Musculoskeletal Infection Research Interest Group

International consensus meeting on preclinical models of MSKI

Revised list of questions and answers March 23rd, 2023.

Chaired by:
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Sections:

Please click on the Table of Contents entries to navigate the document.

Back to top

01. Host Immunity

QUESTION 1

Do joints have a natural microbiome, and does this affect joint infection?

Christopher J. Hernandez, Steven Gill & Emanuele Chisari

Response/Recommendation: Yes

Microbial DNA has been detected in samples collected from joints of patients. The presence of microbial DNA in a region is not unexpected, however, since bacterial cell free DNA is present in the systemic circulation. Whether or not live bacteria are present within the joint is unclear given the ease with which environmental contamination can influence measurement of samples with low bacterial load.

Strength of Recommendation: Low

Rationale

The human microbiome is defined as the genetic components of microorganisms that reside within the body. The term microbiota is used to refer to the microscopic organisms themselves which may include bacteria, archaea, viruses and single celled eukaryotes that inhabit the body[1]. Most of the organisms within the microbiota have a commensal or mutualistic relationship with the host[2]. However, an organism within the natural microbiota that plays a symbiotic or commensal function may also cause an infection, as is seen with the pathogens *Staphylococcus aureus* and *Clostridium difficile* which are also common human commensals. The microbiota can prevent infection by: 1) competing for nutrients with organisms that cause infection, thereby preventing sufficient growth of those organisms within the gut; and 2) by providing steady stimulus to enhance the response to infection by local immune cell populations [3]. To assess our knowledge to date on the presence of a natural microbiome or microbiota within the joint space we performed a peer-reviewed literature search on PubMed (August 27, 2022) “microbiome” and the medical subjects headings (MeSH) “joints” identified 84 references. Nine of these references provided analyses of the microbiome in in joint tissues[4–12]. Of those, two examined patients with prostheses[4,8]. The summary of the results is shown here.

These studies report the presence of microbial DNA within joint tissues (synovial fluid, synovium or deep tissues) in patients with rheumatoid arthritis, osteoarthritis or controls. Detection of low copy number microbes or microbial DNA within a sample, however, is challenging and prone to false positives caused by specimen contamination during collection, contamination during sample processing or analysis of the sequencing results [5,13,14]. By strict definition, a natural “microbiome” requires only the presence of microbial genetic elements. Together with other work demonstrating a common presence of bacterial cell free DNA is blood plasma[15,16], these studies suggest that it is likely that microbial genetic elements are often present within the joint.

Findings that a natural microbiota (living microbes) within the joint space are sparse. Such a study requires rigorous control mechanisms and confirmation of bacterial viability through culture[13]. Even samples demonstrating presence of an organism through culturing may show negative results for the DNA of the cultured organism[4]. Rigorous control samples can reduce the likelihood of false positives, one study used tissue from germ free mice as a negative control[5]. Of the studies found in this review, only one[10] included immunohistochemical and

Back to top

culture (but did not perform sequencing, n=23 patients). None of the studies tested the idea that a natural joint microbiota influences infection.

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Back to top

QUESTION 2

Does gut microbiome affect host immunity during MSKI?

Christopher J. Hernandez, Steven Gill & Emanuele Chisari

Response/Recommendation: Yes

The microbiome is in constant contact with host immune cells at endothelial barriers. Interactions between immune cells and microbes or microbial proteins is known to cause immune responses that can be signaled around the body. Mouse models indicate the modifications to the gut microbiome can have a strong influence on osteomyelitis and periprosthetic infection.

Strength of Recommendation: Strong

Rationale

The gut microbiome consists of microscopic organisms that inhabit the gut including bacteria, archaea, single celled eukaryotes and viruses [1]. Microbes present within the gut are in constant contact with host endothelial cells and immune cell populations at the gut lining [2]. The gut microbiota therefore stimulate and thereby regulate innate and adaptive immune mechanisms within the host [2]. In mice, the absence of a microbiome has been associated with impaired response to systemic infection with *Listeria monocytogenes* or *Staphylococcus aureus* [3]. Interactions between the gut microbiota, host immunity and infection are therefore well recognized. To assess the relevance of this known influence on immunity to musculoskeletal infection a literature search was performed on PubMed on August 28, 2022 using the MeSH keywords “Microbiota” and one of the following: “Bone Disease, Infectious,” “Prosthetic-Related Infections,” or “Soft Tissue Infection.” Together these searches resulted in 54 references. Seventeen of these references were relevant to the gut microbiome and infections within the musculoskeletal system [4–20] and their findings are reported below.

Modification of the dysbiotic gut microbiome in obesity-related type 2 diabetic mice with tibial *S. aureus* infections, resulted in significant reductions in *S. aureus* colonization and proinflammatory signaling[4]. Another study in mice indicated that alteration to the gut microbiota reduced survival of the animals in a model of ostoemyelitis[12]. Additionally, a mouse model of periprosthetic joint infection indicated increased susceptibility to periprosthetic joint infection in animals in which the gut microbiota was disrupted by chronic oral antibiotics [9]. Examination of circulating markers of inflammation and flow cytometry showed that the systemic immune response to bacterial challenge was muted in animals with an altered gut microbiota. These findings suggest that the gut microbiome, by modulating the host immunity, can influence infections localized to musculoskeletal tissues.

These findings in mice support the idea that the composition of the gut microbiota can influence host immunity in ways that regulate the response to musculoskeletal infection.

Back to top

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Back to top

QUESTION 3

Does antibiotic therapy affect host immunity to MSKI?

Nathan K. Archer & Debora Coraca-Huber

Response/Recommendation: No

There is limited and conflicted data to support a role for antibiotics to affect host immunity during implant infections. There was no evidence to support a role for antibiotics in systemic cytokine responses and limited or conflicting evidence to support an effect of antibiotics on immune cell recruitment and implanted bone. There is evidence suggesting a negative impact of antibiotic therapy on the efficacy of immune responses by altering the gut microbiome. Although there is less evidence on the role of the microbiome on the incidence of implant infections. Given these results, further studies are warranted.

Strength of Recommendation: Low

Rationale

Orthopaedic implants are highly susceptible to bacterial infections[1, 2]. Treatment of implant infections includes the use of systemic and local antibiotic therapy[3, 4]. Host immune responses have been implicated in the resolution or recalcitrance of implant infections[5-8]. However, whether antibiotic therapy affects host immunity during the implant infection is not entirely clear.

There is evidence suggesting a negative impact of antibiotic therapy on the efficacy of immune responses by altering the gut microbiome[9]. The influence on the immune system by the gastrointestinal microbiome has long been underestimated. Modern molecular biological methods can now be used to examine the microbiome in detail and to work out the interindividual differences. As a result, various influences of the microbiome on the innate and adaptive immune system have already been demonstrated: In germ-free mice, a reduced number of granulocytes and an increased rate of severe infections have been shown[10]. Furthermore, the gut microbiome has an influence on both immune enhancing and immune inhibitory T cells, resulting in homeostasis[11].

The microbiome is formed in the first years of life and depends on factors such as genetics, environmental influences, and diet. Although the microbiome varies widely between people, it remains relatively stable within individuals[12]. However, antibiotic use significantly alters the microbiome[13]. Restoration of the microbiome after treatment can take up to six months[14]. Therefore, we set out to determine how antibiotic therapy influences host immunity during implant-associated infections.

To identify how antibiotic therapy affects host immunity during orthopedic implant infections, a systematic review was completed on peer reviewed literature identified by a PubMed search performed on August 18, 2022 using the key words “(orthopedic implant infection or implant-associated infection or prosthetic joint infection) and (animal model or mouse model or rabbit model or rat model or pig model or in vivo) and (immune response or host response or immunity) and (antibiotic or antibiotic therapy)”. This literature search identified 85 references from 1998 to 2022. After eliminating 76 articles that did not contain information directly addressing the question, there remained 9 relevant pre-clinical research articles.

In pre-clinical models of implant infections, antibiotic therapies were routinely evaluated for their effect on cytokines in circulation. For example, treatment with kanamycin, gentamicin, colistin, metronidazole, and vancomycin had no effect on TNF and IL-6 in sera during a prosthetic joint implant infection[15]. Similarly, gentamycin or vancomycin treatment during a fracture fixation model had no effect on IL-6 in circulation[16], whereas cefazolin treatment during a tibial implant infection had no effect on systemic C reactive protein levels[17]. In contrast, doxycycline treatment reduced C reactive protein during an implant infection with methicillin-sensitive *S. aureus*, although no difference was found during a MRSA infection[18].

Antibiotics were also routinely evaluated for their effect on immune cells in response to implant infections. Vancomycin treatment was found to increase neutrophil recruitment during the implant infection[16], whereas minocycline treatment reduced numbers of neutrophils, lymphocytes, and fibroblasts in the infected tissue[19]. However, erythromycin and doxycycline treatment had no effect on immune cell infiltration into the infected tissue or white blood counts in circulation, respectively[18, 20].

Lastly, antibiotics were examined for their effect on the implanted bone. Although rifampin/cefazolin combination therapy had no effect on the osteolytic response[21], minocycline treatment increased mineralized tissue and bone-to-implant contact[19].

Given that (1) multiple studies with various antibiotic treatments had no effect on cytokine levels in circulation, (2) there is conflicting evidence regarding the nature of antibiotic therapy on immune cells during implant infections, and (3) there is a paucity of data on the effect of antibiotics on the implanted bone, we conclude that there is limited and conflicted data to support a role for antibiotics to affect host immunity during implant infections. The influence of antibiotic substances on the immune responses should be carried out in a global approach involving the evaluation of gut, skin and joint microbiomes.

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Back to top

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Back to top

QUESTION 4

Are there validated models of host immunity in MSKI with quantitative outcome measures?

Nathan K. Archer & Jan Harro

Response/Recommendation: Yes

There are validated models to study host immunity during orthopedic implant infections, with strong evidence for mouse models of prosthetic joint, tibial implant, osteomyelitis, and fracture fixation infections. However, there is only moderate evidence in rats, pigs, and rabbits for validation of orthopedic implant infection models. Frequent outcome measures in the validated animal models include measurements for cytokines production, immune cells populations, and antibody responses. However, our findings warrant further investigation to validate host immunity studies in medium-to-large animal models (e.g., rats, pigs, rabbits, and sheep) and their immunological outcomes.

Strength of Recommendation: Strong

Rationale

It is well established that orthopedic implants are susceptible to chronic infections mediated by biofilm forming bacteria[1, 2], which are resistant to antibiotic therapy[3, 4]. With the emergence of antibiotic resistance bacteria outpacing the development of novel antibiotics, it is important to understand the host immune responses that promote clearance for the development of novel therapies. Therefore, we explored the literature to understand the validated models of host immunity and outcome measures to study orthopedic implant infections.

To identify validated models of host immunity and studies defining immunological measures used to evaluate orthopedic infections, a systematic review was completed searching the peer-reviewed literature available from PubMed on June 15, 2022, and EMBASE on June 17, 2022. The search was performed using the key words “immune + animal or *in vivo* + bone or orthopedic + infection + arthroplasty or internal fixation or fracture or osteomyelitis or implant”. The PubMed search identified 420 references from 1960 to 2022. After eliminating references that were reviews, *in vitro* or *ex vivo* studies, human or animal case reports, and non-orthopedic implant models, the remaining 105 articles related to orthopedic-specific animal models were reviewed for relevance to the question based on the evaluation of host immunity following infection. Further down-selection to remove articles lacking evaluation of infection and/or immunological response identified 67 articles of interest, of which, 43 articles detailed mouse studies, and 24 detailed other animal models (rats, rabbits, pigs, etc.). The EMBASE search yielded 132 references after down-selection for articles limited to animal experiments, animal model, and in vivo study from the primary search with the above terms. The EMBASE database yielded an additional 19 relevant studies in mice and other species, for a total of 86 relevant articles between the two database searches.

Preclinical animal studies were reviewed for models that evaluated host immunity during orthopedic implant infections with an emphasis on immune outcome measures. We found that mouse models were routinely used to study host immunity, including models of orthopedic prosthetic joint[5-8], tibial implant[9-12], osteomyelitis[13-15], and fracture fixation infections[16-19], with *Staphylococcus aureus* as the predominant infectious agent. To a lesser extent, Sprague-Dawley rats were used in the prosthetic joint[20-22] and tibial implant models[23, 24], whereas pigs

Back to top

were used in the fracture fixation[25, 26] and tibial implant[27, 28] models and rabbits were used in prosthetic joint[29] and fracture fixation[30] infection models to study host immunity.

We also evaluated the immunological assays used to study host immunity in the during orthopedic implant infection models. A frequent outcome measure of the host response was cytokine production in the infected tissue and sera, which was evaluated using enzyme-linked immunosorbent assay (ELISA)[31-33], Multiplex systems[12, 34, 35], RNA sequencing[8, 25, 36, 37], and transgenic fluorescent reporter mice[33, 35, 38-40]. Furthermore, localized or systemic levels of immune cells, including polymorphonuclear leukocytes (PMNs), B cells, and subsets of T cells were routinely evaluated using fluorescent activated cell sorting (FACS) analysis[6, 20, 26, 34, 35, 41, 42] and immunohistochemistry[9, 28, 29, 43-45]. Lastly, antibodies were frequently measured by ELISA and Multiplex assays[12, 46-48].

Given that (1) there are abundant studies using mouse orthopedic implant models to study host immunity, (2) there are a few studies supporting the use of rat, pig, and rabbit models to study host immunity, and (3) there are routine outcome measures to evaluate host immunity during implant infections, we conclude that there are validated mouse, rat, pig, and rabbit models to study host immunity, especially to evaluate cytokine, immune cell, and antibody responses.

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Back to top

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Back to top

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Back to top

QUESTION 5

Are there rigorous approaches for detecting and quantifying intracellular bacterial reservoirs?

Karen L. de Mesy Bentley, Chao Xie, Motoo Saito

Response/Recommendation: Yes

For in vitro experiments, current standards of detecting and quantifying intracellular bacterial include time lapse, dark field, and/or fluorescent microscopy of fluorescent mammalian cells (GFP, RFP, YFP, etc.) infected with a different fluorescent color bacteria[1-3], cultured with non-permeable antibiotic (i.e. gentamycin) to kill extracellular bacteria. These data should be validated with static outcomes including colony forming unit (CFU) assay, flow cytometry (i.e., Amnis Imagestream™ imaging flow cytometry) for analysis of host cell-pathogen uptake in intracellular infections to provide bacterial counts together with statistical analyses to quantify bacterial load within mammalian cells. Transmission electron microscopy (TEM) utilized as a follow up to flow cytometry is useful for correlating occupation of bacteria within mammalian cell cytosolic compartments. For in vivo experiments, multiphoton intravital microscopy of a fluorescent transgenic mouse (i.e., CMV-GFP) infected with compatible fluorescent bacteria (i.e., RFP, GFP) should be performed as the primary outcome with validation by stained histology (i.e., Gram stain) paraffin sections to define regions of interest (ROI) to be analyzed by TEM. For ex vivo assessment of clinical biopsies, histochemical staining again should be used to identify a region of interested to be interrogated by TEM. CFU assays and other methods to detect bacterial load (i.e., PCR) cannot be used to detect and quantify intracellular bacteria in tissue.

Strength of Recommendation: Strong

Rationale

There is a very rich literature for detecting and quantifying intracellular bacteria in vitro, and in vivo in experimental bone infection animal models to also include human tissue. To specifically answer this question, a PubMed search performed on August 25, 2022, using the keywords “osteomyelitis”, “microscopy” and “bacteria” was performed which identified 199 publications of which a subset, were studies directly related to microscopy methods for identification of cellular bacterial uptake and host-microorganism interaction within bone[1-27]. We eliminated two studies related to fungal osteomyelitis and experimental osteoarthritis[16,17]. In the 1980’s both in vitro and in vivo studies of osteomyelitis were initiated to evaluate bacterial biofilm growth on implants models of infection in rat or rabbit tibiae. However, a scanning electron microscopy (SEM) study was eliminated as only surface topography of biofilm formation and not intracellular occupation can be evaluated[1].

For in vivo studies, only through the use of transmission electron microscopy (TEM), which is considered the “gold standard” can validate light microscopy or SEM studies of bacterial invasion and occupation of host bone tissue[2-3]. For in vitro studies of bacterial invasion of native osteoblasts, osteoclasts, or mast cells, mesenchymal stem cells, TEM (unlike the limitations of confocal microscopy analysis) are needed to document any subcellular effects, which could include sensitivity imaging assays of antibiotic resistant strains treated with antibiotics within cell types[4-13,15,19,22-24,27]. TEM imaging is an important imaging modality in the development of experimental mouse models of *S. aureus* bone infections used to mimic the pathology of human disease for future testing of novel treatment modalities[14,20].

Back to top

The development of a methicillin-resistant *S. aureus* (MRSA) vaccine to evaluate a neutralizing anti-glucosominidase (Gmd) monoclonal antibody (1C11), for treatment of bone infection also utilized TEM imaging of macrophage uptake and analysis of intracellular bacterial aggregation found to be significantly increased with 1C11 treatment[18]. A feasibility in vitro SEM study of the conjugation of bisphosphonate to antibiotics importantly revealed significant *S. aureus* outer wall damage leading to cell death[25]. A major bacterial reservoir was discovered utilizing TEM imaging of mouse bone from an implant study of osteomyelitis documented a novel mechanism of invasion by *S. aureus,* deformation into rod-shaped bacteria, to enter submicron spaces of the Osteocyte-Lacuno-Canalicular-Network (OLCN)[20]. This was followed by a TEM case study of *S. aureus* infected human bone confirmed in the OLCN providing the clinical significance of the previous finding[21]. Finally, a TEM 3D study of *S. aureus* invasion into the OLCN of mouse bone revealed the paradox of infected osteocyte canaliculi adjacent to non-infected, leading to the hypothesis, that viable osteocytes may be able to resist infection[26].

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Back to top

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Back to top

QUESTION 6

Can human immune responses be fully recapitulated in animal models of MSKI?

Mina A. Botros, Gowrishankar Muthukrishnan, James E. Cassat, Nathan K. Archer

Response/Recommendation: No

There is extensive literature that examines how the murine animal model has significantly contributed to our understanding of the immune responses and host-pathogen interactions during musculoskeletal infections (MSKI). However, the murine model has limitations in studying specific human immune responses. Mice engrafted with human immune cells i.e., humanized mice are a better alternative. Recently, humanized mice have gained prominence as small animal surrogates to study human immune responses to infection. Indeed, MSKI studies with humanized mice are now being performed. But humanized mice also have limitations and cannot fully recapitulate human immunity. Large animal models (sheep, rabbit, horse, pig, dog) are also routinely used in the examination of MSKI and for testing orthopaedic implants, biomaterials and antibiotics. However, immune responses to infection in these animals are yet to be compared to human responses.

Strength of Recommendation: Low

Rationale

To answer the question listed above, we performed a literature review search for PubMed articles that included “bacterial osteomyelitis,” and “animal models,” which identified 374 papers. Almost all these papers demonstrate the usefulness of animal models in examining musculoskeletal infection[1-5]. Several of the 374 papers included infection studies using ex vivo human immune cells. However, none was related to examining human immune responses in animal models. A biased search using the terms “human immunity”, “bacterial osteomyelitis” and “animal models” yielded 3 papers that were not relevant to the topic, but a biased search that included “bacterial osteomyelitis” and “humanized mice” yielded one paper[6]. Additionally, for the rationale below, the authors selected 15 articles, that were not identified in any systematic review literature search. Our systematic review revealed that the closest animal model to partly mimic human immune response in MSKI is the humanized mice model.

Murine animal model

Murine animal models have been essential tools for many studies evaluating immune responses and has provided tremendous insight into the inner workings of various immune cells during infection[1,5,7]. However, there have been several discrepancies between mice and human innate and the adaptive immunity[8]. The overall structure of the immune system and mice and human is quite similar, however, there are differences in the composition of white blood cells[8,9]. For instance, human blood is rich in neutrophils (50-70% vs. 10-25% in mice) while mouse blood is rich in lymphocytes (75-90% vs. 30-50% in humans)[9].

Murine animal models have significantly contributed to our understanding of the *S. aureus* pathogenesis and identification of critical virulence factors such as iron-scavenging proteins [IsdB], Staphylococcus protein A, fibrinogen binding proteins, penicillin-binding proteins, hemolysis, autolysis, etc.,[2,10-18]. Unfortunately, murine immune responses to *S. aureus* don’t always translate to human disease. A case-in-point is the failure of Merck’s IsdB vaccine. In mice, IsdB vaccination reduced infection lethality and protected mice from mortality induced due to *S. aureus* bacteremia[7,19-21]. In sharp contrast, an active vaccination human

clinical trial involving ~8000 patients, which was motivated by the murine data, failed to provide any protection to humans. In fact, the vaccination trial was halted by the FDA due to a 5-fold increase in fatal outcomes in the vaccinated patients due to *S. aureus* bacteremia[22].

It is now acknowledged that human-adapted pathogens such as *S. aureus* have numerous virulence proteins, including biocomponent leukotoxins that exhibit a high degree of tropism to the receptors expressed in human leukocytes and not to murine leukocytes[23-28]. This is a particularly important caveat for evaluating immune responses in murine models, given that studies of humoral immunity in humans with musculoskeletal infection reveal strong responses to staphylococcal leukotoxins[29,30]. Hence, we expect the infection phenotypes to be different in these hosts and there is a need for small animal models that better mimic the human immune system.

Humanized mice animal model

Humanized mice, which are generated by engrafting human immune cells into immunodeficient mice[31-33], can be a great small animal surrogate to study human immune responses to MSKI. They have been utilized to examine the safety of drugs that target human immune cells [34]. There has been a few preclinical studies utilizing humanized mice model, which led to successful human clinical trials. These mice are now being utilized to study MSKI as well. A recent study reported that humanized mice suffer exacerbated *S. aureus* osteomyelitis compared to non-humanized mice and that engrafted human T cell response determined the severity in these humanized mice[6]. However, humanized mice cannot fully recapitulate the human immune system and have several limitations that limit their ability to examine specific human immune responses. These include susceptibility to acute xenogeneic graft-versus-host disease, differences in Major Histocompatibility complex [MHC] molecules, cytokines, and hemopoietic growth factors, impaired antibody class switching, and suboptimal architecture of the lymphoid structures[32,35-37]. In addition, another important limitation is that you cannot fully recapitulate human stromal and immune cell crosstalk in humanized mice as stromal cells are of murine origin in these mice [38-40].

Large animal model

Additionally, large animal models have been utilized in the examination of musculoskeletal infections[5]. The rabbit model of osteomyelitis has been used to examine bone infection secondary to open fractures, hematogenous spread, and periprosthetic infection[5,41-44]. The advantage of the rabbit model is that it is easy to handle, manipulate, and maintain[5,41]. Relative to mice, rabbit leukocytes also display enhanced susceptibility to key *S. aureus* leukotoxins such as the Panton-Valentine Leukocidin (PVL), which has been demonstrated to contribute to the pathogenesis of experimental osteomyelitis in rabbits[45]. Further, rabbit bone size remains large enough to be able to perform screw and plate fixation; and the medullary canal of both the tibia and the femur can accommodate an implant or a nail[5,42]. Literature also reports the utilization of a dog model for chronic osteomyelitis, where the authors created a cortical window[38] and injected *S. aureus* and fill the defect with intramedullary cement[46]. Additionally, guinea pigs have also been utilized to study bacterial colonization of titanium mini-plate implants and screws[47]. Further, it has been reported that sheep model has also been used to examine staphylococcal osteomyelitis secondary to open fracture[48]. Even though there are several animal models to mimic human osteomyelitis, as reported above, there is no published literature comparing the immune response of these large animal models to human responses during MSKI.

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Back to top

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Back to top

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Back to top

QUESTION 7

Does anti-inflammatory medication influence MSKI in preclinical models?

Nathan K. Archer & Stuart B. Goodman

Response/Recommendation: Yes

The use of NSAIDs as an adjunct to antibiotics in mitigating the adverse effects of implant infections is supported by the evidence in rodent models with various infectious agents, including *S. aureus*. The influence of NSAIDs during implant infections included reduced bacterial burdens, decreased pro-inflammatory responses, and improved bone formation.

Strength of Recommendation: Strong

Rationale

Implant-associated infections are marked by biofilm formation that leads to chronic inflammation and bone destruction[1, 2]. Mouse studies have shown that staphylococcal biofilms skew the host immune response towards anti-inflammatory responses, thus impeding clearance of the bacterial infection[2-5]. Beyond their role in immune suppression, anti-inflammatory medications have been shown to have effects on bacteria. For example, aspirin promotes the effectiveness of antibiotics against *S. aureus* and biofilm-associated infections[6, 7]. Moreover, diflunisal suppresses the quorum-sensing agr system in *S. aureus* to reduce virulence factor production[8, 9]. Therefore, we explored how anti-inflammatory medications influence implant-associated infections.

To identify how anti-inflammatory medication influences implant infections, a systematic review was completed on peer reviewed literature identified by a PubMed search performed on August 14, 2022 using the key words "(osteomyelitis or orthopedic implant infection or implant-associated infection or prosthetic joint infection) and (anti-inflammatory or anti-inflammatory medication or anti-inflammatory drug) and (preclinical model or animal model or mouse model or rabbit model or rat model or pig model or in vivo)". This literature search identified 135 references from 1976 to 2022. After eliminating 124 articles that did not contain information directly addressing the question, there remained 11 relevant pre-clinical research articles.

We set out to determine the influence of anti-inflammatory medication on orthopaedic implant infections in pre-clinical models. Using a murine subcutaneous implant model of *S. aureus* infection in mice, treatment with the anti-inflammatory drug diclofenac was associated with reduced bacterial burden and biofilm formation on the implants[10]. Similarly, curcumin treatment in a *S. aureus* tibial implant model in Wistar rats resulted in a modest suppression of bacterial burden, but a substantial reduction in inflammation associated with decreased TNF and IL-6 in circulation[11]. These results were amplified when curcumin was co-administered with erythromycin, which had a greater effect than each monotherapy alone[11]. Furthermore, in a murine model of prosthetic joint infection with *S. aureus*, curcumin treatment reduced signs of inflammation and anti-inflammatory monocyte-derived suppresser cells (MDSCs) in the circulation when given in combination with vancomycin[12]. Similar to diclofenac and curcumin, treatment with dexamethasone reduced bacterial burden and diminished inflammatory cell influx and improved bone formation in a Wistar rat model of a subcutaneous implant infection with *S. aureus* [13]. In an osteomyelitis model due to *S. aureus*, treatment with diflunisal diminished bone destruction[9, 14, 15], but had no effect on bacterial burden[14]. Importantly,

diflunisal did not inhibit efficacy of systemic treatment with vancomycin[15]. Jiang et al[16] found that treatment with aspirin reduced osteolysis and the periosteal reaction, inhibited osteoclast activation, promoted osteoblast activation, and facilitated healing of a tibial implant infection model with *S. aureus* in mice. Treatment with the non-steroidal anti-inflammatory drug, carprofen resulted in diminished osteolysis in Wistar rats with a tibial implant infection due to *S. epidermidis*. However, carprofen therapy alone had no effect on bacterial burden and was associated with diminished reparative bone formation and reduced the efficacy of rifampicin and cefazolin treatment[17]. In a mouse model of periodontitis with *P. gingivalis*, the anti-inflammatory compound, cinnamoyloxy-mammeisin attenuated bone loss and reduced expression of the osteoclast activation markers, Trap/Acpp5 and Ctsk[18]. Finally, Wu et. al. showed that baicalin, the major active constituent of the isolated root of Scutellaria lateriflora Georgi, reduced bone destruction, attenuated pro-inflammatory markers in serum (e.g., IL-1β, IL-6, and C-reactive protein), and increased osteogenic markers in the tibia of mice with a prosthetic joint implant infection due to *S. aureus* [19].

Given that (1) diclofenac, dexamethasone, diflunisal, aspirin, cinnamoyloxy-mammeisin, and baicalin improved bone formation, diminished osteolysis, and attenuated osteoclast markers, (2) diclofenac, curcumin, and dexamethasome reduced bacterial burdens, and (3) curcumin, baicalin, and dexamethasome decreased pro-inflammatory responses, including immune cell influx and cytokine production, we conclude that there is strong evidence to support a role for anti-inflammatory medications to influence the outcome of implant infections.

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Back to top

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Back to top

QUESTION 8

Does autoimmunity affect musculoskeletal infection in animal models?

Edward M. Schwarz & Stuart B. Goodman

Response/Recommendation: No

Limited evidence suggests that autoimmunity does not exacerbate MSKI in murine models.

Strength of Recommendation: Low

Rationale

Autoimmunity is a known risk factor for infection in general,[1; 2] and patients with rheumatoid arthritis have a 50% increased risk of prosthetic joint infection (PJI) compared to those with osteoarthritis.[3; 4] Thus, animal models with predictive validity of human autoimmune susceptibilities to musculoskeletal infection (MSKI) are of great value to elucidated mechanisms and evaluated interventions through regulatory approval processes. To assess our knowledge to date on the interactions between autoimmunity and MSKI in animal models, a peer-reviewed literature search on PubMed was performed on June 22, 2022, using the keywords “autoimmunity” and “musculoskeletal infection” and “animal model”, which identified 164 references. Among these only 4 were deemed to be somewhat related to the question, but did not provide evidence to support an answer.[5-8] Thus, a more focused PubMed search was performed on specific autoimmune diseases that identified: 47 paper for “arthritis”, 40 papers for “diabetes”, 13 papers for “lupus”, 36 papers for “multiple sclerosis”, 48 papers for “inflammatory bowel disease” (IBD), 0 papers for “thyroiditis”, and 3 papers for “psoriasis”. Of these, only 4 were deemed highly relevant to the question, [9-12] and the data and conclusions are summarized below.

Mice with active collagen-induced arthritis do not have exacerbated *S. aureus* PJI.[9] Mice with hyperglycemic streptozotocin-induced type-1 diabetes have a very modest increase in *S. aureus* implant-associated osteomyelitis.[12] No information on MSKI in animal models of lupus (i.e. MRL/lpr and NZBxNZW mice) was found. No information on MSKI in animal models of multiple sclerosis (i.e., mice with experimental autoimmune encephalomyelitis) was found. No information on MSKI in animal models of IBD (i.e., chemical or Helicobacter induced) was found. In conclusion, the literature on MSKI in the setting of autoimmunity in animal models is scant, and prospective research efforts are warranted to develop animal models that faithfully reflect the known susceptibilities to MSKI in patients with autoimmunity.

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Back to top

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Back to top

QUESTION 9

Is there an established immunization protocol (adjuvant, route of administration, time between boost) to assess novel vaccines for MSKI?

Edward M. Schwarz, Gowrishankar Muthukrishnan & John L. Daiss

Response/Recommendation: Yes

There is extensive literature on immunization protocols to assess novel vaccines in research animals. These protocols are specific for active immunization with: 1) biochemical (e.g., proteins, polysaccharides, peptides, oligosaccharides, or hapten-conjugate vaccines); 2) recombinant viral vectors, 3) DNA, and 4) mRNA lipid nanoparticles (LNP). There is also a very strong veterinary literature (peer reviewed and commercial) for vaccination of domesticated animals,[1-3] which was not considered here.

Strength of Recommendation: Strong

Rationale

To the end of a "Guidance Document" for investigators evaluating the safety and efficacy of novel vaccines for MSKI, we performed literature searches for PubMed article titles that included "immunization protocol (n=68)," "novel vaccination (n=360)," and "animal model + vaccine (n=60)." These articles were then scrutinized for detailed methods (adjuvant, route of administration, time between boost) for immunizing mice, rats, hamsters, and rabbits, which are the most used mammals for assessing novel vaccines. This search identified five very high-quality methodology papers with extensive citations dating back to the 1950s, which serve as the basis of the recommendations for active immunization with purified biochemicals.[4-8] Articles that provide specific immunization protocols for viral vectors,[3; 9] DNA and mRNA LNP[10; 11] were reviewed to provide detailed recommendations for these approaches respectively.

Biochemical Vaccines. Immunizations in laboratory animals have been performed for a wide variety of reasons, but it should be noted that most standardized protocols are for induction of specific B cells for the generation of polyclonal antibody sera and hybridomas for monoclonal antibodies (mAb). Thus, immunization protocols aimed at generating vaccines for life-long protective T-cell immunity may vary. With respect to vaccines for mAb production, it should be noted that the Institute for Laboratory Animal Research (ILAR) Committee on Methods of Producing mAb concluded in 1999 that the intraperitoneal accumulation of ascites is likely to be associated with pain and distress,[7] and consequently the use of the ascites method has been banned or strongly discouraged by most institutional animal care and use committees (IACUCs) world-wide. The critical steps in the entire production process and recommendations on how to optimize and refine protocols, including: selection of animal species, adjuvant selection and preparation, injection protocol with booster immunization, and post-injection observation of laboratory animals, have been published.[12]

Most experimental vaccines use adjuvants in the immunization protocol. However, it is well known that many adjuvants (e.g. Complete Freund's adjuvant (CFA), which contains heat-killed *Mycobacterium tuberculosis*) are quite irritating or toxic, and engenders animal safety issues that need specific IACUC approval.[7; 8] The major adjuvant products used in research animals, their mode of action, immunology, pathology, and pain and suffering have been formally reviewed.[13]

A consensus protocol for vaccinating mice commences with the priming immunization in which the emulsion containing 25 to 100 µg of antigen plus adjuvant is injected intraperitoneal (i.p.) (<0.3 ml) or subcutaneously (s.c.) (<0.2 ml) using a 22-gauge needle to reduces the risk of leakage after injection and limit pain associated with the use of larger bore needles (e.g., 18-G or 20-G).[8] Vaccine emulsions are typically too viscous for higher gauge needles (e.g., 25-G or 27-G). Up to three booster immunizations 2 to 3 weeks after the prior immunization are recommended with 10 to 50 µg of emulsified antigen in Incomplete Freund's adjuvant via the same i.p. or s.c. route as the priming dose.[8] CFA should never be given in a booster vaccine, but non-CFA adjuvants (e.g. Ribi, Hunter's TiterMax, ImmunEasy, or Alum) may be used in boosters after IACUC approval.

Consensus protocols for vaccinating rats, hamsters and rabbits are very similar to mice, except for the dose and regimens. For rats and hamsters, a dose of 100–200 µg is sufficient.[5] For rabbits, the minimum dose rages from 10-100 µg per injection, but can be increased to 0.5-1 mg if a pure, soluble protein antigen is being used.[6] Adjuvants (Freund's, Ribi, Hunter's TiterMax, ImmunEasy, or Alum) should be mixed with the immunizing antigen for the first two immunizations only; and CFA can only be used with the first immunization. Subsequent immunizations are performed in phosphate-buffered saline (PBS) or normal saline, with or without adjuvant. For rats and hamsters, boosts should be spaced every 2–3 weeks, and serum samples of 400–500 µL should be collected 10–12 d after each boost. For rabbits, it is important to shave and disinfect the injection site before immunization, and boosts should be spaced every 6 weeks, with serum samples of 20-40 mL collected approximately 10-12 d after each boost; typically, a single sample bleed from a rabbit will yield 25 mL of serum.

Viral vector vaccines. Many vaccine antigens are very challenging to produce as purified soluble biochemical for various reasons including size, insolubility, glycosylation, and their native form as transmembrane proteins (ref?). Gene transfer vaccination approaches overcome these limitations by eliminating biochemical synthesis and purification steps and allow for successful vaccination by very small amounts of antigen (picograms) that are produced within the host. As viruses are the most efficient vectors for gene transfer into cells, recombinant viral vectors have been used as vaccines for over half a century.[3] Recombinant adenovirus (rAd) gained popularity following the successful and safe immunization of millions of US military recruits in 1971 with enterically coated Ad4 and Ad7 as a preventative against acute respiratory disease outbreaks.[14] Following these first trials, a number of rAd have recently been constructed and tested not only for humans but also for veterinary vaccination.[15; 16] Unfortunately, the rAd vector itself is very immunogenic, which elicits neutralizing antibodies within a few weeks that prohibits boosting.[17] There is also a lack of consensus on dosing and route of administration, however, an appropriate guideline for intramuscular injection is 10^9 viral particles for mice, 10^{10} viral particles for rats and 5.6×10^{10} viral particles for rabbits.[18; 19]

DNA vaccines. Although the clinical utility of DNA vaccines has diminished due to the advancement of more effective alternatives, they are still commonly used for eliciting mAbs against challenging targets.[20] Additionally, DNA immunization is particularly useful for the *in vivo* expression of structurally native full-length proteins in the membrane-bound state, such as GPCRs, providing an attractive alternative for generating mAbs against membrane proteins.[21] In contrast to the other approaches, DNA vaccines can be administered via different approaches including: 1) conventional needle injection of DNA plasmids dissolved in various buffers or lipids and nanoparticles, 2) the gene gun, which uses a "ballistic" force to deliver DNA plasmids coated on gold particles, and 3) electroporation delivery of DNA plasmid.[22]

A study conducted a side-by-side comparison of the efficacy of DNA vaccine delivery among intramuscular (IM) needle injection, electroporation (EP) following IM injection, and gene gun (GG), and found that GG and EP delivery methods were more effective than IM injection alone.[23] Additional details on DNA dosing with the various DNA vaccine delivery approaches for mice, rabbits and humans have been published.[22] The immunization schedule for the priming dose and boosters is variable, however, a consensus regimen for small animal models has been established.[24] The minimum number of immunizations for a DNA vaccine is two regardless of the type of delivery approach used. The maximum number of immunizations for a DNA vaccine is 3–4 if the antigen is reasonably immunogenic. The common resting period between DNA immunizations is 2 weeks but can be increased to 4 weeks or longer in larger animals. The frequency of immunizations may not need to be equally distributed.

mRNA/LNP vaccines. Shortly after the first successful experimental injections of mRNA into murine muscle cells in vitro in the early 1990s,[25] researchers began testing mRNA vaccines, which have an efficiency advantage over DNA vaccines since they do not need to enter the nucleus and can facilitate protein synthesis immediately after entering the cell.[10] However, it was not until critical mRNA modifications (e.g. pseudouridine) to increase stability and translational capacity, while decreasing host immunity, were developed that the field of mRNA vaccination expanded.[26] Now, following the remarkable success of the COVID-19 vaccines, this approach is considered mainstream, and perhaps the preferred approach for human vaccines.

The majority of mRNA vaccines are currently packaged in biodegradable ionizable lipid nanoparticles (LNPs) consisting of variants of phospholipids, cholesterol, and polyethylene glycol (PEG) containing lipids.[27; 28] The ionizable lipid is positively charged to form complexes with the negatively charged mRNA for protection of the mRNA and may also help with cellular uptake and endosomal escape.[28] PEG–lipids significantly increase the bioavailability, i.e., time of mRNA in the circulation, which greatly improves the prospects for therapeutic use, but this can be at the expense of reduced transfection efficiency.[29] Alternative core-shell structured lipopolyplex (LPP) nanoparticles have also been used in which mRNA binds to a positively charged protein or polymer to form a dense core structure that is encapsulated in a lipid shell.[30]

A consensus mRNA/LNP protocol has emerged largely based on the COVID-19 vaccines. The doses range from 1-30mg of mRNA mixed with LNP, and injected IM. The priming dose can be given with CFA, and up to 3 boosts are given between 2 and 4 weeks apart.[31; 32]

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Back to top

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Back to top

QUESTION 10

Are there interventions beyond vaccination known to boost host immunity to MSKI in animal models?

Edward M. Schwarz, Gowrishankar Muthukrishnan & John L. Daiss

Response/Recommendation: Yes

There is peer reviewed literature that largely demonstrates the efficacy of nutritional supplements, prebiotic and probiotic treatments, and moderate exercise on infection reduction with various microbes in animal models. However, the literature on animal models of MSKI is too limited to make a strong recommendation.

Strength of Recommendation: Low

Rationale

To answer the question, we performed a literature search for PubMed articles that included "boost immunity" and "animal model" and "infection", which identified 359 papers. Almost all of these papers focused on immunizations, and only one was specific for MSKI animal models.[1] However, a biased literature search using nutrition, microbiome, and exercise revealed 51 papers.

Nutritional supplementation. There is extensive literature on vitamin supplementation to enhance immunity and protection in pre-clinical models. These studies include treatments with vitamins A,[2] B3,[3] C,[4] D,[1] and E.[5] In terms of antioxidants, Al Azzaz, et al showed that resveratrol is able to boost xenophagy in zebrafish, and enhances clearance of *Salmonella typhimurium* and Crohn's disease-associated adherent-invasive *Escherichia coli*.[6] Sepahi et al showed that short-chain fatty acids (SCFAs), produced by the commensal microbiota from dietary fibers, function to maintain optimal numbers of ILCs in peripheral tissues during infection and inflammatory responses in mice.[7]

Prebiotic and probiotic treatments. As it is now known that the host microbiome significantly impacts immune responses [8], researchers are now investigating the composition of gut microbiota in disease and efficacy of treatments that alter gut microbiota. Recent studies showed that prebiotic and probiotic treatment protects mice from Salmonella.[9-11] In contrast, Spinler et al found that administration of probiotic kefir to mice with *Clostridium difficile* infection exacerbates disease.[12] These results suggest that alterations of gut microbiota are pathogen- and treatment-dependent. Interestingly, a recent study demonstrated that treatment with oligofructose (SCFAs) markedly shifted the diversity of the gut microbiome from a pro-inflammatory to anti-inflammatory state in obese/type 2 diabetic mice, and reduced *S. aureus* osteomyelitis severity in these mice [13].

Exercise. There are a few published studies on the role of exercise in boosting host immunity in animal models going back to 1965.[14] A recent study found that a single bout of prolonged high-intensity exercise can be either deleterious or beneficial to antiviral immunity.[15] One interesting study in lower organisms demonstrated that indirect flight muscles of Drosophila and trunk muscles of zebrafish are capable of mounting a potent humoral immune response, suggesting that physiologically fit muscles might boost the innate immune response of an individual.[16] Other studies found that moderate exercise protects mice from *Trypanosoma cruzi* and *Salmonella Typhimurium.*[17; 18]

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Back to top

QUESTION 11

Has the immune proteome been well-defined in animal models of MSKI?

Mina A. Botros, Gowrishankar Muthukrishnan, Edward M. Schwarz, and John L. Daiss

Response/Recommendation: No

Current literature examines the advancement in our understanding of the immune proteome in the setting of musculoskeletal infection (MSKI). Circulating antibodies represent a very important marker, which reflect the repertoire of antigens to which the immune system identifies as foreign (non-self). Antibodies are good biomarkers for diagnostic applications as they are stable to examine in an experimental and clinical application. Essentially, immune proteomics is utilized for antigen discovery, which is effective in monitoring and diagnosing disease state, and even vaccine development.

Strength of Recommendation: Low

Rationale

To answer the question listed above, we performed a literature review search for PubMed articles that included “immune proteome,” and “musculoskeletal infection,” which identified 64 papers. Almost all these papers demonstrate the effectiveness of the immune proteome in the setting of MSKI, which focuses on the validation and translation of immunoproteomics biomarkers to monitoring disease progression, development of diagnostic tests and vaccines. Authors selected 6 articles that are referenced below, that were not identified in the systemic review search.

Immune Proteome

Immune proteome is a very powerful tool for evaluating the adaptive immune system to natural encounters between a microorganism and a host, specifically in musculoskeletal infection (MSKI)[1,2]. The knowledge acquired from immune proteome is essential for the development of effective vaccines, as well as the discovery of diagnostic and novel therapeutic tools[3]. Additionally, the immune proteome illuminates the human adaptive immune response in the setting of an MSKI[3,4]. The utilization of enzyme linked immune absorbent assay (ELISA), agglutination, or Western blots techniques has been used previously for many years to examine the immune response to vaccination or diseases[5]. However, with the advancements in immune proteomics, researchers can further understand a pathological condition, its progression, identify vaccine candidates, and important biomarkers[6]. Immunoproteomics is rapidly expanding due to its increasing techniques that identify various immune response triggering proteins, which are derived from invading microorganisms or immune-signaling molecule[6,7].

One of the most used techniques in immune proteome examination is the 2D–PAGE, which separates protein based upon their physical characteristics[2,7]. When combined with Western blot's, the technique is referred to as Serological Proteome Analysis (SPA)[1]. To identify at antigenic protein within a proteome, 2D–PAGE can be utilized; where gels are then transferred to a membrane and probed with animal or human model sera, per the traditional Western blot technique[8]. Many gels can be run in parallel to provide a reference map and the identification of immunoreactive proteins[1,8].

Immune proteome assays have been reported to be effective in monitoring, or diagnosing, disease state, and even determining vaccine efficacy or antigens involved[2-4].

Back to top

Bacterial infections especially in the setting of osteomyelitis are highly preventable through vaccination, where immunoproteomics techniques are utilized for antigen discovery for vaccine development[9]. The goal of vaccination against an infectious disease is to stimulate a protective immune response, which can be measured and correlated with the protection of the receiving host against a microorganism[10,11]. The success of formulating a vaccine is based on the identification of immunoprotective proteins such as antibodies, cytokines, etc. Therefore, with the advancements of Immunoproteomics, the identification of these immunoprotective proteins is correlated with the formulation of effective vaccination[12,13]. Additionally, circulating antibodies represents a very important marker, which reflects the repertoire of antigens to which the immune system identifies as foreign (non-self)[13]. Antibodies provide a good biomarker for diagnostic applications as it amplifies a signal of a low abundance disease, have half-life of days to months, and are stable to examine in an experimental/clinical application[14]. Current MSKI research focuses on the validation and translation of immunoproteomics biomarkers to monitoring disease progression, development of diagnostic tests and vaccines[1,2,10,11,14].

