenges, burdens, and costs associated with FRIs, novel preventive and therapeutic interventions that are effective in the reversible stages of FRI are needed to improve patient outcomes. To ethically test the safety and efficacy of potential interventions, valid and applicable preclinical animal models for early and delayed FRI are needed [13]. Previous preclinical animal models for FRI have not consistently included clinically relevant time points, management strategies, or outcome assessments, and only 21% of previously described preclinical FRI models document the critical important bacterial biofilm component of the problematic FRIs encountered in patients [13]. Therefore, the purpose of this research was to develop and validate a canine ulna model for delayed FRIs that accurately mimics the clinical course noted in patients with translational rigor sufficient for evaluating strategies designed to improve patient outcomes and mitigate costs and burdens associated with FRI.

| Confirmatory Criteria                                                                                                                 | Suggestive Criteria                                                                                         |
|---------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------|
| <i>Clinical</i> : fistula, sinus tract, wound breakdown, purulent drainage, and/or presence of pus                                    | <i>Clinical</i> : local redness or fever, new onset wound drainage                                          |
| <i>Bacteriologic</i> : phenotypically identical pathogens identified from at least two separate deep tissue/implant specimens         | <i>Radiographic</i> : imaging consistent with bone healing complications, infection, and/or implant failure |
| <i>Histological</i> : presence of microorganisms in deep tissue specimens and/or presence of >5 PMNs in chronic/late-onset cases      | <i>Laboratory</i> : blood test parameters consistent with infection (CRP, ESR, WBC)                         |
| Key: CRP: C-Reactive Protein, ESR: Erythrocyte Sedimentation Rate, Pmns: Polymorphonuclear Neutrophil(S), WBC: White Blood Cell Count |                                                                                                             |

**Table 1:** Confirmatory and suggestive criteria for fracture-related infection diagnosis.

## Methodology

### Animals

All procedures were approved by our institution's Animal Care and Use Committee. Purpose-bred female research hounds (1-2 years of age, 20.2-25.7 kg) (n=8) were studied. Prior to inclusion, a complete orthopaedic examination by a board-certified veterinary surgeon was performed on each dog to ensure that no pre-existing musculoskeletal disorders were present.

### Implant Preparation

A known biofilm-producing strain of MRSA (OJ1) was prepared at a suspension of  $1 \times 10^5$  Colony-Forming Units (CFUs) per milliliter. Prior to implantation, 6-hole, 2.7 mm Limited Contact Dynamic Compression Plates (LC-DCP) and 2.7 mm cortical screws (DePuy Synthes, Raynham, MA) were immersed in this bacterial suspension in conical tubes and incubated at 37°C for 48 hours.

The dogs were premedicated (dexmedetomidine [5-10 µg/kg IV] and morphine [0.5 mg/kg IM]), anesthetized (propofol [4-8 mg/kg IV] and isoflurane [1-4% inhaled in O<sub>2</sub>]), and placed in dorsal recumbency to prepare both forelimbs for aseptic surgery of the ulnas. Dogs were also administered perioperative antibiotics (cefazolin [22 mg/kg IV] at induction of anesthesia and every 90 minutes during surgery). Through a caudal open approach, a 1 cm segment of each distal ulna was osteotomized using a sagittal saw to create an “open fracture” at risk for delayed union [14-17]. The radius remained fully intact in each limb of each animal. The OJ1-incubated plates and screws were washed with phosphate-buffered saline solution to remove planktonic bacteria while leaving biofilm, and then used to stabilize each ulna with one plate and four screws. Subcutaneous tissues and skin were closed routinely.

Dogs were recovered from anesthesia (atipamezole HCl IM; same volume as dexmedetomidine), directly monitored daily for general health with assessment and documentation of pain and function and provided analgesics (carprofen [4.4 mg/kg SQ] and 2 doses of morphine [0.5 mg/kg IM] given within 6 hours of the preceding dose and followed by tramadol [2-7 mg/kg PO] within 6 hours of second postoperative dose of morphine and then every 12 hours for 3 days and carprofen [4.4 mg/kg PO] once a day for 7 days and then as indicated based on documented assessments of pain) [18-22]. Soft padded bandages were placed on both forelimbs and maintained for 7 days. The dogs were housed individually and limited to activity in their 25-square-foot runs with enrichment items provided. Dogs were evaluated each day by a licensed Doctor of Veterinary Medicine (DVM) or Registered Veterinary Technician (RVT) with advanced training in laboratory animal medicine to assess each animal's general health, pain, attitude, appetite, and activity level [18-22]. Specifically, a complete examination was performed to determine and record Heart Rate (HR), Respiratory Rate (RR), temperature, and hydration status. Function was evaluated based on visual examination of gait using a 10 cm Visual Analogue Scale (VAS) based on assessment of weightbearing, stance time, stride length, head movement, and load distribution, which has been validated based on strong correlations with pressure mat kinetics [18-22]. Pain was evaluated in each dog by assessing responses associated with pain in dogs (i.e., tensing muscles, resisting, flinching, yelping, turning to look, turning to bite) with the observer recording level of pain using a dynamic (during movement) interactive (associated with palpation of the surgical sites) VAS scale [18-23]. When VAS pain score was > 2 after the initial standardized analgesic protocol was complete, tramadol and carprofen were administered as described above until documented resolution (VAS pain score <2). Water and food consumption were recorded. Surgical incisions were examined to document suggestive (e.g., local redness or fever, new onset wound drainage) and/or confirmatory (e.g., fistula, sinus tract, wound breakdown, purulent drainage, and/or presence of pus) signs of infection wound healing concerns, and other observable complications [7]. Participation in daily social interaction and enrichment activities was also evaluated and recorded.

Intervention and evaluation time points were determined based on clinically relevant milestones for standard-of-care I&D when symptomatic FRI was diagnosed, postoperative wound checks, suture removal, and clinical and radiographic assessments for healing [2,4,5,14-17].

#### *Irrigation and Debridement (I&D)*

Three weeks after FRI surgery, dogs were premedicated, anesthetized, and prepared for aseptic surgery of the ulnas as previously described. Surgical sites were re-opened, grossly devitalized and/or infected tissues were debrided using scalpel, scissors, and curette, and the wounds were thoroughly irrigated (1 L 0.9% saline) using pulsed lavage (InterPulse; Stryker Corp., Kalamazoo, MI). Subcutaneous tissues and skin were closed routinely. Dogs were recovered from anesthesia (atipamezole HCl IM; same volume as dexmedetomidine), directly monitored, and provided analgesics (morphine [0.5 mg/kg IM] within 6 hours of recovery and then as indicated based on documented assessments of pain). Neoprene sleeves (Coodeo Dog Recovery Sleeves; Coodeo, Zhuhai City, China) were maintained on both forelimbs to protect the surgical sites. The dogs were housed individually and limited to activity in their 25-square-foot runs for the duration of the study with monitored daily social interaction and enrichment items provided.

#### *Clinical Evaluation*

Following the FRI surgery, dogs were assessed daily for general health, pain, appetite, and activity level [18-22]. Surgical incisions were examined to document signs of infection, wound healing concerns, and other complications, as described above. Whole blood samples were collected for Complete Blood Counts (CBC) at the time of primary surgery, and weeks 3, 4, and 11 to assess for indications of systemic infection.

### Radiographic Evaluation

Craniocaudal and mediolateral radiograph views of the antebrachium were obtained at the time of I&D (week 3), and weeks 4, 5, and 11. Radiographs were assessed by a board-certified veterinary radiologist to subjectively characterize ulnar bone, implants, and associated soft tissues.