Immune Proteome of *Staphylococcus aureus in MSKI*

Staphylococcus aureus is a ubiquitous microorganism, most newborns contact the microorganism within a few hours from birth[15]. Approximately 80% of infants is colonized by this microorganism in the first year of their life[16]. It is reported that as we age, the colonization rate gradually decreases in the adult population, with only 20% having continuous *S. aureus* colonization in their nares[17]. It is important to note that the bacterial colonization between *S. aureus* and its human host is not a hostile relationship. However, this balance is easily altered by a weakening of the immune system or an increase in bacterial virulence[17,18]. *S. aureus* is the most common microorganism associated with osteomyelitis and sepsis[19,20]. Unfortunately, carriers are frequently more affected than non-carriers[21,22]. Historically, the success of *S. aureus* as a human pathogen has been significantly influenced by its ability to formulate antibiotic resistance, such as methicillin–resistant staph aureus [MRSA], which now ranks is one of the leading causes of nosocomial infections[23]. Additionally, these multidrug-resistant *S. aureus* species of also been associated with osteomyelitis and has the ability to negate virtually any antibiotics of clinical value[23,24]. Therefore, it is essential that we understand how the immune-system controlled S. aureus, and why under certain conditions, it fails to create a balance between the carrier-host and this microorganism.

S. aureus genome includes approximately 2700 proteins, either membrane attached, cytoplasmic, cell wall component, or released into the extracellular space[25]. The adaptive immune system formulates an antibody response against extracellular and cell wall-associated proteins and non-protein antigens [i.e. lipoteichoic acid and peptidoglycans][18]. The ability to treat S. aureus is limited due to the paucity of new classic antimicrobial agents in the pharmaceutical discovery pipeline. Therefore, there is an essential benefit for an effective vaccine to target these multi-drug-resistant pathogens.

S. aureus immune invasive proteins include hemolysins (i.e. alpha hemolysins, beta–hemolysins, gamma-hemolysins), leukocidins (i.e. LukAB, LukDE, PVL), enzymes (glucosaminidase, Aminidase, Aureolysin, Saphlyokinase, Nuclease), Phenol-soluble modulins, superantigenic exotoxins (toxic shock syndrome toxin), Wall teichoic acids[2,4,18]. Glucosaminidase (Gmd), a subunit of autolysin (Atl), which several groups of identified as an immunodominant antigen[18]. Functionality of autolysin has been essential for cell wall biosynthesis and degradation during binary fission[2]. Anti-Gmd antibodies utilized as serum

Back to top

biomarkers of protective immunity against S. aureus in patients with orthopedic infections[3,9]. Varrone et al. l. 2014 have demonstrated the potential use of anti-Gmd passive immunization to protect patients from implant-associated osteomyelitis by facilitating opsonophagocytosis[26].

Utilizing Multianalyte Immunoassay on medium enriched for newly synthesized antibodies (MENSA) from antibody-secreting cells (ASC) to evaluate the anti-*S. aureus* immunoglobulins (IgG) response in the setting of a musculoskeletal infections[27,28]. MENSA IgG levels can be utilized to assess and identify the presence and type of *S. aureus* MSKI[27]. Examining the MENSA IgG response among patients with bone infection, antigens predictive of an active MSKI (IsdB, SCIN, Gmd), and antigens predictable MSK type (IsdB, IsdH, Amd, Hla)[27,29]. When combined these antigens, they are highly discriminatory of S. aureus MSKI[29]. This demonstrates the value of utilizing the patient's active immune protein home against *S. aureus* diagnosed challenging MSKI.

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Back to top

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Back to top

QUESTION 12

Are there vaccines/passive immunizations for MSKI in animal models? (e.g. *S. aureus*)?

Edward M. Schwarz, Gowrishankar Muthukrishnan & John L. Daiss

Response/Recommendation: Yes

There is extensive peer reviewed literature on vaccines and passive immunizations for MSKI including *S. aureus* in experimental animals. There are also some veterinary commercial vaccines and passive immunizations for MSKI that are used in domesticated animals. Unfortunately, no such vaccines exist for humans.

Strength of Recommendation: Strong

Rationale

As there is great demand for vaccines against the most prevalent organisms that cause MSKI (e.g. Staphylococcus, Streptococcus, Pseudomonas), there is rich literature on this, and current progress is reviewed annually.[1-3] However, to formally address this question, a PubMed search was performed on July 28, 2022, with the keywords “animal model”, “musculoskeletal infection” and “vaccine”, which identified 101 references dating back to 1979. The same search replacing “vaccine” with “passive immunization” identified 10 references dating back to 1981.

It is also of note that there are commercially available vaccines for MSKI. Septic arthritis can be caused by *Borrelia burgdorferi* and commercial vaccines for domesticated animals are available (e.g., LymeVax®, Zoetis; Nobivac® Lyme, Merck Animal Health, RECOMBITEK® Lyme, Boehringer Ingelheim, and VANGUARD® crLyme, Zoetis)[4; 5]

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Back to top

QUESTION 13

Does prior MSKI shape immune responses to subsequent bacterial infection?

Edward M. Schwarz, Gowrishankar Muthukrishnan & John L. Daiss

Response/Recommendation: Yes

MSKI follows the theory of "original antigenic sin", which posits that prior exposure to pathogens shapes the immune response and affects the outcome of subsequent bacterial infection. The affect(s) can be positive (protective) or negative (pathogenic), and depends on the antigen(s), host genetics, and pathogenic circumstances.

Strength of Recommendation: Very Strong

Rationale

Conventional thinking about how prior infection shapes immunity to subsequent infections derives from the theory of "original antigenic sin",[1] which was first proposed by Thomas Francis Jr., in his treatise "On the Doctrine of Original Antigenic Sin" and has been utilized to explain adaptive immunity following initial infection.[2] Interestingly, "original antigenic sin" was used in the 1940s to explain the Spanish flu pandemic and influenza vaccines,[1; 2] and is now a focal discussion point to understand the SARS-CoV-2 pandemic and vaccine strategies to prevent or contain the rapidly emerging COVID-19 variant strains.[3-9] The basis of this theory is that survival from an initial pathogenic exposure provides protective immunological memory against a subsequent challenge from the exact same pathogen, and that the immune system can autocorrect to provide significant protection from a variant. Indeed, this theory is personified by the SARS-CoV-2 pandemic, as hospitalizations and death from COVID-19 are significantly decreased in patients who were previously infected and/or immunized.[10-12] Regrettably, the "original antigenic sin" hypothesis also posits that immunity against an initial exposure can exacerbate disease following a subsequent infection via antibody-dependent enhancement of the disease or induced anergy to protective antigens.[2; 13] A prime example of this is dengue fever, which only occurs after the virus has induced a non-neutralizing antibody response that it uses to facilitate viral replication in macrophages.[14; 15]

Experimental investigation of the "Original antigenic sin" hypothesis in animal models commenced in the 1960s,[16] and included influenza antigen immunization experiments in rats,[17] and classic studies demonstrating that rabbits primed with beef myoglobulin, and boosted with myoglobulin from other species generate an increased antibody response to the original beef myoglobulin.[18] In terms of pathogen types, "original antigenic sin" is known to affect a wide array of viruses, bacteria, and parasites.[2] However, a PubMed search performed on July 13, 2022, using the keywords "original antigenic sin" and "musculoskeletal infection" or "orthopaedic" or "bone" failed to identify any peer reviewed literature on this subject. We also performed PubMed searches with the keywords "animal model" and "reinfection" or "musculoskeletal" or "bone" that identified three papers that did not address the question.[19-21] Lastly, we performed PubMed searches for the most common bacteria in musculoskeletal infections and "reinfection" and "animal model", which yielded 506 papers. 13 papers were identified for *S. aureus* of which only six addressed the question and concluded that prior infection of mice is protective.[22-27] In contrast, it was recently shown that mice actively and passively immunized against iron surface determinant protein B (IsdB) have increased susceptibility to *S. aureus* implant-associated surgical site infections due to increase in Trojan horse macrophage formation and sepsis,[28] which is consistent with the failed phase 2 clinical

Back to top

trial of active IsdB immunization in patients undergoing cardiothoracic surgery.[29] It is also known that chronic *S. aureus* prosthetic joint infection in mice is associated with feedback mechanisms involving T-cell inhibitory receptors and exhaustion markers, suppressive cytokines, regulatory T cells and decreased T-cell proliferation, all of which could have downstream effects on anti-*S. aureus* protective antibody responses in mice.[30] Thus, prior infection has the potential to be pathogenic due to anergy, as predicted by the “original antigenic sin” hypothesis.

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Back to top

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Back to top

QUESTION 14

Is the antibody response to MSKI pathogen-specific?

Elysia Masters & Dina Raafat

Response/Recommendation: Yes

The antibody-mediated immune response to MSKIs is highly pathogen-specific. There are several first-class studies characterizing the human and murine humoral response to *Staphylococcus aureus* (*S. aureus*)-related MSKIs. Such studies feature highly specific immune responses, which are dependent on many factors including among other things: specific pathogen strain, site of infection, infection stage, host immune status and comorbidities. While fewer studies have focused on pathogens outside of *S. aureus* in the setting of MSKI, there is strong evidence for the development of pathogen-specific antibodies during infection with a range of bacteria and fungi. Despite the specificity of host antibody-response to MSKI, such responses are rarely sufficient for microbial clearance, and hence might be of more value in the development of new diagnostic tools.

Strength of Recommendation: Strong

Rationale

A search of the English language literature related to the question was conducted using PubMed, Web of Science and Google Scholar, during the period from January 1950 to August 2022. Search words included: osteomyelitis, humoral response, antibody response, prosthetic joint infection (PJI), musculoskeletal infection, MSKI and pathogen-specific, with no restrictions. Authors found no controlled clinical studies related to this question. Given the limited literature on antibody response to pathogens beyond *S. aureus* in MSKI, authors performed a structured review focusing on the differences in pathogen-specific host immune responses in MSKI. After review, selection, and inclusion of relevant articles, 21 were included in this response.

Several microorganisms have been implicated in MSKI, whether as a monomicrobial infection, or even in a polymicrobial setting. Because of difficulties associated with diagnosing MSKI and more specifically, identifying the causative pathogen, emphasis has been given to the development of antibody-based serological assays to diagnose patients. Serology-based immunoassays have also been developed to diagnose and prognose MSKIs. Marmor et al. demonstrated the use of a multiplex antibody detection-based immunoassay for the diagnosis of PJIs and for the genus-level identification of causative pathogens in humans [1]. In this work, authors utilized 16 antigens from staphylococcal species, *Streptococcus agalactiae* and *Propionibacterium acnes,* and observed sensitivity/specificity values of 72.3%/80.7% for staphylococci, 75%/92.6% for *S. agalactiae*, and 38.5%/84.8% for *P. acnes*. Additionally, Sulovari et al. developed species-specific immunoassays for the identification of *S. aureus* and *S. agalactiae* in active MSKI [2]. This work shows that the human antibody response is pathogen-specific and can be utilized to diagnose the causative pathogen.

It is well-known that *S. aureus* is the most common etiologic agent in MSKIs and consequently most MSKI-related research studies focus on *S. aureus*. During MSKI, humans produce antibodies targeting numerous *S. aureus*-specifc antigens, which are predominantly secreted products or antigens associated with the cell wall [3]. In a study investigating the human humoral response to *S. aureus* osteomyelitis, researchers found that cross-reacting antibodies to *S. aureus* teichoic acid (TA) were only detected in 6% of patients infected with non-*S. aureus* organisms [4]. Further, several groups have developed immunoassays for the diagnosis and prognosis of *S. aureus* MSKIs [5-9]. In these studies, authors have confirmed the absence of

interspecies cross reactivity of selected *S. aureus*-specific antigens to *S. epidermidis*, *S. lugdunenesis*, or *E. coli* in Balb/c mice [5], thereby emphasizing the specificity of the host antibody response.

Indeed, a commercial product (BJI InoPlex™; Diaxonhit) is currently available, which allows the measurement of the presence of specific serum IgG to a panel of recombinant antigens from pathogens frequently implicated in MSKI including staphylococci (8 antigens), *Streptococcus agalactiae* (4 antigens) and *Cutibacterium acnes* (4 antigens), by means of a multiplex ELISA [10-12]. Whereas the test is simple and relatively fast, providing results within a few hours, it is relatively expensive, and more importantly, it does not cover the whole spectrum of pathogens responsible for MSKI, and his hence insufficient to affirm an MSKI diagnosis [12, 13].
Several research groups have attempted the diagnosis of MSKI using minimally invasive serological tests. Among the *S. aureus* targets of promising potential as markers of staphylococcal infection are: (i) staphylococcal slime polysaccharide antigens [14]; (ii) SACOL0688 (the manganese transporter MntC) [15]; (iii) IsdA, IsdB, IsdH, Gmd, Amd, Hla and SCIN [7, 16]; and (iv) lipoteichoic acid [3, 17, 18].

Notably, researchers have identified many factors which influence the antibody-response to *S. aureus* MSKIs, resulting in a remarkably heterogenous antibody response within one pathogen species. Such factors include (1) pathogen strain, (2) infection site, (3) infection stage, (4) host comorbidities (5) previous exposure. First, the antibody response can be dependent on the specific strain of *S. aureus.* Niemann et al. have shown that patients diagnosed with PVL-positive *S. aureus*-osteomyelitis produce specific anti-PVL antibodies [19]. Further, using a serologic assay (ELISA) to detect antibodies targeting the leukocidin LukAB has also been investigated as a potential marker for the diagnosis of invasive *S. aureus* infections [20, 21]. Next, antibody response can be dependent on infection site and stage. Examination of the IgG response in human MSKIs demonstrated that responses specific to *S. aureus* antigens can distinguish between patients with PJI versus septic arthritis, and between patients with diabetic foot ulcers versus skin/soft tissue infections [7]. Further, researchers have identified immunogens specifically associated with *S. aureus* biofilms, that are distinct from those expressed during the planktonic phase [15, 22]. Finally, antibody response is heavily influenced by host health and comorbidities. A comprehensive study characterizing the human antibody response to *S. aureus* across many diseases showed a strong influence of sex, smoking, age, body mass index and serum glucose [23]. Together, these studies prove that while the host response is pathogen-specific, many factors influence the host antibody response.

Despite of the limited number of studies focusing on the antibody-response to non-*S. aureus* MSKI, there are numerous studies attempting to characterize the antibody response to pathogens which are involved not only in MSKI but also other infectious diseases. Sellman et al. identified and recombinantly expressed 27 immunogenic proteins of *S. epidermidis* as vaccine candidates [16]. Of which, only 3 were nonspecifically associated with the staphylococcal cell surface suggesting a species-specific immune response to *S. epidermidis.* Interestingly, in an old case report of intervertebral candidiosis from the year 1990, anti-*C. albicans* antibodies were found in high titers in the earlier stage of the infection [17].

It should be noted that MSKIs is associated with a significantly subdued immune response. For instance, Bansal et al. showed that serum immunoglobulins from patients with chronic osteomyelitis failed to rise above the normal (healthy) level in the presence of infection [18]. Further, pathogen-specific antibodies are often detected in non-infected patients [19]. In fact, some individuals are more susceptible to *S. aureus* PJI than others likely due to the protective

Back to top

vs. susceptible nature of their immune proteome [20]. Therefore, it appears that the well-documented presence of antibodies in response to MSKI is by itself not sufficient to ensure microbial clearance, probably because of immune evasion mechanisms [21]. Ultimately, understanding the pathogen-specific immune response in MSKI can be a useful tool for both diagnostic and therapeutic purposes [19].

In conclusion, given that (1) pathogen-specific antibody profiles can be used to diagnose MSKI causative pathogens, (2) the antibody response can be influenced by pathogen strain, and (3) pathogens beyond *S. aureus* elicit a specific antibody-response in infections other than MSKI, we conclude that the host antibody response to MSKI is highly pathogen-specific. Further high-level studies are needed to directly outline the pathogen-specificity in the antibody response to MSKI.

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Back to top

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Back to top

QUESTION 15

Are certain species more susceptible and resistant to MSKI based on host immunity than others?

Elysia Masters & Dina Raafat

Response/Recommendation: Yes

Based on host immunity, we can assume that certain host species are more susceptible and resistant to MSKI than others. Specifically, larger animals such as pigs, sheep and goats tend to replicate human disease more accurately than smaller animals such as rodents and rabbits. However, there is limited high-level evidence of this, and more studies must be performed.

Strength of Recommendation: Moderate

Rationale

A search of the English language literature related to the question was conducted using PubMed and Google Scholar, during the period January 1950 - August 2022. Search words included: osteomyelitis, species, susceptibility, host immunity, musculoskeletal infections. Authors found no controlled studies related to this question. Given the limited literature directed comparing host species susceptibility to MSKI, authors performed a structured review focusing on the differences in MSKI prevalence and host immunity across animals. After review, selection, and inclusion of relevant articles, 41 were included in this response.

Since it has proved difficult to translate vaccination success from mouse models to humans – it stands to reason that the host response to infections varies. While rodents are invaluable for research MSKI research of many immunological and pathogenic mechanisms, it is understood that they are not a perfect model for human infection. The characterization inflammatory genes during prosthetic joint infections and implant-associated osteomyelitis has been limited to rodent models [1]. However, the immune system of mice differs greatly from humans in several aspects. Firstly, the rat and mouse are known to possess a strong immune system that can at times complicate infection models [2]. Relative to humans, mice have been proven to be highly resistant to inflammatory challenge, where a lethal dose of endotoxin in mice is about 1,000,000-fold more than what is reported to cause lethal shock in humans [3]. Second, no correlation was found between the genes regulated in inflammatory conditions in humans compared to orthologue genes in mice [1] and human genes IL-26, CXCL8 and CXCR2 have no homolog in mice [4, 5], thereby complicating comparisons between murine and human immune responses.

In order to induce a reproducible MSKI, murine and rat models typically involve a fracture, drill hole or implant alongside pathogen inoculation [2, 6-10]. Together, these studies suggest that mice and rats are more resistant to MSKI than humans in experimental models. Notably, studies have shown that inbred strains of mice show varying immune responses to inflammatory stimuli and infection, making some strains more susceptible (ex: BALB/c) and others more resistant (ex: C57BL/6) to infection [11].

Like mice, rabbits are oftentimes used in animal models of MSKI. Rabbits are an attractive choice for infectious disease models because they are more similar to humans in their sensitivity to LPS, unlike mice [12]. However, rabbits are often characterized as being hyperimmune and thereby more resistant to infection. Rabbits with experimental osteomyelitis

Back to top

show elevated levels of anti-teichoic acid and peptidoglycan IgGs in 72% and 28% of serum samples, respectively [13]. On the other hand, humans with confirmed *S. aureus* osteomyelitis showed elevated of anti-teichoic acid and peptidoglycan in 44% and 3% of serum samples, respectively [13]. This study suggests the rabbit elicits more of a humoral immune response than humans.

As a result, a sclerosing agent is oftentimes required at the time of bacterial inoculation in order to induce osteomyelitis [14]. The addition of the sclerosing agent de-vascularizes the site of infection, thereby limiting the host immune response and encouraging bacterial proliferation. Alternatively a fracture or drill hole is created in the bone at the time of inoculation [15]. Together, these results suggest that rabbits show increased humoral immune response to MSKI and may be more resistant to infection than humans.

The use of porcine models of infectious diseases are becoming increasingly popular, as the porcine immune system is the third best characterize after human and murine systems [16] and tends to resembles humans more than that of rodents [17]. Specifically, the porcine immune system shares 80% similarity to humans in function and structural parameters, while mice share less than 10% similarity to humans [17]. Studies have shown that porcine experimental models of infection are more predictive of therapeutic efficacy in humans than rodents [18]. Pigs have also been used as models for hematogenous osteomyelitis [19]. Taken together, pigs might be more similar to humans in their susceptibility to MSKI based on host immunity.

Similar to small animal models, large animals such as sheep or goats are often administered a sclerosing agent or a drill hole is created at the time of infection. However, such large animals are also frequently administered prophylactic doses of antibiotics 1 hour after surgery to prevent fatal sepsis [20, 21], suggesting their increased susceptibility to fatal infection compared to rodents, rabbits and pigs. It is also important to consider previous exposure as a factor affecting species susceptibility to MSKI. Especially in experimental conditions, most small animals are bred and raised in pathogen-free conditions. On the other hand, larger animal studies are not always in pathogen-free conditions and have been previously exposed to common pathogens [16], such as *S. aureus*, thereby eliciting a conditioned immune response and possibly influencing susceptibility to infection.

Finally, an important factor that must be considered when investigating animal susceptibility to MSKI and infections in general is the pathogen strain selected for inoculation. It is well known that pathogens evolve specifically for infection in a particular host and in experimental conditions. While in many cases using human-adapted pathogens is acceptable in animal models, the animal immune susceptibility is drastically different than compared to a murine, rabbit or pig adapted pathogen. For example, Panton-Valentine Leukocidin (PVL), a cytotoxin produced by *S. aureus* that causes leukocyte death and tissue necrosis, has been shown to have high species specificity. Niemann et al. demonstrated that PVL targets human and rabbit neutrophils but does not affect neutrophils from mice or from Java monkeys [22].

Outside of experimental conditions, several species are commonly affected by MSKIs. Livestock animals are frequently afflicted with various forms of osteomyelitis including: bacterial chondronecrosis with osteomyelitis in chickens [23]., turkey osteomyelitis complex [24]., hematogenous, and vertebral and mandibular osteomyelitis in cattle [25-27]. And in the wild, many different species are afflicted with MSKI including: turtles [28], snakes [29], lemurs

Back to top

[30], anteaters [31], seals [32], dolphins [33], foals [34], red deer [35], sheep [36, 37], dogs and cats [38]. It is apparent that many species are susceptible to MSKI, however very little is known on the differences in host immune responses to MSKI. In veterinary medicine, prosthetic joint and implant-related infections are oftentimes reported in dogs [39]. Like humans, osteomyelitis is usually induced by tissue lesions, fracture and reduced host defenses [27]. Therefore, it can only be assumed that many species are similarly susceptible to MSKI, without knowing their specific immune responses to infection.

Given that (1) different species immune responses vary in their similarity to humans and (2) different species require sclerosing agents and/or antibiotic therapies to replicate human disease, we conclude that there are some species more susceptible/resistant to MSKI than others. Further high-level studies supporting are needed to provide direct evidence of host species susceptibility/resistance based on host immunity to MSKI.

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Back to top

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Back to top

02. Treatment

Back to top

QUESTION 16

Is there a standard timeline or duration of treatment to best evaluate resolution of infection in animal models of MSKI?

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Response/Recommendation: No

Timelines for studying infection resolution in animal models of osteomyelitis, fracture-related infection, and PJI are highly dependent on the model, i.e. infectious agent, the extent of bone infection, anatomical location, and the species of animal used. Even in human clinical scenarios, these timelines are highly variable from patient to patient under similar circumstances. Currently, there is no definitive evidence to indicate the optimal timelines of infection treatment for *in vivo* models of osteomyelitis, facture-related infection, and PJI. We recommend using existing clinical paradigms to guide model selection and study timelines. This is guided by four considerations: clinical scenario being modelled, inoculum type, bone remodelling/response, and current recommended practices.

Strength of Recommendation: Moderate

Rationale

Animal models are critical to the development of devices and therapeutics for treating orthopaedic-related infections.[1] Yet there is currently no single *in vivo* model that defines an optimal timeline to study infection resolution of osteomyelitis, fracture-related infection (FRI), or peri-prosthetic joint infection (PJI). Similarly, timelines of clinical paradigms remain unclear; multiple researchers have highlighted the absence of high-quality investigations into optimal timelines for antibiotic treatments.[2,3] Guidelines exist to navigate antimicrobial prophylaxis, but no consensus exists on the appropriate course of action after infection develops.[4]

Systemic antibiotic therapy is the typical clinical response to acute osteomyelitis. Treatment of chronic osteomyelitis is less defined, with recent publications failing to support long-duration antibiotic therapy,[5] despite research identifying a typical antibiotic treatment course with a mean of 90 days.[6] In cases of acute bone infections, recent analyses point to the clinical practice of antibiotic therapy anywhere from four to six weeks or, in some cases, up to twelve weeks.[7-10] Clinical corroboration waivers and the literature consensus indicates six weeks as the standard treatment for osteomyelitis. This result is corroborated by Roblot et al., who suggested that antibiotic therapy for treating vertebral osteomyelitis may be safely shortened to six weeks without risking relapse.[11] Similar research demonstrated that six weeks of antibiotic treatment is non-inferior to twelve weeks.[12] Benkabouche et al. found no statistically significant difference in patients' microbiological remission rates when looking at four versus six weeks of systemic therapy after infected osteoarticular implant removal,[3] which was corroborated in other studies.[8,12] The uncertainty of systemic antibiotic treatment duration complicates optimal timeline selection for *in vivo* research and points to the need to customize model development for specific cases. Arriving at the optimal antibiotic treatment is further complicated by uncertainty regarding the route of administration. Current clinical recommendations suggest daily parental antibiotics, most often delivered intravenously (IV).[13] Yet emerging evidence suggests oral antibiotics are non-inferior to IV antibiotics.[14]

While the timeline recommendations of systemic treatment are generally similar, local antibiotic therapy may influence the optimal timeline by delivering therapeutic payload directly to the nidus of infection.[15] Local antibiotic therapies are increasingly prevalent in orthopaedic infection treatment. These interventions are well-documented, with low levels of antibiotics documented in serum and overall low toxicity.[16] The earliest treatment modalities, such as polymethylmethacrylate (PMMA) beads, require a second surgical intervention to remove the treatment, potentially increasing optimal timelines.[15] No clear empirical research dictates the proper course and treatment regimen for post-explant surgeries, with most recommendations being guided by expert opinion or consortia of infectious disease experts such as recommended by the IDSA.[3,17,18] Newer methods, like antibiotic-loaded calcium sulfate beads, are degradable *in vivo* and do not require a second intervention. In these cases, timelines may need to be shortened or paired with suppressive antibiotics for the study duration to reflect the direct therapeutic action of these treatments. Studies combining an investigation of systemic and local therapeutics may further affect treatment duration and require comparison to delineate effect.

The nature of contamination and injury are also critical to appropriate model development.[19,20] For example, in the case of open fractures, while most are contaminated with bacteria at the point of injury, a smaller percentage progress to infection.[21] The risk of infection is highly correlated with the extent of soft tissue injury in the fracture site.[15] In the model design, therefore, the timeline may be altered by soft tissue injury, if any. Activities such as periosteal stripping may affect the ability of the infection to take hold, the rate at which it does so, and, consequently, the remodelling rate of the associated bone.

Similarly, the method of initial inoculation can impact timeline to infection.[20] Free-floating, planktonic bacteria are primarily used as initial inocula in animal models of osteomyelitis, facture-related infection, and PJI. Planktonic inocula can rapidly develop raging infections,[20,22] failing to mimic chronic and difficult-to-treat scenarios. It is well established that bacteria in natural ecosystems dwell in the biofilm phenotype.[23] *In vivo* models of infection, then, can benefit from the use of biofilms as initial inocula,[19,20] which may influence infection timelines.[24,25] Models using biofilms as initial inocula are sparse yet may allow for lengthier longitudinal studies, shorten optimal timelines by reducing the 'incubation' period, provide a model with immediate recalcitrance to antibiotic therapies, and more closely model contamination events and clinical paradigms of infection.[20]

The end of an antibiotic therapy window is not necessarily the sole endpoint of investigation within a timeline of study. Critical areas of inquiry after the cessation of treatment include bone healing and injury site/soft tissue recovery, which almost certainly extend beyond the end of treatment. Bone response is highly dependent on the model selected. Rats, rabbits, pigs, sheep, and goats are all commonly used species in orthopedic research. Yet the bone remodeling rate between these species may be highly variable, and difficulty may arise in translating the results in some of these models to the human condition. Bone turnover is also affected by age, the health of the animal, and the type/location of bone.[26] In therapeutic studies investigating bone healing, it is essential to consider the implications of different treatments on bone remodeling and the individual species' specific characteristics.[24,27-29] From there, more informed decisions toward an optimal timeline may be made. Consider that osteomyelitis is a bone defect which needs to heal even if all bacteria are eradicated.

Selecting improper timelines may adversely influence research outcomes. Timelines that do not allow a therapeutic to run its indicated course risk overlooking solutions that require longer time points to exert their intended effect. Further, shorter investigations may not fully capture bone healing, which may extend several weeks past the cessation of treatment. Studies that extend beyond the optimum are also more costly, can potentially endanger an animal, or may

fail to identify the point at which infection is cleared. Choosing the ideal timeline is not straightforward and no single timeline will answer all questions. Clinical factors should weigh into the decision for what is 'optimal' and thus guide the model to be as close as possible to the clinical paradigm in question.

In summary, no definitive guidelines dictate the optimal timeline for studying infection *in vivo* and no single timeline covers all orthopedic infection research. Proper timeline selection may be particular to the clinical question trying to be answered, which should influence the specific animal model, inocula type, mode of therapeutic treatment, and study endpoints at a minimum. Models and studies should be designed in such a way that they replicate the clinical scenario as closely as possible. For example, if the rate of bone remodeling is a primary outcome measure, sheep may be a more relevant model than a rodent as sheep have bone remodeling rates that are more similar to humans;[30-32] or, if a traumatic injury with soil contamination is to be replicated, using biofilms as initial inocula may be more relevant than planktonic bacteria.[20] A holistic approach must be taken in selecting a justified timeline that incorporates the various elements of infection progression. Fortunately, a large body of existing research, documenting *in vivo* treatments, exists for orthopedic-related infections. These studies can be used as a guide in circumstances where the species model and endpoints require delineation.

In conclusion, clinical paradigms should guide timeline and model selection with microbiological improvement (bioburden reduction) and bone healing being two crucial outcome measures.

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Back to top

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Back to top

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Back to top

QUESTION 17

Does radiography score correlate to infection and treatment efficacy for bone?

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RECOMMENDATION: Yes

Radiographic scoring strongly correlates to bone infection when X-ray imaging is performed 1-2 weeks or greater following infection. Radiographic scoring also strongly correlates to treatment efficacy for bone infection when X-ray imaging is performed following substantial differences in infection burden between groups over a duration of approximately 3 weeks or greater. Limitations in radiographic scoring include inability to detect differences in early infection and quantify early treatment efficacy. Limitations in current literature assessment includes variability in radiographic scoring methodology and animal model used.

STRENGTH OF RECOMMENDATION: Moderate

RATIONALE

A conventional/plain radiograph/X-ray provides a 2-dimensional image of internal structures of the body [1]. Computed-tomography (CT) utilizes specialized X-ray techniques to create 3-dimensional or cross-sectional images and is often categorized separately from conventional/plain radiography [2]. For this discussion, radiography will be defined as conventional/plain radiographs/X-ray.

Radiography is utilized clinically for diagnosing and monitoring infection of the bone and can be utilized as a marker of treatment efficacy. Clinical radiographic abnormalities associated with osteomyelitis include: i) soft tissue swelling; ii) periosteal reaction; iii) focal cortical and trabecular lysis; iv) bone necrosis/sequestration; and v) sinus tracts [3]. In the context of infected orthopedic implants, other specific clinical features may be present, such as: i) bone loss around the bone-implant interface and ii) implant loosening [4]. In the context of pre-clinical animal studies, scoring criteria for radiographic features have been devised to evaluate and quantify responses to infection and/or treatment efficacy for bone. Radiographical scoring is advantageous for preclinical models of infection because it provides a minimally invasive longitudinal measure to compare experimental groups over time, with equipment that is more readily available to most facilities than other specialized equipment such as microCT or in vivo fluorescence or luminescence imaging. Challenges with using radiographs to evaluate bone infection are that changes may initially be subtle, and obvious findings of bone infection may take up to a week or longer to be present [3, 4]. Further, after antimicrobial therapy, signs of healing may be delayed [5].

For the purpose of this question, we focused our search on animal models of infection related to bone. We evaluated whether subjective scoring of radiographs providing quantitative results correlates to infection and/or treatment efficacy for bone. Studies compared infected vs. non-infected groups or infected treatment groups vs. infected control groups. Studies that did not investigate antimicrobial agents, such as those investigating bone anabolic agents, were excluded. Furthermore, studies that did not provide a quantitative radiographic score were excluded. Our primary search included: 1) Pubmed search with the following keywords:

"radiography AND score AND bacteria AND orthopedic"; filter used was "other animal"; 2) Scopus search with the following keywords: "radiography AND scoring AND bacteria AND orthopedic"; filter used was "Non-human". We acquired a total of 100 results, and 29 manuscripts met criteria for further review. Animal models of bone infection included osteomyelitis alone, fracture-related infection, and implant associated infection. The most common animals used included rabbit, rat, and mouse.

In a rat model with infected femoral fracture vs non-infected fracture, radiographic scoring was performed using a modified criteria originally provided by Lane and Sandhu [6, 7].This scoring criteria is based on degree of fracture healing, with the lowest score of 0 (no fracture callus present) and highest score of 4 (complete bony union) [6, 7]. Radiographic scoring using Lane and Sandhu criteria demonstrated significantly worsened healing (lower score) in the infected group at 6 weeks post fracture and infection [6]. A limitation of this study is that it evaluated radiographic scoring for fracture healing rather than specific infection related pathology [6]. In a study of fracture-related infection in the rat femur, Robinson et al. used a 4-point scale for radiographic scoring developed by Lucke et al. and found that animals treated with antibiotic had decreases in severity scores that correlated with reductions of other markers of infection [8, 9].

The most common animal models of bone infection have been models of osteomyelitis without usage of an implant or creation of fracture [10-19]. Two of the most common radiographic scoring methods were scoring criteria provided by Norden et al. or Rissing et al. [10-16]. As outlined by Norden et al, radiographic scoring of osteomyelitis can be determined by: i) sequestrum formation, ii) presence of periosteal new bone, iii) presence of bone destruction, and iv) the extent of involvement. In Rissing et al, scores are based on degree of the following: i) raised periosteum, ii) destruction of architecture; iii) widening of shaft; and iv) new bone formation [16]. Smeltzer et al. used radiographic scoring to characterize a rabbit model of osteomyelitis and to correlate other infection outcome measures to dose and strain of *S. aureus* inoculation [17]; similar radiographic scoring criteria have been used to investigate virulence factors and treatment efficacy [18, 19]. In the Norden et al, Rissing et al, and Smeltzer et al scoring methods and associated modifications in following manuscripts, a cumulative score is provided for the combined indices [10-19]. In summary, in our review of animal models of osteomyelitis, radiographic scoring correlated to infection (vs. no infection) between week 1-4 [15-18]; furthemore, radiographic scoring correlated to antimicrobial treatment efficacy between 3 and 6 weeks following treatment [10-14, 19].

Animal models of periprosthetic joint infection (PJI) and/or implant-associated infection[9, 20-27]commonly used scoring methods modified from Norden et al and Rissing et al [15, 16, 21, 23]. In addition, implant models with infection location in or in communication with diaphyseal region of the bone commonly utilized scoring criteria provided by An and Friedman [9, 20, 22, 24], a modification of Norden et al. Original criteria included by An and Friedman, 1998 include the following scoring: i) diaphyseal periosteal reaction, ii) osteolysis; iii) sequestrum formation; iv) joint effusion; and v) soft tissue swelling [28]. In summary, in animal models of PJI and/or bone-implant associated infection, radiographic scoring correlated to infection (vs. no infection) between 2-6 weeks post infection [20-2225]; furthermore, radiographic scoring correlated to antimicrobial treatment efficacy between 3 and 6 weeks following treatment [23, 24, 26, 27].

Limitations:

While there are often-used methodologies for radiographic scoring, they are repeatedly modified and there is no standard technique. Scoring methodologies routinely use cumulative scores across parameters and seldom report individual scoring criteria, making it unclear how individually scored radiographic features contribute to the cumulative score. Rissing et al. classically described that of their 4 defined radiographic features of osteomyelitis, there was a

Back to top

65% chance of finding any 2 radiographic findings as compared to 11% in the non-infected surgical control group [16]. Norden et al. found a greater proportion of incidence of any radiographic variable the longer the duration of infection and found the least obvious radiographic feature was periosteal new bone formation [15]. Kraft et al found that the probability of individual scoring differences such as implant loosening, peri-implant reaction, and soft-tissue swelling were directly proportional to bacterial burden at the infection site, with lower probability for low colony forming units (CFUs) (0-11%) and high probability with higher CFUs (up to 100%) [21]. Harrison et al. used scoring methods from Smeltzer et al. to show that lower composite scores correlated with treatment efficacy, and in particular scores for soft tissue deformation, bone shaft widening, and periosteal elevation [26].

As discussed in known limitations of clinical radiography, evidence of infection can present more at a week or longer post-infection [3, 4]. Similarly in all animal models of infection, radiographic scoring was not performed until 1-6 weeks and more commonly 3-6 weeks post infection or treatment. Therefore, radiograph is limited in capturing early infection and effective treatment early in the time course. Another limitation of using radiographic scoring as a measure of infection status is that materials or therapeutics that cause inflammation and lysis of the bone could lead to confounding results. In a study by Croes et al., silver-releasing coatings caused cytotoxicity and impaired neutrophil function in a rat implant associated infection model, which correlated to an increased radiographic score on a 4 point scale [27]. Odekerken et al. observed early artifacts from the surgical procedure in non-infected controls [25].

The majority of manuscripts used blinded scoring; however, a number of manuscripts do not describe blinded scoring. A number of studies report only one scorer. Numerous manuscripts discuss using an experienced scorer, such as a trained physician; however, some do not report experience of the scorers. A study by Aktekin et al. compared different radiographic scoring scales reported by Mader et al., Lucke et al. and An and Friedman, as well as inter-rater reliability, and found good agreement between raters (kappa values greater than 0.84 for all individual measures) and recommended that individual measures of radiological criteria are superior to generalized composite scores [9, 28-30]. Other studies using modified scales found that inter-rater reliability was fair with a kappa score of 0.489 [31]. Having multiple blinded observers and evaluating inter-rater reliability would strengthen confidence in radiographic scoring, overcoming nuances and biases in interpretation.

Summary

Prior radiographic scoring methods have been developed to effectively provide correlation between

radiographic scoring and infection; these methods include but are not limited to those provided by Norden et al, Rissing et al., An and Friedman, and Smeltzer et al. These radiographic scoring methods have been further validated and optimized to suit a wide range of infection models including focus on osteomyelitis alone, fracture-related infection, as well as PJI and/or bone-implant associated infection. While these scoring methods provide a strong correlation to infection; there are limitations to using radiographic scoring assessments: including i) requirement of substantial infection burden, location, duration; ii) type of infection; ii) variation in scoring criteria and animal model used; iii) methodology of the scoring and experience of the scorer.

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Back to top

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Back to top

QUESTION 18

Are there any imaging techniques available that can effectively indicate the degree of infection or monitor the advancement of the disease in animal models of MSKI?

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RECOMMENDATION: Yes

There are imaging techniques available that can effectively indicate the degree of infection or monitor the advancement of the disease in animal models of MSKI. X-ray can provide basic assessment of trabecular and cortical bone structure changes over longitudinal assessment during the development of MSKI. Micro-computed tomography (CT) is valuable for revealing microarchitecture of periosteal reaction, medullary low-attenuation areas, trabecular coarsening, and focal cortical erosions with high precision in small animal models. However, both X-ray and micro-CT are limited in detecting features of disease progression often days to a week after the initiation of infection and treatment. Magnetic resonance imaging (MRI) is more sensitive to the presence of soft-tissue abnormalities and acute osteomyelitis than X-ray and CT, due to its in-depth determination of tissue swelling or fluid formation, formation of ducti, evaluation of soft tissue involvement in infection, and assessment of angiogenesis. Positron emission tomography (PET)/CT shows promise in detecting infection boundaries and likely has the potential to define the scope of lesions more accurately. Additionally, PET/CT can calculate blood flow through a specific area of interest in real-time. Finally, some novel imaging techniques, such as nano-CT, are expected to improve the detection of MKSI advancement in *in vivo* models.

STRENGTH OF RECOMMENDATION: Strong

RATIONALE

Early and accurate detection of bone infection is essential if appropriate therapy is to be started before osteolytic destruction occurrs[1]. Currently, non-invasive *in vivo* imaging techniques such as conventional radiography, CT, MRI and PET have been employed to visualize infections over time.

There is a rich literature reporting the current imaging techniques to follow disease progression in animal models of OM, FRI and PJI. To specifically answer this question, a PubMed search was performed on July 29, 2022, using the keywords “bone infection” AND “*S. aureus*” AND “imaging”. We also performed PubMed searches with the keywords “animal model” AND “imaging” AND “bone infection”. Abstracts of records were screened based on the inclusion of: 1) the use of *in vivo* animal models 2) the use of bone infection 3) the evaluation of imaging techniques. Exclusion criteria included: 1) no *in vivo* imaging; 2) not osteomyelitis, fracture related infection, or PJI; 3) no full text available; and 4) review articles. To answer the question stated above, a total number of 94 records were analyzed and a qualitative review was performed.

Several imaging modalities can be applied to gather insight in the local morphology of orthopedic infections. This regards two questions: whether an infection is present and where it is located[2]. Typical findings of X-ray are nonspecific periosteal reactions, bone destruction and osteolysis; however, these features are often not clearly obvious until several weeks have passed since the infection has formed, and this technique has low sensitivity and specificity. It is well-known that CT can provide three-dimensional (3D) images to reveal bone density and micro-architecture. Features of bacterial osteomyelitis with CT images include overlying soft-tissue swelling, periosteal reaction, medullary low-attenuation areas or trabecular coarsening, and focal cortical erosions[3]; however, these features are of more value in the later stages of the infection once significant structural changes are obvious. A limitation of CT imaging for the diagnosis of infection may be that there is no known specific feature that distinguishes early-stage bone infection at a time early enough to impact early treatment. As a result, X-ray and CT only describe bone morphological changes as a result of an infection; they do not provide direct insight on the activity or the progression of an infection. Additionally, in the evaluation of a potential musculoskeletal infection, CT is invaluable for detecting deep complications of cellulitis and pinpointing the anatomic compartment that is involved in an infection[4]. On the other hand, the application of CT is limited to the large animal models. Fortunately, micro-CT has had a considerable success for the investigation of trabecular and cortical bone micro-architecture in small laboratory animals with bone infection. Currently, by combining micro-CT images from subsequent time points, it is possible to compute histomorphometric indices, such as bone formation and resorption rates, noninvasively[5]. Recently, some authors noted that time-lapsed micro-CT—the combination of spatial and temporal CT data—is a potent imaging method for increasing the sensitivity for small bone changes and, therefore, may enable the detection of signs related to potential infections earlier than “static” methods[6]. Specifically, bone-implant contact, bone fraction, and bone changes (quiescent, resorbed, and new bone) could be calculated from consecutive scans and validated against histomorphometry[5]. Nanoscale CT (nano-CT) is engineered to be versatile enough to be able to scan at ultra-high resolution[7]; it generally uses a nano focal spot source (<400 nm). Recent applications of nano-CT for the analysis of osteocyte lacunae and the lacunar-canalicular network were found. Nano-CT can provide reliable and innovative information on bone nano-porosities, bone mineral, and extra cellular matrix at the cellular scale, which may be crucial to learning about the pathophysiological properties of bone tissue and more generally to gain a better understanding of bone mechanical properties[7, 8]. Even though there are fewer publications about its application on musculoskeletal infection models, we believe it can provide new insights into detecting the bacteria colonized within osteocyte lacuno-canalicular network (OLCN) of live bone.

Based on its high spatial resolution and excellent soft tissue contrast, MRI is a versatile method to image angiogenesis and inflammatory processes during bacterial infections non-invasively. Unlike micro-CT rely on lead-based contrast perfusion to evaluate the vasculature structure on a sacrificed animal model, MRI could perform a live scan and is more sensitive to the presence of soft-tissue abnormalities and acute osteomyelitis than CT, which are mainly manifested as low signal on T1WI, high signal on T2WI, high signal of fat suppression sequence, and enhanced scanning[9]. Both image acquisition in micro-CT and MRI are in dicom format which allows image post-processing for quantitative 3D reconstruction and histogram analysis. However, disadvantages of MRI include its occasional inability to distinguish infectious from reactive inflammation, and it is more time consuming and more expensive than CT in the diagnosis of musculoskeletal infection. Interestingly, some scholars demonstrated that labelling *S. aureus* with iron oxide particles and detecting *S. aureus* colonies by MRI in infection models provides a feasible and versatile tool to follow bacterial infections. The established cell labeling strategy can be transferred to other bacterial species and provides a conceptual advance in the field of molecular MRI[10].

Back to top

Unfortunately, CT and MRI are occasionally limited by metal hardware-induced artifacts. Functional imaging modalities have been extensively studied and applied for the diagnosis of periprosthetic joint infection. Some authors noted that Fluorine-18-fluoro-2-deoxy-D-glucose positron emission tomography combined with computed tomography (^{18}F-FDG PET/CT) provides cutting-edge functional imaging, as it provides images with higher resolution with concomitant anatomical information[11]. It is a sensitive and specific tool in the diagnosis of experimental foreign-body osteomyelitis[3] and in monitoring the therapeutic response of systemic antibacterial treatment of experimental *S. aureus* osteomyelitis after removal of a foreign body[11, 12]. It is even useful to determine when an antimicrobial technology is failing by monitoring longitudinal disease progression[13, 14]. Recently, ^{68}Ga-fibroblast activation protein (FAP) inhibitor (FAPI) has emerged as another promising radiopharmaceutical in recent years[15] and has been addressed by an increasing number of scholars in the field of bone inflammation[16]. The mechanism of ^{68}Ga-FAPI is different from ^{18}F-FDG, and the property of ^{68}Ga-FAPI showed promising prospects in detecting infection boundaries and likely had the potential to define the scope of lesions more accurately[15].

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Back to top

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Back to top

QUESTION 19

Are In Vivo Imaging Systems (IVIS) Using Fluorescence or Luminescence Complementary to Other Methods Such as Culture or PCR?

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RECOMMENDATION: Yes

We conclude that IVIS provides complementary information on the bacterial burden to culture and PCR, and its usage is particularly valuable in small animal models for in vivo longitudinal assessments. Limitations of IVIS, compared to CFU analysis or PCR, include less reliable detection and quantification of infection at low bacterial concentrations and infections localized deep underneath tissue. Furthermore, since the production of bioluminescent signals relies on metabolically active bacteria, data obtained by IVIS are less reliable when metabolic activity is decreased (such as when biofilm is present). This should be taken into consideration in the design of musculoskeletal infection studies.

STRENGTH OF RECOMMENDATION: Strong

RATIONALE

In vivo imaging systems (IVIS) have become widely used in animal models of musculoskeletal infection. In vivo bioluminescent imaging (BLI) detects light signals produced by luciferase enzymes. Several bacterial species have been genetically modified to express luciferase enzymes, allowing longitudinal monitoring of metabolically active bacteria in anesthetized animals. The most common bioluminescent system used in infectious diseases research involves the luxCDABE operon derived from the bacterial insect pathogen *Photorhabdus luminescens* [1, 2]. If the lux operon is inserted into a bacterial plasmid or the bacterial chromosome, bioluminescent light with a peak wavelength of 490 nm will be produced from live and actively metabolizing bacteria [1-3]. In vivo fluorescent imaging (FLI) utilizes the excitation of a fluorophore by an external light source and detection of the emitted light upon relaxation of the molecule [4]. Fluorescent proteins can be conjugated to many molecules to be expressed both by bacteria and mammalian cells. BLI and FLI can be applied simultaneously, for example to detect bioluminescent pathogens and fluorescently labelled host immune cells in the same animal.

Conventional methods to quantify bacterial burden include colony forming unit (CFU) analysis, where bacteria are detached from the harvested implants and surrounding tissue and plated on agar. Quantification of the live and culturable (proliferating, colony forming) bacteria is achieved by counting the individual colonies. Another method is the real time polymerase chain reaction (RT-PCR), which involves DNA isolation from the harvested tissues and biofilm and determination of bacteria-specific gene copies in the sample.

While both CFU analysis and the above-described RT-PCR requires terminal euthanasia of the experimental subject, the advantage of IVIS is that bacterial activity can be monitored directly, in a less invasive manner, significantly reducing the number of animals required. However, neither BLI nor FLI are without limitations. In animal models of infectious diseases, accurate determination of bacterial burden is crucial in order to study the pathogenesis and to determine the antimicrobial effect of potential interventions.

The aim of this review was to critically examine existing data and determine if IVIS using luminescence or fluorescence are complementary to other conventional methods of evaluating and quantifying infection, such as culture or PCR.

For the purpose of this question, we focused our literature search on animal models of musculoskeletal infection. We searched PubMed using the following combinations of MeSH terms and keywords: 1) “bioluminescence AND (bacterial load) AND orthopaedic”; 2) “bacteria AND (bioluminescence OR fluorescence) AND (CFU OR PCR) AND bone AND orthopaedic” using “other animal” filter. In addition, we searched Scopus using the following keywords: “bioluminescence AND (bacterial AND load) AND orthopaedic” using “medicine” filter. Abstracts of records were screened based on inclusion: 1) use of in vivo animal models 2) use of BLI or FLI and culture or PCR 3) musculoskeletal disease model and exclusion criteria: 1) no in vivo imaging 2) not musculoskeletal disease model 3) no full text available 4) review articles. Records included from each search have been reviewed in full text for data extraction. Between searches we had 108 results, of which 39 were used for data extraction and analysis.

We found that in vivo imaging systems using fluorescence or luminescence are complementary to other methods such as culture or PCR. IVIS provides supplementary, dynamic information about the course of infection compared to the detection of bacterial load by CFU analysis and PCR, being terminal data acquisition methods, usually performed only a few time points of the study. We conclude that utilization of IVIS is highly recommended as an additional method to longitudinally monitor musculoskeletal infection, with respect to its limitations. The main advantages of BLI and FLI are their less invasive character and rapid processing time. Since IVIS is performed on live, anesthetized animals, living animals can be tracked longitudinally for infection burden over time with multiple time points in the same animal; this can reduce sample size and associated cost when evaluating for multiple end-points related to time. The main limitation of IVIS stems from its different detection mechanism, i.e., bioluminescent and fluorescent light is emitted only by live, actively metabolizing bacteria, whereas CFU analysis accounts for the number of viable, culturable bacteria, and PCR quantifies the number of bacterial gene copies regardless of metabolic activity or culturability. This difference in detection method should be taken into account, especially at later (chronic) stages of implant-associated infections, when biofilm is formed. In addition, due to the limited penetration of light through tissues, the use of BLI and FLI is often restricted to studies with small animals and more superficial infections in relation to the skin surface. BLI signals are also influenced by environmental conditions such as temperature and oxygen.

Comparing BLI to CFU analysis, we found that in most of the records considered, there was a good correlation between bacterial burden indicated by bioluminescent intensity and quantified by CFU analysis[5-10]. However, discrepancy was observed in the following occasions: 1) Biofilm growth phase. Acute and peak infection can often be readily identified on IVIS imaging. At later stages of chronic infection, when host innate and in particular adaptive immune responses have been able to substantially diminish bacterial burden, BLI signals can return to baseline (or close to the level of detection) falsely indicating that infection has cleared even though remaining bacterial biofilm may be present. At later stages of infection, challenges in identifying primarily bacterial biofilm alone with IVIS include lower overall bacterial burden compared to peak infection, and bacteria residing in mature biofilms are often characterized by a low metabolic rate[11]. This can lead to misinterpretation of the data. CFU analysis performed at this stage on the implant or surrounding tissue often identifies viable bacteria not detected by BLI[12, 13]. Hence, BLI is more reliable to predict bacterial presence in the acute phase of infection, without providing accurate estimate of bacterial load in the later stage when biofilm formation occurs. 2) Pathogens with unstable luciferase gene expression. Incorporation of the bioluminescent gene construct into bacteria can happen through integration into unstable plasmids, stable plasmids, or integration into the bacterial chromosome. For studies using BLI, luciferase expression by stable plasmids or integration into the chromosome is recommended. One example of unstable luciferase expression is when the construct is integrated into plasmids maintained by antibiotic selection. In this case, luciferase gene expression might be lost during in vivo replication of the microorganism in the absence of antibiotic selection, leading to decreased or lost bioluminescent signal and underestimation of bacterial burden after the early phase of infection[14, 15]. 3) Induction with suboptimal inoculum concentration. In some cases, BLI was not able to detect significant difference between non-infection and infection initiated by a low number of inoculums, or between infections initiated by a high vs low number of inoculums[16, 17]. For optimal signal generation, the minimum CFU

Back to top

content of the inoculum should be carefully chosen. 4) Deep tissue infection. The detection of bioluminescent signal is highly influenced by tissue density. IVIS is limited by the ability of light to penetrate through the tissue, which restricts the reliable usage of this system to superficial infection in small animals with 1-2 cm infection depth. Another consideration related to the localization of infection is that bacterial metabolic activity is higher in the soft tissue compared to bone or to the surface of metallic implants. Hence, BLI correlates more with soft tissue infection as compared to bone infection. This makes some orthopaedic models less suitable for in vivo imaging, such as in the intramedullary pin-induced osteomyelitis model[18].

Determination of the copy number of bacterial genes is a sensitive method to estimate bacterial burden, but it cannot distinguish between metabolically active and dormant (such as in biofilm) bacteria. Contrary to CFU analysis, PCR is able to detect viable but non-culturable (not proliferating) bacteria. An example of discrepancy between BLI and RT-PCR outcome is reported by Li and colleagues [19], where the correlation between BLI and *nuc* gene copy determination by PCR was highly dependent on the time of sampling. They reported no correlation during concomitant planktonic and colonized bacterial growth, while a significant correlation was shown when bacteria were restricted to biofilm growth. In addition, PCR might underestimate real bacterial burden depending on the yield of DNA extraction from biofilm residing bacteria.

FLI was used in most records to monitor host immune responses by fluorescently labelled immune cells[5, 15]. One study reported by Shepard and colleagues[17] used fluorescent antibody targeting *S. aureus* in biofilm to detect low grade, chronic infection. In this study, FLI appeared to be a sensitive method to pick up signals of biofilm residing *S. aureus* and was able to detect low grade infection. On the other hand, like BLI, FLI is also limited by the penetration of signal through tissues, which is in the case of FLI, not more than a few millimeters. Furthermore, due to the bidirectional nature of light detection by excitation-emission, autofluorescence of host tissues with FLI represents an additional challenge. However, with recent advances in fluorescent detection methods [20, 21], larger future application of FLI can be expected in musculoskeletal infection research.

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Back to top

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Back to top

QUESTION 20

Can techniques be employed to precisely evaluate the formation of biofilms on implants or infected bone in in vivo models of osteomyelitis?

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RECOMMENDATION: Yes.

Biofilm formation in vivo differs structurally from in vitro biofilm, as the latter lacks the mushroom-like structure and is smaller in size[1]. In vivo models of implant-related bone infection demonstrate complex interactions between the host, the implants and microbes, leading to milieu bacterial adhesion.[2] Various techniques are available to assess the formation of biofilms on implants or infected bone *in vivo* models of osteomyelitis. These include microscopic analysis, biomolecular assays, bioluminescence imaging (BLI), X-ray imaging, and histological analysis. Microscopic analysis, such as scanning electron microscopy (SEM), field-emission scanning electron microscopy (FE-SEM) and confocal laser scanning microscopy (CLSM), allows for detailed visualization and characterization of biofilm structure and composition. Biomolecular assays, like enzyme-linked immunosorbent assays (ELISAs), enable the detection and quantification of specific molecules associated with biofilm formation. BLI provides real-time monitoring of biofilm formation using genetically engineered bacteria that emit light under specific conditions. X-ray imaging, specifically micro-CT, allows for visualization of biofilm distribution on implants or infected bone. The histological analysis enables visualization of infection extent and biofilm formation in surrounding tissue. The selection of technique(s) depends on the research question and the type of implant or infected bone being studied. Combining multiple techniques can provide a more comprehensive understanding of biofilm formation in vivo.[3-7]

STRENGTH OF RECOMMENDATION: Strong

RATIONALE: The literature search from the PubMed database based on the keywords "bone infection", "in vivo model", and "biofilm assess", and "methods" was performed on January 30, 2023.

Biofilm plays a critical role in the resistance of chronic osteomyelitis to antibiotic therapy by serving as a dominant protective barrier from the action of antibiotics, which can only be studied with in vivo models.[8] Currently, *S. aureus* is the most prevalent pathogen. Besides biofilms formation on the implants surface, Staphylococcal abscess communities (SACs) and invasion of the osteocyte lacuno-canalicular network (OLCN) are the other two main biofilms of existence within the infected bone. The main reasons which cause *S. aureus* osteomyelitis pathogenesis are considered to be incurable [9]. To date, numerous in vivo models including mice, rats, and rabbits have been well-established [10]. While rare these models contain quantitative endpoints that can determine bacterial load or growth of biofilm on implants or infected bone *in vivo*. The main roadblock to the development of a quantitative osteomyelitis model is the difficulty in extracting individual live bacteria, classically known as colony-forming

units (CFU) from infected bone. It is well-known that CFU assays from implants has been used to quantify biofilm bacterial burden *in vivo*[11]. Some authors mentioned that CFU levels remain constant during the in vivo phases of adhesion, proliferation, and early stasis[12]. However, this traditional and widely used method requires careful interpretation of data generated. BLI permits the noninvasive sequential monitoring of cell growth and gene expression *in vivo*, which has emerged as the only longitudinal *in vivo* biomarker of infection[6, 12], it was able to monitor the infectious processes throughout the course of the disease in both the acute and chronic phases without sacrificing the animals[13]. Real-time quantitative PCR (RTQ-PCR) is a highly specific and sensitive method that has been successfully used to quantify *S. aureus* levels in contaminated cheese[14]. Dr. Schwarz proved that the combination of RTQ-PCR for the detection of *S. aureus-specific nuc* gene with BLI can demonstrate the quantitative model of implant-associated osteomyelitis that defines the kinetics of microbial growth[6]. However, the extraction of *nuc* genes from infected tibiae becomes less efficient when the bacteria are residing in dense biofilm and the rather low *nuc* gene levels observed in latent infections (day 18) may be an underrepresentation of the actual bacterial load[6].

Additionally, some scholars also quantified the biofilm colonization via confocal laser scanning microscopy. Biofilm samples were tested for presence of the administered bacteria via PCR analysis[15, 16].

Scanning electron microscopy (SEM) have provided important insights into the observation of biofilm formation on implant surfaces, maturation, and accessory gene regulator-dependent bacterial emigration to perpetuate implant-associated *S. aureus* infection in animal models [12, 17]. Some scholars have utilized cross-sectional SEM studies to reveal that biofilms incorporate host components, including fibrin, and are approximately 0.1μm in diameter[12]. Interestingly, host cells, made up a large percentage of the biofilm volume. Surprisingly, the biofilm growth ceases at only 40% surface coverage, suggesting an inhibitory host interaction and biofilm growth may be dependent on bacterial replication and/or the kinetics of innate immune response.

One of the most notable discoveries is that the colonization of the OLCN [4] and SACs in cortical bone by S. aureus can be accessed via transmission electron microscopy (TEM) [5, 18]. Ren YL and colleagues also acquired images of the *S. aureus* autolysis morphology within OLCN by TEM. They provided the evidence of live bacteria *in vitro* and *in vivo,* presented as dense cocci of approximately 1 μm in diameter, as well as the morphology features of remnant cell walls of dead bacteria or "ghosts" and degenerating (non-dense) bacteria[5]. Therefore, we consider TEM the first-choice approach to assessing the morphology features of pathogens and biofilm within SACs and OLCN.

Additionally, based on recent publications, certain novel scientific strategies may be effective tools to assess biofilm formation on implants in *in vivo* models of osteomyelitis in the further. For instance, intravital two-photon microscopy has significantly advanced our understanding of dynamic processes within the immune system[19, 20]. For *in vivo* models, we are considering the use of multiphoton intravital microscopy of a fluorescent transgenic mouse infected with compatible fluorescent bacteria (i.e. RFP) as primary approach to assessing biofilm formation on implants. Although there are few publications on this approach, it is worth further exploration.

Magnetic resonance imaging (MRI) is a versatile method to non-invasively image inflammatory processes upon bacterial infections. Some scholars have described using iron oxide pre-labeled bacteria[21], and *in vivo* MRI detection of small *S. aureus* colonies in infection models as feasible, providing a versatile tool to follow bacterial infections *in vivo* without being subject to limited penetration depth[22, 23]. However, it's still unclear whether this method will be

Back to top

limited by hardware-induced artifacts for those with implant-associated osteomyelitis, so further testing and verification are necessary.

Following implant extraction from in vivo studies, molecular imaging techniques can be employed to obtain information on biofilm and its formation. These techniques can not only detect the presence of biofilm but also provide valuable molecular insights into its chemical and morphological properties. Various imaging methods such as Confocal Raman Microscopy (CRM), Confocal Laser Scanning Microscopy (CLSM), and mass spectrometry imaging (MSI) have been utilized to map the material distribution and micro-processes within the biofilm matrix.[24]

Raman imaging is a non-nondestructive, non-noninvasive, and label-free technique that can visualize the chemical composition of the biofilm with a resolution of 10^3 nm.[25] Surface-enhanced Raman scattering (SERS) is a technique based on Raman imaging that has been developed to enhance its sensitivity.[26] Confocal laser scanning microscopy (CLSM), on the other hand, utilizes a laser source and fluorescence microscopy to provide molecular information on the biofilm.[27] Mass spectrometry is an increasingly popular technique that visualizes metabolite distribution in the biofilm in two or three dimensions, offering valuable insights into biofilm and its formation. The literature suggests that the mass spectrometry methodology is continually optimized and fine-tuned to increase the molecular coverage that this technique offers.

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Back to top

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Back to top

QUESTION 21

Is there an animal model representative of DAIR (debridement, antibiotics, and implant retention)?

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RECOMMENDATION: Yes

There have been several *in vivo* murine and rabbit models for DAIR; however, the literature is limited when it comes to other animals used. Additional work is needed to develop a DAIR-relevant animal model and should include implant resemblance, inoculation technique, and time to DAIR intervention as considerations.

LEVEL OF EVIDENCE: Moderate

RATIONALE

With any surgery there is the potential for infection, one of the most dreaded complications for orthopaedic surgeons because of increased morbidity and surgical challenges [1]. Prosthetic joint infection (PJI) is a complication of arthroplasty that results in extended and expensive hospital stays [2]. Treatments for prosthetic joint infection (PJI) include debridement, antibiotics, and implant retention (DAIR), TKA revision, and amputation [1]. The gold standard treatment is two stage revision: the 1st stage involves debridement, surgical removal of the prothesis, and antibiotic-loaded spacers accompanied by systemic antibiotic therapy; the 2nd stage is new implant placement, which takes place six weeks post-therapy or once infection is cleared. Debridement and irrigation with implant retention (DAIR) begins with identification of the microorganism present in the joint followed by debridement surgery and replacement of removable implant parts such as polyethylene inserts [1]. Intravenous or oral antibiotics are given for six weeks, as determined by the type of pathogen and the patient's individual needs and restrictions [1]. Implant retention without re-infection is the ideal result of treatment for an infected total knee arthroplasty due to removal creating substantial increase in morbidity; however, in the case of established PJI, the success rate could be 28-62% and recurrent infection is common [1, 3]. However, in a 2014 study on the outcome of DAIR for gram-negative PJI, there was a 79% success rate [4]. Because of the wide variability in patient outcomes with DAIR, the use of the procedure is best supported when the following conditions are met: well-fixed implant; short time period between symptom onset and treatment (3–4 weeks; acute infection); patient is not immunocompromised; organism is not resistant to antibiotic being used; when possible, use of open arthrotomy and extensive debridement and irrigation; use of antibiotic regimens tailored to the specific organism; use of intravenous antibiotics for no longer than 6-8 weeks; extended oral antibiotic use, although life-long use has not been studied in

depth [5]. DAIR has mixed clinical results compared to one and two stage revisions and there is still no consensus on its benefit [6-9]. Animal models may be useful to assess DAIR efficacy, guide product and procedural development, and clinical decisions. There are a number of animal models that attempt to replicate a debridement and implant retention procedure, although they are limited in evaluation and have significant limitations relative to the clinical setting.

For the purpose of this question, we focused our search on animal models of infection related to bone. Studies compared infected vs. non-infected groups or infected treatment groups vs. infected control groups; both types of investigation were well represented in the literature. Our primary search included: 1) Scopus search with the following keywords: “DAIR AND PJI AND in vivo”; 2) Pubmed search with the following keywords: “Debridement antibiotics animal PJI”. We acquired a total of 38 results, and 18 manuscripts met criteria for further review. All manuscripts were primary articles.

Multiple PJI animal models exist and have been reviewed [10-13]. An ideal PJI animal model should be as clinically relevant to the human condition as possible. However, in 2019 the International Consensus on Orthopaedic Infections found a lack of ideal prosthesis PJI prosthetic design for animal models [14]. Indeed, delegates were unable to find any animal model in the literature wherein a fully replicated total knee or hip replacement device was utilized in a PJI or DAIR model. The challenge to mimic clinically relevant devices with complex surgical procedures in small animal models is understandable. Yet while such a complete design may be lacking, proof-of-concept and clinically relevant data can be obtained with infection progression and severity, inocula type, implant materials, anatomical position, and fit being carefully considered. For example, if the effect of DAIR and PJI in the context of osseointegration is to be studied, correct anatomical placement of hardware would be crucial [15].

Mice are commonly used in PJI models. Bernthal et al. developed a murine arthroplasty model with bioluminescent *S. aureus* [16]. They found *in vivo* luminescent signals to correlate with *ex vivo* tissue bioburden, which could be used to screen for appropriate timepoints to start DAIR intervention.

Another murine DAIR model was used to model PJI treatment with bacteriophage-derived lysin [17] in a PJI model developed by Carli et al.[17, 18]. Mice with Ti-6Al-4V implants were inoculated with Xen 36 S. *aureus* (10^4 CFU) [17] and infection was allowed to progress for five days before dividing into three groups (n=7): no intervention, irrigation and debridement w/ saline, and irrigation and debridement combined with the study compound [17]. This same murine model of DAIR was used in a follow-up study, combining the study compound with vancomycin [17]. Strengths of this model include the use of common biomaterials in clinical implants (titanium), placement of the implant in an anatomic location for prosthetic joint infection. Weaknesses include the lack of modular parts for exchange.

Other murine models look at the setting of a fracture-associated infection. In a model using plated femoral osteotomy,a single screw was inoculated with bacteria prior to insertion of a titanium four hole plate [19]. At seven days after implantation, an irrigation and debridement procedure was performed with removal of all implants and placement of two more screws outside the previously infected area [19]. While this model mimics the exchange of modular parts, it does not retain any existing implants so the chronic biofilm is removed, which may simulate more a two-stage procedure than a debridement and implant retention. A similar model also used plate fixation around an osteotomy site in a murine model [20, 21]. This model

Back to top

involved a repeat irrigation and debridement of soft tissue and periosteum at 7 and 14 days after implantation with no exchange of the original plate [20, 21]. This model may better mimic the clinical scenario where plate removal will destabilize the existing fracture and may be avoided.

Rats are also popular animals for PJI models. A murine model for PJI during implantation observed the progress of *S. aureus* infection over 4 weeks using multiple analysis methods in a study by Fan et al. [22]. Holes were drilled into the lateral femoral condyle and injected with *S. aureus* [22]. The tibial hole was then filled with a titanium screw, the wound was closed, and second injection of *S. aureus* was also administered into the joints [22]. This model suggested that postoperative recovery for PJI can be monitored by assessing gait, weight-bearing, and pain with touch [22]. However, a major limitation of this model is that the implants are not articulating and are not true joint replacement implants. SØe et al. developed a rat sized simulated knee replacement, which was one of the closest to a clinically relevant implant we found [23]. The prosthetic was composed of metal for the femoral component and high-density polyethylene for the tibial component. The authors found that inoculating the condylar holes prior to prosthetic placement produced reliable infections within the bone marrow, although the rats were able to clear infection by 2 weeks Other murine models look at the setting of a fracture-associated infection. In a model using plated femoral osteotomy,a single screw was inoculated with bacteria prior to insertion of a titanium four hole plate [24]. In this model, biofilm was grown on the head of the titanium femoral implant, which was non-lethal and resulted in infection in 100% of the animals. Further, this study used a realistic implant model with bone cement fixation. Others have shown that all materials, including PMMA bone cement, can harbor bacteria in clinical implants [25, 26]. Localized infection, increased inflammatory signals, and loosening identified radiologically were evident on the titanium implants and in surrounding tissue even though gentamicin loaded bone cement was used in all animals [24].

Rabbits provide easier surgical approaches than rodents and are more affordable than large animals [27]. In 2005, Craig et al. created a rabbit knee arthroplasty model by cementing a stainless-steel screw with a ultra-high molecular weight polyethylene (UHMWPE) washer in the femoral condyle; only when bacteria were inoculated intraarticularly at concentrations greater than 1×10^4 CFU did infection occur in 100% of animals [28].

A rabbit model was developed using a stainless-steel screw with an UHMWPE washer implanted into holes in the lateral condyles of knee joints and inoculated with *S. epidermidis* [29]. After 14 days, the knee joint was then irrigated by an intra-articular lavage device without direct exposure of the implant [29]. The limitations of this model include the lack of modular implant exchange and direct mechanical debridement of the joint cavity, which may be important factors in infection eradication. A major limitation of this model was the tradeoff between microbiological analysis and prosthetic loosening; the prosthetic must be carefully excised to evaluate bioburden.

A DAIR model using New-Zealand White (NZW) rabbits to compare vancomycin administration and DAIR to non-treated controls [30]. A DAIR model using New-Zealand White (NZW) rabbits to compare vancomycin administration and DAIR to non-treated controls [30]. “Day 0” consisted of tibial arthroplasty for all groups, where the infection and DAIR groups were infected with MRSA [30]. The DAIR group was given a buffer injection on Day 7 to represent surgical debridement, followed by vancomycin injections from Day 7 to Day 13 for vancomycin and DAIR groups with the endpoint of 14 days. The DAIR treatment group had significantly less CFU than the infection group on the implant and bone, but no animal was completely free

Back to top

of infection following DAIR [30]. This work was presented at ECCMID in 2021, and discussed that DAIR did not work well, but the authors proposed that the model may be promising for testing DAIR-related therapies [31].

Similarly, a different rabbit model was used for a study of biofilm quantification [32]. A DAIR model using New-Zealand White (NZW) rabbits to compare vancomycin administration and DAIR to non-treated controls [32]. After 2 weeks, the DAIR group received treatment of DAIR, with cefazolin given for the next 2 weeks [32]. After another 2 weeks, it was found that the DAIR treatment group had an average of 61% of the implant surface covered with biofilm, compared to 90% coverage for the sham treatment group [32]. A limitation of this model is that it does not involve a true metal-on-polyethylene articulation common in human total knee arthroplasty patients [32].

Another example of a DAIR rabbit model is in a study on the use of shockwave therapy in fracture-related infection [33] A mid-diaphyseal osteotomy of the rabbit humerus was created and fixed with a compression plate after *S. aureus* was inoculated in the screw holes [33]. Revision surgery was performed after 2 weeks, which included debridement, saline irrigation, and implant retention. Similar to the clinical scenario and other animal studies, none of the treatment strategies completely cleared bacterial burden in soft tissue, bone, or on the implant, when implants were retained.

Biofilm is increasingly being used as initial inocula in orthopaedic infection models [24, 34-38]. Using biofilms as initial inocula improves the robustness of the model and increases positive signals of infection, when compared with models wherein planktonic bacteria are used as initial inocula [39] . Further, injection of planktonic bacteria may increase systemic toxicity and form irregular biofilms *in vivo* [40]. Direct inoculation with biofilm can more confidently ensure infection severity at a given time point and may be ideal for DAIR model development as biofilms underpin PJI [24, 41-44]. Gristina and Costerton found as many at 76% of biomaterials to have surface biofilm colonization [45]. Biofilms used as initial inocula form clinically relevant infections [46]. Multiple large animal models that use biofilms as initial innocula have been developed [37, 47-51]. Developing DAIR-related animal models with biofilms as initial inocula could be adapted to standardize infection in PJI models.

Conclusions

In summary, much has been published on PJI and DAIR models in rodents and rabbits, however little information is available on DAIR models for other animals. Few PJI models contain implants materially similar to those used clinically. Further, the amount and strain of inoculation vastly differs between studies, which makes determination of an appropriate DAIR intervention timepoint difficult. A move towards biofilm grown *in vitro* prior to surgery may standardize *in vivo* infection signals and reduce risk of systemic toxicity or non-infection. Furthermore, biofilm inocula can reduce the waiting period between initial PJI surgery to DAIR intervention, which can reduce animal housing costs and ethical concerns. The inoculation amount and method, infection signal, time to intervention, material selection, and anatomical positioning should be influenced by the clinical paradigm in question.

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Back to top

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Back to top

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Back to top

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Back to top

QUESTION 22

Is there a single predominant combination of bacterial species in humans that should be studied in animal models of polymicrobial MSKI?

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RECOMMENDATION: No

There is no particular combination of pathogens predominant in polymicrobial infections. Animal models in which polymicrobial infections are studied should not be limited to a particular pair of pathogens, although consideration of a Gram-negative microorganism may be useful. Polymicrobial infections are relatively rare in the context of musculoskeletal infections in humans and are associated with a wide range of pathogens with no particular combination dominating. Gram-negative bacteria may be slightly more common in polymicrobial infections. In animals, only a very limited number of models are available investigating polymicrobial infections. An overview of these studies is provided in Table 1.

LEVEL OF EVIDENCE: Low

SEARCH STRATEGY:

The PubMed and Web of Science databases (date last accessed 7 October 2022) were searched. The keywords used for search criteria were ("fracture*" OR "musculoskeletal infection" OR "surgical site infection") AND "animal*" AND "polymicrobial", yielding 43 results. Identified studies were screened based on the titles and abstract followed by a review of cited literature of relevant articles. Inclusion criteria were (1) preclinical studies; (2) use of an animal model; (3) studies on bone or joint infection; and (4) presence of polymicrobial infection with a minimum of two pathogens. The exclusion criteria were: (1) studies on chronic wounds or ulcers; (2) conference papers; and (3) not in English language.

RATIONALE

In polymicrobial infections, a complex environment may be formed in which microbiological synergistic interactions exist between microorganisms. This microbial synergism in line with the need for broad-spectrum antimicrobial therapy may result in a more challenging treatment, but also adverse outcomes for patients suffering from musculoskeletal infections [1–3]. Depending on the etiology (osteomyelitis, periprosthetic joint infections, fracture-related infections, spondylodiscitis, and infected non-joints) and local epidemiology, the prevalence of polymicrobial infections ranges from about 10% to 25% [4–6]. In about 65% of the cases these infections are caused by Gram-negative bacilli, followed by non-epidermidis coagulase-negative Staphylococci, *Staphylococcus epidermidis* and *Staphylococcus aureus* [5].

Limited literature exists on polymicrobial infections of the musculoskeletal system in animal models. Available models include polymicrobial infections induced by *Staphylococcus aureus* in combination with *Pseudomonas aeruginosa*, while two models instead examine co-infection with *Escherichia coli* (Table 1) [3,7–9]. Difficulties were identified in finding the appropriate concentration of bacterial inoculum to establish infection while avoiding severe adverse events in animal welfare. However, none of these studies mentioned serious complications or high rates of animal exclusion [3,7–9]. One study of a murine infection model indicates that

synergistic interactions between *S. aureus and P. aeruginosa such that* increased *pseudomonas* colonization on the bone was associated with the presence of s*. aureus* [11]. Nevertheless, animal models for polymicrobial musculoskeletal infections should be limited to specific research questions such as pathogen interactions or specific treatment approaches for polymicrobial infections. If such a model is planned, it may contain a Gram-negative pathogen, consistent with the current microbial etiology in musculoskeletal infections.

Back to top

Table 1: Overview of available animal models on polymicrobial musculoskeletal infections.

Animals	Bacteria species and concentration (CFU)	Model	Method	Study type	Outcome	Source
Mouse	*Staphylococcus aureus* (1.0×10^4 CFU*) *Pseudomonas aeruginosa* (1.0×10^5 CFU)	Osteomyelitis	Insertion of a soaked silk thread into the tibial metaphysis	Experimental study	Both pathogens were present post euthanasia.	[9]
Mouse	*Staphylococcus aureus* (1.0×10^4 CFU) *Escherichia coli* (1.0×10^2 CFU)	Orthopedic implant-related infection	K-wire insertion in the femur	Treatment study	Chitosan sponges loaded with vancomycin and amikacin were effective against both pathogens.	[7]
Mouse	*Staphylococcus aureus* (1.0×10^5 CFU) *Pseudomonas aeruginosa* (1.0×10^5 CFU)	Orthopedic implant-related infection	Wound contamination	Treatment study	The combination of *Staphylococcus aureus* and *Pseudomonas aeruginosa* mimics clinical infections and responded to antibiotics but antimicrobial therapy alone is not effective at clearing the infection.	[11]
Mouse	*Staphylococcus aureus* (1.0×10^5 CFU) *Pseudomonas aeruginosa* (1.0×10^5 CFU)	Orthopedic implant-related infection	Wound contamination	Treatment study	Chitosan sponges loaded with amikacin and vancomycin significantly reduced CFUs on retrieved implant.	[12]
Rat	*Staphylococcus aureus* (1.0×10^3 CFU) *Pseudomonas aeruginosa* (1.0×10^3 CFU)	Orthopedic implant-related infection	K-wire insertion in the spinous process of a lumbar vertebra	Experimental study	The combination of *Staphylococcus aureus* with *Pseudomonas aeruginosa* yielded infection rates higher than either organism alone. Both pathogens were present post euthanasia.	[3]
Rat	*Staphylococcus aureus* (1.0×10^4 CFU) *Escherichia coli* (1.0×10^2 CFU)	Traumatic orthopedic wound infection	Wound contamination	Treatment study	Biodegradable scaffolds loaded with Gentamicin prevented osteomyelitis. *E. Coli* was only detected in one control rat (out of 32) and that rat died of sepsis two days post-operation.	[10]
Boer goats	*Staphylococcus aureus* (1.0×10^8 CFU) *Pseudomonas aeruginosa* (1.0×10^8 CFU)	Traumatic orthopedic wound infection	Wound contamination	Prevention study	Significant reductions in overall bacterial load of *Staphylococcus aureus* and *Pseudomonas aeruginosa* comparing wet-to-dry dressings alone and chitosan sponges loaded with vancomycin and tobramycin. *Pseudomonas aeruginosa* was not isolated from the wounds.	[8]

* Colony forming units

Back to top

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QUESTION 23

Are there immunological plasma biomarkers that are useful to measure infection or treatment effects in rat models of musculoskeletal infection?

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RECOMMENDATION: Yes

There are markers which have demonstrated efficacy in longitudinal rat inflammation and infection studies. Haptoglobin, alpha2-macroglobulin (α2M), fibrinogen, tumor necrosis factor alpha (TNF-α), and c-reactive protein (CRP) have demonstrated correlation with infection and inflammation in rats shortly (~1-7 days) after infection, with or without injury. Data on these markers as indicators of treatment effect are limited, with a few studies reporting utility of haptoglobin. Due to varying pathogeneses of infectious agents and levels of inflammation, there is likely not a single superior marker for tracking infection or treatment efficacy in rats. Recent studies analyzing multiple plasma markers and cell types have begun to enable a more complete understanding of the immune response to infection.

LEVEL OF EVIDENCE: Moderate

SEARCH STRATEGY: A literature search was conducted in Google Scholar using the following terms: rat, rat model, acute phase response, acute phase proteins, blood collection, immunological markers, serum markers, plasma markers, haptoglobin, C-reactive protein, white blood cell count, cytokine, haptoglobin, macroglobulin, fibrinogen, and amyloid. A review article summarizing these findings was published in March 2022.2 A subsequent search in December 2022 did not identify any additional papers which fit the criteria of question 3.02-3.03.

RATIONALE:

Clinically, blood markers are used for diagnoses of infection in humans (e.g., elevated white blood cell counts, CRP).[1] In contrast, the gold standard measure for treatment efficacy in preclinical infection models is bacteriology with colony forming unit (CFU) counts. As a terminal measure, this requires many animals and may not capture discrete information which occurs throughout a study. While longitudinal imaging provides structural information about the tissues, it does not detect cellular or biochemical responses to infection. Furthermore, markers which are strong indicators in humans are not necessarily strong indicators in rats (or other species); therefore, species specific markers must be used to effectively elucidate treatment effects. These markers participate in complex signaling pathways, and their expression changes over time following infection and/or inflammation. [2] Summarized below are blood biomarkers which were effective indicators of infection (with or without injury) status in rat models.

Haptoglobin levels differentiated between sterile inflammation and infection, as indicated by different concentrations and peak timepoints.[3,4] Following injection of methicillin-susceptible *S. aureus* (MSSA) into the femoral intramedullary cavity (injury with infection), haptoglobin levels were lower in the presence of gentamicin coated k-wire, which was associated with lower bacterial loads, compared to uncoated k-wire.[5,6] Similarly, localized treatment of femoral MSSA osteomyelitis with chitosan hydrogel containing fosfomycin led to lower haptoglobin levels and bacterial loads compared to blank gels.[7]

Alpha-2-macroglobulin also showed differences between infection and inflammation due to injury, as indicated by differing concentrations and peak timepoints.[8–10] In models of sterile injury or *S. aureus* infection, α2M was elevated for both injured and infected groups; however, the infected group showed significantly higher levels of α2M, which remained elevated longer than that of the injured group.[8] As shown in another model with injury and infection, α2M may not be a good marker for studies longer than 28 days, as the injury with infection group had returned to baseline levels by then.[11]

Fibrinogen levels were higher than both baseline and saline controls for days 1, 4, and 7 post induction of either infection or inflammation via injection.[4] To our knowledge, there is not a study measuring fibrinogen in a model of injury and infection.

In one study, CRP levels were not different among infection, inflammation, or saline control.[4] In two other studies, both tumor necrosis factor alpha and CRP differentiated between injury with infection and injury only groups.[12,13] Cui et al. showed an effect of treatment over time using these markers.[13]

More data is needed to confirm each biomarker's effectiveness in distinguishing between infection models, and between treated and untreated groups in infection models, as different pathogens modulate the immune response through various mechanisms which may alter bacterial load and mask treatment effect.[14] There are still many markers which do not have sufficient data supporting or refuting their use as an indicator of infection.

Methods for quantifying these biomarkers include electrophoresis, enzyme-linked immunosorbent assays (ELISAs), and multiplex assays, but their use is inconsistent across studies, which makes interpreting results challenging. Electrophoresis and gel staining can distinguish multiple molecules by size from the same sample. ELISAs are highly accurate and sensitive but are generally designed for singular proteins or macromolecules, requiring large volumes of blood to quantify multiple markers. Multiple markers can be analyzed from the same sample at the same time in bead-based multiplex arrays, but these require specialized instrumentation and can be less sensitive than single-factor ELISAs. For example, TNF-α and interferon gamma (INF-γ) were undetectable in a multiplex panel in an injury with infection model.[12]

In some studies, statistical analyses were not performed on both blood markers and traditional outcome measures (i.e., CFU counts). In other studies, blood markers and traditional outcome measures were statistically analyzed, but effects of infection and/or treatment were not detected. Commonly measured blood markers and their utility in indicating infection or injury + infection is summarized in Table 1.[2] Green boxes indicate strong agreement with other outcomes, red boxes indicate weak agreement or no statistical testing, and white boxes indicate an absence of data for the model type.

Table 1: Scoring of immunological plasma markers reported in Gautreaux et al.[14]

	Marker	Infection	Injury + Infection
Cytokines	CINC-1	-	-
	IFN-γ	-	? [11]
	IL-1β	-	? [11]
	IL-2	-	? [15]
	IL-4	-	? [11]
	IL-6	-	? [11]
	IL-8	-	-
	IL-10	-	? [11]
	TGF-β	-	-
	TNF-α	-	✓ [11,12]
Acute Phase Proteins	α2M	? [7*]	✓ [10]
	AAG	-	-
	Alb	? [3]	-
	Cp	? [3]	-
	CRP	? [3]	✓ [11,12]
	Fb	✓ [3]	-
	Hp	✓ [3]	✓ [4,5]
	LCN-2	-	-
	SAA	? [3]	-
Other	Cortisol	-	-
	Hb	-	? [11,16,17]
	SAP	-	-
	SRM	-	-
	WBC	-	? [11,12,16,17]

*indicates articles where statistical methods were not reported

✓ in green denotes markers that have shown changes in response to infection or injury + infection and/or markers that displayed similar responses as other longitudinal/terminal measurements (e.g., bacterial counts, imaging scores, etc.)

? in red denotes markers for which there has been limited testing, or results either are not consistent between models or indicate little to no changes in response to infection/inflammation

- denotes markers that were not reviewed within a model category

Abbreviations: α2M, α2-macroglobulin; AAG, α1-acid glycoprotein; Alb, albumin; CINC, cytokine-induced neutrophil chemoattractant; Cp, ceruloplasmin; CRP, C-reactive protein; Fb, fibrinogen; Hb, hemoglobin; Hp, haptoglobin; IFN, interferon; IL, interleukin; LCN, lipocalin; SAA, serum amyloid A; SAP, serum amyloid P; SRM, seromucoid; TGF, transforming growth factor; TNF, tumor necrosis factor; WBC, white blood cell.

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Back to top

QUESTION 24

Can all standard-of-care antibiotics included in the clinical treatment guidelines for musculoskeletal infection be used in animal models?

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RECOMMENDATION: Yes

The optimal choice on antibiotic strategy for treating musculoskeletal infection in animal models depends on multiple factors such as the animal included, the aim of the study, the presence of a foreign body and the causative pathogen. All first line antibiotic types in the treatment of musculoskeletal infection have been used in animal studies, often for multiple weeks. This suggests that substantial antibiotic therapy is possible with standard of care (SOC) antibiotics in animals. To provide useful information for basic research studies in animals, we provide an overview of these antibiotics in Table I. This table does not include any information on study outcome and tolerability.

LEVEL OF EVIDENCE: Strong

RATIONALE

Systemic antibiotic administration is a central aspect of the treatment of musculoskeletal infections in human patients. Most often, treatment is started with empiric broad-spectrum antibiotics and switched to a more targeted approach as soon as the causative pathogen is known. Furthermore, in many cases, therapy commences in the hospital with intravenous therapy, and continues at home with oral antibiotic therapy.

Previous preclinical research on systemic antibiotic treatment of musculoskeletal infections were mostly performed in small animal models (e.g., guinea pig, mice, rat, rabbit) and focused on infections caused by (methicillin-resistant) *Staphylococcus* species, Enterobacteriaceae and *Pseudomonas aeruginosa*. Clinical guidelines are available to direct targeted antibiotic therapy against these pathogens in human patients[1-4].

For methicillin-sensitive *Staphylococcal* infections, treatment with a narrow-spectrum penicillin antibiotic (e.g., nafcillin or flucloxacillin), or alternatively a cephalosporin (e.g., cefazolin) is recommended. For infections involving an implant, rifampicin should be added. After initial intravenous (IV) treatment, a combination regimen of rifampicin with levofloxacin, cotrimoxazole or doxycycline can be started. Vancomycin or Daptomycin are the antibiotics of choice for methicillin-resistant *Staphylococcal* infections. For Enterobacteriaceae a combination of a penicillin and a beta-lactamase inhibitor or Meropenem is the treatment of choice. Ciprofloxacin serves as an alternative when no implant is present or as an oral option after initial IV treatment. The treatment of infections caused by *Pseudomonas aeruginosa* is largely similar to that of Enterobacteriaceae, however often an aminoglycoside antibiotic is added.

A search for relevant articles that included the concepts "antibiotics", "musculoskeletal infection" and "preclinical models" in their title or abstract was performed in Medline, Embase, Web of Science and Scopus. For the concept "musculoskeletal infection", the main focus was on osteomyelitis, discospondylitis, fracture-related infection and periprosthetic joint infection. Articles describing the administration of any of the SOC antibiotics in a preclinical *in vivo* model are displayed in table I. This table also provides information of the daily dose in clinical practice, the daily dose in animal studies and the total duration of antibiotic treatment in animal studies.

Table I. Overview of standard of care antibiotic utilized in small animal studies.

Antibiotic Class	Antibiotic	Daily dose in clinical practice (mg/kg)*	Daily dose in animal studies (mg/kg)	Duration of antibiotic administration in animal studies (days)
Aminoglycoside	Gentamicin	5-7 IV	6-30 IM[5-7] 48 SC[8]	4[6] 7[5,7] 14[8]
	Tobramycin	5-7 IV	3 IM[9] 20 SC[10,11]	14[10] 21[9] 28[11]
Carbapenem	Meropenem	21-86 IV	240 SC[5,7,12]	7[5,7,12]
Cephalosporin	Cephalotin	43-171 IV	150 SC[13]	14[13] 28[13]
	Cefazolin	14--86 IV	50-150 IM[14,15] 220 SC[8]	14[8] 21[14,15]
	Ceftaroline	17 IV	120 IM[16] 40 IP[17] 200 SC[18]	3[17] 7[16,17] 14[17] 42[18]
	Ceftazidime	14-86 IV	1500 SC[19]	20[19]
	Cefuroxime	32-129 IV 4-14 PO	60 IM[9]	21[9]
Glycopeptide	Teicoplanin	6-12 IV	20 IM[20]	28[20]
	Vancomycin	40-45 IV	16-120 IM[9,16,21,22] 30-200 IP[17,23-34] 40-100 IV[35] 50-360 SC[8,18,36-42]	3[17] 4[29] 7[16,17,22,25,35,41] 10[40] 14[8,17,21,23,26,31,37,39] 15[27] 21[9,24,28,30,33,34,36] 28[32,36,39,42] 42[18,38]
Fluoroquinolone	Ciprofloxacin	11-17 IV 7-21 PO	16 IM[9] 100 IP[25] 60-120 SC[11,14,36]	7[25] 14[11] 21[9,14,36] 28[11,36]
	Levofloxacin	4-14 IV 4-14 PO	100 IP[43] 30 PO[44]	7[43] 28[44]
	Moxifloxacin	6 IV 6 PO	20 IP[23,26,45] 45 IV[35]	7[35] 14[23,26,45]
Lipopeptide	Daptomycin	4-6 IV	45-100 IP[17,29,43,46] 50-60 SC[18,37,47]	3[17] 4[29] 7[17,43] 14[17,37] 21[46] 28[47] 42[18]

Back to top

Penicillin	Amoxicillin (clavulanate)	57-171 (9-11) IV 21 (5) PO	25-50 (5-10) IM[6] 600 (150) SC[48]	4[6] 28[48]
	Ampicillin (sulbactam)	57-114 (29-57) IV	600 (300) SC[49]	28[49]
	Flucloxacillin	21-171 IV 21 PO	600 IP[45] 600 SC[48]	14[45] 28[48]
	Nafcillin	29-171 IV	120 SC[44,50,51]	28[44,50,51]
Rifamycin	Rifabutin	2-9 PO	2 PO[27]	15[27]
	Rifampicin	9 IV 9 PO	20 IM[16,22] 20-50 IP[17,24,28-31,33,34,43,45] 10-100 PO[8,18,21,27,42,50,52] 20-50 SC[13,36-39,53,54]	3[17] 4[29,52] 7[16,17,22,43,53] 14[8,13,17,21,31,37,39,45,53] 15[27] 21[24,28,30,34,36,54] 28[13,36,39,42,50,53] 42[18,38]
Tetracycline	Doxycycline	1-3 IV 1-3 PO	200 PO[18]	42[18]
	Minocycline	1-3 PO	20 PO[21]	14[21]
	Omadacycline	1 IV 5 PO	20 IP[28]	21[28]
	Tigecycline	2 IV	28 IM[5,7] 20-28 IP[29,34] 28 SC[20,42]	4[29] 7[5,7] 21[34] 28[20,42]
Trimethoprim	Trimethoprim	4-6 PO	160 SC[13,53]	7[53] 14[13,53] 28[13,53]

* For a human patient with a weight of 70kg
IM, intramuscular; IP, intraperitoneal; IV, intravenous; PO, per os; SC, subcutaneous

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Back to top

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Back to top

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Back to top

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Back to top

QUESTION 25

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Back to top

Question 26

Is a statistically significant reduction in bacterial burden clinically significant, if infection remains after treatment in animal models of infection?

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Recommendation: Yes

The literature linking changes in CFU counts in animal models with clinically significant changes in infection is weak and circumstantial. Despite these weaknesses, there does appear to be some evidence that bacterial loads generated in current in vivo models does replicate findings from clinical studies in the chronic wound literature in humans. Additionally, thresholds below 10^3 - 10^4 CFU, which would correlate in most studies to a 2-log reduction in bacterial load would be below the threshold that has been used clinically to roughly define appropriate wound healing and skin colonization versus active infection in chronic wound healing studies in humans. This would probably be a minimum reasonable threshold to use for in vivo studies.