### Quantitative Microbial Cultures

Bone and soft tissue samples from one surgical site in each dog (n=8) were collected prior to I&D at the 3-week timepoint and the 11-week study endpoint for quantitative microbial culture. Samples were retrieved from the proximal end of the ulnar “fracture” site immediately subjacent to the bone plate using 7x13 mm-jaw single-action Adson rongeurs. These samples were placed in pre-weighed Eppendorf tubes. Tubes were re-weighed to calculate tissue weight (0.1-0.5 g). One milliliter of thioglycollate broth (Remel) was added to the Eppendorf tube and vortexed for 15 seconds. The contents of the tube were transferred into a sterile single-use tissue grinder (Covidien) and ground into a slurry. Six serial dilutions were made by transferring 100µL of the slurry into an Eppendorf tube containing 900µL of phosphate-buffered saline, vortexing, and further diluting via the same procedure. Ten microliters of each dilution were struck in duplicate onto Tryptic Soy Agar (TSA) with 5% Sheep Blood (SB) (Remel) and pre-reduced TSA + 5% SB using a calibrated loop and striking for enumeration. Aerobic plates were incubated at 36°C in room air for 48 hours; anaerobic plates were incubated at 36°C for 48 hours under anaerobic conditions using Mitsubishi boxes and Anaero-Pak sachets (Remel). After the 48-hour incubation, colony numbers were counted, and CFU/gram of tissue were calculated. Identification of bacterial isolates from each specimen was confirmed with a Bruker MALDI-TOF instrument (Bruker Corp., Billerica, MA).

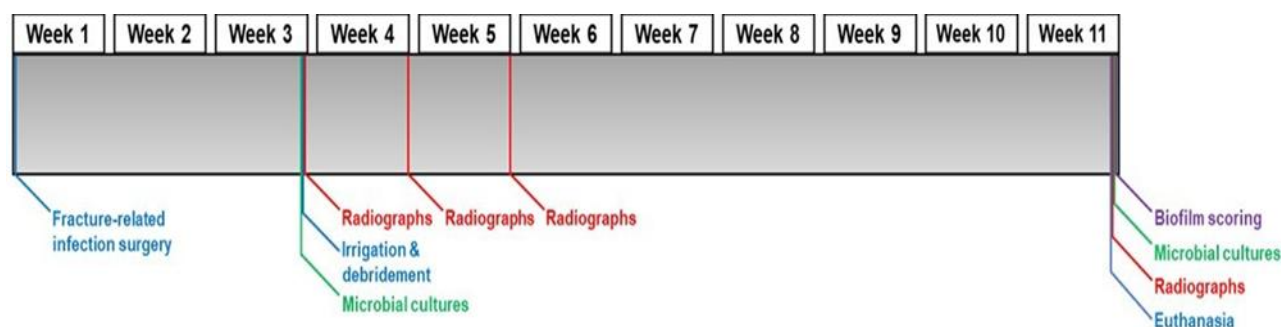
All animals were humanely euthanized with pentobarbital sodium (Fatal Plus 390 mg/ml, 1-2 ml/5 kg IV; Vortech Pharmaceuticals, Ltd., Dearborn, MI) at the 11-week study endpoint (Fig. 1).

### Histologic Evaluation

Undecalcified and methyl methacrylate-embedded ulna specimens at one osteotomy site in each dog (n=8) were prepared, longitudinally ground sectioned through the approximate center of the plate and screws to encompass the entire “fracture” site including the proximal and distal ends of the ulna and associated bone plate and screws. Sections were stained with Stevenel’s blue van Gieson. Fracture-fixation hardware was assessed for biofilm formation by two board-certified veterinary pathologists using a categorical scoring system. The mean of the two scores was calculated and used for reporting (Table 2).

| Biofilm Score | Description                                                                                         |
|---------------|-----------------------------------------------------------------------------------------------------|
| 0             | No biofilm noted                                                                                    |
| 0.5           | Rare biofilm associated with implants                                                               |
| 1             | Small areas of biofilm closely associated with implants                                             |
| 1.5           | Small areas of biofilm with a few moderately sized clusters closely associated with implants        |
| 2             | Small to moderate areas of biofilm closely associated with implants                                 |
| 2.5           | Small to moderate areas of biofilm with a few large-sized clusters closely associated with implants |
| 3             | Moderate to large areas of biofilm closely associated with implants                                 |