Strength of Evidence: Limited

Rationale

This is a challenging question to answer because direct comparisons of bacterial burden that are achieved in an animal model have not been/probably can't be evaluated in the corresponding human setting. In my opinion, this question is really asking something to the effect of do bacterial burdens that are achieved in animal models of infection replicate the human clinical setting. There does seem to be some literature to address this question. Unfortunately, the literature in clinical infection regarding bacterial burdens in the setting of infection is mostly older literature and derives substantially from chronic open wounds in the setting of trauma, chronic ulcers, and burn literature. Studies beginning in the 1960s examining chronic wounds such as decubitus ulcers, traumatic wounds, burns, and delayed healing surgical wounds used wound swabs and soft tissue biopsies to suggest that bacteria loads above 10^5 - 10^6 CFU/g tissue cultured from the wound prevented appropriate healing [1-8]. Methods in these studies to evaluate bacterial loads included wound swabs, tissue biopsy, and rapid gram stain [1-8]. Later studies supported these findings of prior studies suggesting that minimum intrawound bacterial loads of $\geq 10^4$ CFU/g tissue were needed to cause infection of complex extremity wounds and post-surgical wounds, and this also appeared to be the approximate threshold for successful skin grafting [9,10,11]. Given these findings in the clinical literature, I evaluated: 1) the bacterial load changes that are achieved in animal models of osteomyelitis and implant-associated infection to determine whether these approximate the evidence that exists in the clinical setting and 2) the association of bacterial load changes seen in these animal models with histological evidence of persistent infection. In general, studies in different animal models of both direct inoculation resulting in long bone osteomyelitis, hematogenous osteomyelitis, implant associated osteomyelitis, and fracture associated osteomyelitis all tend to result in bone and soft tissue CFU counts in the 10^5 range with the vast majority of these studies using *Staphylococcus aureus*. In the setting of an implant, the implant CFUs are typically around 10^2 or 10^3. This appears to be at least somewhat consistent with reported CFU counts from human clinical infections as described above. The CFU needed to consider an infection eradicated in these animal models is a bit less clear with most studies using a 2 log or even 3 log change as being a significant decrease in bacterial burden. This would also be at least somewhat consistent with the human studies described above that looked at minimum bacterial colonization that allowed for successful tissue healing. For

Back to top

example, one rat model of *S. aureus* osteomyelitis using direct inoculation required at least 0.5 x 10^5 CFU/100mg tibia to create any histological or radiological evidence of osteomyelitis with 3x10^5 CFU/100mg necessary to create histological scores of osteomyelitis that were significantly different than control groups [12]. In a rat post-traumatic, implant associated infection model using *S. epidermidis* with an inoculum of 10^3 showed that 2/5 animals with mean 1.5 [1] CFU/g explant were culture negative and overall bacterial growth was not different than control groups. In this group, findings of positive gram stain for bacteria correlated with successful fracture healing or the development of nonunion. In contrast, animals with an inoculum of 10^5 all developed nonunion, histological evidence of severe osteomyelitis, and had mean 10.33 [9.5] CFU/g explant bacterial counts. Another rat model of implant associated *S. aureus* osteomyelitis showed that even with low innoculums of 10^2 CFU, histological osteomyelitis developed with bone CFU counts of mean 2.87 x 10^5 CFU/g bone [14]. This is also consistent with other models such as infected open fractures with one rat model using *S. aureus* showed a mean 4.4 x 10^4 CFU/g tissue in retrieved bone tissue in infected animals regardless of initial inoculum (1 x 10^2 CFU was minimum inoculum to result in infection) [15]. Mouse models appear to be consistent with the findings from rat models. For instance, one mouse model of implant associated osteomyelitis with *S. aureus* showed low innoculums of 10^3 CFU resulted in implant associated infection with mean 7.58 x 10^4 CFU in wild type mice and 2.91 x 10^2 mean CFU on implant [18]. Similarly, *S. aureus* infected implant associated open fracture model in mice showed mean 3.7 x 10^5 CFU in the periimplant tissue and 2.8 x 10^2 CFU from implant when untreated by day 42 [20]. Another mouse model of implant-associated osteomyelitis with *S. aureus* found that in animals with no evidence of implant-associated infection, defined as CFU 0-20 at the implant surface, had corresponding bacterial loads in the surrounding knee joint tissue were median 267 CFU (range from 0-5.1 x 10^4 CFU) [19]. Similarly, a mouse model of MRSA osteomyelitis that was not implant-associated, found that approximately 10^5 CFU/g remained in samples that showed persistent positive cultures with histological evidence of osteomyelitis [17].

Large animal models also appear to be consistent with these prior findings in smaller animal models. For instance, a rabbit model of Methicillin resistance *S. aureus* osteomyelitis with intramedullary injection showed uninfected controls with mean 9.21 x 10^4 CFU/g bone. In contrast, antibiotic treated animals with vancomycin had mean 1.4 x 10^2 CFU/g of bone with 2 of 11 samples with positive cultures; tigecycline treated animals with mean 20 CFU/g of bone with 1 out of 10 positive culture [16].

In summary, the literature linking changes in CFU counts in animal models with clinically significant changes in infection is weak and circumstantial. Despite these weaknesses, there does appear to be some evidence that bacterial loads generated in current in vivo models does replicate findings from clinical studies in the chronic wound literature in humans. Current weaknesses in the literature include: need more studies directly from human cases of bone and joint infection to define active infection versus colonization and threshold CFU counts, most studies examine *S. aureus*, and it is not clear if other more indolent bacterial infections follow the same patterns.

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Back to top

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Back to top

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Back to top

Question 27

After using an antimicrobial-loaded biomaterial in an animal study, can you prevent false negative culture results due to antimicrobial carryover during sample processing in the lab?

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Response/Recommendation: Yes

False negative cultures occur when study antibiotics or antiseptics kill bacteria or inhibit culture after the time of sampling. In the case of antibiotics, super-minimum inhibitory concentration (MIC) antibiotic concentrations accompany bacteria into culture plates. To this end, preventing false negatives requires one of several techniques to either dilute or remove antibiotics such that antibiotic concentrations are below MIC values when culturing: e.g., pelleting/washing or neutralization in combination with good laboratory practices and controls.

Strength of Recommendation: Strong

Rationale:
While implanted biomaterials have revolutionized medical treatments, their presence in a host significantly reduces the minimum infectious dose of bacteria.[1-3] Asa result, research in antimicrobial-loaded biomaterials has exploded since the early 1950s.[4,5] Countless combinations of materials loaded with antiseptics, antibiotics, or other antimicrobial technologies exist.[4] To bring this promising technology out the laboratory and into patients, appropriate preclinical animal models and laboratory methodology are critical for determining efficacy. However, a concerning trend has emerged: laboratory processing of antimicrobial-loaded biomaterials from various animal models produces false negative culture results.[6] This trend also extends to the clinical environment and thus, a consensus on mitigating false negative culture results is critical to advancing research in this field.[7]

False negative cultures result when study antibiotics or antiseptics kill bacteria or inhibit culture after the time of sampling. In a hypothetical experiment, a researcher produces an active release antimicrobial coating and incorporates it onto a device surface. The implant is surgically placed in an animal model and inoculated with biofilm. After a set duration of time, the implant is removed surgically. During explanation, the researcher sees several signs of infection including inflammation and pus. The infected implant is placed in stagnant broth and sent to the laboratory for processing. Following vortexing and sonication, aliquots of broth are plated on agar using a 10-fold dilution series. The agar plates are incubated, and the researcher counts the remaining colony forming units (CFU) after 24 h. No growth is observed. This represents a false negative culture result.

As an active release antimicrobial coating resides in stagnant broth solution, it releases its payload into a fluid environment and volume that are not physiologically relevant. Within a living system, fluids flow and as such the active agent of a coated device is diluted as it elutes from the coating; agent is diluted immediately upon release. In stagnant broth, active agent accumulates as it elutes into the fluid environment; its concentration increases as opposed to decreases. Higher concentrations of agent improve antimicrobial activity, increasing the

likelihood that bacteria within the test tube will be killed, and contributing to a false negative culture result. Furthermore, as the biofilm is broken up by vortexing and sonication, the cells are transitioned from a biofilm phenotype to planktonic (persisters notwithstanding). Planktonic bacteria are more susceptible to antimicrobials than those in biofilms. As broth aliquots with high concentrations of antimicrobial are collected from the test tube in the hypothetical experiment, those high concentrations of antimicrobial are carried through the 10-fold dilution and plating process. The combination of the phenotypic state of the bacteria and elevated levels of antimicrobial lead to false negative culture results as the bacteria are killed during the process Thus, washing techniques, appropriate neutralizers, and laboratory controls are necessary (discussed below) to mitigate false negative culture results. Where possible, microbiological analysis should be tethered with imaging to confirm the presence and quantity of bacteria.

Washing techniques can be applied to mitigate false negative culture results by reducing antibiotic culture concentrations to below MIC levels. For example, our lab has developed several flow cell systems to take dilution into account when developing active release antimicrobial technologies and performing biofilm testing.[8-12]While a variety of procedures exist, alternating cell washing, dilution, and centrifugation is a fundamental technique in microbiology and can effectively dilute active agent in a broth solution. The washing step normally begins with suspending bacteria and/or active agent in broth, PBS or other buffer, then vortexing and sonicating,[13] followed by centrifugation. In centrifugation, the speed and time depend on the type of cell culture.[14] Some types of bacteria are more sensitive than others to gravitational forces. In our experience, a triplicate process of washing and centrifugation is typically adequate to dilute residual antimicrobial and prevent it from producing a false negative result.

Various neutralizers (e.g., Dey-Engley, Letheen, and HiCap™) exist to counteract antimicrobial biomaterials. Neutralizers are commonly used to inactivate antiseptics such as chlorhexidine gluconate and iodine-based complexes.[15] Current work is also being done in the field of antibiotic neutralizers.[15] It should be noted that all types of neutralizers will have effects on cells. Mackinnon provides certain criteria for these inactivators.[16] They must “neutralize the disinfectant it is used against. not give rise to any inhibiting effect, either of its own or as a result of any products formed when it is combined with the disinfectant . . . [and should be] fairly rapid.”[16] Few if any neutralizers meet all these criteria. Therefore, the best option should be considered.[16] A variety of resources including ASTM E1054-21 and ISO-14698 address appropriate neutralizers and should be consulted and cited in work to not select a neutralizer that is also a biocide.[17,18]

Laboratory processing techniques should also be considered. Two key factors include time and temperature. The length of time after an animal is sacrificed, before an implant is removed, and any duration of time the explanted biomaterial sits in solution containing antimicrobial should be considered before quantification begins. Additionally, the temperature of the neutralizing solution, buffer, or wash/bath may change the kinetics of the environment.[19] Keeping samples on ice or in a cooler while transporting and processing is recommended. Finally, appropriate controls should be implemented at each point during a study. Some infection models self-resolve, necessitating negative infection controls in all pre-clinical animal models.[20] Additional controls for all relevant laboratory variables are encouraged.

Even with optimal microbiological techniques, there is no perfect way to represent the bacterial condition using culture on explanted devices. Therefore, appropriate histology and imaging are critical to confirming negative culture results.[20] Many preclinical studies involve implantation of two experimental pieces of hardware such that one can be used for microbiological analysis and the other for histological assessment.[12,21-23] Stoodley et al. said, “Microscopic examination

Back to top

of the devices and associated tissue themselves, are still the only way to definitely demonstrate the presence of biofilm."[24] Other methods include fluorescence for *in situ* real-time monitoring.[25] As the field of imaging continues to progress, additional options will arise. There are other ways of generating microbial information through genetic exploration; polymerase chain reaction (PCR), DNA Extraction, and 16S rRNA Gene Sequencing are used less frequently, but can be used to verify a false negative culture results as they can amplify even small amounts of genetic material.[24,26] Ultimately, it is up to each laboratory to develop protocols for mitigating false negative culture results upon harvesting antimicrobial-loaded biomaterials. These methods must be validated using a known inoculum and other necessary controls. If processes are considered carefully, a negative culture result may be a promising conclusion instead of a dubious question mark.

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Back to top

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Back to top

QUESTION 28

Should treatment of fracture and/or implant related infection always include debridement in animal models?

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RECOMMENDATION: Yes

Debridement should always be considered as part of the standard of care for treating fracture and/or implant related infections in animal models.

LEVEL OF EVIDENCE: moderate

RATIONALE

Debridement is considered as an essential part of the multistep treatment approach when treating a fracture or implant related infection. Infection can be a significant complication for patients and a burden for surgeons to treat. Definitive treatment should be considered and initialized as soon as the suspicion of an infection is made and confirmed and should not be delayed1.

Treatment of necrotic infected tissue may be challenging if solely managed with an antibiotic regimen due to low penetration rates, bacterial adherence, expression of surface molecules, virulence factors, antibiotic resistance2,7,8,10,11 and biofilm formation3-5. Consequently, the use of debridement as part of management for this type of infection is essential since it helps to remove necrotic tissue, reduces bacterial count, and prevents the progression of biofilm formation as well as the potential removal of biofilm matrix6-9.

Treatment success rates in animal models with the use of debridement or antibiotic alone have not been successful in diminishing bacterial colony forming units (CFU), reducing bone lysis formation, stimulating bone formation, or eradicating infection if solely used.

As seen in the rat animal model of chronic osteomyelitis (OM) from Dernell et al. (2001)2 in which they compared the use of antibiotics only with local or systemic administration vs the use of debridement alone. It which they found that it did not show any difference when they examined for radiographic signs of OM, in terms of improvement of infection or regarding the quantitative culture result when measured for CFU per gram of bone2.

In a rat model of chronic OM conducted by Sener et al. (2009)12, they compared no treatment, debridement only, and debridement with local antibiotics in a cement carrier and in bone graft. Results showed reduced CFU/g in the harvested bone from the groups treated with debridement and local antibiotics in a carrier. The conclusions in this study are the as a clinical; study on implant related infections that debridement, local and systemic antibiotics is optimal treatment. 12, 13

Although the findings from Sener et al. found macroscopic findings of infection in the group with local and systemic antibiotics, Inzana et al. (2015)3 used a mice animal model with chronic OM in which they compared the use of debridement without antibiotic, the use of systemic antibiotic plus debridement, the use of local antibiotic plus debridement or the combination of debridement with systemic plus local antibiotic. In all the groups, tissue debridement significantly reduced bacterial burden as evidenced on bioluminescent imaging (BLI). Additionally, the groups that had received debridement and local or systemic antibiotic also

showed significant BLI reduction. Moreso, when comparing the use of local and systemic antibiotics with the use of only systemic regimen, there were no significant differences in BLI values. This suggests that local therapy did not augment the systemic regimen and, observing the high BLI values at the beginning of the study from the local antibiotic treated mice, it indicates that antibiotic concentration was low compared with the mice treated with the systemic antibiotics.3

CFU assays used on the samples collected from the mice treated with local or systemic antibiotics showed a reduction in bacteria for the tissue, implant, and the fixation hardware but no statistical difference was observed. When soft tissue and bone from the mice treated with local plus systemic antibiotics were analyzed for bacterial colonization, only one of the soft tissue samples collected revealed a negative culture and, even, when examining the implants used from these group as well as from the group treated with only local antibiotic therapy, they revealed a high bacterial burden.3

Moriarty et al. (2017)5 used a sheep animal model to assimilate an orthopaedic device-related infection. This model consisted of a two-stage procedure: At the initial stage, implant was removed, debridement was done and, depending on the treatment group, they added local, systemic or both antibiotic regimens. At the second stage, local or systemic antibiotics were removed -if used-, definitive treatment with a nail was inserted and minimal debridement was made.

Furthermore, when the removed hardware and nail at both the different time points were analyzed for quantitative bacteriology, the group with debridement alone and nail exchange did not show any signs of improvement of the infection.5 This suggests that debridement and nail exchange alone does not treat the infection.

Moreover, the group that received systemic antibiotic alone was analyzed at the end of the second stage and showed that all cultures collected were negative. Although, when new samples were collected after euthanized, bacterial growth was present. Samples were also collected and analyzed for the sheep that received local antibiotics alone, When the cultures collected from the sheep that received both local and systemic antibiotics were analyzed, for both -at the end of stage 2 and euthanization-, all cultures were negative.5

Interestingly, when looking at the rat model used by Chen et al. (2007)14 which compares the different treatments in rats with a chronic infected segmental defect of their femur with the treatments being: debridement with or without systemic antibiotic and with the addition of different doses of rhBMP-2 (0, 20 and 200 micrograms). Results show that the use of systemic antibiotic in combination with debridement had fewer number of bony lysis in the femoral cortices when comparing to the rats in the group that did not receive systemic antibiotic.14 Moreover, Brunotte et al. (2019)15 used a tibia rabbit implant-related infected model to determine the utility of the two-stage revision procedure regarding implant-related infections. The first stage consisted of irrigation and debridement, removal of hardware and implantation of a vancomycin impregnated-PMMA spacer with a second stage procedure consisting of irrigation and debridement, removal of antibiotic spacer and reimplantation of hardware. When the rabbits were clinically assessed after stage one, they showed improvement of clinical infection signs. After re-examination when stage two was completed, there was evidence of clinical signs of recurrence of the infection suggesting that the debridement from stage one had helped improve the clinical signs for infection. Even so, when bacterial culture growth was examined at the end of stage one (debridement and irrigation), they found eradication rates of 67% in group 1, 50% in group 2 and 33% in both group 3 and 4. 15

Another model used by Shiels et al. (2016)16 in which they compared the use of local and systemic antibiotic with concomitant debridement and irrigation against debridement alone

Back to top

showed the benefits of using both local and systemic antibiotic regimen. They found that there were fewer bacterial counts and inflammatory markers (TNF-a and IL-6) in the femoral rats sample that received the dual regimen.16

A chronic osteomyelitis model in beagles was used by Huneault et al. (2004)17 to determine the effectiveness of the use of debridement alone, debridement and systemic antibiotic or debridement with local antibiotic to eradicate infection. Fewer bone lytic lesions were found in both the local and systemic antibiotics when comparing to only debridement in which it was also found a greater cortical remodeling of the bone defect.17

Likewise, based on macroscopic evaluation, bone healing of the defect was greater in both antibiotic groups than compared with the debridement group alone. Bacterial cultures were analyzed which showed fewer positives in both groups of antibiotics when compared to the debridement group alone. As well, under histologic examination, it was found that there was more endosteal bone proliferation in both groups of systemic and local antibiotic than the debridement group alone with higher neutrophilic and lymphoplasmocytic infiltration in the latter group.17 Findings that suggest similar results from Shiels et al.

Foremost, the model used by Wagner et al. (2016)18 consisting of an infected tibial defect in mice was utilized to evaluate different treatment therapies for posttraumatic OM. The different methods evaluated in this model were: use of debridement with systemic antibiotic or systemic antibiotic without debridement. After completion, mice were euthanized and examined for persistence or resolution of infection in two different time points: one and two weeks.

Based on microbiotic assessment, only one sample from the debridement group at week one showed CFUs of Staphylococcus aureus; meanwhile, the agar plates from the mice of week two showed no CFU compatible with the formation of S. aureus. On the other hand, smears from the mice from week one and two who did not receive debridement showed characteristic CFU that were compatible with S. aureus.18

Tibial samples were also GRAM-stained to identify the presence of bacteria. The samples collected from the debridement group from week one showed very few bacteria; meanwhile, mice from week two detected no bacteria. Additionally, samples from the no debridement group from week one and week two detected multiple clusters of GRAM-positive bacteria.

Also, when tibial samples were examined for new bone formation, the mice from the debridement group from week one and week two had decreased bone formation when compared to the control group (mice that received debridement but had no inoculation of the bacteria). Comparatively,

mice from week one and two from the no debridement group had similar results regarding bone formation.18

These data support our conclusion that “Debridement should always be considered as part of the standard of care for treating fracture and/or implant related infections in animal models.”

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Back to top

03. Clinical

QUESTION 29

Should synovial biomarkers be investigated for MSKI in large animal models?

Leonard C Marais, Robin Patel

Response/Recommendation: Yes

The most used synovial fluid markers of septic arthritis are leukocyte counts and bacterial counts. Other synovial fluid biomarkers have been associated with infected joints in large animal model experimental studies including IL-1β, TNF-α, stromelysin and keratan sulfate (lapine model). In equine animal models, serum amyloid A, D-dimer, glycylproline, neutrophil viability and myeloperoxidase levels may be useful as synovial biomarkers for MSKI.

Strength of Recommendation: Moderate

Rationale

Large animal models are increasingly being used to further our understanding of the pathophysiology of bone infections.[1] Furthermore, these models are then applied to investigate novel diagnostic and therapeutic interventions in haematogenous osteomyelitis and orthopaedic device-related infection (ODRI), which encompasses periprosthetic joint infection (PJI) and fracture-related infections (FRI). The aim of this systematic review was to determine which synovial biomarkers should be investigated for musculoskeletal infection (MSKI) studies using large animal models.

Methods

A systematic literature search was conducted using MEDLINE via Pubmed, Web of Science and Scopus. We also searched our own files, reviewed reference lists from identified articles and searched for cited references of key publications. The following combination of keywords and Medical Subject Headings (MeSH) terms were used: (“osteomyelitis” OR “musculoskeletal infection” OR “infectious arthritis” OR “periprosthetic joint infection” OR “septic arthritis” OR “fracture-related infection,” OR “septic non-union” OR “spondylodiscitis” OR “implant-related infection” OR “orthopaedic device-related infection” OR “osteoarticular infection” OR “infected total knee replacement” OR “infected total hip replacement”) AND [(“synovial” OR “synovial fluid”) AND (“biomarkers” OR “inflammatory markers” OR” acute phase proteins”)] AND (“animal” OR “animal model” OR “pre-clinical”). Publications describing synovial biomarkers used in in vivo large animal models of musculoskeletal infections or veterinary studies of musculoskeletal infections were considered eligible. (Figure 1) The primary outcome of interest was the biomarkers used in large animal studies investigating bone and orthopaedic device related infections. Large animals were defined as involving rabbits, dogs, goats, sheep, cattle, horses, donkeys or non-human primates.[1] Studies involving chickens, rats, mice and other rodents were excluded.

Only studies involving human subjects and published in English were considered. Case reports, congress proceedings, and abstract-only and conference reports were excluded. Due to the explorative nature of the project, and the outcomes of interest, a qualitative description of the data is reported in the form of a scoping review.

Results

The most used synovial fluid markers of septic arthritis are leukocyte counts and bacterial counts. However, several other biomarkers have also been used in large animal models (Table1).

In a lapine model of Haemophilus influenzae type B septic arthritis Jafari et al., measured the following synovial biomarkers as a measure of the degree of response to certain interventions: IL-1β, TNF-α, stromelysin and keratan sulfate.[2]

Several synovial fluid biomarkers have been identified that aid in differentiating septic arthritis from other causes of arthritis in horses. These include the metabolite glycylproline detected by nuclear magnetic resonance (NMR) spectroscopy,[3] and beta defensin-3 ELISA.[4] Wauter et al showed that flow cytometric analysis of neutrophil viability could also be used to differentiate infected from non-infected equine joints, with increased viability in infected cases.[5] Another study, by the same group of authors, found that synovial fluid from infected joints contained significantly more total and active myeloperoxidase than samples from non-infected joints.[6] Several researchers showed the value of serial analysis of serum amyloid A in synovial fluid sample to discriminate between infected and non-infected causes of arthritis in horses.[7-9] Ribera et al. demonstrated elevation of D-dimer levels in synovial fluid from septic joints in foals.[10] Finally, Kozyi et al, recently performed a proteomic analysis of synovial fluid, using liquid chromatography with tandem mass spectrometry (LC-MS-MS). Twenty-six differentially abundant proteins from cellular origin were found at higher levels in the synovial fluid of horses with septic arthritis compared to the non-infected controls. These were also shown to serve as markers for the elimination of infection from the joint.[11]

Conclusion

An array of synovial fluid biomarkers has been associated with infected joints in large animal model experimental studies. IL-1β, TNF-α, stromelysin and keratan sulfate, have been shown to increase in infected joints in a lapine model. Several biomarkers may serve to differentiate infected from non-infected joints in equine animal models, including serum amyloid A, D-dimer, glycylproline, neutrophil viability and myeloperoxidase levels.

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Back to top

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Back to top

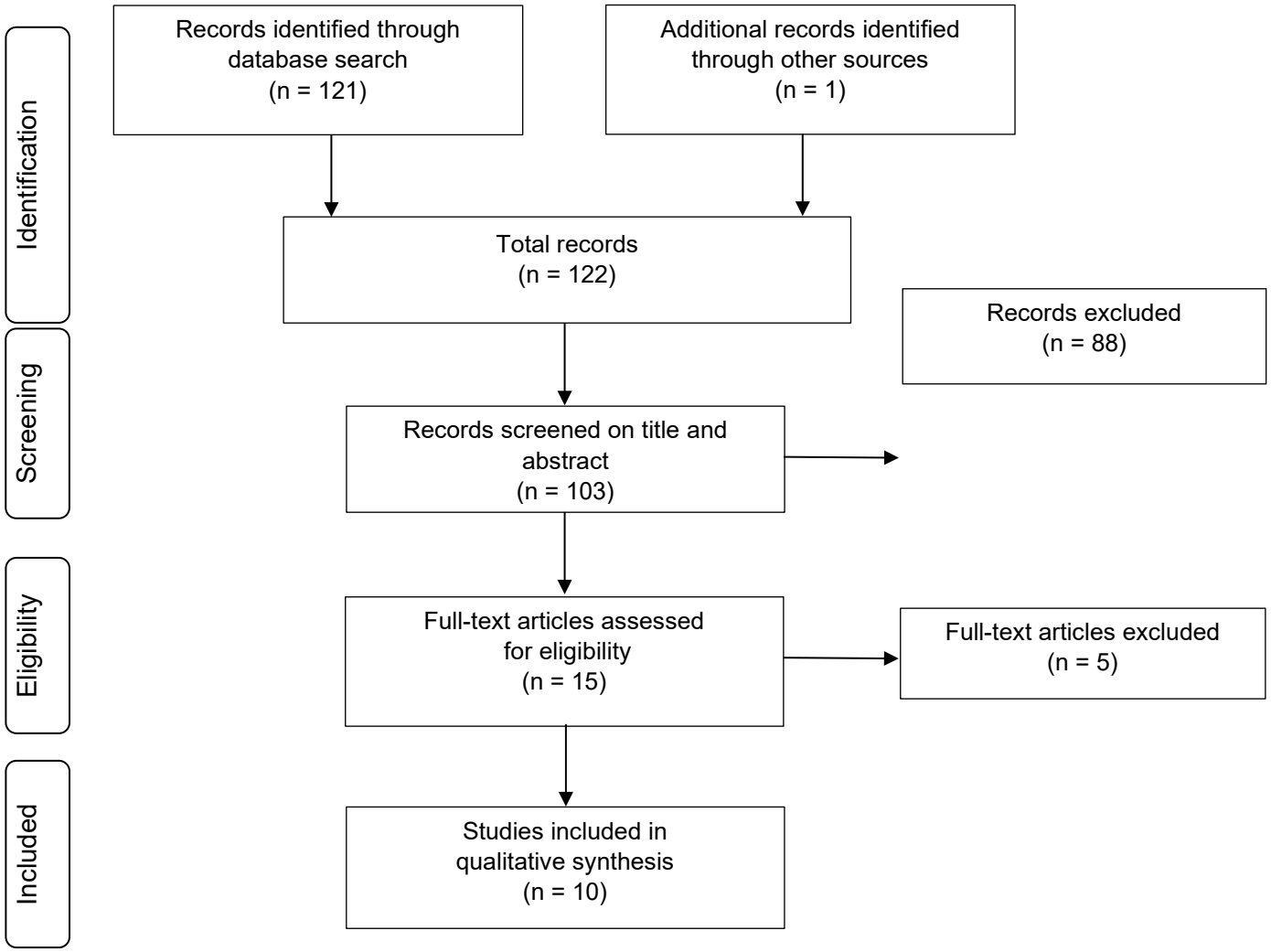

Figure.1 Flow diagram showing selection of included studies.

Back to top

Table 1: Study characteristics

Author Date of publication	Animal model	Sample size	Study design and methods	Synovial fluid biomarker associated with infection	Comments
Anderson, 2018	Equine	19	Horses with and without infection [haematogenous sepsis (n=1) and wound sepsis (n=6)]	Glycylproline	Glycylproline is increased in infected cases
Boger, 2022	Equine	14	Horses with and without septic joints.	Beta defensin-3	Beta defensin-3 is increased in infected cases
Jafari, 1993	Lapine	40 joints	Induced Haemophilus influenzae type b arthritis with 4 groups (3 groups included an intervention).	Leukocyte count Bacterial counts IL-1β, TNF- α Stromelysin Keratan sulfate	Assayed biomarkers are increased in infected cases
Wauters, 2012	Equine	31	Compared horses with culture-confirmed infected joints (n=13), pronounced infectious synovitis (n=11) and healthy controls (n=14)	Neutrophil count Neutrophil viability	Neutrophil viability is higher in infected cases
Wauters, 2013	Equine	82	Compared horses with cultured-confirmed infectious arthritis, osteochondritis dissecans, trauma and healthy controls.	Myeloperoxidase	Total and active myeloperoxidase are increase in infected cases
Yoshimura, 2020	Equine	17	Three groups of horses: Saline injection into joint (n=3), lipopolysaccharide injections (n=6) and Escherichia coli septic arthritis group (n=8)	Serum amyloid A	Synovial fluid Serum Amyloid A is increased in septic arthritis
Ribera, 2011	Foals	39	Observational clinical study of foals with septic arthritis (n=18), septic foals without septic joints (n-9), systemically healthy foals with septic joints (n=9), and control foals (n=3)	D-dimer	Synovial fluid D-dimer levels are elevated in septic joints

Back to top

Robinson, 2017	Horses	112	Observational clinical study of horses with synovial fluid contamination or sepsis (n=38), non-septic intra-synovial pathology (n=66), and controls (n=8)	Serum amyloid A	Synovial fluid serum amyloid A levels are elevated in septic joints
Sanchez-Teran, 2016	Horses	6	Experimental study in which one healthy tarsocrural joint in each horse was randomly assigned to repeat through and through lavage at three time points	Serum amyloid A	Repeat through and through lavage did not affect synovial fluid serum amyloid A levels
Koziy, 2022	Horses	17	Proteomic analysis performed on horses with experimental septic arthritis (n=8), non-septic lipopolysaccharide-induced arthritis (n=6), and controls (n=3).	26 differentially abundant proteins identified	Septic joints have specific synovial fluid proteomic patterns

Back to top

QUESTION 30

Are there any recommended diagnostics to monitor the safety of antibiotic therapy in animal models?

Jerry Tsang, Leonard C. Marais, Antonia F. Chen

Response/Recommendation: No

Although there is no set standard of diagnostics for monitoring antibiotic therapy safety in animal models, a combination of clinical evaluation and serum biomarkers (leukocyte count, C-reactive protein, fibrinogen, haptoglobin, serum amyloid A, ceruloplasmin, albumin, osteocalcin, bone-specific alkaline phosphatase, and deoxypyridinoline) may be useful.

Strength of Recommendation: Low

Rationale

Animal models are increasingly being used to further our understanding of the pathophysiology of bone infections. Furthermore, these models are then applied to investigate novel diagnostic and therapeutic interventions in hematogenous osteomyelitis and orthopaedic device-related infection (ODRI), which encompasses periprosthetic joint infection (PJI) and fracture-related infections (FRI). The aim of this review was to determine which diagnostic techniques and biomarkers can be used to monitor the safety of antibiotic therapy in animal models.

Methods

A literature search was conducted on 25 August 2022 using MEDLINE, Web of Science and The Cochrane Library. We also searched our own files, reviewed reference lists from identified articles and searched for cited references of key publications. The following combination of keywords and Medical Subject Headings (MeSH) terms were used: “arthroplasty, replacement, knee,” OR “prosthesis-related Infections” OR “osteomyelitis,” OR “musculoskeletal infection,” “infectious arthritis,” OR “Arthroplasty, Replacement, Hip,” OR “periprosthetic joint infection,” OR “septic arthritis,” OR “fracture-related infection,” OR “septic non-union,” OR “spondylodiscitis AND ““C-reactive protein, CRP,” ”biomarkers, ” OR serum inflammatory markers” OR.” acute phase proteins,” OR “ESR,” OR “antibiotics” OR “antibiotic monitoring” AND “animal,” OR “animal model,” OR “pre-clinical.” Publications describing serum biomarker or antibiotic monitoring of in vivo animal models of musculoskeletal infections or veterinary studies of musculoskeletal infections were considered eligible. The primary outcome of interest was diagnostic tests and modalities used to monitor the safety of local and systemic antibiotic therapy in animal studies investigating bone and orthopaedic device related infections. (Figure 1).

Only studies involving human subjects and published in English were considered. Case reports, congress proceedings, abstract-only and conference reports were excluded. Studies dealing with the evaluation of novel agents as potential antimicrobial agents were considered beyond the scope of this review and were also excluded. Discovery studies involving potentially new antibiotics had to comply with regulations as prescribed by the Food and Drug Administration (FDA) and European Medicines Agency (EMA), including toxicokinetics, immunotoxicity, reproductive and developmental toxicity, gene toxicity, carcinogenicity, and organ-level toxicity.(1, 2) Due to the exploratory nature of the question and the outcomes of interest, a qualitative description of the data was reported as a review.

Results

Moriarty et al. noted that current preclinical in vivo models and studies are highly disparate, irregularly conducted and reported, and without standardization and validation.(3) A recent systematic review identified 316 studies involving large animal models of bone infection (254 Lapine, 23 Canine, 23 Ovine, 16 Porcine).(4) In 171 of these studies, the effect of systemic or local antimicrobials were investigated. The authors echoed these sentiments, concluding that published studies frequently exhibit substantial shortcomings in terms methodological quality and missing outcome quantification. They went on to recommend a standard study guideline template for animal bone infection studies. However, this template does not provide guidance on safety monitoring in case of antibiotic administration.

Both small and large animal models have been used in an effort to advance our knowledge of bacterial osteomyelitis. It is relevant to acknowledge the differences in terms of immune and inflammatory responses to implants and infection represent a limitation of in vivo studies. Furthermore, different animal species may exhibit different responses to antibiotic therapy. Rabbits, for example, may experience severe side effects, including diarrhea and dehydration, when exposed to high doses or prolonged therapy with certain antibiotics like penicillin and vancomycin.(5) Similarly, the toxicity and side effects of antibiotics may be different in animals than that seen in humans due to differences in pharmacokinetics, drug metabolism, susceptibility of nontarget bacterial flora, and physical or anatomic characteristics.(6)

Investigations into antibiotic therapeutics in animal models typically involve either local or systemic administration. In the case of systemic application, it would seem prudent to focus screening on evidence of systemic toxicity. The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) consist of a checklist of information to include in publication of in vivo experiments to enable others to scrutinize the work adequately, evaluate its methodological rigor, and reproduce the methods.(7) These guidelines advise that authors report all expected and unexpected adverse events that had a negative impact on the welfare of the animals in the study (e.g., cardiovascular and respiratory depression, central nervous system disturbance, hypothermia, reduction of food intake).(8) Authors should also indicate whether these events were expected or unexpected. Reporting adverse events allows future researchers to plan their studies appropriately and monitor these events. Furthermore, studies should aim not to only examine efficacy but rather assess treatment benefit and risk. The clinical parameters and observations that should be considered as minimum requirements include: vitals, weight loss, wound healing issues, behavioral changes, lameness and changes in behavior or body condition.(3, 9) All studies should state if any animals excluded from the study and state the reason for exclusion (e.g. severe symptoms, death, sepsis).(3) At time of autopsy, the liver and kidney is typically examined histologically for signs of injury.(10, 11)

On the other hand, when local application is investigated, monitoring of local toxic effects would be advisable. However, this is much less well defined in the literature. In general, it is advisable that fracture healing, bone regeneration and osteointegration, or lack thereof, should be reported.(3) The potential for nephrotoxicity with agents like vancomycin and tobramycin should also be considered.(12) For the purposes of gauging efficacy, these studies typically monitor local antibiotic levels. Traditionally, this has been done by determining the concentration in bone samples. Recently, microdialysis techniques have been used to selectively sample the unbound extracellular fraction of antibiotics.(13) In order to detect systemic uptake, blood concentration may also need to be monitored. At the site of application, histological evaluation is recommended and studies should report neutrophil and macrophage

Back to top

counts, degree of necrosis, osteolysis or osteoclast activation, formation of granulation tissue or fibrosis, and new bone formation or osteoblast activation.(4).

Aside from therapeutic interventions, the introduction if infection in an animal may also result in unwanted effects. It is advisable that the smallest bacterial load to introduce infection should be used without overwhelming the animal immune system. While low inoculums may not reliably produce infection, higher levels might result in septic shock and mortality.(14) Furthermore, it is important to monitor for signs of local or systemic spread of the infection. This may take the form of spread to an adjacent joint (with swelling and reluctance to bear weight on the involved extremity) or systemic emboli that may only be discovered at autopsy.

It has been recommended that commonly used human biomarkers of infection may not be appropriate for pre-clinical models.(3) The most commonly reported biomarkers of infection used monitor disease progression/response to treatment in pre-clinical models are the **leukocyte count** and **C-reactive protein** (CRP).(4) However. it should be remembered that acute phase proteins, such as CRP, are more sensitive than hematological parameters for detecting infection and inflammation in ruminants and pigs where changes in the leukocyte count and neutrophil response after inflammation are less evident than in companion animals (e.g. dogs and cats).(4) Furthermore, acute phase proteins have the advantage of being much more stable than hematological cells.(4)

Although CRP is a widely used biomarker in the most popular models of musculoskeletal infection, such as rabbits and pigs,(5, 15, 16) its usefulness as a biomarker of infection has been much debated, particularly in murine models of these diseases.(17) Historic studies reported early and substantial increases in CRP titres following induced injury and inflammation.(18-20) However, more recent work in the context of infection found alternative acute phase proteins, such as **fibrinogen** and **haptoglobin** to be more sensitive.(21) Supporting evidence for the use of alternative acute phase proteins can be found for **haptoglobin** (mice and rats, horses, ruminants),(17, 21, 22) **serum amyloid A** (mice and rats, dogs, pigs, horses and ruminants),(8, 16, 17, 21-25) **ceruloplasmin** (mice and rats and pigs),(21) and **albumin** (mice and rats).(21) However during the interpretation of serum biomarkers, there should be an awareness of the inter-breed variation in reference ranges amongst species, as well as the inter-species variation.(26)

The discovery of novel plasma biomarkers will not only help improve the monitoring of pre-clinical models of musculoskeletal infections, but will help to improve the early clinical diagnosis of these conditions.(27) Approaches at a transcriptomic, proteomic, and metabolomic level have been described to identify candidate molecules. A veterinary transcriptomic study in horses reported a distinct difference in synovial expression of proteins in septic arthritis compared with aseptic arthropathies,(28) with upregulation in cellular movement, hematological system development, inflammatory response, cell-to-cell signalling and immune cell trafficking in the proteome of synovial fluid from septic joints, with downregulation in these pathways in aseptic cases.(28) Similar findings were reported using the same disease model with proteomic analysis.(29) It has been shown that there is often a discrepancy in the “inflammasome” of local tissue concentrations and that found within serum in musculoskeletal infections.(30, 31) A microdialysis study in a porcine model of osteomyelitis identified upregulation of cytokines and chemokines involved in angiogenesis and bone remodelling (e.g. OPG, TGFα, MCP-1, VEGFA, and uPA) during disease development.(32) Using plasma metabolome analysis in a S. aureus model of murine osteomyelitis, Isogai et al identified twelve

Back to top

metabolites as candidate positive biomarkers and a further two candidate negative biomarkers for osteomyelitis.(33) A study in rabbits found that the combined use of serum **osteocalcin**, bone-specific **alkaline phosphatase**, and **deoxypyridinoline** concentrations provided an accuracy of 96% in the prediction of infected fracture non-union.(34) Through a variety of modern molecular techniques, there is an untapped potential for the development of diagnostic and prognostic biomarkers in clinical and pre-clinical musculoskeletal infection.

General overviews on this subject are available from Zak & O'Reilly (1991) and Morris (1995).(6, 35) To the authors' knowledge, there is no contemporary guidance focused on the field of antimicrobial monitoring and toxicity in musculoskeletal infection.

Conclusion

There is a lack of standardization of monitoring of the safety of antibiotic therapy in animal models investigating bone infections. Studies often focus on efficacy with limited exploration of local or systemic toxicity. Therefore, there is a need for the development of pragmatic contemporary guidelines focused on monitoring toxicity and side effects in animal models of musculoskeletal infection.

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Back to top

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Back to top

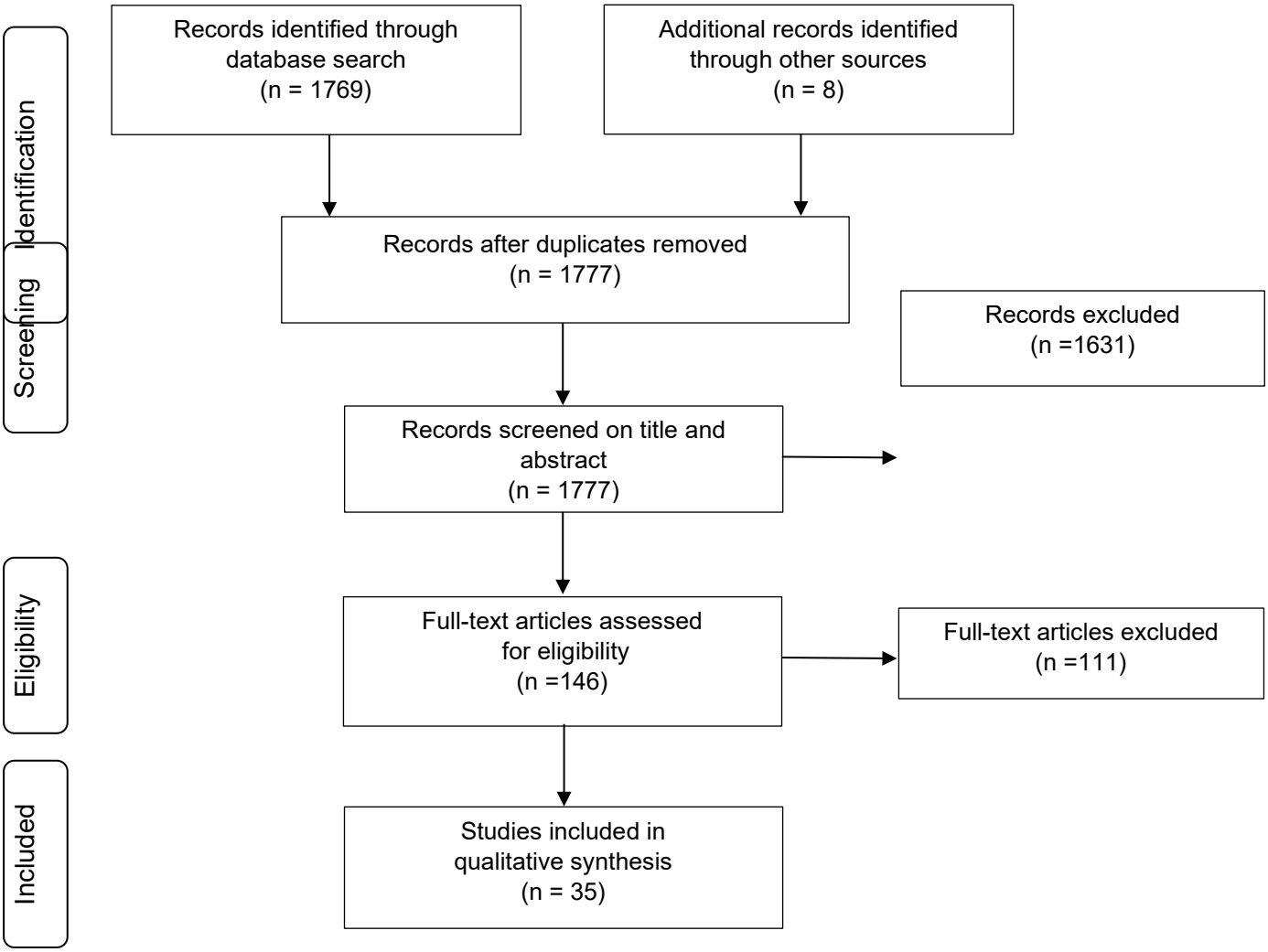

Figure.1 Flow diagram showing selection of included studies.

Back to top

QUESTION 31

Do X-ray and advanced imaging have a role in diagnosing PJI using animal models?

Kohei Nishitani, Alex C. McLaren, Michelle Ghert

Response/Recommendation: Yes

X-ray is the most common imaging modality used in animal models of PJI (approximately half of the identified studies), and its use in these models is applicable to the diagnosis of PJI in humans. Studies may not be consistent with standard clinical practice and therefore eventual translation beyond preclinical studies will be challenging.

Strength of Recommendation: Moderate

Rationale

The quality and applicability of animal models in the study of prosthetic joint infections (PJIs) are important for successful translation of research findings from preclinical research to clinical research and eventually to clinical practice. This pertains to all aspects of animal research, including imaging in the diagnosis of PJI. We systematically reviewed the available literature to identify the most commonly used imaging modalities and approaches for diagnosing PJIs in animal models in order to determine the most likely to translate successfully beyond preclinical research.

Literature Search Strategy

Articles on PJI using an animal model in which x-ray or advance imaging was used were searched using PubMed. Searching strategy was shown in Table 1. First, for searching imaging related articles, the following 8 terms (radiography, radiograph, Xray, X-ray, computed tomography, magnetic resonance imaging, MRI, and Imaging) were searched, and 3,574,664 articles were extracted. Next, for searching for PJI, 80,204 articles of “joint infection“ were extracted by searching (infection and joint, infectious arthritis, and septic arthritis). Next, 662,273 “implanted related studies” were extracted using (implant, prosthesis, arthroplasty). Then 15,039 articles of “implant associated join infection” were extracted by searching (“joint infection” and “implanted related study”). Finally, 16,349 articles of PJI were extracted by searching (“implant associated join infection” or “prosthetic joint infection”). And then, 3,938,202 “Animal related study”, were extracted, by searching ((animals) NOT (human) or (animal) NOT (human) or animal model). Finally, 123 articles of PJI animal model with imaging were extracted using (Imaging and PJI and animal model).
One-hundred twenty-three extracted articles were reviewed (Figure 1) There were 88 exclusions because of 3 non-original studies, 15 clinical studies, 1 non-English study, 8 in vitro studies, 33 animal studies without in vivo infection, 11 animal infection studies without implant associated infection, 13 animal implant associated infection studies without joint involvement, or 4 animal PJI studies without imaging. Finally, 35 articles were remained for review (1 – 35). The 35 articles were summarized in Table 2.

Animal and Bacterial Species and Anatomic Joint

A total of three animal species were used in the 35 studies. The most common model was a mouse model which was used in 19 studies. Eight studies used a rabbit model, and 6 studies used a rat model. In terms of the causative bacteria, the majority of studies (30 studies) used *S. aureus*. There were 3 studies which used *E. coli*, and 1 study each that used *C. albicans,*

Back to top

P. aeruginosa, or *S. epidermidis*. Most studies involved the knee joint (32 articles), with the hip and shoulder joints involved in 2 and 1 studies, respectively.

Implant Model

The implant used to create a PJI was predominantly with a K-wire (18 articles) with retrograde intramedullary insertion. This surgical procedure was used in two other studies in which an implant other than a K-wire was used. More clinically relevant models were also used. Three studies used a titanium tibial implant to mimic a tibial implant (12, 15, 17). One study modelled a total knee arthroplasty, in which a rat-sized, non-constrained knee prosthesis in which the femoral component was made from a metal alloy and the tibial component was milled from high-density polyethylene stock was used. Notably, in contrast to the common use PMMA cement in human hip arthroplasty, only one hip hemiarthroplasty study used cement for implant fixation (4).

Imaging Modality

As for the imaging modality used for the diagnosis of PJI ,17 studies used X-ray, 3 studies used MRI and 2 studies used Tc scintigraphy. These imaging modalities are commonly used in clinical practice. However, we found that it is not uncommon to use non-clinically relevant imaging in animal studies of PJI. These included bioluminescent imaging (BLI) or fluorescent imaging in14 studies and microCT in 11 studies.

Imaging Criteria for the Diagnosis of PJI

The role of X-ray in animal studies in the diagnosis of infection is similar to that in human clinical evaluation, such as the identification of radiolucencies, osteolysis, osteointegration, bone destruction or implant loosening. Some studies used a radiographic score to quantitate PJI (12, 25). X-rays are generally taken longitudinally in *in vivo* fashion. Other predominant *in vivo* imaging used was BLI, which can detect bacterial burden of the infection longitudinally, although it requires a special bacterial strain which contains bioluminescence operon such as luxICDABE operon. MicroCT was mostly performed after sacrifice. Different from computed tomography (CT) for human PJI, which usually assesses for implant loosening or osteolysis, or reactive bone formation, microCT is also used for the bone morphometric analysis in animal models (19, 20. 31) to evaluate parameters such as bone volume/ total volume or trabecular thickness, and the measurement of bone mineral density (BMD) (3, 23) around the infection site. Probably because of its time-consuming feature, MRI is not often used in animal models of PJI. Two studies used MRI to evaluate for an inflammatory signal, similar to the identification of inflammation by MRI in humans. One study used MRI to confirm the reduction of the hip joint. Finally, Tc scintigraphy and Photoacoustic imaging were also examined for experimental purposes.

Conclusion

In conclusion, we found that X-ray is the most common imaging modality used in animal models of PJI (approximately half of the identified studies), and its use in these models is applicable to the diagnosis of PJI in humans. Animal specific BLI and microCT are also frequently used for animal models of PJI to follow longitudinal bacterial burden in PJI and to perform bone morphometric analysis and identify bone- implant loosening. These latter studies are not consistent with standard clinical practice and therefore eventual translation beyond preclinical studies will be challenging.

Back to top

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Back to top

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Back to top

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Back to top

Table 1. Searching strategy for PJI animal model with imaging		
Search for imaging including radiograph		
1	radiography	1,909,738
2	radiograph	237,429
3	Xray	409,066
4	X-ray	2,115,021
5	computed tomography	632,042
6	magnetic resonance imaging	642,411
7	MRI	707,852
8	Imaging	2,753,134
A. Imaging	1 or 2 or 3 or 4 or 5 or 6 or 7 or 8	3,574,664
Search for PJI		
1	infection	3,910,010
2	joint	660,408
3	1 and 2	65,218
4	infectious arthritis	22,279
5	septic arthritis	24,882
B. joint infection	3 or 4 or 5	80,204
7	implant	577,331
8	prosthesis	626,579
9	arthroplasty	112,231
C. implant related study	7 or 8 or 9	662,273
D. implant associated join infection	B and C	15,039
10.	prosthetic joint infection	4,266
E. PJI	E or 10	16,349
Search for animal experiment		
1	(animals) NOT (human)	3,500,397
2	(animal) NOT (human)	3,550,930
3	animal model	770,780
F. animal related study	1 or 2 or 3	3,938,202
Search for PJI animal model with imaging		
	A and E and F	123

Back to top

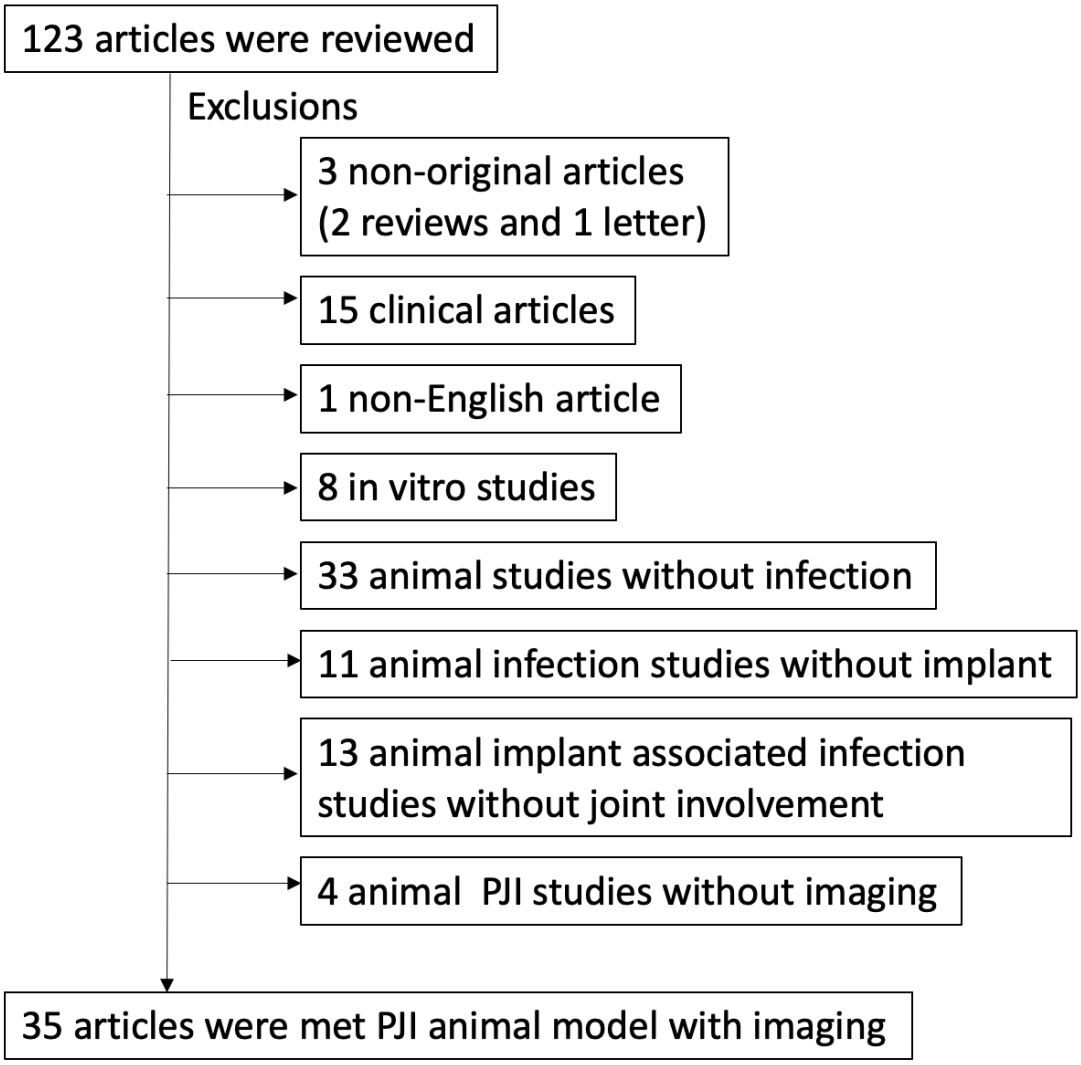

Figure 1. Flow-chart of the narrowing down process

Back to top

Table 2. Articles of PJI animal model with imaging									
	animal	Bacteria	implant	Joint	surgery	Imaging	Treatment	Study aim	Role of imaging
1	mouse	*S. aureus*	Titanium K-wire	Knee	Retrograde insertion into the distal femoral canal	X-ray, BLI	Observation	To evaluate the role of the physis on influencing the development of S. aureus hematogenous orthopaedic implant-associated infections in a mouse model comparing younger versus older mice	BLI: in vivo longitudinal monitoring of bacterial burden, X-ray: evaluate length and width of the femur (ex vivo)
2	mouse	*S. aureus*	Titanium K-wire	Knee	Retrograde insertion to distal femur	BLI	CIA mouse vs control mouse	To interrogate a well-validated mouse model of PJI as well as a well-validated model of RA to determine how RA alone affects perioperative infectious burden	In vivo longitudinal monitoring of bacterial burden
3	mouse	*C. albicans*	Titanium K-wire	Knee	Retrograde insertion to distal femur	microCT	Anidulafungin-loaded organic-inorganic sol-gel	To evaluate the efficacy of an anidulafungin-loaded, organic–inorganic sol-gel in preventing PJI caused by C. albicans using an in vivo murine model	microCT: bone mineral content (BMC) and bone mineral density (BMD)
4	rat	*S. aureus*	Cemented titanium prosthesis	Hip	Hip hemiarthroplasty	microCT, MRI, BLI	Observation	To develop a clinically representative cemented hip hemiarthroplasty PJI model in rats using a 3D-printed titanium implant	MRI: Maintenance of reduction of hemiarthroplasty (in vivo), microCT: to evaluate bone-cement, cement-implant interfaces (ex vivo) BLI: In vivo longitudinal monitoring of bacterial burden
5	mouse	*S. aureus*	Titanium K-wire	Knee	Placed retrograde into	BLI	Anti-CD41 antibody	To determine the mechanistic relationship between platelet count	BLI: in vivo longitudinal monitoring of bacterial burden

Back to top

					the distal part of the femur			and infectious burden and to assess the modifiability of this factor	
6	rabbit	*S. aureus*	Screw	Knee	intraosseous injection of planktonic bacterial suspension into a predrilled bone tunnel in femoral epicondyle	X-ray,	Observation	To develop and characterize a rabbit model of chronic PJI using common radiological and clinical markers.	X-ray: radiolucency, osteolysis (in vivo and ex vivo)
7	mouse	*S. aureus*	Custom steel implant	Shoulder	A surgical implant was press-fit into the proximal humerus	X-ray, BLI	Observation	To develop a longitudinal and noninvasive model of periprosthetic SII that can be used as a platform to analyze the pathophysiology of these infections as well as test and guide future diagnostic and treatment strategies	X-Ray: monitoring for osteolysis (in vivo), BLI: in vivo longitudinal monitoring of bacterial burden
8	mouse	*S. aureus, E. coli*	Titanium K-wire	Knee	Retrograde insertion to the femur	microCT	Moxifloxacin-loaded organic-inorganic sol-gel with different antibiotic concentration	To evaluate the efficacy of a moxifloxacin-loaded organic-inorganic sol-gel in preventing PJI caused by S. aureus and E. coli using an in vivo murine model	microCT: Bone mineral content (BMC) (a) and bone mineral density (BMD) (ex vivo)
9	rat	*S. aureus*	A 3D printed porous titanium implant	Knee	Inserted press-fit into the distal femur	X-ray,	Ceragenin-90 (CSA-90), a synthetic compound based on endogenous antibacterial peptides	To investigate whether treating porous titanium implants with CSA-90 would prevent PJI when challenged with S. aureus	X-Ray: implant osseointegration or osteolysis (in vivo), microCT: to examine the pattern of lysis (ex vivo)

Back to top

10	rat	*S. aureus*	UHMWPE plug, titanium maxillofacial screw	Knee	PE to femoral epicodlye, titanium screw to tibia	X-Ray, microCT	Observation	To establish a novel PJI model in rats incorporating clinically relevant materials (a UHMWPE plug and a Ti6Al4V tibial screw)	X-Ray: septic loosening including periosteal reaction, grading (in vivo, ex vivo), microCT: to evaluate bone volume loss
11	rabbit	*S. aureus*	Steel K-wire	Knee	Intramedullary insertion to canal of the left tibia	X-ray,	Two-stage revision in implant-related infection	To establish a preclinical small animal model to simulate a two-stage revision in implant-related MRSA infection	X-Ray: lytic lesions and new periosteal bone formation
12	mouse	*S. aureus*	Titanium tibial implant to mimic a joint implant	Knee	Tibial implant to mimic a joint implant	X-ray,	Gut microbiota	To consider the role of the gut microbiome as a risk factor for PJI	X-Ray: bony destruction and quantitative score for PJI
13	rabbit	*S. aureus*	Titanium locking peg	Knee	Retrograde insertion of the bacterial coated implant to the femur	BLI	Nanofiber coating loaded with or without linezolid-rifampin	To develop a rabbit model of implant associated infection in which in vivo BLI was used to assess the preclinical efficacy of an antibiotic-releasing implant coating	BLI: in vivo longitudinal monitoring of bacterial burden
14	mouse	*P. aeruginosa, E. coli.*	Titanium K-wire	Knee	Retrograde insertion into the distal femoral canal	X-ray	Bispecific antibody targeting P. aeruginosa virulence factors	To develop a mouse model of gram negative-PJI to evaluate the pathogenesis of a more virulent infection caused by P. aeruginosa, and a less virulent infection caused by E. coli	X-ray: width and area of the infected femur (ex vivo) BLI: in vivo longitudinal monitoring of bacterial burden

Back to top

15	mouse	*S. aureus*	3D printed titanium implant	Knee	Tibial implant to mimic a joint implant	X-ray,	Vancomycin mixed with PMMA cement	To develop a mouse model of the first-stage surgery of a 2-stage revision for PJI involving a 3D printed titanium implant and a mouse-sized cement spacer that elutes vancomycin.	X-ray: radiolucency, metaphyseal fragmentation, and cystic change (in vivo)
16	rat	*S. aureus*	Titanium K-wires	Knee	Retrograde insertion to femur	BLI, microCT	Vitamin E phosphate-coating of titanium K-wire	To demonstrate the synergistic activity of vitamin E in preventing bacterial adhesion to orthopaedic implants	microCT: bone resorption and osteomyelitis (ex vivo), BLI: in vivo longitudinal monitoring of bacterial burden
17	mouse	*S. aureus*	3D printed titanium implant	Knee	Tbial implant to mimic a joint implant	X-ray,	Observation	To provide a load-bearing translational representation of clinical PJI that effectively recreates the periprosthetic space	X-ray: implant loosening, radiolucency, metaphyseal fragmentation (in vivo)
18	mouse	*S. aureus*	Titanium K-wire	Knee	Retrograde insertion into the distal femoral canal	BLI, Photoacoustic imaging (PAI)	Indocyanine green (ICG) conjugated to β-cyclodextrin (CDX-ICG) or teicoplanin (Teic-ICG)	To provide proof-of-concept for detecting PJI noninvasively with PAI.	BLI: in vivo longitudinal monitoring of bacterial burden, PAI: to distinguish and quantify the burden of infection from surrounding tissue
19	rat	*S. aureus*	Titanium rod	Knee	Retrograde insertion to femur	X-ray, microCT	Bacitracin-modified Titanium	To evaluate the in vivo performance of bacitracin functionalized on Ti, since the complex biological environment may influence its bioactivity	X-ray: to evaluate the cortical bone destruction (in vivo), microCT: for bone morphometric analysis (ex vivo)

Back to top

20	rat	*S. aureus* *S. epidermidis* *E. coli*	Titanium implant	Knee	Femoral medullary cavity	X-ray, microCT	Enoxacin-modified PEGylated titaium	To examine the antibacterial properties of enoxacin-modified PEGylated titanium alloys	X-ray: cortical bone destruction (in vivo) microCT: Bone morphologic analysis (ex vivo)
21	mouse	*S. aureus*	Titanium K-wire	Knee	Retrograde insertion into the distal femoral canal	BLI, X-ray	Biodegradable coating using branched poly(ethylene glycol)-poly(propylene sulfide) (PEG-PPS) polymer	To test the efficacy of the coating as a vehicle for the delivery of antibiotics to eradicate infection and prevent biofilm formation.	X-ray: periprosthetic osteolysis (in vivo), BLI: in vivo longitudinal monitoring of bacterial burden
22	mouse	*S. aureus*	Titanium K-wire	Knee	Retrograde insertion into the distal femoral canal	BLI, fluorescent imaging, microCT	Observation	To model an orthopaedic prosthetic joint infection in mice, 2D and 3D in vivo optical imaging procedures	microCT: outer bone volume change, BLI: in vivo longitudinal monitoring of bacterial burden, fluorescent imaging: to track in vivo EGFP-neutrophil fluorescence
23	mouse	*S. aureus*	K-wire	Knee	Retrograde insertion to femur	microCT	Third generation cephalosporin alone or associated with a PGE_1 vasodilator	To investigate the effects of a PGE1 on implant-related infections in a diabetic mouse model	microCT: bone volume and BMD (ex vivo)
24	rabbit	*S. aureus*	Titanium implant	Knee	Retrograde insertion to femur	X-ray	Silver ion-doped ceramic nano-Bpowder coating	To determine whether silver ion-containing calcium phosphate-based ceramic nanopowder-coated implants prevented implant-related infection by comparing silver-	X-ray: periprosthetic osteolysis (ex vivo)

Back to top

								coated, hydroxyapatite (HA)-coated, and uncoated titanium implants in vivo	
25	rat	*S. aureus*	Non-constrained knee prosthesis (metal tibial component and high-density PE femoral component	Knee	TKA	X-ray	Observation	To establish an implant model of acute osteomyelitis associated with metallic implants without any promoters of infection other than the implant itself.	X-Ray: To assess development and progression of bone infection (osteolysis, radiographic score)
26	mouse	*S. aureus*	Titanium K-wire	Knee	Retrograde insertion into the distal femoral canal	BLI, fluorescence imaging, X-ray, microCT	Observation	Attempting to use multimodality optical and anatomical imaging to better evaluate noninvasively and longitudinally the bacterial burden and neutrophilic inflammation in the context of the pathologic changes that occur in bone in mouse model of orthopaedic implant infection	microCT: outer bone volume change (in vivo), X-ray: longitudinal osteolysis (in vivo), BLI: in vivo longitudinal monitoring of bacterial burden, fluorescent imaging: to track in vivo EGFP-neutrophil fluorescence
27	mouse	*S. aureus*	Titanium K-wire	Knee	Retrograde insertion into the distal femoral canal	BLI	Vancomycin, daptomycin, and tigecycline	To compare the efficacies of daptomycin, tigecycline, and vancomycin prophylaxis using a preclinical in vivo mouse model of surgical implant infection	BLI: in vivo longitudinal monitoring of bacterial burden,

Back to top

28	mouse	*S. aureus*	Titanium K-wire	Knee	Retrograde insertion into the distal femoral canal	BLI, fluorescence imaging	Observation	To compare four available bioluminescent S. aureus strains to determine whether a model of chronic post-arthroplasty infection	BLI: in vivo longitudinal monitoring of bacterial burden, fluorescent imaging: to track in vivo EGFP-neutrophil fluorescence
29	mouse	*S. aureus*	Stainless steel K-wire	Knee	Retrograde insertion into the distal femoral canal	BLI	IL-1β–deficient mice (F8) , TLR2-deficient mice (B6.129-Tlr2tm1Kir/J) (F7) , wildtype (wt) C57BL/6J mice	To evaluate the mechanism by which TLR2 and IL-1β play a role in host defense using an in vivo mouse model of post-arthroplasty S. aureus infection	BLI: in vivo longitudinal monitoring of bacterial burden,
30	mouse	*S. aureus*	Stainless steel K-wire	Knee	Retrograde insertion into the distal femoral canal	BLI	Minocycline/rifampin-impregnated bioresorbable polymer implant coating	To evaluate novel preventative therapeutic strategies against post-arthroplasty infections	BLI: in vivo longitudinal monitoring of bacterial burden, fluorescent imaging: to track in vivo EGFP-neutrophil fluorescence
31	rat	*S. aureus*	Titanium K-wire	Knee	antegrade insertion	X-ray, microCT	Vancomycin-containing sol-gel film	To demonstrate whether a vancomycin-containing sol-gel film on Titanium alloy rods can successfully treat bacterial infections in an animal model.	Xray: osteolysis and bone destruction (in vivo) microCT: total bone volume and bone morphometric analysis (ex vivo)
32	rabbit	*S. aureus*	Tibial silicone-elastomer implant (C Carbon)	Knee	Partial knee arthroplasty	^{99m}Tc-UBI 29-41 scintigraphy	Observation	To test the ability of ^{99m}Tc-UBI29-41 to discriminate between infected and uninfected prosthetic joints using a previously validated rabbit model of prosthetic joint infection	Tc scintigraphy: ^{99m}Tc-UBI 29-41 uptake in infected prosthetic knees longitudinally (in vivo)
33	rabbit	*S. aureus*	Stainless steel screw	Hip	Stainless steel screw was inserted into the	MRI	Vancomycin-loaded acrylic bone cement	To investigate whether continuous wave ultrasound could	MRI: inflammatory signal and the inflammatory area

Back to top

					proximal femur for simulating the primary metal implant			enhance vancomycin release and antimicrobial efficacy of antibiotic-loaded bone cement (ALBC).	
34	rabbit	*S. aureus*	Tibial silicone-elastomer implant (C Carbon)	Knee	Partial knee arthroplasty	^{99m}Tc-UBI 29-41 scintigraphy	Observation	To test the ability of ^{99m}Tc-ciprofloxacin imaging to discriminate between infected and uninfected prosthetic joints, using a rabbit model of prosthetic joint infection	Tc scintigraphy: ^{99m}Tc-UBI 29-41 uptake in infected prosthetic knees longitudinally (in vivo)
35	rabbit	*S. aureus*	Tibial silicone-elastomer implant (C Carbon)	Knee	Partial knee arthroplasty	MRI	Observation	To test high-field high-resolution chemical shift MRI to study Norden's rabbit model of osteomyelitis (chemical shift-specific slice selection) to study Norden's rabbit model of osteomyelitis	MRI: to evaluate inflammatory signal and the inflammatory area

Back to top

QUESTION 32

Are there specific animal tissues that need to be studied to diagnose MSKI and treatment outcomes?

Bryan Springer, Benjamin Ricciardi and Kordo Saeed

Response/Recommendation: Yes.

Generally mixed tissues should be studied to diagnose MSKI and treatment outcomes. It depends on the study, which may include bone, soft tissue or a mixture of these. MSK infections are not typically isolated to a single tissue type or compartment, so examining multiple tissue types provides a better overview of the pathological mechanisms of infection and subsequently treatment. These can be included with prosthetic materials as well as lymphatics and blood, if necessary.

Strength of Recommendation: Moderate

Rationale

The literature does not directly address the necessity of particular tissues to study MSK infection appropriately, however, some inferences can be made based on prior literature. The most important consideration for answering this question is the goal of the individual study. Clearly, a detailed assessment of the infected tissue (bone tissue for osteomyelitis and soft tissue for a soft tissue infection) would be important for any study. Many MSK infection models involve infection of multiple tissue types (bone and joint, bone and soft tissue, etc..). There is some variation in analysis within these studies: some studies focus their analysis on a particular tissue type of interest (bone in an osteomyelitis model) while other studies broaden their analysis to surrounding affected tissue (the entire femoral compartment including muscle and other soft tissue structures excluding skin in a femoral osteomyelitis model) depending on the goals of the study [1-14, 22-29]. MSK infection is not typically isolated to a single tissue type or compartment so examining multiple tissue types provides a better overview of the histopathological mechanisms of infection and treatment. For instance, important host components of the local immune response to osteomyelitis occur within the surrounding soft tissue suggesting that these tissues should be included within mechanistic studies examining host response to infection [10,11,20]. The study aims should likely dictate tissue analysis at the local site of infection, but consideration to including all surrounding tissue should be given.

Many studies of MSK infection involve an implant-associated infection. It is critical in these studies to evaluate the implant, in addition to the surrounding tissue, because independent mechanisms of implant adherence, colonization, biofilm formation, and interaction with host immunity exist for the implant relative to host tissue [9,15-17]. For example, the presence of an implant can change the propensity for developing an implant associated infection in a hematogenous murine infection model, and in the setting of low virulence organisms like *Cutibacterium acnes* [9,17]. Additionally, some bacteria including *Streptococcus agalactiae* may not use the implant as a bacterial reservoir with associated robust biofilm formation in contrast to organisms such as *Staphylococcus aureus* [15].

In order to study the pathophysiology of MSK infection and treatment, analysis of regional tissue can be beneficial. A number of studies have shown regional effects of MSK infection on the draining lymph node, which may help be useful to study host immune response in this setting [18,19,20]. In localized osteomyelitis models, enlargement of regional draining lymph nodes have been demonstrated with alterations in the relative lymphocyte distributions and cytokine production suggesting a systemic response is generated in these models [18,19,20]. Additionally, organs such as the spleen, kidney, lung, and liver may also be useful to examine in models to evaluate systemic infection and further study systemic host immune response in the setting of the spleen [18-21,30].

Another important tissue type to assess systemic response in MSK infection assessment is peripheral blood sampling. Studies have used blood samples for a wide range of outcomes including assessing for bacteremia, measuring serum systemic inflammatory markers that parallel those in clinical use (C-reactive protein, white blood cell count), assessing serum inflammatory markers in animals that may be used to measure the systemic response to infection and treatment (serum amyloid a, haptoglobin, fibrinogen), assessing systemic circulating inflammatory cytokine production, systemic measurements of antibiotic concentrations, identifying systemic circulating antibodies and antibody producing cells in response to infection, and assessing the peripheral circulation of relevant immune cells [28,31-41]. Some of these outcome measures are used clinically to diagnose infection and quantitatively follow response to treatment, and incorporating them into existing animal models may be beneficial to improve their clinical relevance.

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QUESTION 33

Are there unique pathophysiologic features of MSKI in the pediatric population?

Benjamin Ricciardi, Robin Patel, Alberto Carli

Response/Recommendation: Yes

Pediatric patients have a higher incidence of *Kingella kingae*, which is more likely to be present in acute osteoarticular infection, more likely to cause septic arthritis, and more likely to be present in younger (<4 years) children. Nucleic acid amplification tests should be performed to detect this microorganism, compared to traditional tissue and synovial fluid culture. There is also greater variation in microbiology and pathogenesis based on age. Finally, pediatric MSKI has greater variability between pathogenesis of infection, including the incidence of septic arthritis versus osteomyelitis.

Strength of Recommendation: Moderate

Rationale

While the overall quality of studies examining the pathophysiology of pediatric musculoskeletal infection is relatively low, limited mostly to single center or small multicenter retrospective studies, there are several unique themes that be elucidated relative to adult MSKI.

The first unique feature of pediatric MSKI is the higher incidence of *Kingella kingae* relative to adult MSKI. While methicillin-susceptible and -resistant *Staphylococcus aureus* are common microorganisms in osteoarticular and spinal infections in pediatric patients similar to adults, one of the most common organisms in many anatomic locations is *K. kingae,* a Gram-negative facultative anaerobic coccobacillus that colonizes the oropharynx in children [1,5,7,8,11,12,15,19,47,48]. *K. kingae* is more likely to be a causative microorganism in acute *versus* chronic osteoarticular infection, more likely to cause septic arthritis *versus* osteomyelitis, and more likely to be present in younger (<4 years) *versus* older children [1,2,4,8,12,27]. Unique features of *K. kingae* include the high rates of negative traditional tissue and synovial fluid cultures, with the use of nucleic acid amplification tests needed to detect this microorganism in many cases [4,16,1,28,29]. This stresses the importance of use of diagnostic methods beyond traditional tissue culture in this patient population [37,29]. *S. aureus* appears to be more common in chronic osteomyelitis, the upper extremity, older children, and cases of pyomyositis [6,9,10,14,32]. In children with hematological or immunological issues, other less common microorganisms may be major sources of osteoarticular infection. For instance, in pediatric patients with sickle cell disease, *Salmonella* species are the most frequent cause of osteoarticular infection [17,34]. Additionally, in endemic areas, microorganisms such as *Mycobacterium tuberculosis* and *Brucella* species should be considered [18,23,25,33].

The second important feature unique to pediatric MSKI is the variation in microbiology and pathogenesis based on age. Age at presentation for pediatric MSKI affects the microbiology of infection, with infants less than three to six months of age having a higher incidence of infection with microorganisms such as *Streptococcus agalactiae* and *Escherichia coli* relative to older children [3,21]. For children ages 6 months to 4 years, *K. kingae* is very common in addition to

more typical microorganisms such as *S. aureus* [21,26]. In older children, the microbiology becomes more like adult MSKI, with *S. aureus* being most common [21,26]. In sexually active children, *Neisseria gonorrhoeae* MSKI must be considered [21].

The third unique feature of pediatric MSKI is variability between pathogenesis of infection, including the incidence of septic arthritis *versus* osteomyelitis, which is associated with unique host and microorganism features predisposing to different patterns of infection and virulence. For instance, older children are more likely to have isolated osteomyelitis, or osteomyelitis and septic arthritis combined than younger children, who are more likely to have an isolated septic arthritis [22,24]. Some studies have attempted to create decision tools based on clinical criteria to determine the need for advanced imaging to rule out adjacent septic arthritis in the setting of osteomyelitis, and to different noninfectious diagnoses like transient synovitis *versus* infectious etiologies [40-45]. Other studies have attempted to identify differences in host immunological response and unique bacterial features that correlate with severity of illness. For instance, a serum cytokine assay consisting of transforming growth factor alpha, interleukin (IL)-7, IL-33, and IL-28A correctly classified 20 of the 22 cases of septic arthritis from transient synovitis with a sensitivity and specificity of 90.9% (95% confidence interval: 73.9%-100.0%) [46]. Additionally, cytokine assays may be useful for differentiating septic arthritis from osteomyelitis in pediatric patients, with an assay of CTx-II, transforming growth factor alpha (TGF-α), monocyte chemoattractant protein 1 (MCP-1), B cell-attracting chemokine 1 showing promise [49]. In acute hematogenous osteomyelitis (AHO) due to methicillin-resistant *S. aureus* (MRSA), underexpression of adaptive immunity (lymphocyte activation, and T-cell/NK cell/ and B-cell activity) and STAT4 downregulation and genomic heterogeneity of MRSA strains were correlated with increased severity of illness [30,31]. Another study found a vancomycin MIC >1.5 microgram/ml for methicillin-susceptible *S. aureus* (MSSA) as an increased risk for post-treatment complications, such as multiple débridements and venous thromboembolism, suggesting it may be a marker of microbial virulence [36]. A third study found that MSSA isolates with cefazolin inoculum effect (which confers some resistance to first generation cephalosporins) and characterized by *agr*III were more likely to progress to chronic osteomyelitis relative to other strains, suggesting that antibiotic resistance patterns may be a marker of virulence [38]. Another study found associations with certain single nucleotide polymorphisms (SNPs) and susceptibility to AHO [35]. These microorganism specific pathological features may account for the spectrum of infection seen in the pediatric population. For instance, one USA100 isolate of MRSA from pediatric musculoskeletal infection showed a predilection for septic arthritis in an acute hematogenous mouse model of infection relative to other isolates tested, which more frequently resulted in osteomyelitis or a mixed osteomyelitis and septic arthritis [39].

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QUESTION 34

Does antibiotic use in patients with periprosthetic joint infection (PJI) increase the risk of antibiotic resistance compared to the general population?

Hyonmin Choe.

Response/Recommendation: Yes

A higher percentage of antimicrobial-resistant organisms has been reported for PJI than in the general population. Furthermore, causative organisms of PJI reportedly acquire antimicrobial resistance after treatment. These findings suggest that the antimicrobials used in the treatment of PJI may contribute to the acquisition of further antimicrobial resistance in the causative organisms of PJI compared with the general population.

Strength of Recommendation: Strong

Rationale

1. Background:

The treatment of musculoskeletal infections (MSKI) has been a constant battle against antimicrobial resistant (AMR) bacteria. Although the use of antibiotics is an important therapeutic option in the treatment of bacterial infections in the orthopedic field, unnecessary use of antibiotics can lead to the development of resistant bacteria. With the current high prevalence of multidrug resistant (MDR) bacteria[1], it is necessary to confirm antibiotic susceptibility before using antibiotic agents for the treatment of MSKI.

PJI is often difficult to treat due to the formation of a biofilm around the implant, as well as the invasion of the causative organism into the bone and soft tissue [2]. Therefore, longer-term antibiotic treatment after surgery is recommended to improve postoperative outcomes and only the necessary antibiotics should be continuously administered. However, the continuous administration of unnecessary broad-spectrum antibiotics that may accelerate AMR was often observed in the treatment of PJI due to the lack of identification of the causative organism.

The purpose of this systematic review was to search the literature for the clinical question, “Does antibiotic use in patients with PJI increase the risk of antibiotic resistance compared to the general population?”

Papers were searched by using the following formula in PubMed and Cochrane Library:

("Periprosthetic joint infection"[Title/Abstract] OR "prosthesis related infections/drug therapy"[MeSH Terms]) AND ("Anti-Bacterial Agents"[MeSH Terms] OR "Anti-Bacterial Agents"[Title/Abstract] OR "agents anti bacterial"[Title/Abstract] OR "Anti-Bacterial Agents"[Title/Abstract] OR "Antibacterial Agents"[Title/Abstract] OR "agents antibacterial"[Title/Abstract] OR "Antibacterial Agent"[Title/Abstract] OR "agent antibacterial"[Title/Abstract] OR "anti bacterial compounds"[Title/Abstract] OR "anti bacterial compounds"[Title/Abstract] OR "anti bacterial agent"[Title/Abstract] OR "anti bacterial agent"[Title/Abstract] OR "anti bacterial compound"[Title/Abstract] OR "anti bacterial

compound"[Title/Abstract] OR "Bacteriocidal Agents"[Title/Abstract] OR "Bacteriocidal Agent"[Title/Abstract] OR "Bacteriocide"[Title/Abstract] OR "Bacteriocides"[Title/Abstract] OR "anti mycobacterial agents"[Title/Abstract] OR "anti mycobacterial agents"[Title/Abstract] OR "anti mycobacterial agent"[Title/Abstract] OR "anti mycobacterial agent"[Title/Abstract] OR "Antimycobacterial Agent"[Title/Abstract] OR "Antimycobacterial Agents"[Title/Abstract] OR "agents antimycobacterial"[Title/Abstract] OR "Antibiotics"[Title/Abstract] OR "Antibiotic"[Title/Abstract]) AND ("Antimicrobial Drug Resistance"[Title/Abstract] OR "Antimicrobial Drug Resistances"[Title/Abstract] OR "antibiotic resistance microbial"[Title/Abstract] OR "Antimicrobial resistance"[Title/Abstract] OR "drug resistance, microbial"[MeSH Terms])

As a result, 237 papers were identified in PubMed and 2 papers in Cochrane Library. Among them, 38 papers were considered relevant to the question based on their titles and abstracts and were reviewed.

2. Profile of causative bacteria in PJI

The use of cephalosporins during initial THA and TKA is important to prevent the occurrence of surgical site infection (SSI), whereas methicillin resistant *staphylococcus* (MRS) is the causative organism in cases of early infection after THA and TKA[3]. In a survey of North American and European hospitals with admissions of patients with PJI, *S. aureus* was the most common causative organism, followed by *S. epidermidis*, and 58% of these organisms were resistant to at least one antimicrobial agent, with hips (62.3%) being particularly resistant compared to knees (52.6%)[4]. It has been reported that the use of low-dose antimicrobials is associated with antimicrobial resistance of bacteria and the formation of a biofilm[5]. In addition, the coagulase-negative *Staphylococcus* (CNS), which is endemic in the nasal cavity and inguinal region, acquires antimicrobial resistance after THA and TKA due to prophylactic antibiotic use[6].

One of the characteristics of the antimicrobial use in orthopedic surgery is local antimicrobial therapy using antimicrobial cement. Several studies have demonstrated that the use of antibiotic-loaded bone cement (ALBC) in primary THA and TKA is associated with the development of antimicrobial resistance[7,8], although one study indicated that ALBC use was not associated with the acquisition of AMR [9]. Further studies are needed to determine the association between the use of topical slow-release antimicrobial agents and the acquisition of antimicrobial resistance[10].

Gram-positive bacteria are the most common species of PJI, with *S. aureus* being one of the most common causative bacteria[11-13]. However, the bacterial profile of PJI has changed over time, with reportedly increased CNS infections [14 15]. In addition, the proportion of causative organisms and AMR vary widely among regions and countries[4,16-18]. Polymicrobial infections or gram-negative bacteria (GNB) infections become not uncommon in PJI[12]. In PJI due to GNB, reports of progressive resistance to antimicrobial agents have been widely recognized[17,19,20] and MDR has been associated with a history of orthopedic surgery in GNB[21]. The frequency of resistance varies among antibiotics in the causative bacteria of PJI[11,12,15,17,22] and CNS and GNB frequently possess AMR with an increasing trend of MDR[19,20].

3. Acquisition of antimicrobial resistance of bacteria in PJI treatment

In recent years, MDR bacteria have been on the rise worldwide, and have been implicated in the failure and increased cost of treatment of orthopedic infections[1]. Particular attention should be

paid to the increasing resistance of CNS infections to antimicrobial agents, especially in the older adults[1,23]. Furthermore, difficulties in the identification of causative organisms and acquisition of AMR have been widely reported in orthopedic infections caused by a subtype of bacteria called the small colony variant (SCV) [24-29]. Furthermore, heterogeneity of AMR, especially to gentamicin and vancomycin, has been observed in the same patient with the same strain[30]. In addition, PJI caused by a subtype of bacteria called SCV has been reported as a factor in poor outcomes of PJI treatment [29].

Although various reports have been published on the mechanisms of antimicrobial resistance, there are two major mechanisms by which causative organisms become resistant to antimicrobial agents: intrinsic and acquired resistance[23,31,32]. Bacteria can be intrinsically resistant to antibiotics owing to their inherent structural or functional characteristics[1,23]. Bacteria can acquire resistance to certain antibiotics through chromosomal mutations or obtain antibiotic resistance genes from other bacteria. Based on previous data, rifampicin, which is a commonly used antimicrobial agent for PJI [33,34], can easily cause antibiotic resistance when used alone. It has also been reported that bacteria can acquire resistance to RFP even when treated in combination with vancomycin, and this is thought to be due to the fact that RFP is the only antibiotic agent that can reach the local area in combination therapy with RFP and antibiotic with poor tissue migration, such as vancomycin[35]. Another report demonstrated that the use of rifampicin during treatment tends to cause the causative organism to acquire resistance to rifampicin in patients who have undergone three or more revision surgeries for PJI, or in patients with a high bacterial load at the time of PJI surgery [36].

In a two-stage revision procedure for 30 patients with PJI who had persistent *S. aureus* infection, nine patients (30%) were reported to have increased minimum inhibitory concentration (MIC) values against vancomycin. Although vancomycin resistant *staphylococcus* did not appear, changes in MIC against vancomycin suggest that bacteria remaining in PJI treatment may acquire resistance to antimicrobials during a two-stage revision procedure [37]. Another report demonstrated that 10 of 142 patients with PJI (7%) who underwent a two-stage revision had emergence of antibiotic resistance during that time [38]. Genotype and phenotype changes and mutations in antimicrobial resistance were also observed in PJI caused by *S. epidermidis* before and after treatment for PJI [39]. In addition to the conventional method based on MIC values, a recent study reported the utility of multiplex polymerase chain reaction and next-generation sequencing for the detection and monitoring of AMR [40,41]. Further studies on the genetic profiling of bacteria are required to clarify the mechanism of resistance acquisition in PJI.

4. Conclusion

Prolonged use of inappropriate antimicrobials may contribute to the emergence of further AMR through intrinsic or acquired mechanisms for the causative bacteria in PJI, in which CNS and GNB with MDR are increasingly prevalent. Further studies with genetic profiling of causative organisms before and after PJI are required to clarify the effect of PJI treatment on the emergence of AMR.

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04. In vitro

QUESTION 35

Are there general[izable] negative and positive controls that be incorporated within all biofilm experiments to allow cross-referenc[ing] between experiments?

Kapil Raghuraman, Matthew Libera

RECOMMENDATION: Unknown

Generally, unmodified controls are indicated, where cross-referencing is not well-established and standard testing protocols need to be established for control surfaces to benchmark experiments.

Strength of evidence: Limited

RATIONALE

Background

Mechanisms to inhibit bacterial colonization and biofilm formation have recently gained popularity amongst both academics and industry stakeholders because infection remains one of the most common but poorly addressed modalities for failure of orthopedic implants. Although there is a large literature base describing novel surface modifications or materials intended to prevent microbial colonization, this work has largely been isolated in nature, as studies are published on individual agents. The lack of systematic reviews on antimicrobial technology or the consistency in how these studies are carried out has resulted in a growing field with no well-established positive or negative controls for benchmarking the performance of a new antimicrobial surface.

As such the question was restated as follows:

Restatement of the Questions, with associated Response, Strength of Recommendation and Rationales:

1a. Should negative and/or positive controls be incorporated into biofilm experiments aimed to demonstrate colonization resistance of a candidate antimicrobial surface, whether it is eluting or non-eluting?

- Response/Recommendation: Although the available literature is wide and variable based on anatomy, application of various antimicrobial surfaces and target pathogens, consistency

exists in the use of controls to benchmark antimicrobial efficacy to unmodified surfaces or uninoculated surfaces. As such, it is recommended that positive and negative controls be incorporated into biofilm experiments, despite the underlying mechanism of action of the surface considered.

- Strength of the Recommendation: The strength of the recommendation that negative and/or positive controls should be included in the evaluation of candidate antimicrobial surfaces is strong.
- Rationale: Experimentally, those studies that include well-constructed controls are generally more credible and impactful than those that do not. A systematic literature review for this question was complicated by the absence of comparative studies of various novel antimicrobial surfaces and the dual meaning of the word “control” meaning “prevention” with respect to biofilm prevention and the alternative meaning of “experimental baseline group”. Cursory searches performed on PubMed between July and August 2022 for “novel antimicrobial surface” yielded 10520 results, with 4065 within the past 5 years, indicating a steady rise in the exploration of biofilm prevention technologies. More specifically, narrowing the scope to “narrow antimicrobial surface orthopedic” yields 186 results, with 103 in the past year. A cursory review of the 103 papers in this search suggested that, in general, well cited studies utilized an unmodified surface control. This was independent of the application in craniofacial, spinal or large joint interventions, and regardless of substrate.

1b. Are there general negative and/or positive controls that should be incorporated within all biofilm experiments to allow cross-referencing regarding the relative antimicrobial performance of different candidate surfaces?

- Response/Recommendation: While there is some literature that addresses the need to develop standards, there is relatively little published literature addressing the issue of specific protocols and which communities these might serve.
- Strength of the Recommendation: The strength of the recommendation that general control experiments and protocols are currently available to enable cross-referencing between different laboratories and different candidate materials systems is weak.
- Rationale: While there is substantial anecdotal agreement that generalizable protocols and test methods for benchmarking materials development is desirable, there currently is little consensus on what those protocols might be given the diversity of interests and applications. As suggested in the rationale for 1a, the literature is fragmented in its approach to standardization of evaluation for antimicrobial surfaces. Many studies have pointed to the use of an unmodified surface as a positive control to benchmark comparisons to the subject group, though given the diversity of the surfaces considered, are not consistent [1-7]. Similarly, there exists a lack of consistency in the dosing and measurement of antimicrobial efficacy across these studies, suggesting a lack of consensus. It is recommended that a positive control be included with an unmodified surface, though further consideration must be given to understand whether a similar negative control can be incorporated. Additionally, further study is required to establish whether a control group exists with universal applicability to any novel antimicrobial surface.

1c. Can general negative and positive controls be developed to incorporate within all biofilm experiments to allow cross-referencing regarding the relative antimicrobial performance of different candidate surfaces?

- Response/Recommendation: Following 1b, there is recognition in the community, particularly those seeking or providing regulatory approval, that standard testing protocols will enhance the ability to make claims regarding the ability of a surface to resist colonization and biofilm formation.
- Strength of the Recommendation: The strength of the recommendation that general control experiments can be developed is moderate.
- Rationale: There are ongoing efforts internationally by subcommittees within such organizations as the ISO and the ASTM to develop standards. The process is nevertheless slowed by competing interests and needs of various stakeholders working in different sectors of orthopedics.

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Although the references included in these rationales are limited in their utility, as the focus of these studies is largely on the novel materials rather than the associated experimental controls, there is an identified consistency in the use of an unmodified surface as a positive control.

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QUESTION 36: Can antibiotic tolerance be used to support the presence and maturity of a biofilm?

Dacheng Ren and Paul Stoodley

RECOMMENDATION: No

We found consistent lab data showing that antibiotic tolerance increased with biofilm age. However, since the degree of tolerance depends not only on the age of the biofilm but many other factors, a single measurement cannot be used to determine whether a biofilm is mature. This emphasizes the needs for future studies to better understand the correlation between antibiotic susceptibility and biofilm growth in vivo.