**Table 2:** Biofilm characterization scoring system.



**Figure 1:** Flowchart of the 11-week period with the research canines monitored in this study (n=8).

## Results

### *Clinical Evaluation*

All animals survived the 11-week study period with retained implants. Heart rates remained within the normal reference range for medium- to large-breed dogs with a mean of 84, median of 85, standard deviation of 18, and range of 36-138 beats per minute (bpm) throughout the study period. No individual dog varied more than 10% from baseline HR at any time point when not anesthetized or recovering from anesthesia. Respiratory rates remained within the normal reference range for medium- to large-breed dogs with a mean of 22, median of 24, standard deviation of 6, and range of 10-36 Respirations Per Minute (rpm). As expected, body temperatures decreased immediately following anesthesia, however, body temperatures returned to the normal reference range by the day following anesthesia and remained there throughout the study with a mean of 100.9 °F, median of 101°F, standard deviation of 0.8 °F, and range of 98.8-102.7 °F. White Blood Cell (WBC) counts remained within the laboratory reference range ( $4.8 \times 10^3/\mu\text{L}$ - $13.4 \times 10^3/\mu\text{L}$ ) for all animals at all time points apart from two dogs with slightly elevated WBC counts ( $13.8 \times 10^3/\mu\text{L}$  and  $14.4 \times 10^3/\mu\text{L}$ ) at the 4-week timepoint. Documented dynamic VAS pain scores ranged from 0 to 1, attitudes of all animals were documented as alert and responsive, and appetites were documented as either slightly decreased or within normal limits throughout the study period. Daily participation in socialization and enrichment activities was documented for each dog. Consistent with stabilized ulnar osteotomies with intact radii, there was no evidence of unmanaged pain (VAS score >2, elevated HR, inappetence, inactivity, unwillingness to engage in socialization and enrichment activities), pain-related dysfunction (function scores less than 90% of baseline), or systemic infection (elevated HR, neutrophilia with degenerative left shift, inappetence, inactivity, unwillingness to engage in socialization and enrichment activities). Therefore, no additional analgesics or antibiotics were necessary for ethical care of the animals beyond the comprehensive pain management and perioperative intravenous antibiotic treatment provided to all dogs, as described above.

From week 2 through week 11, all surgical sites (n=16) displayed consistent evidence of mild local infection including suggestive signs of redness, swelling, edema, and minimal to moderate quantities of serosanguinous drainage [7]. However, all incisions remained intact without dehiscence.

### *Radiographic Evaluation*

Subjective radiographic assessments were consistent among dogs with evidence of progressive soft tissue swelling, implant-associated osteolysis and implant loosening, and non-bridging callus formation (Fig. 2). Bone union was not achieved in any animal during the study period, consistent with delayed union [14-17].