Strength of evidence: Limited

RATIONALE

Main findings and insights: Multiple laboratory-based studies report that older biofilms have higher tolerance to antibiotics than early-stage biofilms. Changes in antibiotic susceptibility have been attributed to presence and changes in the biofilm matrix, change in bacterial metabolism, increases in efflux pump activity, and alterations of antibiotic target and bacterial membrane permeability to antibiotics. The degree of tolerance is dependent on the antimicrobial agent, the species, the treatment exposure time and the model system. Detection of increased tolerance ranged from 24 hrs to 3 weeks of biofilm age. Many studies only had two time points or did not reach a plateau, so it was not possible to determine what age was required for maximum tolerance. In addition the identified articles did not provide a clear definition of what time scale or phenotype constitutes as being “mature”. Also the methods and metrics used to quantify tolerance varied between the studies. Meanwhile, there are reports showing no significant difference or the opposite trend of biofilm age vs. antimicrobial susceptibility. No clinical information apart from an *ex vivo* clinical wound debridement containing biofilm (Wolcott *et al.*, 2010), was identified by our search. Caution should be taken when predicting *in vivo* antimicrobial susceptibility using *in vitro* platforms especially those that lack the factors that mimic the *in vivo* environment in the host.

Can antibiotic tolerance be used to support the presence and maturity of a biofilm? We found consistent lab data showing that antibiotic tolerance increased with biofilm age. However, since the degree of tolerance depends not only on the age of the biofilm but many other factors, a single measurement cannot be used to determine whether a biofilm is mature. Our search found no direct clinical evidence that antibiotic tolerance supported the presence of a biofilm, although extrapolations from laboratory studies coupled with a poor response to antibiotic therapy as predicted by susceptibility frequently leads to an assumption that a biofilm must be present. This emphasizes the needs for future studies to better understand the correlation between antibiotic susceptibility and biofilm growth *in vivo*.

Search strategy and parameters: This question had two parts, can antibiotic tolerance be used to support the *presence* and *maturity* of a biofilm? To simplify the search we focused on biofilm maturity and antibiotic tolerance. Relevant literature was collected by searching PubMed. English language was used as a filter. A search on June 16, 2022, using the keywords ["biofilm age" AND antibiotic tolerance] yielded 6 articles (Donlan, 2008, Cheow *et al.*, 2010, Bernier *et al.*, 2013, Flores-Treviño *et al.*, 2019, Hiltunen *et al.*, 2019, Chen *et al.*, 2020). Another search on July 12, 2022 using the keywords ["biofilm maturity" OR "biofilm age" AND antibiotic susceptibility] yielded 12 articles (Korber *et al.*, 1997, Monzón *et al.*, 2002, Leriche *et al.*, 2003, Cheow *et al.*, 2010, Wolcott *et al.*, 2010, Kwiecińska-Piróg *et al.*, 2013, Olsen, 2015, Fallatah *et al.*, 2019, Flores-Treviño *et al.*, 2019, Hiltunen *et al.*, 2019, Babushkina *et al.*, 2021, Swimberghe *et al.*, 2021). Two articles appeared in both searches (Flores-Treviño *et al.*, 2019, Hiltunen *et al.*, 2019) and the two Cheow et al. 2010 articles did not directly address biofilm maturity and antimicrobial susceptibility and so were excluded. The Flores-Treviño article was a review article that did not specifically review papers dealing with biofilm susceptibility as a function of age but did note that as biofilm ages the structure and chemistry of the EPS changes and this will likely affect antibiotic susceptibility. The Donlan, 2008 (Donlan, 2008) article was a review presenting no direct evidence and so was also excluded but identified two articles of relevance (Anwar *et al.*, 1992, Amorena *et al.*, 1999). In addition two other relevant articles directly addressing the question were identified in related literature searches and reference checking (Shen *et al.*, 2011, Babushkina *et al.*, 2021) for a total of 17 articles from which we drew our main conclusions.

Brief summary of the articles

Amorena et al. (Amorena *et al.*, 1999) found that 48 hr *S. aureus* biofilms were more tolerant to a range of antibiotics than 6 hr biofilms. Similarly, in a series of 3 papers Anwar et al. (Anwar *et al.*, 1992) found that 7 days *P. aeruginosa* biofilms were more tolerant of tobramycin and piperacillin than 2 day biofilms. Babushkina et al. (Babushkina *et al.*, 2021) compared the antibiotic susceptibility of 15 clinical isolates of *S. aureus* and found 48 hr biofilms were more tolerant to ceftriaxone, vancomycin, ciprofloxacin, and levofloxacin than 24 hr biofilms by a factor of between 2 and 5 times (Babushkina et al., 2021). Similar results from the same group were found for clinical isolates of *P. aeruginosa* exposed to levofloxacin, ciprofloxacin, ceftriaxone cefoperazone or amikacin, as assessed by “minimum inhibition concentrations inducing death of 90% bacterial cells (MIC90)” (Babushkina *et al.*, 2021). Bernier et al. (Bernier *et al.*, 2013) found that over 24, 48 and 72 hr biofilms of *E. coli* became more tolerant of oxacillin but there was no further increase in (??) tolerance exhibited at 96 hrs suggesting that 4 days were required to achieve maturity in this system. The increasing tolerance was related to starvation and the SOS response. Chen et al. (Chen *et al.*, 2020) reported a general decreased susceptibility in 72 hr vs 24 hr biofilms formed by strains of *S. aureus* and *P. aeruginosa*, treated with vancomycin or tobramycin respectively. However, the degree of tolerance was influenced by media type, antibiotic concentration and treatment duration, as well as biofilm age. Besides antibiotics, Fallatah et al. (Fallatah *et al.*, 2019) noticed that biofilm age affects bacterial susceptibility to graphene oxide (GO). Interestingly, GO detached 48-hr *P. putida* biofilms and reduced bacterial viability by causing membrane damage. However, the viability of 24- and 72-hr biofilm cells and detached cells was not affected (Fallatah *et al.*, 2019). Biofilm matrix also plays a role in antibiotic tolerance, as speculated by Flores-Treviño et al. (Flores-Treviño *et al.*, 2019) because the matrix can block or absorb certain antimicrobials, e.g., positively charge aminoglycosides and polypeptides. Biofilm age affects its

structure and matrix composition, and thus, the susceptibility to antimicrobials (Olsen, 2015). Similar, biofilm age was found to affect the matrix composition of *S. aureus* biofilms, although its younger biofilms did not also show higher antibiotic susceptibility than more aged biofilms (Hiltunen *et al.*, 2019). In a study of clinical isolates of *Proteus mirabilis*, ciprofloxacin was more effective in eradicating 24-hour biofilm than 12-hr biofilms, while the opposite was observed for ceftazidime (Kwiecińska-Piróg *et al.*, 2013). In a study of clinical isolates of *S. epidermidis*, resistance profiles obtained by broth microdilution and diffusion did not provide a good estimate of biofilm killing. In addition, only vancomycin but not rifampicin or tetracycline showed a major decrease in killing aged biofilms (48 hrs) than younger biofilms (24 hrs) (Monzón *et al.*, 2002). The role of biofilm age in antimicrobial tolerance was also reported for non-pathogenic bacteria. For example, Leriche et al. (Leriche *et al.*, 2003) studied the effects of a commercial alkaline chlorine solution (22 mg/L of free chlorine, pH 11) mixed-species biofilms of *Kocuria sp*. C714.1, *Brevibacterium linens* B337.1 and *Staphylococcus sciuri* CCL101. The results showed that aged biofilms were more tolerant to the treatment and the production of exoprotein was important for the protection by neutralizing chlorine. This also affected the dynamic change in the population of the three species (Leriche *et al.*, 2003). One day biofilms of *E. faecalis* was found more susceptible to NaOCl than 11-day biofilms, although the number of CFU did not differ significantly (Swimberghe *et al.*, 2021). In glass flow cells, 10% trisodium phosphate (TSP) solution was found to kill 48-hr *Salmonella enteritidis* biofilms more effectively than 72-hr biofilms, and the crevices on the glass surface protect biofilm cells (Korber *et al.*, 1997). Shen et. al. (Shen *et al.*, 2011) specifically investigated the influence of developmental age of multi species biofilms grown from dental plaque on susceptibility to chlorhexidine. Viability was assessed microscopically using live/dead differential staining and scanning electron microscopy. The proportion of killed bacteria in mature biofilms (3 weeks) was lower than in young biofilms (of 2 days, 1 and 2 weeks). Under nutrient limitation the resistance profile was similar in 3, 6 and 12 week old biofilms. Wolcott et al. (Wolcott *et al.*, 2010) studied the antibiotic susceptibility of *S. aureus* and *P. aeruginosa* biofilms using four different models: an *in vitro* drip-flow biofilm model where biofilms were either exposed to gentamicin or “hydrodebridement” in which they were sprayed for 20 s with a jet of saline, a mouse surgical excision wound model where biofilms were exposed to gentamicin or bleach treatment, an *ex vivo* porcine explant biofilm model where *P. aeruginosa* biofilms were treated with gentamicin and *S. aureus* biofilms were treated with oxacillin, and a clinical longitudinal debridement study. In the drip flow biofilm study 12 hr *P. aeruginosa* biofilms were reduced by 3 logs after treatment with gentamicin; however, for 24 hr biofilms there was no log reduction relative to the untreated controls. *S. aureus* showed a similar response but over a longer ageing scale with the 24 hr biofilm being reduced by 4 logs with a steady reduction in the log reduction as a function of biofilm age when for 96 hr biofilms there was no reduction compared to the untreated control. In the mouse study the susceptibility of *P. aeruginosa* biofilms diminished with age up to 4 days but there was no further tolerance after 5 days. Similarly in the porcine model *P. aeruginosa* and *S. aureus* 24 hr biofilms showed a 4 and 6 log reduction respectively compared to untreated controls but as the biofilms aged the log reduction diminished until by day 5 there was only an 0.5 log reduction. Interestingly, in the debrided material from 3 venous leg ulcers showed that 24 hr after excision the biofilms were more susceptible to gentamicin, presumably because of disruption of the biofilm; however, they became less susceptible with aging between 2 (1 patient) and 3 (2 patients) days. It was notable that gentamicin achieved a 4 to 5 log reduction even in the mature and 3 day old “less susceptible” specimens.

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QUESTION 37. Can drug clearance and protein binding be modeled in an in vitro system to predict efficacy of drugs?

Ebru Oral and Bingyun Li

RECOMMENDATION: Yes

Although there are many factors which can change the relationship between predicted concentration and efficacy for antibiotics, modelling work may present a good correlation between modelling and in vitro and/or in vivo experiments. There is currently no standard methodology for simulating these factors, especially for intra-articular/target site administration of drugs.

Strength of evidence: Moderate

RATIONALE

Summary: Yes. PK/PD modeling of antibiotic drugs based on route of administration, clearance, and other factors such as protein binding are necessary to evaluate the clinically relevant concentrations of drugs in vitro. Although there are many factors which can change the relationship between predicted concentration and efficacy for antibiotics, modeling work may present a good correlation between modeling and in vitro and/or in vivo experiments. There is currently no standard methodology for simulating these factors, especially for intra-articular/target site administration of drugs.

Pharmacokinetic/pharmacodynamic parameter determination in humans and in animals with local (musculoskeletal) administration

To predict the concentration of given drugs in the plasma and local tissues of interest, one or two-compartment pharmacokinetic/pharmacodynamic (PK/PD) models with variations for route of administration and/or clearance mechanism are commonly used and are necessary as part of the development of new antibiotic drugs [1]. In these models, the 'compartments' are areas where monitoring of concentration is desired. The volume of distribution for the drug in these compartments is a theoretical number that is calculated by the extrapolation of the dose (in mass) over the measured concentration (mass/volume) in the compartment of interest. In general, more hydrophobic drugs tend to have higher volumes of distribution. These PK/PD models are constructed by using mathematical descriptions of feed amounts into the desired volume (such as intestinal absorption for oral route of administration into the blood stream) and clearance from the desired volume (such as by hepatic metabolism or excretion clearance through the kidney) [2-4]. There were no PK/PD models for antibiotics using intra-articular administration.

Relationship between concentration and efficacy

To predict the efficacy of an antibiotic *in vivo*, other dependent factors may be important. The type of antibiotic itself (solubility) and its route of administration determines the concentration at the desired site of infection. For a known concentration, the efficacy is dependent on the organism in question and the mode of action of the antibiotic. The relationship between drug concentration (dose) and exposure duration are described in different ways with respect to the minimum inhibitory concentration (MIC) of the drug for the strain of bacteria of interest. For example, time above MIC (t>MIC), the ratio of area under the concentration-time curve (AUC) to MIC (AUC/MIC) can be used [4-6]. For aminoglycosides, the maximum plasma concentration (C_{max}) to MIC ratio of 8-10 is used [7]. Clinical data remains limited for some target site-antibiotic exposure relationships.

Factors shown to have effects on PK/PD parameters for consideration

To have a good correlation with in vivo data, the PK/PD models must consider various variables including host factors (e.g., host's cellular response), protein binding, the site of drug release, nature of the disease, and the pharmacokinetics of the drug and its penetration into areas of disease [8].

The fraction of unbound drug is very important in predicting the efficacy and clearance of antibiotics from vascular spaces. In plasma, the major proteins that are involved in binding to drugs are albumin and alpha1 acid-glycoprotein (AGP). Protein binding depends on the types of proteins [9-11], and may prolong the drug clearance while it may also make some drugs less effective or have no effect on the efficacy of some antibiotics such as Fosfomycin [12]. Extended plasma protein binding can also be associated with adverse effects, like low clearance, low brain penetration, and drug–drug interactions. The fraction of antibiotic binding to plasma proteins can vary greatly (Table 1). While the major factor for protein binding in plasma is albumin concentration, whether protein binding can affect the efficacy of antibiotics with local administration is unknown.

Table 1. Literature on plasma protein binding fractions of different antibiotics

Antibiotic	**PPB fraction**	**Model/Route (IV or IM)**	**Reference**
Moxifloxacin	28.4+/-3.77%	Buffalo calves	[13]
Cefuroxime	13.1-21.6%	Goats	[14]
Difloxacin	28-43%	Dromedary camels	[15]
Orbifloxacin	14.76%	Korean Hanwoo cattle	[16]
Danofloxacin		Camels	[17]
Gentamicin sulphate	16.8, 11.0, 8.0%	Lambs, calves, foals	[18]
Ceftazidime	13.1-21.6%	Rabbits	[19]
Enrofloxacin		Pigs	
Marbofloxacin		Sheep	177
Meropenem	42.8%	Ewes	[20]
Ampicillin	20%	In vitro	[21]
Moxifloxacin	26%		
Oxacillin	60-94%		

Solubility of antibiotics in bone are variable: beta-lactams display bone: serum concentration ratios ranging from ~0.1 for oxacillin to ~1 for cefepime with vancomycin around 0.2. Fluoroquinolones, which are hydrophobic and have high volumes of distribution, have higher bone:serum ratios ranging up to ~0.75 for levofloxacin. Studies are lacking for aminoglycosides [4]. In addition to plasma protein binding of drugs having an effect on concentration at target site, the infectious state may also alter drug concentration [4].

In vitro and preclinical models for PK prediction

The effects of binding in in-vitro medium are studied by the addition of serum or serum proteins, most commonly albumin to the growth or drug exposure medium. A commonly used percentage is 4% albumin by other concentrations have been shown to affect efficacy without reaching protein binding levels observed in pure serum [22]. The temperature and pH of the medium as well as the type of medium itself can have an effect on the efficacy of the drug in combination with the presence of albumin in the medium [12].

In-vitro models of efficacy testing have been developed by applying PK parameters obtained from in-vivo plasma concentrations and using different chamber set-ups with flowrates based on plasma half lives [23-26]. This type of in-vitro model was able to predict the in vivo antimicrobial effects of multiple antibiotics (i.e., cefuroxime, cefamandole, ceftriaxone, and ceftazidime) [27]. PK/PD models may also be developed and applied to predict potential therapeutic outcomes in vivo, although the models are more complex and the correlation is less reliable (compared to in vitro), even though some progress has been made.

In general, pharmacokinetic/pharmacodynamics (PK/PD) models have been studied to reasonably reveal the relationship between drug levels and in vitro biological responses. Some PK/PD modeling work showed good correlation between modeling and in vitro experimental data. For instance, an in vitro physiologically based PK model showed accurate correlation in the depletion of methyl-3-quinoxaline-2-carboxylic acid (marker residue of olaquindox) in pig tissues at long time (e.g., 23 days) antibiotic treatment [28]. PK/PD model was also found to be useful in describing and predicting the in vitro bacterial inoculum effect of certain antibiotics like ceftazidime against *Pseudomonas aeruginosa* [29]. Moreover, PK and PK/PD models were successfully applied to reliably correlate the in vitro therapeutic outcomes with drug uses including both a monotherapeutic treatment with vertilmicin and combination therapies of vertilmicin and ceftazidime [30]. In the combination therapy, the drug-drug interaction was considered in the PD model. Overall, in vitro modeling can be useful in guiding the design and optimization of therapeutic regimens for maximal therapeutic effects.

A common preclinical model used in the determination of the pharmacodynamics of antibiotics is the murine thigh model where bacteria are inoculated in the thigh and bacterial eradication efficacy is determined as a function of plasma concentration via different routes [26, 31-33]. Correlations may be possible from this animal model to the human [34].

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QUESTION 38. Can in vitro antimicrobial efficacy (CFU log 2 or log 3 reduction) be used to achieve the minimum rationale for moving into the animal?

Tianhong Dai and Britt Wildemann

RECOMMENDATION: Yes

Although no clear recommendation can be given on how to translate in vitro results of antibiotic treatment into in vivo efficacy, in vitro data provide important information before proceeding to animal models. The commonly used in vitro models are very limited and do not take into account the complex in vivo situation (host environment, inflammation, bacterial susceptibility, etc.). We agree with the statement within the limitations of the models used.

Strength of evidence: Moderate

RATIONALE

In vitro tests are recommended and often performed before animal testing. The majority of the studies screened demonstrated in vitro and in vivo efficacy, while only few studies showed in vitro efficacy only. The commonly used tests to determine in vitro antimicrobial effects are Zone of Inhibition-test (Kirby-Bauer test) on agar plates, reduction of CFU in suspension or CFU count on agar plates. The in vitro models are very restricted and are far from reflecting the in vivo situation in animal models or even the human situation. Improved in vitro models could use co-culture or organ-on-chip models to mimic also e.g. the race for the surface or inflammation. We recommend the review from Moriarty et al. 2014, which gives a great overview on the current knowledge and highlights the limitations and strength of in vitro and in vivo models to investigate antimicrobial effects in the context of device-associated infections. A clear recommendation for the necessary CFU reduction in vitro cannot be given, but a reduction of log 2 or log 3 is often described. Whether this is sufficient to prevent or eliminate infection in vivo cannot be predicted based on the current data. Furthermore, it is a point of discussion, whether a reduction of the bacteria is sufficient to allow the immune system to eliminate the remaining bacteria, or whether complete eradication in vivo is necessary. A PubMed search with BOOLEAN operators (orthopedics AND infection AND in vitro AND CFU AND in vivo) and a free google search was performed. This resulted in 53 publications, which were further screened for eligibility. After exclusion of 35 publications (not in vitro and in vivo) and further 8 publications (not suitable), 10 publications were included in this statement. [1-10]

To correlate in vitro efficacy with in vivo efficacy and to make the results in different studies comparable, in vitro and in vivo models will need to be standardized (also optimized) by the ORS for future studies.

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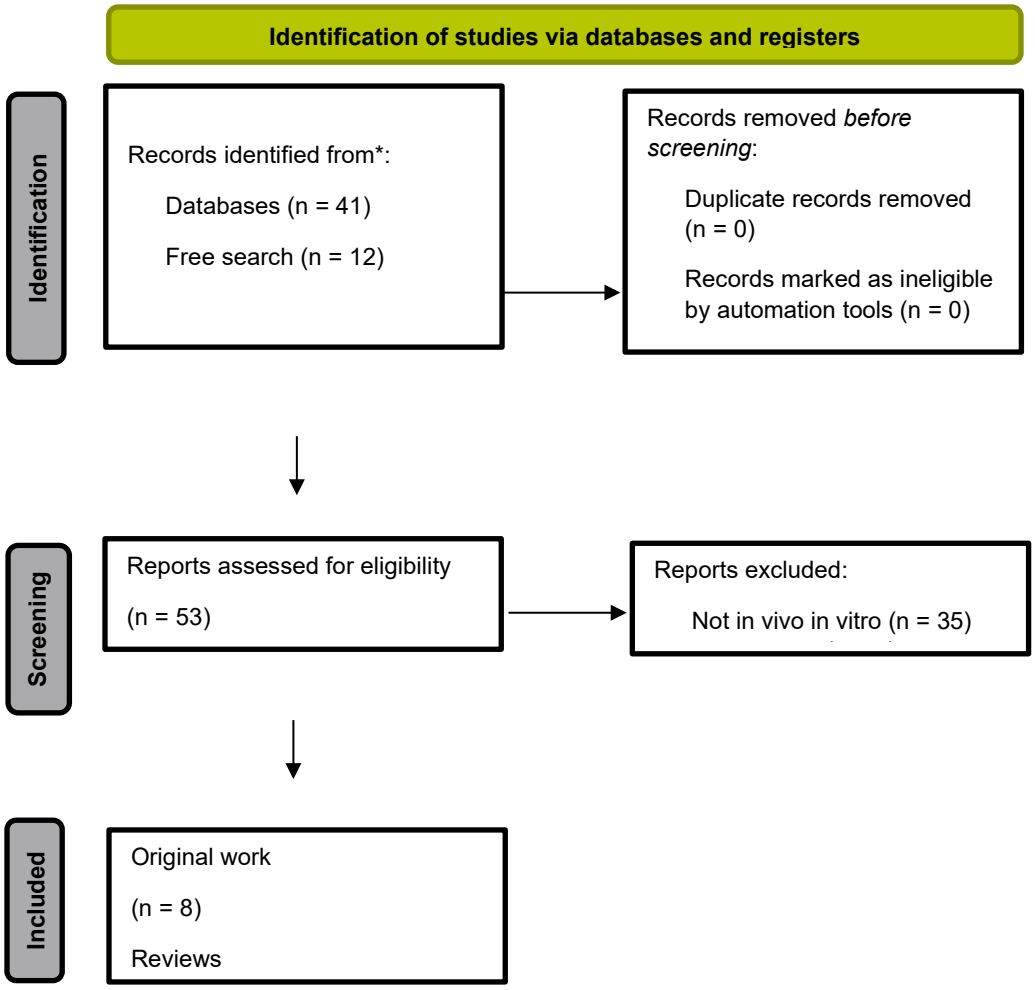
Search strategies performed May 2022

BOOLEAN operators: orthopedics AND infection AND in vitro AND CFU AND in vivo

1. PubMed search → 41 Paper; 2. Free search google, → 12 Paper



QUESTION 39: Is there a best method for assessing MBEC in vitro?

Bas Zaat and Ed Greenfield

RECOMMENDATION: No

Systematic comparison of MBEC methods for particular antimicrobials against biofilms of selected bacterial test strains have not been performed. The final aim of standardized methods is to be able to predict clinical efficacy. To the best of our knowledge no correlations between MBEC values for antimicrobial agents assessed by different methods, and their efficacy in treatment of orthopedic device infections have been reported, nor have in vivo studies related to this question been performed.

Strength of evidence: Limited

RATIONALE

MBEC assays require exposure of a pre-formed biofilm to antibiotics or other agents of interest. The biofilms can be formed in either batch or dynamic platforms.[1] The two most commonly used batch platforms are microtiter plates and the Calgary biofilm device, which is based on a microplate lid with 96 polystyrene pegs that can be inoculated by immersion into individual wells of a 96-well microtiter plate.[2] Both formats can provide highly reproducible results in inter-laboratory studies if the protocols are carefully calibrated between the laboratories.[3,4] and the Calgary device is ASTM standardized for determining disinfectant efficacy against *Pseudomonas aeruginosa* biofilms grown on the polystyrene pegs (ASTM E2799). Both microtiter plates and the Calgary device can provide relatively high throughput, which is especially important when assessing a large number of bacterial species/strains or a large number of antibiotics and/or antibiotic combinations. The Calgary device facilitates removal of planktonic bacteria by gravity during culture and by immersing the pegs in sterile buffer in 96-well plates at multiple steps in the protocol.[1,2] The Calgary device is available commercially as MBEC Assay® Kits (Innovotech, Edmonton, Canada) and MBEC analysis using this device is also offered by commercial service providers. The Calgary device is fairly expensive. However, lower-cost home-made versions have been reported recently.[5] Removal of planktonic bacteria is also facilitated in the Amsterdam Active Attachment biofilm model that has primarily been used in the dental literature.[6] In this model, coupons of various materials are suspended from clamps into culture media in a 24-well microtiter plate. Dynamic platforms for biofilm formation are generally lower throughput and include the CDC Biofilm Reactor, the Drip Flow Biofilm Reactor, and a number of recently described micro-fluidic approaches.[1] The CDC Biofilm Reactor and the Drip Flow Biofilm Reactor can provide highly reproducible results in inter-laboratory studies [7,8] and the CDC Biofilm Reactor is ASTM standardized for determining disinfectant efficacy against *S. aureus* and *P. aeruginosa* biofilms on non-porous polymer surfaces, but not for medical device surfaces (ASTM E2871 and E3161). A commercially available microfluidics device (BioFlux device, Fluxion

Biosciences, South San Francisco, CA) can also be used to measure MBECs in a plate reader format.[9,10]

MBEC results are highly method-dependent and therefore are difficult to standardize or directly relate to clinical situations.[11-13] Moreover, many important MBEC research questions are not covered by the ASTM standards described in the previous paragraph. It is therefore extremely important to follow the recognized guidelines for reporting biofilm experiments.[14,15] Crucial methodological variables to consider include the choice of bacterial species and strains (clinical isolates *versus* laboratory acclimated), inoculum preparation and quantitation, and conditions for biofilm formation, antibiotic challenge, and recovery (time, media, pH, temperature, fluid dynamics, etc.). Those variables control biofilm maturity which has dramatic effects on MBEC results.[16-23] Although biofilm formation can be documented by microscopy methods, it is difficult to assess relative levels of biofilm maturity by those methods.[1] Relative biofilm maturity can however be assessed functionally by measuring MBECs for an antibiotic whose activity is known to vary with biofilm maturity.[16-23] With regard to choice of bacterial species and strains, use of strains often used for *in vivo* infection models might provide better concordance between *in vitro* MBEC measurements and *in vivo* antibiotic effectiveness.[24,25]

Crucial methodological variables to consider also include methods to remove planktonic bacteria and to measure bacteria viability, either in intact biofilms or after disaggregation (see question #12 and [1] for details on methods to assess bacteria viability). For example, CFU assays performed better than Crystal Violet or resazurin reduction assays in the inter-laboratory study of the microtiter plate format.[4] It is also important to include a post-challenge recovery period to allow antibiotic washout/neutralization and re-activation of persister cells.[2,26] Fluorescence-activated cell sorting (FACS) based methods have been developed to characterize and quantify persister cells from planktonic cultures [27,28] but whether those methods are feasible with persister cells in biofilms is not known. Quantification of the pre-treatment biofilms is important to differentiate between reducing biofilm viability *versus* inhibiting biofilm growth during the antibiotic challenge.[29] Moreover, the criteria for effectiveness in different MBEC test systems vary from a certain fold-log reduction of biofilm cfu to complete eradication of the biofilm. For both methods the starting numbers of biofilm CFU are very important,[29] but not always standardized. When aiming for instance for a 6 log-fold reduction of biofilm CFU, at least 10^E6 CFU need to be present in the biofilms in the system, which is not always possible. In many studies the numbers of biofilm CFU per sample are not specified, making quantitative claims difficult.

Of particular interest to MSKI research, the type of substrate used for biofilm formation prior can substantially alter MBEC results. Polymeric substrates are most commonly used by the wider biofilm community. MBECs can however be measured after biofilm formation on coupons of orthopaedically-relevant substrates (titanium alloys, stainless steel, polyethylene, etc.) on disks in 24-well plates, in the CDC Biofilm Reactor, or in the Drip Flow Biofilm Reactor. Additionally, the polystyrene growth substrates in the Calgary biofilm device can be coated with a variety of materials, including titanium dioxide or hydroxyapatite. MBECs measured on pieces of cortical bone or frozen muscle can be substantially different than MBECs measured on polymeric substrates.[30,31] A recent study measured *ex vivo* MBECs using biofilms formed *in vivo* on stainless steel screws in rats.[32] It might be possible to extend that approach to clinical samples as *ex vivo* effects of antimicrobial protocols were recently assessed on biofilms formed on percutaneous steel external fixation pins in fracture patients.[33]

Systematic comparison of MBEC methods for particular antimicrobials against biofilms of selected bacterial test strains have not been performed. Therefore, the question "**What is the best method for assessing MBEC in vitro"** is difficult to answer. The final aim of standardized methods is to

be able to predict clinical efficacy. To the best of our knowledge no correlations between MBEC values for antimicrobial agents assessed by different methods, and their efficacy in treatment of orthopedic device infections have been reported, nor have in vivo studies to this question been performed. This would be a very valuable area of future studies to help decide on the best standard method(s) for MBEC testing.

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QUESTION 40: Is there a “race for the surface” between bacteria and host cells that determines the clinical outcome of orthopaedic implant surgery?

Débora C. Coraça-Huber, Christopher Spiegel, Bingyun Li

RECOMMENDATION: No

There is not a race because the host cells usually colonize first. Without clearing by antimicrobial treatment or immune response, bacteria will eventually colonize the surface and result in infection. The environment, type and concentration of bacteria will determine the deleterious effect of the contamination on the human cells layer.

Strength of evidence: Strong

RATIONALE:

Back in 1989, Gristina and collaborators conceptualized the human cells and bacterial cells concurrence for the implant surface as “race for the surface.” The “race for the surface” would involve macromolecules, bacteria, and tissue cells. The adhesive or integrative phenomena for bacteria or tissue cells and substratum surfaces are critical, interrelated, and based on similar molecular mechanisms (1). After implantation, host molecules and cells cover the implant surface and protect it against bacterial colonization. This protection corresponded to a shift in host cell population surrounding the implant. Initially, cells present are primarily non-differentiated stem cells, such as bone marrow mesenchymal stem cells, or immature haematopoietic cells. After approximately 7 days, a mature monocyte/macrophage population takes place (2).

There are a significant number of studies on the concept of “race for the surface,” most were *in vitro* studies (3-15) along with very limited *in vivo* studies (16). These studies may pre-conditioned the implant surfaces with macromolecules from body fluids (14, 17), pre-attached with host cells (7, 11, 13, 14), pre-colonized with bacteria (3-6, 9, 10, 13), or cultured in the presence of both host cells and bacteria (8, 12, 13, 15). It was found that the pre-culturing of either cohort compromised the subsequent adhesion of the other (13). The synergistic effect of preferential cell adhesion and antibacterial activity of the bi-functional surface led to the predominant colonization and survival of osteoblasts, effectively inhibiting the bacterial adhesion and biofilm formation of *Staphylococcus aureus* (*S. aureus*) in the co-culture systems with both cohorts (13). Some authors suggest that there is a pinpoint moment after the colonization by human cells, where the presence of bacteria could have a deleterious effect on the host cells – 12 hours post contamination. A metabolomics analysis provided new insights into the pathophysiology of infection. In this case, a set of metabolites may be the cause of a switch in the wound environment to an anaerobic metabolism, characteristic for Staphylococcal biofilm co-cultured with fibroblast cells (18). Biofilms, or their secretions, had deleterious effects on wound-healing cells. For example, *S. aureus* biofilms can be detrimental to both keratinocyte and fibroblast migration, proliferation, and viability. *S. aureus* biofilms promote a strong inflammatory response from both keratinocytes and fibroblasts, and has damaging effects on epidermal structures. *S. aureus* biofilms flourished in dermal matrices, while also depleting oxygen from the environment.

Pseudomonas aeruginosa (*P. aeruginosa*) biofilms also affected keratinocyte migration and proliferation, coalesced and adhered to epidermal structures, and multiplied in dermal matrices (19). Both the number and spread area per cell decreased with increasing density of adhering staphylococci. This demonstrates that the outcome of the race for the surface between bacteria and tissue cells is dependent on the number of bacteria present prior to cell seeding (20). The presence of bacteria results in reduced adherence of human cells to the surface of the biomaterials, increased production of reactive oxygen species, and into increased apoptosis. On the other hand, the presence of either type of human cells was associated with a reduction of bacterial colonization of the biomaterial with *S. aureus* (21) .

Besides time, the environment may influence the colonization of the implant surface. Surface colonization of bacteria is significantly enhanced on fibronectin coated surfaces irrespective of whether areas were uncovered or covered with human cells (17). However, a study carried out by Dexter and collaborators (2001) conclude that the increase of fibronectin concentration decreases bacterial adhesion and already low fibronectin concentrations showed the same cell adhesion rates as at high fibronectin concentrations (22). In addition, the concentrations of hyaluronic acid (HA) may play a role on the subject. HA at concentrations equal to or greater than 5% was able to avoid *S. epidermidis* adhesion on chitosan wound dressings. Fibroblasts adhesion also took benefit from the HA presence in the film, especially at 5% content, where the best adhesion and proliferation was found (23). An established and bioactive extracellular matrix (ECM) derived from osteoblast/fibroblast co-culture, may facilitate bone regeneration inhibiting bacteria colonization. ECM enhanced cell-cell communication. Fibroblasts improved osteogenic differentiation of osteoblasts via extracellular vesicles (24). Soft-tissue integration determines the long-term success of dental implants. Earlier good soft-tissue formation and integration helps against pathogenic biofilm formation and long-term inflammation (25). Roughness of the implant may also play a role on the “race.” Zhao and collaborators (2014) showed that smooth implant surfaces provide the best opportunity for a soft tissue formation (26).

Meanwhile, surface properties impact host cell attachment and potentially osteointegration, and can reduce early-stage bacterial adhesion (27-35). However, there are no solid evidence, especially there is a lack of *in vivo* evidences on if “winning the race” will determine the clinical outcomes or avoid infection occurrence. In order to reduce or prevent infection, it is clear that certain antimicrobial mechanisms (e.g., presence of antibiotic, bactericidal surface, induced appropriate immune response) have to present (9, 16) to effectively clear the bacteria, which may or may not win the race. Meanwhile, it is not clear if dominance of host cells (winning the race) on implant surface makes it easier for subsequent antimicrobial treatments.

A “race” may not exist based on the literature. The host cells usually colonize first. A contamination by bacteria and biofilm formation will be responsible for disrupting a host cell layer and ECM priory formed on the implant surfaces. The presence of a host cells monolayer may reduce bacterial colonization (36) or, in some cases, provide substrate for the growth of biofilms (15). Ramirez-Granillo et al (2021) showed that the population of single and mixed biofilm was higher on the limbo-corneal fibroblast monolayer in comparison to abiotic surfaces (37).

The colonization pathways may differ according to the bacterial type. The results from Martinez-Perez and collaborators (2017) show that clinical strains adhere to the material surface at lower concentrations than collection strains. Clinical strains behave differently than collection strains with respect to bacterial adherence (38). In absence of macrophages, highly virulent *S. aureus* or *P. aeruginosa* stimulated cell death within 18 h of simultaneous growth on a surface. Moreover, these strains also caused cell death despite phagocytosis of adhering bacteria in presence of murine macrophages. In contrast, low-virulent *S. epidermidis* did not cause cell death even after 48 h, regardless of the absence or presence of macrophages. Clinically, *S. aureus* and *P. aeruginosa* can yield acute and severe biomaterial-associated infections in contrast to *S. epidermidis*, mostly known to cause more low-grade infection (39). An interesting study from Sanches Jr, *et al.* (2013) show that biofilm conditioned media from clinical strains of *S. aureus* reduced osteoblast viability which was accompanied by an increase in apoptosis. Osteogenic differentiation was significantly inhibited following treatment with biofilm conditioned media as indicated by decreased alkaline phosphatase activity, decreased intracellular accumulation of calcium and inorganic phosphate, as well as reduced expression of transcription factors and genes involved in bone mineralization in viable cells (40). Testing the *in vitro* effects of *Streptococcus oralis* (*S. oralis*) biofilm on peri-implant soft tissue cells, Ingendoh-Tsakmakidis and colleagues (2020) showed that *S. oralis* can actively protect the host tissue. Commensal biofilms can promote homeostatic tissue protection, but only if the implant–mucosa interface is intact and human gingival fibroblasts are not directly exposed (41).

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QUESTION 41 : Is a minimum 1.5 log (95%) reduction in CFU on a surface, in vitro, sufficient for minimal antibacterial activity, in vivo, using a 104-105 CFU/mL inoculum?

Claudia Marques and Noreen Hickok

RESPONSE/RECOMMENDATION: Yes

Our goal was to determine the range of surface antibacterial reductions that were able to inhibit establishment of infection in an animal model. Overall, the number of studies that assayed both *in vitro* and *in vivo* activities were few and among those, the ones that contained plate counts were still fewer. Of those, permanent surfaces that achieved the above reduction in vitro showed meaningful reductions in vivo.

STRENGTH OF EVIDENCE: Limited

RATIONALE

Bacterial surface adhesion is the first step in biofilm formation. Antibacterial surfaces specifically target inhibition of this step. This inhibition can be achieved through a surface that contains antimicrobials that elute with time or surface modifications that either display antimicrobial compounds or have topographical features that inhibit bacterial adhesion. In this question, we have only included studies that characterized changes in surface adhesion in *in vitro* settings and then followed up with an animal study. Our goal was to determine the range of surface antibacterial reductions that were able to inhibit establishment of infection in an animal model. This range of reductions would, according to our reasoning, be designated as the minimal reduction required to be designated as antibacterial, *in vitro*.

Of all the papers reviewed, we ultimately considered 39 that reported clear antibacterial measurements, *in vitro* and *in vivo*. Of those, 9 reported reductions above 3 logs, *in vivo* which we used as a minimum reduction required to be designated as “antimicrobial.” Based on those reports, we examined the accompanying *in vitro* reductions. For these, surfaces that showed as few as 1.5 log reduction can result in the marked *in vivo* antimicrobial properties. The most common surfaces assayed were silver/silver nanoparticles, antibiotic-containing coatings, and quaternary amines/antimicrobial combinations. Most of the surfaces had an eluting activity, but for all reports a determination of surface colonization and clinical signs were a requirement. Most of the studies focused on *S. aureus*, *in vivo*, where *S. aureus*, *P. aeruginosa*, and *E. coli* were the common pathogens assayed, *in vitro*.

Overall, the number of studies that assayed both *in vitro* and *in vivo* activities were few and among those, the ones that contained plate counts were still fewer. This emphasizes the need for (1) more accurate reporting of reductions obtained *in vitro* (whether determined in the same

report or previously) when engaged in *in vivo* studies and (2) the need for more studies that examine both *in vitro* and *in vivo* reductions in the same series of studies.

Table 1: *In vitro* and *in vivo* reductions in surface colonization.

Type of coating	Organism	Reduction in vitro	Animal	Reduction, in vivo
Vancomycin sol-gel[1]	MSSA	4 logs	Rat, femoral canal	4 logs, 1 week; 1 log, 3-4 weeks
Col I/HA/HACC (quaternary ammonia) multilayer modified TCs were prepared by LBL covalent-immobilization.[2]	MSSA, MRSA, MRSE	At 24h, 99.8%, 98.4%, 98.1% (2-3 logs)	Rat, femoral canal	~ 1 log reduction; 10^5 reduced to 10^4
Teicoplanin or clindamycin coating by spraying[3]	MSSA	13-15 mm ZOI	Rabbit tibial intramedullary canal	No at 1 week—100% of 10 animals cleared, teicoplanin; 90% clindamycin
Ag NP in titania nanotubes[4]	MRSA	90% (1 log)	Rat tibial canal	50-70% by PET scan (<1 log)
Ag Ti nanotubes[5]	MRSA and E. coli	80-90% (~1 log)	Rat tibial canal	Lack of signs of infection by histology, microCT
Ag-coated PEEK implant[6]	Bioluminescent MSSA	Reduction in photons: from 1.5 x 10^3 PI to background	Mouse soft tissue	~50% reduction, day 7; complete reduction day 10
HA/tobramycin[7]	MSSA	ZOI constant over 5 days	Male rabbits, femurs; rods	5 Log reduction
Purified phosphatidylcholine was mixed with 25% amikacin or vancomycin or a combination of 12.5% of both[8]	MSSA, PA	All coatings, including PC reduced; PA showed 3 log reduction; up to 6 log for MSSA	Catheter with wire in dorsa of mice	1.3-3.7 log reduction for MSSA/PA mixed
Ag-coated, with or without daptomycin or VAN[9]	Multiple S. epi and S. aureus strains	Zones of inhibition	Mouse back cage	1 log for higher concentration
Combination of 2 papers—Cu-coated Ti[10,11]	*S. epidermidis*	6 log reduction	Femoral condyle, rabbits	2/3 reduction in number of animals infected
ZnO nanowires on SS[12]	MSSA, PA, *E. coli*	~1 log MSSA, PA; ~1.5 log, *E. coli*	Rats with bioluminescent bacteria	Undetectable on treated rod vs. "significant level"
Mo-disulfide/polydopamine with and without NIR[13]	MSSA, *E. coli*	~50% without NIR; 1-2 log with	rabbits	~1 log
Nanosilver coating[14]	MRSA, PA	1-2 logs	Rabbit intramedullary canal	>2 logs—down to single numbers/gram
Polymer brushes with VAN on Ti6Al4V[15]	Luminescent MRSA	~50%	Mice femoral canal	No sig reduction
titanium (Ti) -based implants, TiO2/calcium phosphate coatings (TiCP) doped with various amounts of fluorine (F) (designated as TiCP-F1, TiCP-F6, and TiCP-F9)[16]	MSSA, *E. coli*	TiCPF6 and F9, near 2 log reduction	Rabbits; holes in femur implanted with K wire	~90% reduction on implants; 60% on tissues
Ti structures coated with Ag[17]	MRSA and *S. epidermidis* (ATCC 3598360)	2-log reduction in the numbers of S. epidermidis but	Rat; holes implanted with scaffold	No determination for in vivo

		nothing for S. aureus MRSA		
Ag coated Ti rod[18]	MRSA	Effective at killing colonies	Rat femur rods	No bacterial colonization present
Ag Np or vancomycin Ti[19]	MSSA	Measurements at 24 h and 7 days. Attached bacteria: Eradication with CH + vancomycin at both time points. > than 2.5 Log reduction with Ch+50 mM Ag. Planktonic bacteria - similar for vanc, mas less than 1 Log at 24 hours. 2 or more Logs for CH+ > Ag 50 mM at 7 days	Rat peri-implants	There was a trend towards a decreased bone infection rate in the Ch + vanco group. There was a significant reduction ($p = 0.035$) in the number of culture-positive implants was found for the Ch + vanco group
Chitosan sponges containing 2.0% chitosan or 1.5% chitosan and 0.5% poly(ethylene glycol) (PEG) + ciprofloxacin and rifampin at 10 mg/mL[20]	MSSA and *P. aeruginosa* - polymicrobial mixture *in vitro* MSSA and E. coli in vivo	Additive effect between ciprofloxacin and rifampin against *P. aeruginosa* with a FICI value of 1 and no discernible effect against S. aureus	Mice – femur pin	Eradication of *S. aureus* and *E. coli* at day 7
Ag3PO4 coated fabricated honeycomb (HC) + carbonate apatite (CAp) crystals[21]	MRSA	HC-10 led to eradication. Other decreased to 7.5 +/- 2.5% - aka 1 Log	Rabbits - holes implanted with honeycomb	Week 2 - HC0.1 ($AgNO_3$ 0.1 mmol/L) eradication, HC-0 3x10^5. Week 4 both eradicated
Ag3PO4 coated Ti plate insert[22]	MSSA and *E. coli*	Dark - no difference. Light 808 nm NIR15 min- Bi2S3/Ti and Bi2S3@Ag3PO4/Ti - 80% antibacterial activity with the first and 100% with the second for both bacterial strains - Cell lysis as shown by TEM. S. aureus biofilms: dark 20% or below antibacterial activity; Light - 75% first and around 90% with second (1 log reduction	Rats	Bi2S3@Ag3PO4/Ti - Light 3 days 94.3% reduction
poly(lactic-coglycolic acid) (PLGA) nanofibers embedded PCL film of Vancomycin and Rifampin or Lin + Rif[23]	*S. aureus* strain Xen36 Bioluminescence	Prevents biofilm growth	Mouse – femur holes implanted with honeycomb	4 Log reduction in tissue, eradication. In implant
Amikacin and vancomycin chitosan sponges[24]	*S. aureus* UAMS-1 and *E.*	Only looked at synergistic effect of	Mouse – femur wire	Wire - 2 Log reduction in

	coli – polymicrobial infections	both antibiotics together. Indifferent for P. aeruginosa but synergistic for S. aureus FICI of 0.75		combination compared to half a log or 1 Log alone; in bone: 1.5 Log in combination Vs half Log to 1 log alone. When used with increased dose of combined antibiotics (10 mg/mL)- *E. coli* complete clearance on wire and in bone; *S, aureus* was also cleared ion wire and in bone
PLGA nanofibers - fusidic acid and rifampicin on Ti disk implant[25]	MRSA, MSSA, and *S. epidermidis*	Eradication was recorded after 48 h when *S. epidermidis*, MRSA, and MRSA (Newman) were exposed to the co-loaded nanofiber formulations	Rats – dorsum implant	3 Log reduction in FA/SF-RIF dual-loaded PLGA (50:50) nanofibers against MRSA
Injectable PEG Hydrogels with lysostaphin[26]	*S. aureus* or *S. epidermidis*	eradication	Mice – femur fracture and needle to stabilize the fracture	5 weeks: eradication Quantified in needle, femur, and surrounding tissue: 1 week undetectable levels of bacteria
Titanium intervertebral cages with HACC coating - quaternized chitosan coating[27]	*S. aureus* strain Xen36	Below detection limit of viability and prevention of biofilm formation	Rats – cage implantation in rat tail	Inhibition of bacterial growth with significant lower counts at day 3, Although values are not clear and SD bars are huge. Photons are significantly reduced.
PEEK surface PDA containing the KR-12 peptide (antimicrobial peptide)[28]	MSSA	PI can see at least 0.5 Log reduction;# of colonies was reduced by nearly 50% in PEEK-PDA-K12 group. SEM images are not clear.	Rats with femur rod	2 weeks after surgery. PEEK-PDA-KR12 has less colonies that are countable, while all other plates have TNTC colonies
Titanium covalently attach alkynylated vancomycin through efficient copper-catalyzed azide–alkyne cycloaddition (CuAAC) “click” chemistry to azide-bearing polymethacrylates surface-grafted from Ti6Al4V alloy, a common orthopedic metallic	MSSA and *S. aureus* Xen29-bioluminescence	SEM showed decreased colonization as did bioluminescence but no real values	Mice – femur pin	Bacterial CFU/per pin reduction 1.34 Log

hardware, (23−26) via surface-initiated atom transfer radical polymerization (SI-ATRP)[29]				
Stainless steel with 4.5% copper (00Cr19Ni13Mo3-4.5 wt pct Cu, 317L–Cu)[30]	MSSA and *E. coli*	317L–Cu 2 Log 24 hours to eradication (almost - not clear) at 48 hours	Rabbits with femur screws	No bacterial growth for 317L–Cu, similar to the negative control.
PEEK with Lithium-ion-loaded (Li+)/mussel-inspired antimicrobial peptide (AMP)[31]	E. coli and S. aureus	Reduction between 1 and 2 Log	Rats –femurs with implanted rods	95.03% reduction
Mg-CU alloy implants[32]	MRSA, *E. coli* and *S. epidermidis*	Between 1-2 Log reduction. Significant reduction of bacterial adherence detected by confocal and FESEM	Rabbits – tibia implants	1 Log reduction in bone and nail
Chitosan/nano-hydroxyapatite CS/nHA -Carbon Dots[33]	*S. aureus, E. coli*	The CS/nHA/CD+NIR group had significantly higher antibacterial activity toward pathogenic *S. aureus* and *E. coli* (with an antibacterial rate of 99% and 97%, respectively), whereas the antibacterial rates were approximately 75% in the other groups.	Rats – porous scaffolds – tumor related	1.5 Log *S. aureus*; 2 Log *E. coli*
PEEK with Sulfur[34]	MSSA and *E. coli*	Almost to the point of eradication	Rats – femur implants	Reduction of bacteria but no quantification
Iodine supported Ti implants[35]	MSSA and *E. coli*	Eradication	Rabbits -femur pin	No bacterial quantification
TiO 2 nanotubes loaded with B27polyhexamethylene guanidine (PHMG)[36]	MSSA	After 4 h of culture. 300 cells to around 100 cells/field (< 1Log)	Rabbits – femur rod	no bacteria detected with PHMG-TNT
PEEK-ZN-Mg-MOF74; Dexamethasone-Loaded[37]	MSSA and *E. coli*	Eradication calculated by ImageJ. Biofilm prevention	Rats – femur implant	did GIEMSA stain - no counting. Although it does seem, from the images that there is a reduction. No image analysis presented.
PEEK with brushite (CaHPO(4)·2H(2)O) - CaP-GS*3, *6 and *9[38]	MSSA and *E. coli*	SEM shows bacterial killing and reduction. PEEK/CaP-GS*3 exhibits vigorous antibacterial	Rats – femur implant	No clear numbers

		activity with almost 100% bacteria inhibition on the first day. PEEK/CaP-GS*6 and PEEK/CaP-GS*9 also reduce the bacterial numbers. Biofilm prevention The PEEK/CaP-GS*9 loses the antimicrobial activity on the 7th day.		
PEEK surface with three-dimensional (3D) porous structure coated with mouse beta-defensin-14 (MBD-14)[39]	MSSA and *P. aeruginosa*	SPMBP-5, 10 - resulted in bacterial eradication	Rats – femur cylinder implant	Bacterial reduction but no quantification
Ceramics with ZrO2-ZnO[40]	MSSA and *E. coli*	*E. coli:* 1 Log reduction; *S. aureus* reduction: 2 Log	Rabbit - Femur ceramic hip	The antibacterial rate of the ZrO2–ZnO ceramics was significantly better than that of the pure ZrO2 ceramics group.

Data bases searched:

Ovid, Google Scholar, Pubmed

Search terms:

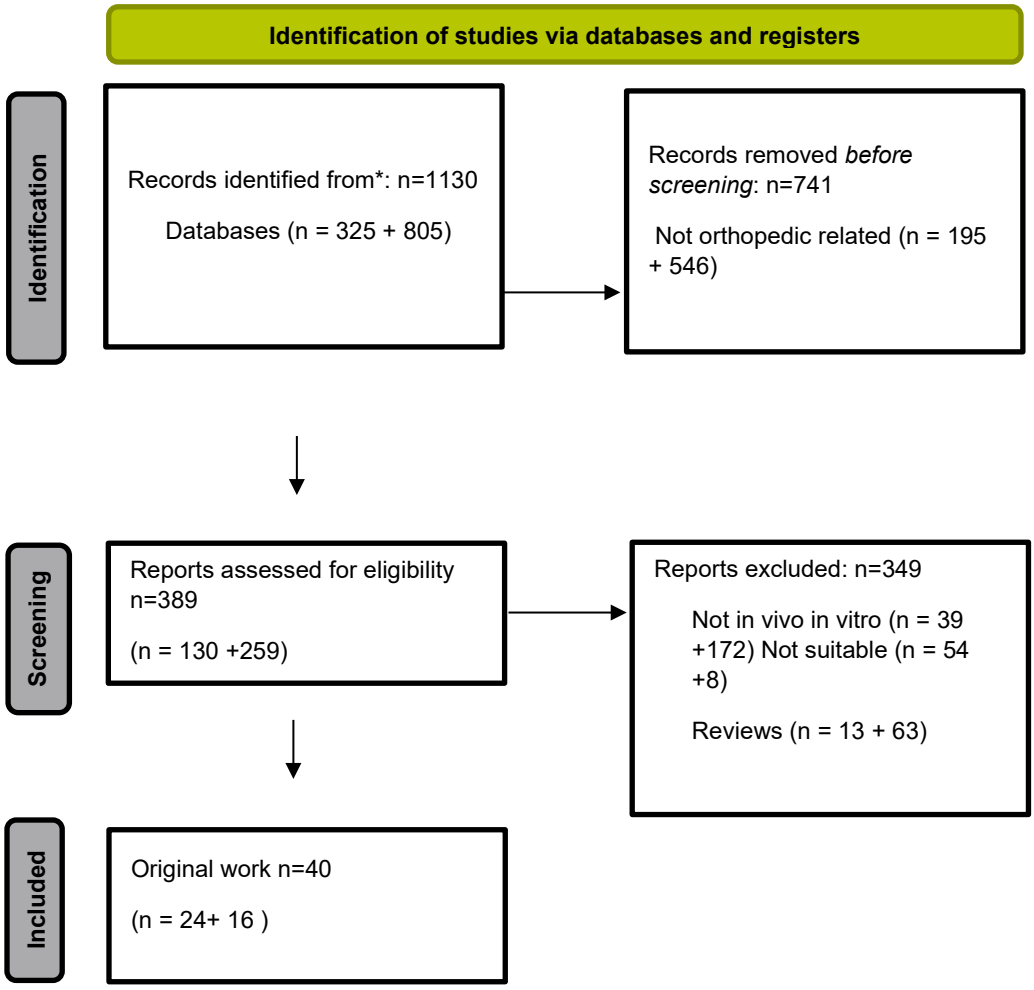
Ovid: 1 exp Coated Materials, Biocompatible/ (16149) AND 2 animal.mp. or exp Animals/ (25725533) AND 3 exp "Prostheses and Implants"/ (571450) AND 4 exp Anti-Bacterial Agents/ (789910)

Number returned: 799 + 6 from additional trees

Pubmed: antimicrobial AND biomaterials AND in vivo AND clinical signs – 313 references

antimicrobial biomaterials AND inflammation And chemistry And biology And materials science – 12 references

Decision trees:



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QUESTION 42 : Does testing against a panel of *S. aureus* (MSSA and MRSA*), S. epidermidis*, GBS (Group B Streptococci), *E. coli*, *P. aeruginosa*, *C. acnes* and *C. albicans* sufficiently capture the minimum required strains to claim universal antimicrobial efficacy when considering a novel prevention technology?

Bas Zaat; Kapil Raghuraman.

RECOMMENDATION: Unknown

The recommendation is based on a narrative review of the literature. Though many studies exist which discuss the virulence and pathogenic cause of MSKI, none propose a universal panel for testing. Also, while a cursory overview of available literature was attempted, further evaluation of existing literature is necessary to ensure that the species chosen in the recommendation cover the geographic and demographic variability of PJI.

STRENGTH OF EVIDENCE: Limited

RATIONALE:

Despite advances in implant and instrument materials and design, PJI continues to remain a poorly-addressed cause of implant failure and revision surgery. As such, creation of a panel to target important species of microorganisms implicated in PJIs may allow standardization of novel antimicrobial solutions. Though the etiology of PJI is regionally variable, S. aureus is a commonly studied and commonly encountered pathogen. In addition to S. aureus, various studies have indicated the prevalence of other gram-positive cocci, C. acnes, coagulase-negative Staphylococci, gram-negative microbes such as E. coli, P. aeruginosa, and fungi such as C. albicans. Since technologies targeting any one of these pathogens is unlikely to effectively target others, a universal panel necessitates that a preventative technology must, at minimum, test against the aforementioned strains. These quantify ~90% of all encountered infections and could provide a strong rationale for a novel technology, if met. However, in addition to targeting the aforementioned pathogens, care must be taken to capture a wide-range of universally available strains for each species such that comparative studies may take place. Due to the difficulty of achieving efficacy against a wide range of microbes, and because the microbes may still not represent potentially common infections in certain localities, it is our belief that a panel does not exist to sufficiently create a universal testing paradigm. However, we do recommend that all preventative technologies test both MSSA and MRSA variants to statistically address the most commonly implicated pathogens in PJI. Additionally, while efficacy is normally captured by MIC measurements of antimicrobial susceptibility, consideration should be given to quantifying the potential reduction of broad spectrum antibiotic use as a more realistic and impactful goal in creation of novel antimicrobial solutions.

a. Background and Available Literature

Though common modalities of implant orthopedic implant failure such as early loosening,

dislocation, joint instability, and fracture can all be resolved by improvements in implant and instrument design and material selection, infection is a common failure modality that requires unique intervention. To that end, many academic institutions and medical device firms are actively investigating preventative technologies to address MSKI, in the form of both eluting and non-eluting surface modifications, coatings or supplemental surgical accessories. In these investigations, no consensus has been reached regarding a common panel of pathogens used in in vitro testing to establish rates of efficacy of novel antimicrobial technology. In this investigation of the epidemiology and pathogenesis of periprosthetic joint infection, a systematic review of available literature was conducted to identify the prevalence of PJI and associated MSKI pathogens, current modalities to address or screen pathogens, and rationale for the determination of a strain panel for use in testing of prevention technologies.
Searches for available literature were performed between July and August 2022, with utilizing Google, PubMed and existing ICM Philly and EUCAST guidance. Cursory searches were performed to identify the EUCAST 2020 guidelines which are referenced. Subsequently, search terms of “orthopedic infections review” on PubMed resulted in 10,690 results dating back to 1951; of these, 4,919 had been published in the last 5 years, indicating a sharp rise in the study of PJI. After duplicates were removed from a study of “PJI epidemiology” (526 results, 370 in the last 5 years), 19 articles were chosen for review. Additional searches for “screening”+”infection”+”orthopedic” yielded 135 articles, of which 28 were identified to be relevant for review. The articles referenced in this document were chosen due to the strength of the study and the recency of reported findings; articles reviewed but not cited were primarily avoided due to the prevalence of literature with similar study design and conclusions to avoid redundancy in this review.

b. Epidemiology, currently reported MSKI pathogens, prevalent, problematic

Although infection incidence and etiology are regionally variable, accepted reports suggest that the incidence of PJI is ~1.5-2% in primary arthroplasties, with the number rising significantly for revision surgeries based on anatomy, present co-morbidities and the number of re-revisions performed [1-3]. Despite the steady rise in year-over-year volume of primary arthroplasty performed, the rate of infection has stayed consistent, though the associated number of infection cases has risen proportionally to the organic growth of surgical volume [2,4].

In general, PJI risk is higher in the immediate post-operative period and decreases as the patient is further removed from the time of surgery. Several studies indicate that up to 70% of infections occur in the first 2 years post-operatively, with the majority of those occurring within 1 year of surgery. Specifically, early-onset PJI refers to infection within 3 months of the last surgery, while delayed-onset PJI occurs between 3-24 months and late-onset PJIs occur after 2 years post-op [4]. In early-onset PJIs, this suggests that despite the prevalence of comorbidities such as diabetes mellitus, obesity and the like, the likely exposure to underlying microorganisms results during the surgery [4]. Due to the relatively low CFUs/mL required to inoculate an implant, colonization at the time of surgery is an important consideration in the epidemiology of MSKI pathogens. The second leading cause of infection is migration from an adjacent site, which means that skin and nasal colonization play a critical role in identifying pathogens of importance in MSKI [1, 3-6].

A clinical review detailing more than 2000 hip and knee infections captures a wide array of surgical strategies, countries, and time points, attempting to resolve the local biases of commonly occurring pathogens [4]. Unsurprisingly, perhaps, it was found that PJIs were largely resultant from gram-positive cocci, and specifically, with S. aureus and coagulase-negative staphylococcus infections making up more than half of studied PJIs in both hip and knee arthroplasty. While local reports of streptococci and enterococci have sometimes been higher, the global aggregate is

closer to 10% [4, 6-8]. Finally, the aerobic gram-negative bacilli comprise <10%, and anerobic bacteria comprise ~4% of reported infections. The aforementioned etiology of PJI is consistent with the virulence of the associated strains [4]. As S. aureus, GNB, multi-drug-resistant organisms and enterococci have been characterized by high virulence, there exists an associated correlation to the prevalence of these organisms in early post-operative infections [4-6, 7-11]. Conversely, species with low virulence such as coagulase-negative staphylococci and Cutibacterium species were more prevalent in late-chronic infections and positive intraoperative cultures. Anatomy, too, played a role in the presence of certain bacterial species—while C. acnes was commonly reported in shoulder surgery cases, comprising up to a quarter of underlying PJIs, there were no reported cases resulting from a C. acnes infection in hip and knee [4]. Though variability does exist based on these anatomical and geographic differences, it is clear that S. aureus is the predominant pathogen driving PJIs [1-11]. Many studies have explored universal screening for both MSSA and MRSA, but mixed results exist for both pre-operative screening and nasal decolonization, because nasal or dermal colonization of underlying pathogens do not successfully predict PJI, and because global PJI incidence has stayed relatively constant despite efforts to screen [23-27]. Additionally, though MSSA and MRSA are studied independently, it is currently inconclusive whether efficacy against MSSA predicts efficacy against MRSA, though the opposite has been shown to be true [24-25, 27]. More conclusive evidence exists for the utility of a longer post-operative course of antibiotics in preventing infection incidence up to 12 months post-operatively [12].

While bacterial species are somewhat well-understood or characterized, limitations arise from the presence of culture-negative infections and the rare cases of fungal infection. Although Candida albicans has been reported as a prevalent cause of fungal infections, the incidence of fungal infections is not well understood, and treatment methodologies are ill-defined [9-11]. Antibiotic treatments utilized in the treatment of more prevalent species responsible for PJI may, in some cases, exacerbate the risk of fungal infection and complicate treatment [10-11]. As such, a preventative technology that addresses commonly encountered microbial species but fails to consider fungal species may be unable to claim broad antimicrobial efficacy.

c. Relevant strain characteristics, resistance, virulence (to link to biofilm formation and final in vivo tests), inclusion in standards (ASTM, ISO) and/or commercial test panels

Since the most commonly encountered pathogens causing PJI are a mixture of gram positive and negative bacteria and fungi, most single antimicrobial agents will not be active against all of these species.

Antimicrobial susceptibility testing is important for 2 different situations. Firstly, susceptibility of isolates from cases of MSKI and from pre-operative screening will need to be assessed. This is done in routine clinical microbiology laboratories using standard protocols and panels of antibiotics tailored to the species of the isolate (EUCAST, FDA, CLSI) [20-23]. This practice is well developed and will not be discussed in this opinion paper. Secondly, in development of novel antimicrobials or novel antimicrobial medical devices, it is desired to determine the spectrum of antimicrobial activity and to assess the expected coverage for the major species of micro-organisms causing MSKI. To the best of our knowledge no standard test panels of bacterial species or strains for this purpose exist.

Novel antimicrobial agents are normally tested with broad panels of microbial species, and for each species a broad collection of strains may be analysed to assess the MIC50 or MIC90 for a species, which is the maximum concentration that inhibits the growth of 50% or 90% of the isolates of that species tested. Often series of at least 100 strains per species are then tested, to include the inter-strain variation within a microbial species [13-19]. This already indicates that single

strains or small panels of strains will not easily represent the susceptibility of a certain species.

Staphylococcus aureus, the species most often causing MSKI, consists of isolates which are either susceptible (Methicillin Susceptible S. aureus, MSSA) or resistant to methicillin (Methicillin Resistant S. aureus, MRSA). The ratio MSSA / MRSA isolates can differ strongly for different geographic locations [4, 16]. In view of testing representative isolates of S. aureus, MRSA strains are often selected, as they are the worst-case pathogens and novel agents are sought which can treat infections of these strains. When an agent is active against an MRSA strain or strains, this does not necessarily mean that it will also be active against MSSA strains since these strains may have different resistances or genetic traits than MRSA strains. So, when selecting strains for a test panel, it is advised to include both MRSA and MSSA strains.

A test panel of S.aureus MRSA and MSSA strains will not necessarily represent the susceptibilities encountered among other gram-positive pathogens, such as S. epidermidis or Enterococcus faecalis, and certainly not of gram-negative species like Escherichia coli or Pseudomonas aeruginosa [4, 16-19, 21]. Treatment of gram positive and gram-negative bacteria often requires different groups of antibiotics, as also does the treatment of anaerobic bacteria such as Cutibacterium acnes [4, 16-23]. The Candida species are yeast/fungi, which require treatment with specific antifungal agents, which are not active against bacteria, just like the antibiotics which target bacteria are mostly not active against fungi [9-11]. So, it is not likely that novel antimicrobials can be tested with a very limited number of microbial species or strains.

d. Proposed panel of test species, and possibly particular strains

Based on the above, a panel of the major pathogens causing MSKI should be selected to be able to assess the width of protection that a novel antimicrobial or antimicrobial biomaterial can provide.

When choosing specific bacterial strains of these and other species, it is advisable to, in addition to antimicrobial resistance profile, consider several strain features of relevance to the pathogenesis of MSKI (virulence factors, biofilm formation, immune evasion) of the bacteria. Moreover, the strains should be available from certified strain collections like ATCC or NCLS to choose the adequate bacterial strain for the intended in vivo osteomyelitis mouse studies.

In addition, it is recommended to use a strain with a well-documented origin, phenotypic and genotypic profile, and to ensure availability of the strain in view of repeatability and reproducibility of the experiments, proved pathogenicity of the strain, and strain characteristics (e.g. biofilm formation, panel of adhesins, bacterial toxins and antimicrobial resistance) [14].

If the conditions are met and a panel for a prevention technology is desired, an antimicrobial technology claiming a high degree of efficacy cannot do so without addressing S. aureus and MDR-S. aureus, though that alone does not satisfy the burden of proof. Additionally, it would be prudent to provide evidence of efficacy against other commonly reported gram-positive pathogens such as S. epidermis and SBG as well as gram-negative microbes such as E. coli and P. aeruginosa and common fungal species such as C. albicans. Still, this suggested panel only defines the minimum testing suggested and, likely, further consideration must be given to the application of the subject device, the anatomy considered and the geography/demographics of the target patient population.

e. Limitations and Conclusion

Current literature carries several limitations with respect to consensus-building a panel for wide-spread use in testing antimicrobial prevention technologies. Though MIC/MBEC appear to be consistent metrics of measuring in vitro efficacy, it remains unclear how well these metrics

correlate to in vivo success. Also, while prevention remains an important consideration in the treatment of PJI, prevention alone may be insufficient in preventing PJI or the recurrence of infection in patients suffering from re-revision. In these instances, presence of biofilm pre-cursors in the joint space or around the implant may require some biofilm eradicating capacity in a successful prevention technology. Therefore, any novel technology would need to be used in concert with some antimicrobial drug (antibiotic/antimycotic).

It is the recommendation of the authors of this study that in future studies involving the study of antimicrobial efficacy a measure of MIC/MBEC be supplemented with an analysis of the potential reduction in the use of broad-spectrum antibiotics. Antibiotic use in treatment is inevitable but can be better targeted by utilizing prevention technologies that provide some statistical reduction in the occurrence or recurrence of common PJI pathogens. Providing evidence for the inhibition of species discussed in this study and supplementing that data by providing an analysis of treatment variability effects may allow surgeons to narrow the scope of potential pathogenic origin and thus limit the amount of broad-spectrum antibiotic use.

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QUESTION 43: Should multiple outcome measures be used for accurately determining antibacterial efficacy in vitro.

Ed Greenfield and K. Scott Phillips

RECOMMENDATION: Yes

Outcome measures for antimicrobial efficacy depends on the format and objectives of the specific study, where measurement of colony forming units (CFUs) is the gold standard. While this method has excellent reproducibility, it has limitations. Therefore, in many studies orthogonal methods that complement each other's weaknesses are needed to reduce potential artifacts and underestimation of performance due to failed assumptions about the reliability of outcome measurements.

STRENGTH OF EVIDENCE: Strong

RATIONALE:

Summary: The most accurate outcome measurement that can be used for tests that determine antimicrobial efficacy in vitro depends on the format and objectives of the specific study. The most frequently used method across all types of studies, and the gold standard in microbiology, is measurement of colony forming units (CFUs) by plating and culturing of microbes. While this method has excellent reproducibility1,2 and can quantify a wide range of bacterial concentrations, it has limitations associated with low throughput, detection of viable but non-culturable (VBNC) bacteria without extended post-treatment culture periods, and assessment of intact biofilms without prior disruption (extraction). Therefore, in many studies the best practice is a combination of multiple outcome measurements that are selected to fit the format and goals of the study. Orthogonal methods that complement each other's weaknesses are ideal to reduce potential artifacts and underestimation of performance due to failed assumptions about the reliability of outcome measurements.

Antimicrobial efficacy testing for medical devices is not equivalent to testing used for antibiotics or disinfectants because it often involves consideration of a complex pathogenesis process over time involving medical devices and their biomaterials, in vivo biology of the patient, and microbes in various forms including dormant/persistent biofilms that have been exposed to systemic antimicrobials. There is not a one-size-fits-all approach to in vitro testing, and all of the above factors need to be considered in developing a preclinical test strategy.3,4 Often, a "log reduction" measurement of the type commonly used for disinfectants or preservatives is also not appropriate. As few as 100 cells have been linked to biomaterials associated infection5, and thus endpoint methods need to have the ability to detect a very small absolute number of pathogenic cells. Because the process of extraction adds additional uncertainty to measurements, this often necessitates the use of additional "in situ" detection methods that directly measure bioburden on biomaterials. Additional modalities used to measure in vitro test outcomes include: direct spectroscopic methods, reagent-based measurements, genetically modified organisms (reporter

assays), molecular biology-based approaches, and microscopy.

Direct spectroscopic measurements: The most common simple method for aqueous suspensions is turbidity, a high throughput but semi quantitative method of measuring light absorbance of a bacterial suspension at 600nm. To detect biofilm in situ, the simplest direct spectroscopic method is intrinsic protein fluorescence6, which detects all proteins using 290nm excitation and 335nm emission settings, but has poor sensitivity for cells.

Reagent-based measurements: These often rely on the binding or derivatization of proteins, nucleic acids or other bacterial cell components to create a fluorescent or luminescent derivative or other reporter molecules through enzymatic interactions. While the entire scope of reagent-based assays is too numerous to discuss here, some of the most common methods are live/dead, resazurin, crystal violet, orthophthalaldehyde (OPA)7, and fluorescent markers (e.g. lectins8, FilmTracer9). They require calibration and validation for specific applications. Crystal violet is commonly used to measure the amount of biofilm. Despite its name, the “live/dead” assay is not reliable for measuring viability10. The use of resazurin may be a better alternative if special measures are taken to “reactivate” quiescent cells.11,12 Fluorescent marker reagents that bind to bacteria or biofilm are often best used in the context of exploratory mechanistic studies using confocal microscopy (discussed below).

Reporter-based assays: Common pathogenic microbes are genetically modified to produce molecules such as fluorescent proteins (e.g. GFP, RFP) and luminescent proteins (e.g. luciferase). Proteins from these bacteria are then directly measured in in vitro assays, providing very high signal/noise characteristics, even directly overlaid on tissue in ex vivo assays13. Luminescent proteins are especially useful for in vivo studies such as mouse models, where bacteria inside the animal can be tracked over time non-invasively in an in vivo imaging system14 . However, both fluorescent and luminescent protein producing bacteria are not reliable to determine bacterial viability due to both false negative and false positive results15.

Molecular biology methods: Quantitative real-time PCR can estimate the overall microbial burden using pan-bacterial primers, usually against 16S DNA. Alternatively, microbial species or strains of interest can be measured with specific primers. Complex microbial mixtures can also be characterized using next generation sequencing of the PCR amplicons. While optimization of sample preparation in these methods to reliably measure microbial viability is still under investigation16, a commonly used surrogate approach to estimate viability is the measurement of bacterial mRNA in intact cells by reverse-transcription PCR.

Microscopy: Due to the multi-faceted nature of microbial biofilm and tendency of many endpoint methods to miss quiescent persister cells deeply embedded in biofilm, microscopy can play an important role in verifying results. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) are two methods that can help elucidate the presence or absence of biofilm and the nature of microbial interactions with materials. Improved cryo-preservation methods for SEM can even capture delicate microbial structures such as nanowires17. Semi-quantitative microscopy can be performed by automated imaging routines with algorithms to count cells or estimate biofilm mass18. Observation of cell movement can provide insight into microbial behavior that is not captured by other methods, such as adhesion, interaction with topography, cell division and biofilm shedding.19 These behaviors may play an important role in pathogenesis and infection, and thus microscopy may provide information about in vitro assay failure modes as well as the antimicrobial treatment being tested.

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QUESTION 44 : Is there a universal concentration for the bacteriological tests of (1) textured surfaces, (2) eluting surfaces, and (3) non-eluting (chemically modified) surfaces?

Vi Khanh Truong and K. Scott Phillips

RECOMMENDATION: No

Starting inocula are often 10^4 CFU – 10^8 CFU depending on the application. It is essential to select the worst-case form of inoculum (planktonic, cell clusters, biofilm, build up biofilm) in order to accurately estimate real-world performance. Differences in performance testing of eluting devices vs. non-eluting and textured biomaterials are related to the differential goals and timeframes of these materials. Evaluating eluting materials requires multiple test methods, including elution profile data, minimal effective concentration (MEC) data, and pharmacologic modeling.

STRENGTH OF EVIDENCE: Strong

RATIONALE

Summary: The selection of inoculum for in vitro performance testing is an important consideration that should take into account the clinical use scenario first and foremost. Where biofilm is involved, use of too small of an inoculum or too large of an inoculum with a “log reduction” measurement can both result in overestimation of effectiveness. The starting inoculum is often in the range of 10^4 CFU – 10^8 CFU depending on the specific application. Measurement of absolute numbers of CFU remaining in a biofilm is preferable to “log reduction”, since as few as 100CFU can cause an infection in the presence of foreign materials. It is also essential to select the worst-case form of inoculum (planktonic, cell clusters, biofilm, build up biofilm) in order to accurately estimate real-world performance. Differences in performance testing of eluting devices vs. non-eluting and textured biomaterials are related to the differential goals and timeframes of these classes of materials (protecting the surgical site vs. protecting only the device surface). Most textured and non-eluting biomaterials have limited potential for long-term implants because of biofouling via the foreign body reaction and Vroman effect. Evaluating eluting materials requires multiple test methods, including elution profile data, minimal effective concentration (MEC) data, and pharmacologic modeling.

Regardless of whether a device will be used to prevent an infection or treat an infection, the anatomic location of the device, the physiologic systems in contact with the device, and the type and number of microbial contamination commonly encountered in the clinic should be considered in determining not only the amount of bioburden but also the form of bacteria to be inoculated.1 Clinical pathogens are often found in biofilms in vivo2, clusters of cells3, desiccated state on skin4, or in nasal or oral secretions5. Bacteria in these forms are hardy and can be resistant to concentrations of antimicrobials (or antimicrobial surfaces) that are 10,000x the normal MIC found

in planktonic testing6.

While there is no universal number (CFU) for inoculum for antimicrobial device performance testing, there are some considerations that differ for performance testing of eluting devices vs. non-eluting biomaterials. The preclinical testing strategy for eluting devices should include characterization of the elution of the antimicrobial from the device, as well as modeling or in vivo testing to translate the in vitro elution profile into expected in vivo concentration-time data. This is important to evaluate effectiveness of protecting both the device and the surgical site tissue from microbial colonization, and the potential for antimicrobial resistance (AMR) by determining the proportion of time that the antimicrobial concentration is in the therapeutic window.1

Where possible, clinical evidence should be used to select the starting inoculum based on the use of the device. If a device is being used to reduce the risk of infection, clinical data on the typical bioburdens encountered in the surgical site and on the device for the indicated surgical procedure are useful as a starting point. For devices used in revision surgery (placed into an infected surgical site), the inoculum is normally greater than those that are used in primary surgery. A worst-case scenario should be chosen, but the amount should not be so excessive that it creates other testing artifacts. Because the amount of antimicrobial needed to kill larger inoculums may increase, this could lead to problems with biocompatibility/toxicity testing. Testing with too high of a bioburden could result in erroneous assumptions about the tradeoffs between biocompatibility of a device and long-term tissue integration with mammalian cells, which is balanced against the goal of protecting the surgical site and device from viable bacterial survival.7 Absolute numbers of CFU used for in vitro testing typically range between 104 to 108 depending on what is known about exposure to bioburden in the clinical use. One of the problems with use of excessively high starting bioburden is the difference between dormant/persister cells and planktonic cells. If a device is inoculated with too many cells, due to limited surface area and surface biofilm formation, eluting antimicrobials may be more effective against the metabolically active portion of the inoculum than the persister cells at the core of the biofilm. This can result in large overestimation of performance, especially if “log reduction” is used as the endpoint metric. A solution to this problem is to create buildup biofilm through exposure to increasing concentrations of the antimicrobial followed by rinsing of dead cells that are loosely bound.8,9 This can create a starting inoculum which has more persister cells. By measuring the number of survivors after treatment instead of the log reduction, this approach provides a more robust test of real-world antimicrobial performance. Because as few as ~100 cells on a biomaterial can result in an infection10, extraction and plating/culturing often do not have adequate sensitivity to detect this threshold of persister cells on devices (See Question 11 for more information), and additional methods may be needed to accurately assess antimicrobial performance.

For non-eluting devices, assuming that chemical characterization/biocompatibility testing has shown that there is no elution of antimicrobials, the surface treatment is only expected to prevent bacterial colonization of the device, and is not expected to protect the surgical site or treat an existing soft-tissue infection. Therefore, non-eluting devices are only appropriate for use where it is demonstrated by clinical evidence that protecting only the device surface has a benefit to patients, such as reduced infection rate or decreased healing time. For antimicrobial performance testing of non-eluting devices, they should be inoculated in a clinically relevant medium, at the clinical temperature, with clinically realistic forms (clusters, biofilm chunks, etc.) and numbers of the targeted pathogenic organism(s), and testing for colonization should be carried out at various time points until failure (colonization detected), and the time until failure should be reported.

For both eluting and non-eluting devices, it is also important to take into account biofouling that can happen with most implantable devices due to the foreign body reaction11 and Vroman

effect12. Because biofouling can reduce antimicrobial effectiveness, the influence of relevant body fluids and their constituents should be studied in order to determine how it impacts the results of the assay.

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QUESTION 45: Should Small Colony Variants or Persisters be detected in clinical samples?

Lia Rimondini and Paul Stoodley

RECOMMENDATION: Unknown

There was little evidence to suggest that the presence of SCVs is associated with virulence or adverse treatment outcomes. The presence of SCVs in clinical isolates by morphology on agar plates is relatively low, between 2 and 20%. Unlike SCVs, since persisters by definition revert to the WT phenotype on culturing in the absence of antibiotics they are difficult to detect in clinical isolates. We conclude at the present time there is no established way to detect persisters. SCVs can be detected by colony size and morphology but there is no compelling evidence that their detection will alter treatment outcome.

STRENGTH OF EVIDENCE: Moderate

RATIONALE

Main findings and insights:

We first used a PubMed search on 7/3/2022 using key words [small colony variants] AND [biofilm] AND [persisters] and got 75 hits. On inspection many of these included the term “persistence” or “persistent” in the context of chronic infections. Next, we removed the term “biofilm” to provide a broader term and using the search [small colony variants] AND/OR ["persister"] on 8/16/2022 we achieved 22 hits1-22. On examination of the papers the emphasis was on persisters with little or no discussion of SCVs. Another PubMed search was performed on 8/25/2022 using the key words “SCV AND infection AND orthopedic”. This resulted in 10 hits23-32. One appeared in both searches Our summary is based on these 31 papers.

How and under what circumstances should Small Colony Variants or Persisters be detected? In these limited number of studies from clinical screening and one animal study there was little evidence to suggest that the presence of SCVs is associated with virulence or adverse treatment outcomes. In the literature SCVs are frequently cited as complicating bone and joint infections but there is little evidence to suggest this. The presence of SCVs in clinical isolates by morphology on agar plates is relatively low, between 2 and 20%. Unlike SCVs, since persisters by definition revert to the WT phenotype on culturing in the absence of antibiotics they are difficult to detect in clinical isolates. One study identified type I and II persister on the basis of growth rate in H. pylori however it was not clear how persisters could be differentiated by slow growing auxotrophs using this method. We conclude at the present time there is no established way to detect persisters. SCVs can be detected by colony size and morphology but there is no compelling evidence that their detection will alter treatment outcome.

What means can be used to identify and target them? There is well supported documentation through clinical and lab strains of staphylococci that SCVs can be distinguished by colony size on an agar plate compared to the normal phenotype after an equivalent incubation time. However there is no strict definition on size difference. Persister cells are more difficult to directly identify although some interesting techniques are being developed based on differential staining. Currently the most common way of identifying and enumerating persisters is by plate count of the surviving population after an antimicrobial challenge and then confirming the return to susceptibility by MIC, e-test strip or other assay after the original 1944 definition by Bigger et al. “Cocci with no greater resistance to penicillin than normal but which happen to be, when exposed to it, in a phase in which they are insusceptible to its action”33. We conclude that persisters and SCVs can be identified and distinguished by culture-based methods.

The clinical and animal studies in our search suggest that SCVs can be treated similarly to normal phenotypes. A more problematic issue is in detecting an infection by SCVs through routine clinical microbiology culture due to their slow growth. For targeting persister cells there were several strategies, ranging from growth independent antimicrobial agents to stimulating activity and simultaneous antibiotic therapy, to interfering with tox-anti-tox systems. However, these studies are still at the laboratory level. Of note a number of in vitro studies not identified by our search have discussed complete eradication (below detection limits) of biofilm bacteria by high concentrations of antibiotics over extended periods suggesting that even persisters and SCVs can be killed34,35. Since dosing of the most currently available antibacterial agents commonly fail to completely eradicate bacterial populations, it is critical to develop novel antibacterial agents that are capable of targeting persister cells.

What are the differences/similarities? SCVs are identified by their small colony size on agar plates. The main difference is that SCVs are slow growing and often present a stable phenotype while persisters by early definition are dormant, even in the presence of nutrients, and revert to the normal phenotype on culture. Auxotrophy is associated with SCVs while tox-anti tox mechanisms36 are generally associated with the persister phenotype.

Summary:

Means to identify persisters.

In a clinical study Bahmaninejad et al.3 found of 50 patients suffering from H. pylori infections 18% (9) were persister cells. A novel protocol for persister cell Isolation37 was used which utilized lysis buffers to differentially lyse rapidly and slowly dividing or dormant cells identified as type I and II persisters. In a laboratory study Micheva-Viteva et al.10 investigated mechanisms of persister cell formation in Burkholderia thailandensis by differentially staining low metabolic activity cells exposed to 1 X MIC antibiotics using a selective dye based on proton motive force then concentrating them using flow cells sorting, or induced persister cells using meropenem. Žiemytė et al.1 identified persister cell populations in P. aeruginosa on the basis of colony morphology and lack of pigment and used mannitol to increase metabolic activity making then susceptible to ciprofloxacin. However, generally it is recognized that persister cells revert to WT phenotype when

they start to grow and so these cells might have been SCVs. Seeger et al.2 in a lab based study assessed the effect of levofloxacin to induce persisters in clinicals isolates of E. coli and measuring them using electronic cell counting. In their lab based study Wang et al.6 did not directly identify persister populations but used increased killing of biofilms formed by an E. coli or S. aureus as indirect evidence assuming that biofilms were enriched for persister cells, as previously described38. However, it is possible that some of the resistant cells were due to hetero-resistant sub-population or other tolerant phenotypes39. Xiao et al.8 assessed the efficacy of essential oils against S. aureus and uropathogenic Escherichia coli. The persister population was defined as the concentration of bacteria that survived antibiotic challenge shown by a plateau in CFU survivors after 3 to 5 days. In lab studies Yam et al.9 used an in vitro lab assay to assess the influence of anti-persister drugs against Mycobacterium abscessus. Persister cells were enriched by starvation or hypoxia. To differentiate “non-replicating persistence” persisters from nutrient starved cells they used a method looking at the ration between chromosome equivalents per mL (CEQ/mL) and CFU using the rationale that the “CEQ/mL of culture should increase over time due to the accumulation of the chromosomes from dead bacteria but for non-replicating persistence, CEQ/mL should be constant. Similarly, Zhu et al.5 assayed the killing of S. epidermidis persisters compared to a baseline of survivor CFUs in a biofilm cells after 24 hr. Cui et al.13 indicated that E. coli persistence was induced by exposing bacteria to a range of antibiotics and stress conditions including starvation and acid pH. The mutants obtained overlapped in both rifampin and tetracycline induced persister screens, and some of them mapped to DNA repair pathway or to global transcriptional regulator (fis) and to efflux (acrB). Similarly Grassi et al.14 suggested a method to obtain in vitro persistent cells of P. aeruginosa and S. aureus with antibiotic-tolerant phenotypes at high efficiency through the exposure of stationary-phase cultures to cyanide m-chlorophenylhydrazone (CCCP). They demonstrated that after CCCP removal, the metabolic activation of induced persisters coincided with the reversion to antibiotic-sensitive phenotypes. Bui and Kidd19 described a method to identify the molecular attributes of quasi-dormant SCV in persistent and recurrent S. aureus infections. They developed a matrix-embedded and stable SCV cell-type of clinical S. aureus strain by growing bacteria under limiting conditions for a prolonged time, and discovered that the stable SCV cell-types possess an increased viability in the presence of antibiotics compared to their non-SCV form, exhibit genomic stability with genetic changes in MgrA (a global regulator) and RsbU (a phosphoserine phosphatase within the regulatory pathway of the sigma factor SigB), and show a shift in the methylome. Velayati et al.17 focused their work on latent M. tuberculosis during which they induced latency in vitro by a double-stress model (oxygen and nutrition) for 26 months, and discovered that persister M. tuberculosis arrest their growth, reduce their size (0.3-0.1μm), lose their acid fastness (85–90%), and change shape. Moreover, whole-genome sequencing (WGS) revealed only a few genetic changes that lead to the conclusion that biological adaptation of these cells was more phenotypic than genomic. Research has also been done on resistant fungal infections. Bojsen at al.18 performed multiplexed barcode sequencing (Bar-seq) screening of a pooled collection of gene-deletion mutants of Candida albicans and Candida glabrata, and demonstrated that decreased TORC1-mediated induction of ribosome biosynthesis via Ras can lead to formation of amphotericin B-persister cells regardless of whether the cells are in planktonic or biofilm growth mode. Two articles were reviews with only general discussion of persister cells 4,7.

Means to identify SCVs.

Colony morphology was commonly used to quantify SCV formation by clinical strains in six of the studies in our search and we found an additional one cited by the authors. Bayston et al.30 showing triclosan stimulated SCVs in three clinical S. aureus MRSA strains which were distinguished on the basis of size compared to WT. Jiang et al.25 used a similar approach to assess amikacin stimulation of persisters in two strains of S. argenteus. Bogut et al.29 found in 31 (19%) coagulase negative Staphylococcus from patients undergoing revision of total hip prosthesis for aseptic loosening or presumed prosthetic-joint infection (PJI). SCVs were defined as pinpoint colonies, 1/10th or less the size of normal colonies after at least 48 h. All the SCVs were auxotrophic for haemin. No correlation was made between the presence of SCVs and treatment outcome, although interestingly they isolated SCVs from patients with subclinical infections. They commented that their results were similar to those of Maduka-Ezeh et al.40 who reported the isolation of SCVs of S. epidermidis in 12 out of 31 patients. Tande et al.28 screened staphylococci isolates from 113 PJI patients and found SCVs in 38 (33.6%) subjects base on phenotype. 75 (66.4%) patients were found to have the normal phenotype. The presence of SCVs was not associated with excess treatment failure where they were primarily managed with two-stage arthroplasty exchange. Similarly, Valour et al.27 screened 90 clinical isolates representing 7 clonal complexes of S. aureus from bone and joint infection using an ex vivo model of human osteoblast infection. The average conversion to a SCV phenotype ranged from between 2 and 4 %. There was no difference in the rate of SCVs between acute and chronic infections or delta toxin production. They did note that “an accurate definition of SCV, involving not only colony sizes but also metabolism markers are lacking”. Joosten et al.31 screened 30 S. aureus isolates from chronic osteomyelitis patients infected with SCVs or normal MRSA strains for virulence in a New Zealand rabbit model. Treatment with hydroxyapatite cement loaded with vancomycin cleared both SCV and MRSA strain infections. Rolauffs et al.32 reported a clinical case study of the difficulty in treating a patient with combined osteopetrosis, femoral fracture, and chronic osteomyelitis where small colony variants (SCVs) were isolated. Trombetta et al.24 reported the development of a 96 well plate rapid screen method for novel antimicrobials that may target SCVs using the stable SCV mutant construct labelled with GFP. The assay was based on a fluorescence viability stain. Yang et al.26 found that in a S. aureus construct that SCVs were associated with internalization by human-osteocyte-like cells. SCVs were identified by gold pigmentation and a diameter of 0.2-mm diameter or less.

Means of targeting persisters

Liebens et al.15 directed their research towards finding small molecules that can eradicate P. aeruginosa persisters in combination with the fluoroquinolone antibiotic ofloxacin, and via screening a small molecule library they were able to identify 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009) as the most promising candidate to kill persisters, without causing erythrocyte damage or major cytotoxicity against mammalian cells. A similar approach was described by Niu et al.21 who screened the clinical drug library of the Food and Drug Administration (FDA) using high throughput drug exposure assay in 96-well plates, and were able to identify 14 drug candidates with high anti-persister activity. Tosufloxacin and colistin showed the highest anti-persister activity due to their capacity to completely eradicate uropathogenic E. coli persisters in a period of 3 days in vitro. Feng et al.20 used the same FDA drug library against stationary-phase B. burgdorferi. They used the newly developed high throughput SYBR Green I/ propidium iodide (PI) assay and identified 27 drug candidates with higher anti-persister activity

than the current frontline antibiotics. They also demonstrated that daptomycin and clofazimine (which had the highest activity against non-growing persisters), had relatively poor activity or a high minimal inhibitory concentration (MIC) against growing B. burgdorferi. With the aim to fight recalcitrant and severe acute chronic and persistent human infections due to P. aeruginosa, Starckey et al.22 targeted the P. aeruginosa quorum sensing (QS) virulence MvfR pathway to isolate robust molecules that specifically inhibit infection without affecting bacterial growth or viability to mitigate selective resistance. For this target, they used a whole-cell high-throughput screen (HTS) and structure-activity relationship (SAR) analysis and were able to identify compounds that block the synthesis of both pro-persistence and pro-acute MvfR-dependent signaling molecules which are active against P. aeruginosa acute and persistent murine infections, and at the same time do not perturb bacterial growth paving the way for a next generation therapeutics. de Miranda Silva at al.12 tested the combination of Pretomanid (PA824) and moxifloxacin (MXF) that are currently under investigation for the treatment of susceptible and resistant Mycobacterium tuberculosis, and concluded that this combination was additive against organisms in the growth phase (log phase), acid phase, and nonreplicating-persister (NRP) phase. Maleki et al.16 focused their research on the bacterial toxin-antitoxin (TA) systems that are promising candidates for the development of antibacterial agents, and they we able to confirm the presence of mazEF and relBE TA systems in N. meningitides.

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QUESTION 46: Should you use the same sterilization method in vitro as you will be using in vivo?

David Armbruster and Rajendra Kasinath

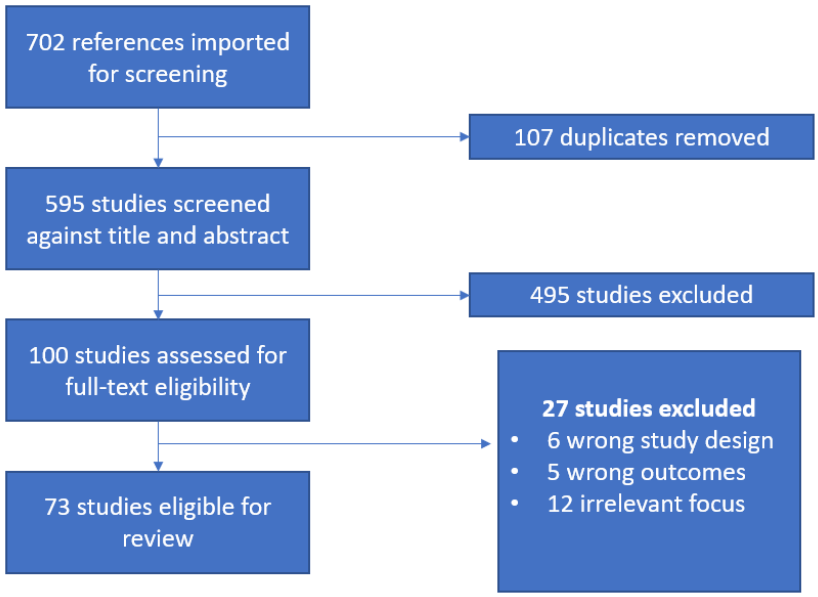
RECOMMENDATION: Yes

Survey of the systematically selected literature revealed that sterilization could affect the physical, chemical and mechanical properties of the intended implants or materials. The effect of sterilization method on *in vivo* performance has been demonstrated specifically for antimicrobial technologies. From the perspective of commercializing a new antimicrobial technology it would make sense to determine sterilization viability as early as possible, as it pertains to device performance, before sterilization of *in vitro* test samples.