### *Quantitative Microbial Cultures*

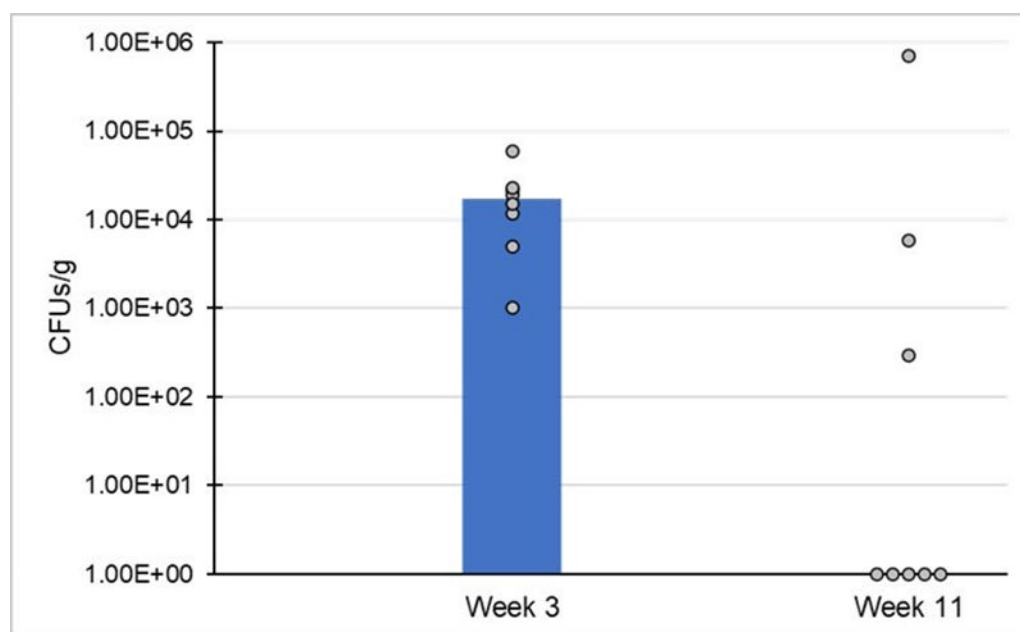
At the 3-week timepoint, all quantitative microbial cultures of surgical-site bone and soft tissue biopsies were positive for MRSA prior to I&D with a range of  $1 \times 10^3$ - $5.9 \times 10^4$  CFUs/g. At the 11-week study endpoint, only 3 of 8 quantitative microbial cultures of surgical-site bone and soft tissue biopsies were positive for MRSA with a range of  $2.9 \times 10^2$ - $7.1 \times 10^5$  CFUs/g (Fig. 3).

### *Histologic Evaluation*

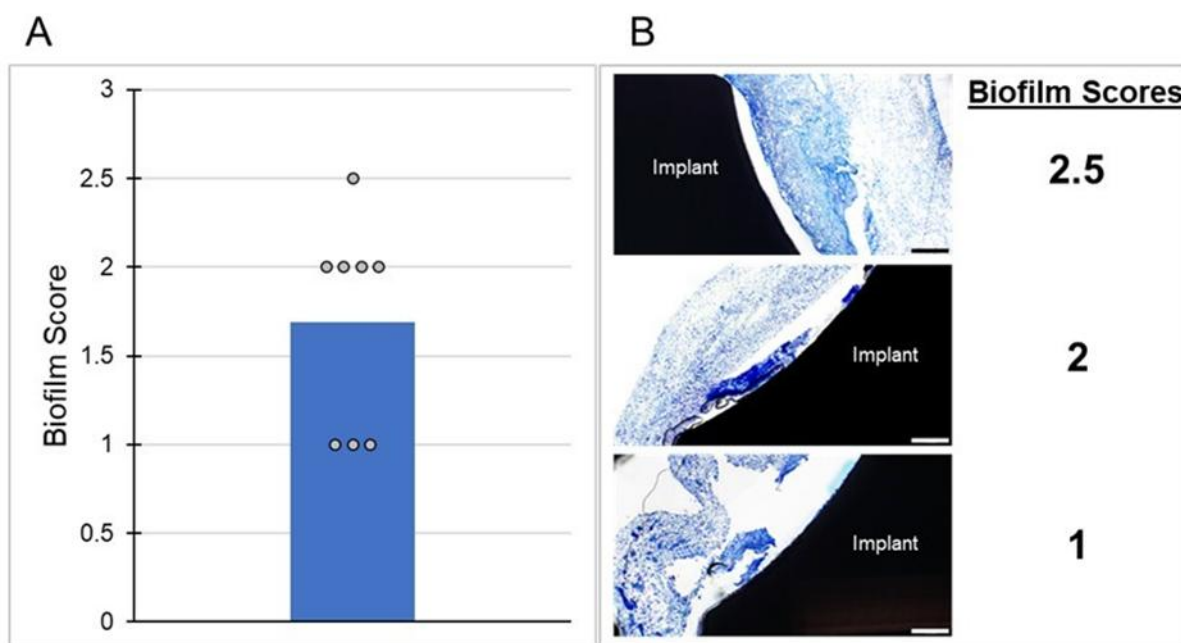
At the 11-week study endpoint, all fracture-fixation plates contained bacterial biofilm formations to varying degrees with scores of 1 (n=3), 2 (n=4), or 2.5 (n=1) (Fig. 4).



**Figure 2:** Serial craniocaudal (top row) and mediolateral (bottom row) radiographic images from a representative dog showing progressive radiographic signs of delayed FRI.



**Figure 3:** Quantitative microbial cultures of bone and soft tissue biopsies at weeks 3 and 11. The median CFUs/g of all animals at each time point is represented by the blue bar. Grey circles represent the individual CFUs/g for each animal at each time point.



**Figure 4:** Histologic evaluation of bacterial biofilms of fracture-fixation implants. (A) Scores of biofilm on fracture-fixation plates using an in-house scoring system. (B) Photomicrographs of *S. aureus* biofilm formations associated with fracture-fixation plates.

## Discussion

In this preclinical model development study, the data supported canine bilateral distal ulnar osteotomies stabilized with MRSA-incubated implants as a valid large animal model for delayed fracture-related infection. This novel model consistently resulted in clinical signs of local infection, radiographic evidence for delayed union with osteomyelitis and implant failure, and implant-associated biofilm formations 3 weeks after “fracture” creation. These findings across clinical, radiographic, bacteriologic, and histologic outcomes closely replicate the clinical scenario for delayed FRI in patients [7].

Delayed FRIs (2-10 weeks) represent a more advanced pathology than early FRIs (<2 weeks) with mature biofilm formations and infection of bone [7,13]. While most FRIs are diagnosed clinically in the late (>10 weeks) stages, modeling early and delayed FRIs is critical for development of effective preventive and therapeutic interventions [3]. For further clinical relevance, return to the operating room for an Irrigation and Debridement (I&D) procedure was performed at the 3-week timepoint [1,2,4-7,13]. Importantly, while this standard-of-care intervention was associated with lack of systemic infection as well as negative tissue cultures in a portion of animals by the study endpoint, clinical and radiographic signs of FRI persisted and implant-associated biofilms were noted in all dogs at the 11-week timepoint, again mimicking the clinical scenario in terms of persistent biofilms and osteomyelitis even when surgical wound infections are addressed by perioperative antibiotics and I&D [1,2,4-7,13]. In addition, following the outcomes for 8 weeks after definitive diagnosis of delayed FRI was critical to clinical application for development of interventions to address the noted progression of pathology, such that implants can be retained, bone union can be achieved, and the limb can be preserved.

There are several limitations to be acknowledged while interpreting the results of this study. The absence of a noninfected control group and the use of a single inoculation dose of  $1 \times 10^5$  colony-forming units limited the ability to determine the effects of bacterial burden on the variables studied. In addition, only a specific species of biofilm-producing MRSA was used such that generalizations regarding other species and/or polymicrobial FRIs cannot be made. The absence of a comparison cohort that did not undergo I&D limited the capabilities for determining this intervention’s contribution to endpoint outcomes. While a more expansive set of variables would allow for more comprehensive data procurement and analyses, this would have required the use of a much larger number of dogs, which would not have honored the 3 Rs of ethical use of research animals based on translational research design aimed at direct clinical relevance and applicability to prevention and treatment of surgically treated (stabilized with implants) FRIs during the reversible stages of disease [7,11,12]. While a canine bilateral distal ulnar osteotomy model may not directly reproduce the open fractures that are at highest risk for FRI in patients, the model involves weightbearing

bones that can be effectively stabilized in the face of infection in large animals, allows for use of standard clinical implants and interventions, and facilitates direct application to veterinary medicine. No non-animal models for preclinical safety and efficacy testing for FRIs has been validated to-date such that Replacement of preclinical animal models for advancing human and veterinary healthcare is not yet possible in this field. As such, this bilateral defect model fully honors the 3 Rs of ethical use of research animals by [24-26]:

- Reducing the number of animals required for unilateral models while maximizing the amount and reproducibility of information gathered, allowing for longitudinal measurements in the same animal, and permitting pairwise and repeated measures comparisons for statistical robustness. Using these criteria in conjunction with typical sample size requirements for pre-study power calculations, the sample size of 8 animals was determined to be the minimum number required for this development and validation study's experimental design
- Refining the methods to include evidence-based efforts to minimize the pain, distress, and dysfunction, and to improve animal welfare. Animal housing, husbandry, social interaction, enrichment, wound care, and pain management were species-specific and met or exceeded clinical standard-of-care as well as all regulatory and ethical policies and procedures for the care and use of research animals

Based on the consistent results noted documenting similarities to FRIs in patients that are characterized by poor wound healing, implant failure, nonunion, and bacterial biofilm-mediated antibiotic resistance, the translational relevance of the model is robust [1,2,4-7,11-13]. As such, use of this model for long-term studies aimed at development of novel preventive and therapeutic strategies for effective management of FRI is warranted. By combining these preclinical data with those targeting early FRI and further developing the models to include other challenging surgical site infections including those associated with osteotomies and arthroplasties, ethical research aimed at reducing treatment failure rates and improving patient outcomes in human and veterinary orthopaedic care can be completed such that clinical application is safe and effective [27].

## Conclusion

This preclinical canine model established a valid representation of delayed FRI in patients based on clinical, radiographic, bacteriologic, and histologic features. Bilateral distal ulnar osteotomies stabilized with MRSA-incubated implants were consistently associated with clinical signs of local infection, radiographic evidence for delayed union with osteomyelitis and implant failure, and implant-associated biofilm formation 3 weeks after "fracture" creation. The translational rigor of the model allows for efficient and effective testing of novel preventive and therapeutic interventions aimed at improving outcomes for FRI patients.

## Conflict of Interests

The authors declare that they have no conflict of interest in this paper.

## Declaration of Interests

The author group also reports the following disclosures:

Bryce Rigden: Nothing to disclose

Aaron M. Stoker: Has the following disclosures

- Musculoskeletal Transplant Foundation: IP royalties

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Kyle Schweser: Has the following disclosures

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- AO North America: Board or committee member
- Arthrex, Inc: Paid presenter or speaker; Research support
- CarboFix: Stock or stock Options
- Johnson & Johnson: Paid consultant; Paid presenter or speaker
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- Musculoskeletal Transplant Foundation/MTF Biologics: Board or committee member; IP royalties; Research support
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### **Ethical Statement**

All procedures were approved by our institution's Animal Care and Use Committee. Institutional Review Board approval was not needed for this study.

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### **Authors' Contributions**

Bryce W. Rigden - Data curation; investigation; formal analysis; writing - original draft; writing - review and editing

Aaron M. Stoker - Project administration; Resources; Supervision; formal analysis; Writing - Original; Writing - review and editing

Chantelle C. Bozynski - Project administration; Investigation; Resources; data curation; formal analysis; Writing - original; writing - review and editing

Kyle M. Schweser - Conceptualization; Supervision; Resources; Writing - Review and editing

Tamara Gull - Investigation; Resources; formal analysis; Writing - Original; Writing - Review and editing

Cristi R. Cook - Investigation; formal analysis; resources; Writing - Review and editing

Keiichi Kuroki - Investigation; formal analysis; Writing - review and editing

James L. Cook - Conceptualization; Supervision; Resources; Project Administration; formal analysis; Writing - original; Writing - review and editing.

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