STRENGTH OF EVIDENCE: Strong

RATIONALE:

The PRISMA method used to conduct a semi-systematic search is presented below:



Searches were conducted between 11 Sept. and 30 Sept. 2022, within the following databases: PubMed, Google Scholar, RightFind, Endnote Click, and the Cochrane Library (Wiley).

The search strategy combined two separate searches which included all appropriate controlled vocabulary and keywords for “sterilization”, “in vivo”, “in vitro”, “protocols ”, “guidance”, “implants”, and “devices” (search 1) and “effect of”, “gamma”, “electron beam”, “autoclave”,

“ethylene oxide”, and “sterilization” (search 2). In order to limit publication bias, there were no language, publication date, or article type restrictions on the search strategy.

Survey of the systematically selected literature revealed that sterilization could affect the physical, chemical and mechanical properties of the intended implants or materials. Evidence strongly suggested this to be critical for softer materials such as polymers, and mainly resorbable ones, however lesser effects have been seen in materials such as commercially pure titanium as well.[1, 2] Some studies demonstrate this directly by measuring properties after sterilization with different techniques. Significant correlation between sterilization method and wear performance have been demonstrated, [3] as was physicochemical property changes for PLGA based scaffolds [4] and SIS (small intestinal submucosa) as a 3D extracellular matrix (ECM) material [5] for tissue engineering applications. Point-of-care (POC) sterilization studies also indicated that the number of sterilization cycles even if the same technique was employed could also be an important property determinant, for example the performance of surgical drill bits. [6]

The effect of sterilization method on *in vivo* performance has also been demonstrated specifically for antimicrobial technologies. As an example, for a crosslinked cyclodextrin drug delivery matrix, the *in vitro* release of antibiotics was significantly reduced by autoclave sterilization as compared to gamma or ethylene oxide sterilization. This was attributed to an increase in crosslinking density due to the autoclave conditions [7]. A chemical sterilant such as ethylene oxide (EtO) can react with active agents, as shown in a study of collagen/PLGA microparticle composites containing gentamicin. NMR revealed that EtO exposure chemically modified the gentamicin, although antimicrobial effectiveness *in vitro* was only slightly reduced. [8] The effect of sterilization method on antimicrobial performance may change with minor differences in formulation chemistry. In a study of hydroxyapatite (HA)-based bone graft substitutes coated with self-assembled monolayers (SAMs) containing silver, there was significant differences in damage to SAMs by different sterilization methods, with the effect of UV and EtO differing only on molecular chain length of the SAM. [9]

Importantly, it has been shown that bacterial colonization of base biomaterials can be affected by sterilization method as well. Studies of bacterial biofilm formation on zirconia disks demonstrated that dry heat sterilized samples showed significantly lower bacterial growth, while ultraviolet and gamma ray irradiation resulted in the highest biofilm growth. [10] A similar study of ultrahigh molecular weight polyethylene showed that fewer bacteria adhered after sterilization with ethylene oxide than after sterilization with gas plasms, especially to the smoothest surfaces. [11] These studies imply that the method of sterilization must be considered even for untreated control samples, and that consistency within a study is important. As such maintaining sterilization technique and protocols between *in vitro* and *in vivo* studies seems critical to ensuring that device performance in not altered during testing.

While this is an important question, it is clear there are additional restrictions on the choice of sterilization method for antimicrobial technologies. Choice of sterilization technique is practically

determined by its impact on clinical performance and commercial viability. For example, if a sterilization technique would negatively or unpredictably affect device antimicrobial performance, then it will not be clinically acceptable. Commercial examples of these instances are triclosan loaded devices or betadine wound washes that cannot be gamma sterilized without damage to the active agent. In these cases, an alternative method is required to ensure sterility and the limitation on sterilization options should be considered when conducting benchtop or *in vitro* product testing. From the perspective of commercializing a new antimicrobial technology it would make sense to determine sterilization viability as early as possible in the research program, as it pertains to device performance, before sterilization of *in vitro* test samples.

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QUESTION 47: Are there rigorous in vitro bone cell models for intracellular infection in osteomyelitis?

Gerald J. Atkins, Anja R. Zelmer, Noreen J. Hickok

RESPONSE/RECOMMENDATION: Yes

The evidence is consistent that *S. aureus*, at least, is able to exist intracellularly in the many different osteoblastic cell types examined. Critical controls for these experiments include non-internalising strains of bacteria to demonstrate extracellular clearance by gentamicin or lysostaphin, characterisation of time and MOI dependence, and characterisation of osteoblastic survival during co-culture. Insufficient data exist for non-osteoblastic MSK cells.

STRENGTH OF EVIDENCE: Moderate

RATIONALE:

Introduction and Approach:

We addressed using a systematic review approach the question, 'Are there rigorous *in vitro* bone cell models for intracellular infection in osteomyelitis?'. A PubMed search strategy was designed to address this question: ((((intracellular infection) AND (bone)) AND (osteomyelitis)) AND (English[Language])) NOT (Sars-cov-2). This translated into the following search:

(("cytoplasm"[MeSH Terms] OR "cytoplasm"[All Fields] OR "intracellular"[All Fields]) AND ("infect"[All Fields] OR "infectability"[All Fields] OR "infectable"[All Fields] OR "infectant"[All Fields] OR "infectants"[All Fields] OR "infected"[All Fields] OR "infecteds"[All Fields] OR "infectibility"[All Fields] OR "infectible"[All Fields] OR "infecting"[All Fields] OR "infection s"[All Fields] OR "infections"[MeSH Terms] OR "infections"[All Fields] OR "infection"[All Fields] OR "infective"[All Fields] OR "infectiveness"[All Fields] OR "infectives"[All Fields] OR "infectivities"[All Fields] OR "infects"[All Fields] OR "pathogenicity"[MeSH Subheading] OR "pathogenicity"[All Fields] OR "infectivity"[All Fields]) AND ("bone and bones"[MeSH Terms] OR ("bone"[All Fields] AND "bones"[All Fields]) OR "bone and bones"[All Fields] OR "bone"[All Fields]) AND ("osteomyelities"[All Fields] OR "osteomyelitis"[MeSH Terms] OR "osteomyelitis"[All Fields] OR "osteomyelitides"[All Fields]) AND "English"[Language]) NOT ("sars cov 2"[MeSH Terms] OR "sars cov 2"[All Fields] OR "sars cov 2"[All Fields])

As outlined in **Figure 1**, this generated 77 hits which were uploaded into the Covidence systematic review program. Abstract screening by 3 independent observers removed 43 studies as irrelevant to the question. Eligibility of the remaining 34 studies removed a further 6 studies based on context (1 study), design (1 study) and non-relatedness to osteomyelitis (4 studies). Twenty eight studies were included for full-text analysis.

Figure 1: PRISMA flowchart.

Full-text analysis considered whether the study utilised a defined bone cell type, i.e. either osteoblast, osteocyte, osteoclast, chondrocyte or synovial fibroblast, and whether the study tested a bacterial pathogen. The number of studies addressing each **bone cell type**, the **host species**, whether **primary cells** or **cell line**, is shown in **Table 1**.

'Rigour' was ascribed to each study according to the following criteria:

- Bacterial number was defined;
- The multiplicity of infection (MOI) was defined;
- At least one reference strain and preferably at least one clinical isolate was used;
- A measure of reproducibility was assessed, e.g. biological replicates, dose/time responses;
- Suitable controls were included;
- There was evidence of intracellular bacterial survival (e.g. recovery from host cell lysates; this would usually necessitate that extracellular bacteria were removed prior to assessment);
- There was evidence of at least some host cell survival;
- Host-pathogen interactions could be examined.

Results and Discussion:

Of the studies fitting the inclusion criteria of our search, four described the use of mouse primary osteoblasts digested from calvariae. These cells are widely used in bone cell biology and recapitulate most if not all features of osteoblasts *in vivo*. To add to the robustness in the context of osteomyelitis, calvarial cells from at least 3 mouse strains have been utilised, including the in-bred strains C57BL/6[1,2] and Balb/c[3] and the outbred strain CD-1[4]. As summarised in Table 1, these studies demonstrate the versatility of calvarial osteoblasts with respect to interactions with a variety of pathogens, including the *S. aureus* osteomyelitis clinical isolate UAMS-1, a number of other *S. aureus* isolates and reference strains, as well Gram-negative salmonellae and *Escherichia coli*. Together, these studies show responses to a range of MOI and utilised controls, mostly uninfected controls. The study by Marriot *et al*.,[3] used additional controls including non-invasive strains, such as the staphylococcal strain *S. carnosus* and Salmonella strain SB136, as well as UV irradiation to render bacteria invasion incompetent. Overall, the mouse calvarial osteoblast model appears robust and suitable to conduct studies of host-pathogen interactions, at least in the context of non-human osteomyelitis.

Three studies [5-7] utilised the mouse calvarial osteoblastic MC3T3-E1 cell line, an extremely well established model used in more than 5,300 published studies to date. Two of these studies were focussed on eradication of internalised *S. aureus* and also tested the coagulase-negative strain *S. epidermidis.* [5,6] The third study focussed on the molecular mechanism of *S. aureus* internalisation [7] While each of the studies defined the number of bacteria added, none examined MOI-dependent effects reflecting the focus away from host-pathogen interactions following infection. However, all included uninfected controls and demonstrated internalised bacteria using CFU assays, suggesting MC3T3-EI is a useful host model, and due to its transformed nature would be suitable for high throughput applications.

Interestingly, human primary osteoblasts were more frequently represented with eight of the included studies utilising various forms of these cells, by their nature immediately addressing the questions of possible species variation and clinical relevance. Nevertheless, it is preferable that reasonable validation of the osteoblastic nature of such preparations is performed, either prospectively or by virtue of the readouts obtained. The seminal explant growth method published by Robey and Termine [8] is the basis of most, if not all, studies utilising cancellous bone-derived human osteoblasts. Two studies used commercially available cell preparations, proprietary validated cells usually derived from a single donor but the characteristics of the donors were not specified [9,10]. Of the included studies that used bone chip explant cultures, one study used mandible [11], one used proximal femur from adult arthroplasty patients [12] while the source of bone in others was not specified [13-16]. Collectively, the human osteoblast studies demonstrated that a wide range of MOI for a number of *S. aureus* clinical and laboratory strains, and in one study salmonella species, could result in intracellular infection over a 24h – 7d infection period (**Table 1**). Overall the studies were suitably controlled. The diverse sources of human bone (and therefore characteristics of the individual donors) for these studies suggests that the model is generally useful, however, might be less reproducible.

Two of the included studies used human mesenchymal progenitor cell preparations [11,17]. As for primary osteoblast studies, validation of the respective cell preparations would seem desirable,

but this was not always provided. Given the use of the few bacterial strains and MOIs reported to date it is not yet clear if a starting population of host cells presumably capable of lineage plasticity represents a rigorous model and further studies are warranted.

Since access to human bone or commercially-derived cells is not always possible, a human osteoblast cell line model is desirable. Two included studies utilised MG-63 osteosarcoma cells[18,19]. This cell line has phenotypic properties of a committed pre- or immature osteoblast, showing limited *in vitro* differentiation and mineralisation potential and interestingly, is the only such line to express the MSC marker STRO-1. The general merits of MG-63 as a model are discussed in more detail by Stracquandanio[20]. Collectively, the two studies examined a number of *S. aureus* strains over a 3h-48h time frame and demonstrated intracellular persistence of at least some of the strains[18,19]. Host cell viability was measured, and Musso and colleagues presented host cytokine release in response to infection[18]. Three studies reported findings with the SaOS-2 human osteosarcoma cell line [21-23]. These cells represent a mature osteoblast phenotype and are capable of *in vitro* differentiation to a mature osteocyte-like stage when cultured under specific osteogenic conditions[24]. These cells have so far only been tested with respect to *S. aureus* infection at a relatively restricted range of MOI, but are clearly capable of bacterial internalisation. Together, MG-63 and SaOS2 seem to be good human cell line models of the immature and mature osteoblast, respectively, with respect to intracellular infection, at least by *S. aureus*.

As the osteocyte is the most numerous and long-lived cell type in bone, their role in infection, particularly chronic disease, appears highly clinically relevant. A recent study by Garcia-Moreno and colleagues, published data using the mouse osteocyte cell line and most utilised cell model of the osteocyte, MLO-Y4[25]. Internalisation of a single strain of *S. aureus* was demonstrated with good experimental replication, and a thorough host proteomics response profile performed, demonstrating the usefulness of the model. The utility of human osteocytes was demonstrated by Yang *et al*.,[12] who differentiated human primary osteoblasts (bone explant-derived) to an osteocyte stage, a model previously validated by the group in a number of studies, and then exposed the cells to various strains of *S. aureus* at various MOI. Host transcriptomic profiling was performed, as well as bacterial intracellular persistence and phenotype switching demonstrated. The model is robust, although technically demanding due to the relatively long culture period required (28d) to generate an osteocyte-like phenotype. The same group subsequently published a human osteocyte-like cell line model using (28d) differentiated SaOS-2 cells[22], and demonstrated similar features to the primary osteocyte infection model. We anticipate this will prove useful for high-throughput applications, such as testing effectivity of antibiotics against intracellular bacteria[2]. It is noteworthy, that in the case of both published human osteocyte models, the cells reside in a multi-layered endogenous type I collagen mineralised matrix, mimicking the arrangement of osteocytes *in vivo*. This is in contrast to MLO-Y4 cells, which do not require differentiation but also do not produce significant organic matrix or lay down mineral.

Only one of the studies included considered infection of the major bone resorbing cell type, the osteoclast. Krauss and colleagues studied infection of osteoclasts differentiated from mouse bone

marrow mononuclear cells or human peripheral blood mononuclear cell preparations[26]. While elegant and excellent intracellular localisation data are shown, further studies of this cell type are required to include greater bacterial strain diversity, MOI range and functional outcome measures. An interesting and complicating aspect of osteoclastogenesis assays is the incomplete nature of differentiation and most cultures contain mature osteoclasts as well as monocyte/macrophages, the contribution of which is potentially difficult to tease out.

None of the included studies considered the chondrocyte as a host cell, and indeed, *in vitro* modelling of this important cell type in general significantly lags behind the other bone cell types. However, as discussed in the review by Alder[27], chondrocytes have been challenged with *S. aureus* in a few studies, which did not internalise bacteria but showed signs of apoptosis. This may indicate that they are not able to internalize bacteria and therefore are not a relevant model for intracellular infection, however further work is warranted to establish this.

Synovial fibroblasts also were not represented in the retrieved studies; these are of added potential relevance since synovial membrane during surgical investigations of PJI is often sent to diagnostic laboratories. Also as discussed by Alder[27], other studies have shown fibroblasts are capable of internalising bacteria, including *S. aureus* [28-30]and *S. epidermidis* [31], and so should also be studied in more detail.

Beyond infection of specific bone cell types, another consideration is that of 3-dimensional (3D) models, more specifically, bone cells arranged in a 3D as opposed to 2D format, to better reflect the hierarchical or structural nature of bone. Only one of the included original research studies also examined bone. Human cancellous bone fragments were subjected to *S. aureus* infection in an *ex vivo* model by Yang *et al*., and findings similar to those obtained for *in vitro* differentiated primary osteocytes were reported, including evidence of intra-osteocytic infection[12]. As reviewed by Hofstee *et al*.[32] and discussed further by Stracquandanio and colleagues[20], a variety of other 3D models have been described but these did not fit the inclusion criteria of this review so perhaps have not yet been validated for studying intracellular infections. Having said that, as discussed above, models that entail long-term osteogenic differentiation of osteoblasts tend to result in multi-layered cultures rather than single (2D) layers of cells, so incorporate the variable of 3D spatiality at least to some extent.

Most studies define the quantitative relationship between bacteria and host cell with a MOI. However, Stracquandanio *et al*.[20] point out that the number of internalised bacteria, often quantified as CFU, strongly depends on the MOI, however this is not necessarily a linear relationship. In contrast, the percentage of internalised bacteria (PIB) is relatively independent of the MOI, once the internalisation minimal inoculum is overcome. Therefore, the PIB should be more comparable between experiments with different MOI and should be determined as well. Furthermore, a range of MOI, at least to determine an appropriate one for the given experiment, should be a standard.

The diversity of experimental design and readouts utilised in the included studies should be acknowledged. While many of the features of each of the studies add value in their own right, deciding on a set of standard design features is desirable and would aid in comparing outcomes between studies. As discussed by Zelmer and colleagues [(2)], the wide heterogeneity between models affects the interpretation of antibiotic effectivity against intracellular pathogens, which is important when deciding how to treat these clinically.

We propose that standard models of intracellular infection for each of the relevant bone cell types is desirable and this may require multi-centre collaborative studies using agreed protocols to standardise these. For optimal clinical relevance, primary cell models are preferred, utilising multiple donor sources in the case of human models, as well as bacterial strains, to allow generalisation of research findings. Cell line models clearly also have a place as they allow ready standardisation and replication between groups and are more amenable to high throughput applications. The choice of cell line should as much as possible reflect the phenotype of the primary cell type under consideration. Cell lines are capable of variable degrees of differentiation, which should be taken into account when interpreting data and are also prone to phenotypic drift and the appearance of sub-lines. Finally, when clinical relevance is a major focus of the study or application, human models are preferred. We conclude that good progress has been made in developing rigorous models of intra-osteoblastic infection in both the mouse and human. Despite the many different osteoblastic cell types examined, the evidence is consistent that *S. aureus*, at least, is able to exist intracellularly in these cells. Critical controls for these experiments include non-internalising strains of bacteria to demonstrate extracellular clearance by gentamicin or lysostaphin, characterisation of time and MOI dependence, and characterisation of osteoblastic survival during the co-culture period. Both primary and cell line infection models of the human osteocyte have also been described but these require repetition by other laboratories. Further work is required to establish rigorous models for osteoclasts, chondrocytes and synovial fibroblasts. Lastly, the bone lining cell remains to be effectively modelled, certainly in the context of osteomyelitis where these cells are, presumably, readily exposed to invading pathogens.

Cell Type	Model Validation *	Bacterial Number Definition	MOI	Species/Strains	Reproducibility (replicates/ time points)	Controls Used	Intracellular Bacterial Survival	Host Cell Survival	Host-Pathogen Interactions	Study
murine primary OB from C57BL/6 and NOD2-deficient mouse neonate calvariae	Not included	yes	25 75 250	*Salmonella enterica serovar Typhimurium* strain SB300; *Salmonella enterica serovar Typhimurium* strain SB136 *S. aureus* UAMS-1 (ATCC 49230)	technical replicate multiple strains	uninfected CTRL 3 ratios of MOI	flow cytometry	flow cytometry	inflammatory cytokines via ELISA Immunoblot analysis NOD2 and Grim-19 Co-immunoprecipitation Rip2 kinase with NOD2	(1)
murine primary OB from BALB/c mouse	Not included		2-3 different MOI per strain	*S. aureus* UAMS-1 (ATCC 49230); *S. carnosus* (ATCC 51365) *Salmonella enterica serovar; Typhimurium* strain 12023 (ATCC 14028); *Salmonella enterica serovar Typhimurium* strain SB300 *Salmonella enterica serovar Typhimurium* strain SB136		*S. carnosus* and *Salmonella enterica serovar Typhimurium* strain SB136 as non-intracellular CTRL UV treated CTRL Multiple MOI and uninfected ctrl	CFU count intracellular bacteria		PCR of RNA Western Blot ELISA	(3)
murine Osteoblasts from 1–2-day-old CD-1 mice	Not included	yes	25 75	*E. coli* HB 101 *S. aureus* UAMS-1 (ATCC 49230); *S. aureus* RN4220 phage and plasmid	only 1 replicate for CFU count	uninfected CTRL	CFU count intracellular bacteria flow cytometry			(4)
Mouse MC3T3-E1 osteoblast, differentiatied (mineralised)	n/a	yes		*S. aureus* ATCC 25923	triplicates 4TP:1/8/24/48h	uninfected, infected untreated CTRL particles with and without Cl	extracellular bacteria were not removed or controlled for CFU count bacteria fluorescent microscopy	MTT assay	PCR of mRNA	(33)
Mouse MC3T3-E1 osteoblast MLO-Y4 osteocyte Co-culture 1:3	n/a	yes	30	*S. aureus* LS1 from a septic arthritis isolate *S. aureus* SH1000 8325–4 with functional rsbU	4 TP: 0/2/4d/7d triplicates	uninfected CTRL co-culture and single culture	CFU count intracellular bacteria and supernatant (including SCV)	Zombie Aqua Fixable Viability Kit (flow cytometry) cell death at different MOIs	cells were exposed to media from infected/ uninfected cells Agr functionality (CAMP assay) psmα RNA expression, Proteomicsl ELISA Lipid Meditators	(25)

									Measured by UPLC-MS–MS	
Mouse MC3T3-E1 osteoblast	n/a	yes	no	*S. aureus* USA 300-0014 (MRSA) *S. aureus* CDC-587 (MSSA) *S. epidermidis* RP-62A	triplicates	multiple drug concentration with different formulations and untreated CTRL	CFU count intracellular bacteria and supernatant	MTT assay only alone and with new component, not with bacteria		(5)
Mouse MC3T3-E1 osteoblast	n/a	yes	no	*S. aureus* USA 300-0014 (MRSA) *S. aureus* CDC-587 (MSSA) *S. epidermidis* RP-62A		multiple drug concentration with different formulations and untreated CTRL	CFU count intracellular bacteria and supernatant	MTT assay only alone and with new component, not with bacteria		(6)
Mouse MC3T3-E1 osteoblast	n/a	yes		*S. aureus* from osteomyelitis patient	infection for 15/ 30/60min before clearing min. triplicates	no treatment and no bacteria vs bacteria no treatment vs bacteria and treatment	fluorescence microscopy CFU count intracellular bacteria		western blot	(34)
differentiated pre-osteoblastic OBβ1 +/+ cell line, obtained after immortalization of mouse primary cells with the T viral oncogene of SV40	Yes, data included		50	Isogenic strains of *S. aureus* 8325-4 and 8325-4ΔfnbAB	3TP: 3/7/120h duplicates	uninfected and heat killed CTRL	CFU count intracellular bacteria	MTT assay	Alkaline phosphatase measurement PCR of RNA	(35)
Normal human osteoblasts (Clonetics, San Diego, CA, USA) - proprietary prepared human osteoblasts - unspecified origin	Proprietary		250	*S. aureus* UAMS-1 (ATCC 49230)	3TP :0/24/28h		CFU count intracellular bacteria			(9)
Normal human osteoblasts (Clonetics, San Diego, CA, USA) - proprietary prepared human osteoblasts - unspecified origin	Proprietary		1 3 10	*Salmonella enterica serovar Typhimurium* strain SB300 *Salmonella enterica serovar Typhimurium* strain SB136		*Salmonella enterica serovar Typhimurium* strain SB136 (invasion defective) uninfected CTRL	flow cytometry	flow cytometry PI	DNA ladderin PCR of RNA Elisa	(10)

Primary human osteoblasts (pHOB) - cancellous bone chip-derived; unspecified origin	Yes, data not shown		50	*S. aureus* isolates were obtained from 41 patients *S. aureus* Cowan I ATCC 12598 *S. aureus* clinical isolate 6850	5TP: 0/1/2/4/7d >40 strains		CFU count intracellular bacteria (including SCV) TEM	PI cytotoxicity assay 24h post infection	ELISA	(13)
Primary human osteoblasts - cancellous bone chip-derived; unspecified origin	No	yes	100	*S. aureus* Cowan I ATCC 12598 *S. aureus* ATCC 49230 *S. carnosus* TM 300 (neg ctrl)	2TP:20/40h after treatment	*S. carnosus* TM 300 (neg) no uninfected CTRL only 1 concentration of ABs, but also combi and alone	CFU count intracellular bacteria			(14)
Primary human osteoblasts - origin not specified or available in cited reference.	No	yes	100	*S.aureus* Cowan *S.aureus* TM 300 *S.aureus* 49230	2TP:20/40h	treatment/ treatment combi	CFU count intracellular bacteria			(16)
primary human osteoblasts: origin not specified and unavailable in cited reference - presumably bone chip derived similar to other studies from this group.	No		50 after testing multiple	*S. aureus* strain 685028 *S. aureus* SH100031 and two clinical osteomyelitis *S. aureus* osteomyelitis clinical isolates x2	2TP:0/7d	MOI testing: 10, 20, 30, 40, 50, 60, 70, 80, 100 at 24/48/72/96h uninfected CTRL	CFU count intracellular bacteria (including SCV) electron micrographs	Tryptan blue staining		(16)
human bone marrow-derived MSC Human bone (mandible) chip derived osteoblasts THP-1 cell line, differentiated into macrophages	No		30 MSC 100 OB 1THP-1	2 Clinical *C. acnes* strains (Cb, Ci)- no defined reference strain	6 human donors for primary cell generation 3 cell types technical duplicates	cytochalasin control	CFU count intracellular bacteria and supernatant imaging of labelled *C. acnes* TEM	flow cytometry	ELISA AFM IR	(11)
human adipose-derived MSC (ADMSCs) differentiated into osteogenic lineage	Yes, data included		30	*S. aureus* (SA113) GFPP	2TP:4/24h 6 replicates	multiple AB conc	flow cytometry CFU count intracellular bacteria	live/dead assay confocal microscopy (uninfected, after 4/24h) infected cells after 4/24h by SEM,		(17)

								confocal microscopy and differentiation		
human osteosarcoma cell line MG-63 OB	n/a		100	4 MRSA CI: 2SA-ST239-III (ST239) 5SA-ST5-II (ST5), 10SA-ST228-I (ST228), 14SA-ST22-IVh (ST22)	2TP:3/24h min triplicates	MOI tested before uninfected CTRL	flow cytometry spot categories	MTT assay	PCR of RNA ELISA	(18)
	n/a		100	*S. aureus* DFU isolate NSA1385 and its isogenic ROSA-like– negative (Δrosa) variant	3TP:3/24/48 h min 3 replicates	*S. aureus* DFU isolate NSA1385 and its isogenic ROSA-like– negative (Δrosa) variant	CFU count intracellular bacteria	LDH assay		(19)
SaOS2 human osteosarcoma cell line	n/a	yes	30	*S. aureus* isolate EDCC 5055 (=DSM 28763)	4TP:2/4/6/24 h and 2TP:4/20h min triplicates	CpG-ODN type-A 2216, type-B 2006, or negative CpGODN 2243 (negative control)	CFU count intracellular bacteria	oxidative stress by flow cytometry	PCR of RNA	(23)
	n/a	yes	10	*S. aureus* BB1279 (GFP)- no defined reference strain		Treatment / No treatment / Treatment combi	CFU count supernatant fluorescence microscopy PI uptake	osteoblast cell viability with WST-1 reagent in every treatment	efflux pump effectivity only tested on host cell alone	(21)
primary human osteoblasts and differentiated osteocyte-like cells: Cancellous bone chip-derived from patients undergoing arthroplasty for end-stage osteoarthritis	Yes, data included		1 10 100	*S. aureus* WCH-SK2 *S. aureus* stain RN6390 (GFP)	5TP:1/2/3/4/ 5d triplicates	uninfected CTRL	CFU count intracellular bacteria (including SCV) Live-cell imaging of osteocyte-like cell infection 4-24h SEM	Live-cell imaging of osteocyte-like cell infection 4-24h	ELISA PCR of mRNA immunohistochemistry gene microarray	(12)
SaOS2 human osteosarcoma cell line either undifferentiated and differentiated to osteocyte-like stage	Yes, data included	yes	Yes, multiple	*S. aureus* WCH-SK2 *S. aureus* DFI clinical isolates GFP expressing *S. aureus* strain RN6390	3TP:2/24/ 96h	uninfected CTRL treatment/ no treatment	fluorescence microscopy CFU count intracellular bacteria and supernatant(including SCVs and "slow g rowers") TEM	LDH-assay	PCR of RNA and DNA gene expression	(22)
Mouse Osteoclasts	Yes, data included	yes	1	*S. aureus* USA300 clinical isolate LAC (45)	2TP:2/18h		CFU count intracellular		Immunoblotting	(26)

differentiated from BMMC; human osteoclasts differentiated from PBMC							bacteria and supernatant flow cytometry			

Table 1: Summary of the literature review results.*Validation refers to evidence contained in the published study that the primary cell model displays the expected phenotypic characteristics; here, ‘not included’ means the cells were generated by a published and previously validated method only; n/a: not applicable.

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Back to top

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Back to top

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QUESTION 48 : Is there a standard method to detach and quantify bacteria attached to surfaces?

Matthew Libera, Britt Wildemann

RECOMMENDATION: No

There are many methods to recover bacteria from surfaces. All have the shortcoming that not all of the bacteria are recovered and not all recovered bacteria are culturable. Hence, these methods are better suited for indicating the presence of bacteria and less well suited for quantifying the total amount of bacteria.

STRENGTH OF EVIDENCE: Moderate

RATIONALE:

The question is best approached in two parts, where our rationale for these is detailed below.

49a. Among the various methods for recovering bacteria from in vitro test coupons or from the surfaces of explants, sonication is the most common.

- Response/Recommendation: The current literature describes a number of methods to recover bacteria from in vitro test coupons. Among these are sonication, enzymatic or chemical treatment, applying electrical current, and hybrid methods that combine imaging with chemical or mechanical treatment. Sonication appears to be the most frequently used of these methods.
- Strength of the Recommendation: The strength of the recommendation that sonication is the most common method is moderate.
- Rationale: The statement is based on a search of the literature which indicates that sonication is the most common method, though not overwhelmingly so. 1-13

Searches using Scopus and PubMed using different combinations of keywords (detachment, bacteria, implants, orthopedics, method, and/or in vitro) returned a total of 126 references. Several of the papers compared subsets of these methods, but none provided an explicit choice of which is the most effective technique from among them all. After removing duplicates and, based on the abstracts, papers that insufficiently addressed bacterial recovery, the number of relevant publications totaled 16. These were then separated into categories based on the method of recovery: Sonication; Enzymatic/chemical treatment; Imaging; Electrical Current; Hybrid methods; and miscellaneous methods difficult to categorize. The total number of papers was further reduced to 14, because three of them were from the same research group and effectively said the same thing. Of the 14 total, 5 addressed some variation of sonication. The remaining 9 papers were scattered amongst the many other methods with no obvious second most popular approach.

49b Among the various methods for recovering bacteria from the tissue surrounding an explant, homogenization is the most common.

- Response/Recommendation: In the current literature, various methods for mechanical processing of the tissue are described that improve the retrieval of bacteria from tissue. A systematic comparison of various methods was done by one research group using different tissues and they found the best retrieval after homogenization.
- Strength of the Recommendation: The recommendation to process the tissue for bacterial retrieval is strong, while the strength of the recommendation for a specific method is moderate.
- Rational: The statement is based on a search of the literature describing several

methods, with homogenization being the most common method, and superiority over other methods was demonstrated in two studies. 14-23

Mechanical tissue processing is the main method used the retrieve bacteria from tissues. The methods used include e.g. homogenization, vortexing, and bead milling. Further factors that influence the microbiological results are the tissue sampling (procedure, location and sample number), the culturing (medium and duration), handling (contamination risk), and antibiotic treatment and should always be considered. Tissue processing methods, such as bead beating, might reduce the viability of bacteria. The statement is based on the recommendations of scientific organizations and studies investigating various methods. Only two studies from one research group are published comparing different methods. A Pubmed and Google scholar search was performed (Boolean search: diagnostics AND infection AND bone AND Implant AND biopsy AND bacteria and microbiology NOT imaging; 2012-2022) resulting in 192 publications. Further selection excluded 132 publications (duplicate, title/abstract check, language). Cross-reading the remaining 60 publications lead to the identification of used/recommended methods for bacterial isolation from tissue. A further search for recommendations from scientific organizations and an individual search was performed. Final screening identified 10 publications included in this statement.

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Back to top

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Back to top

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Back to top

QUESTION 49: Can existing ISTA/ASTM standards be used for constructing biofilm models on medical devices?

Jerry Tsang and Annelies Zinkernagel

RECOMMENDATION: No

ASTM has standardized methods/guidelines for accurate and reproducible biofilm formation and testing of antimicrobials. A previous standard for selecting test methods to determine antimicrobial effectiveness was withdrawn in 2009. Standard methods are mainly used for environmental biofilms, and standardised methods and techniques for the evaluation of clinically-relevant biofilms are needed.

STRENGTH OF EVIDENCE: Strong

RATIONALE:

The American Society for Testing and Materials (ASTM International) Volumes 13.01 and 13.02 include over 380 standards on medical and surgical materials and devices. They cover metals, polymers, and ceramics for implants, prostheses, and medical and surgical devices; silicone elastomers, gels, and foams in medical applications; and tissue engineered medical products. Each standard typically covers manufacture, chemical requirements, mechanical requirements, special tests, and certification1,2. An ATSM symposium on “Antimicrobial combination devices” in 2020 include held a session on methods for quantifying biofilms and methods for assessing antimicrobial efficacy in biofilm eradication3–5.

ATSM has implemented standardised methods, guidelines, and specifications for the accurate and reproducible formation of biofilms and testing of antimicrobial substances6. There are four types of biofilm devices which have been addressed by one or more ASTM standards. The drip flow reactor and rotating disk reactor are used to evaluate biofilm formation in a continuous flow under low and medium shear stress, respectively, (ASTM E2647-20 and ASTM E2196-17)7,8. In the evaluation of disinfectants, the CBD (ASTM E2799-17) and the CDC biofilm reactor (ASTM E2562-17, ASTM E3161-18, ASTM E2871-19) are recommended9–12. The colony biofilm model has also been recently adapted to develop a standard test method (ASTM E3180-18) to grow and quantify Bacillus subtilis biofilms13. These standards methods are mainly used for environmental biofilms, and there remains an unmet need to standardise methods and techniques for the evaluation of clinically-relevant biofilms 14,15. A previously published standard for selecting test methods to determine the effectiveness of antimicrobial agents and other chemicals for the prevention, inactivation and removal of biofilm was withdrawn in 200916.

Medline

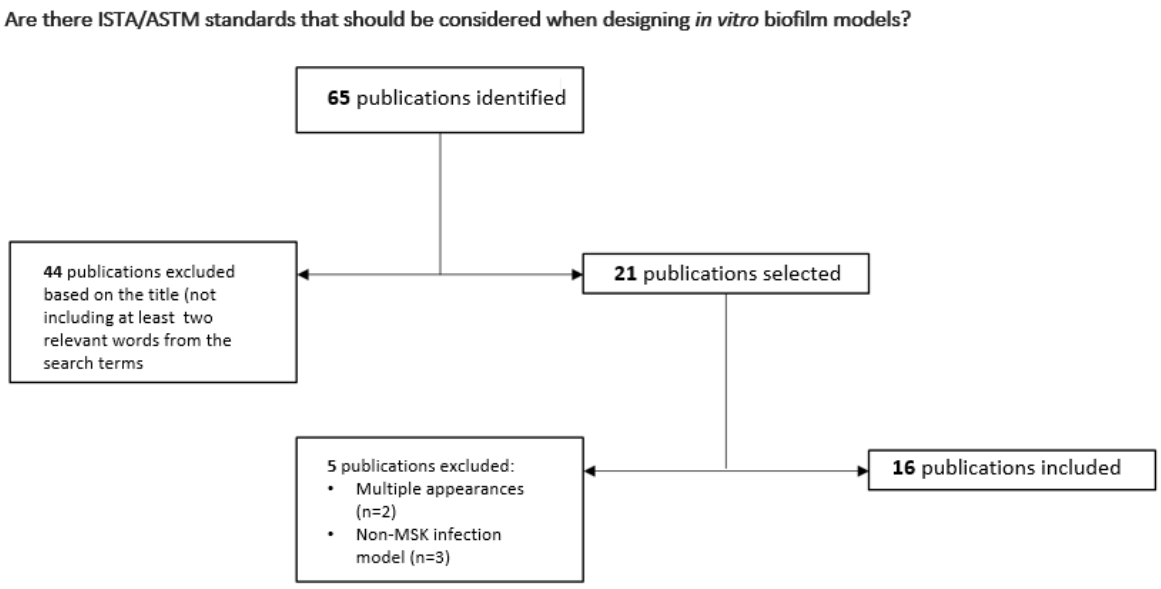
# ▲	Searches	Results	Type	Actions	Annotations
1	exp Bacteria/ or ISTA.mp.	1485999	Advanced	Display Results | More	
2	exp Anti-Bacterial Agents/ or ASTM.mp.	793984	Advanced	Display Results | More	
3	biofilms.mp. or exp Biofilms/	45874	Advanced	Display Results | More	
4	1 and 2 and 3	9253	Advanced	Display Results | More	
5	in vitro models.mp.	10303	Advanced	Display Results | More	
6	4 and 5	21	Advanced	Display Results | More	

Web of Science

5	#4 AND #3	0	Add to query	
4	(ALL=(biofilms)) AND ALL=(In vitro)	9,148	Add to query	
3	#2 OR #1	2,299	Add to query	
2	ALL=(ATSM)	545	Add to query	
1	ALL=(ISTA)	1,754	Add to query	

ASTM website

Using “Biofilms” as a multipurpose search term yielded 44 articles products/services/information articles.



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Back to top

05. Animal

Back to top

QUESTION 50

Should we (as the MSKI group) recommend certain models (and promote them) to have more homogenous experimental settings?

Thomas Schaer, Volker Alt

Response/Recommendation: Yes

There are validated models to study MSKI. Specifically, there is strong evidence for mouse models of MSKI such as osteomyelitis, fracture related infection, prosthetic joint, and tibial implant infections. Conversely, there is only moderate evidence in rats, rabbits, pigs, small ruminants for validation of orthopaedic implant infection models. Frequent outcome measures in the validated animal models include in vivo imaging, measurements for cytokine levels, immune cells, and antibody responses, ex vivo imaging, ex vivo bioburden and histopathology. In conclusion the findings warrant a community-based effort toward establishing basic standards to validate MSKI studies in general and in particular also for higher vertebrate models (rabbits, small ruminants, and pigs). Overall, the literature on animal models of MSKI lacks harmonization and could benefit from guidelines (i.e., clinically relevant outcome measures, histopathology initiative).

Strength of Recommendation: Moderate

Rationale

To answer the question, we performed a systematic literature search for PubMed articles reported on the basis of Systematic Reviews and Meta-Analyzes (PRISMA). Pubmed, EMbase, Cochrane Library, Web of Science for animal models of MSKI, FRI, and PJI from database establishment to October 2022 according to English retrieval words, including “animal model and musculoskeletal infection”, “animal model and fracture-related infection”, animal model and joint infections” “animal model and septic arthritis” “animal model and orthopedic biofilm infections” and “animal model and implant associated infections”. The search parameters resulted in a high number of publications identified in the scientific literature (murine), however few were addressing the question of the utility of specific animal models of infection in a comprehensive way or in a manner allowing to compare between studies. Lack of experimental uniformity is especially the case in higher vertebrate models (small ruminants, pigs, and horses). A specific sub-cohort of studies addressed PJI where representative studies described prosthesis designs used in PJI animal models including the description of prosthesis designs in non-infected animal models which would be suitable for an infection model.[i,ii,iii,iv,v,vi,vii,viii,ix,x,xi,xii]

In recent years there have been several efforts and workshops within the larger scientific community toward consensus building in the space of MSKI. In March 2020 and in June 2022 there were two FDA workshops where animal models were the focus. The March 2020 public workshop (Advancing Animal Models for Antibacterial Drug Development) discussed models for late-stage antimicrobial drugs but failed to discuss topics of MSKI or guidelines.[xiii] The June 2022 workshop (Animal Studies for Orthopaedic Products) concluded that there are no established models for novel device types or devices whose identified risks cannot be mitigated with the existing models. Moreover, the 2015 Draft Guidance “General Considerations for Animal Studies for Medical Devices” states that an animal model should be generally accepted by the field for a certain study for a specific device type. There should be a reasonable amount of scientific evidence supporting the utility for a specific animal model for a specific preclinical study. There is a consensus that there may not be an established or accepted animal model for a given study of a specific device type.[xiv,xv] In 2020, the American Society for

Back to top

Testing and Materials (ASTM) published a series of papers from a symposium discussing Antimicrobial Combination Devices. As with FDA, ICM 2018, ASTM concluded that there is a lack of published standards for non-clinical studies assessing antimicrobial implants and that there is an urgent need for the community to work together toward developing standard methods that can better assess antimicrobial and antibiofilm technologies specifically for medical device applications.[xvi]

The 2018 International Consensus Meeting on orthopedic infections concluded that there is paucity of validated animal models and recommended that the animal model of choice should be robust to address the research questions pursued.[xvii] The scientific literature has seen a marked increase in publications addressing MSKI and there are reviews discussing best practices in preclinical *in vivo* testing using animal models for anti-infective implant technologies and models of osteomyelitis.[xviii,xix,xx,xxi,xxii]

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Back to top

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Back to top

QUESTION 51

Are there specific preclinical animal models of musculoskeletal infection that are accepted by FDA and other regulatory agencies.

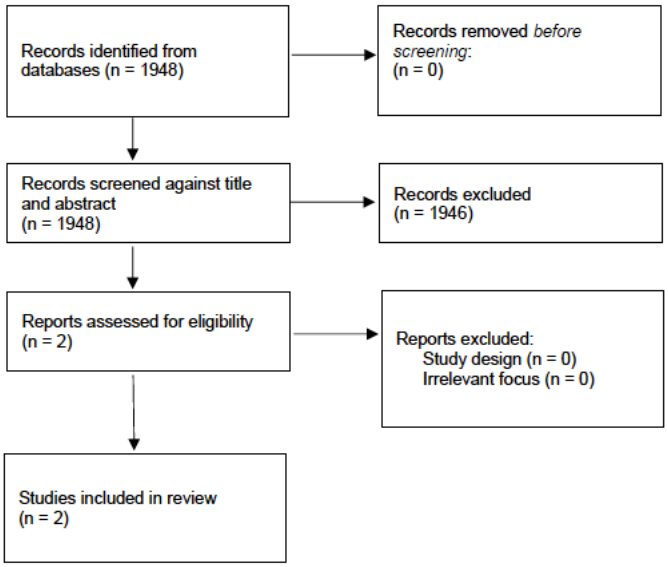
David Armbruster, Robert Harten

Response/Recommendation: No

FDA does not explicitly describe specific animal models of orthopedic infection that are acceptable to support regulatory approval of new technologies. Rather, FDA's approach is to rely on the published preclinical literature to define current best practices in preclinical animal models of musculoskeletal infection, and to engage with submission sponsors on an individual basis to determine a chosen model's appropriateness for a specific technology.

Strength of Recommendation: Low

The PRISMA method used to conduct a search is presented below:



Searches were conducted between October 6th and November 15th 2022, within the following databases: PubMed, Google Scholar, RightFnd.

The search strategy combined six separate searches which included all appropriate controlled vocabulary and keywords for "animal model", "in vivo model", "FDA", "regulatory", "musculoskeletal infection", "bone infection", and "orthopedic infection". In order to limit publication bias, there were no language, publication date, or article type restrictions on the search strategy.

These search parameters resulted in a very low number of relevant publications identified in the scientific literature that dealt directly with the question of acceptable animal models of infection for regulatory filings, especially for the US Food and Drug Administration (FDA). For this reason, an additional search with similar terms was conducted of the FDA web site (www.fda.gov) resulting in four useful references: two public workshops, one draft guidance, and one approval letter for a medical device.

Resources from FDA:

In March 2020, FDA sponsored a public workshop titled "Public Workshop - Advancing Animal Models for Antibacterial Drug Development". This workshop focused primarily on disease- and pathogen- specific models for late-stage evaluation of new antibacterial drugs. None of the presentations in this workshop mentioned musculoskeletal or bone infection, osteomyelitis, orthopedics, or medical devices.[1]

In June 2022, FDA sponsored a public workshop titled "Virtual Public Workshop – Animal Studies for Orthopedic Products". This workshop covered a broad range of topics on the conduct of functional animal studies designed to evaluate device performance and handling. Dr. Lisa Fortier of Cornell University commented that "By far, the most commonly used animal for bone repair and fracture in a large animal, would be the sheep. Dogs are a decent model as well, followed by rabbits". However, none of the presenters indicated that there were specific animal models endorsed or accepted by the FDA for studying musculoskeletal infection. Dr. Sara Thompson, a veterinarian medical officer for FDA commented that "When choosing an animal model, first consider whether there is an established animal model for the type of device being tested. An established animal model is one that has been described in the literature or used to support the clearance or approval of a similar device for the same indications for use. Often, an established model does not exist, particularly with novel device types or devices whose identified risks cannot be mitigated with the existing models."[2]

In 2015 the FDA published a draft guidance titled "General Considerations for Animal Studies for Medical Devices - Draft Guidance for Industry and Food and Drug Administration Staff". This guidance states similarly that "The animal model selected should be generally accepted for the study of the device type. There should be a reasonable amount of scientific evidence that the animal model has utility for the study of the device type. In some cases there may not be an established or accepted animal model for a specific device type. We recognize that the utility of animal testing may be limited in these situations."[3]

On May 17, 2022, FDA approved the direct de novo request from Bonesupport AB for Cerament G, its gentamicin-eluting ceramic bone void filler. The approval letter includes special controls for the newly created device category "resorbable calcium salt bone void filler containing a single approved aminoglycoside antibacterial", including animal performance testing. The requirement states "The model must mimic the identified clinical use, e.g., in a large animal infection model of osteomyelitis.
Testing must characterize aminoglycoside serum levels and characterize product resorption and replacement by new bone". This special control provides some detail as to the required data, however it is specific to ceramic bone void fillers.[4]

Together these indicate that FDA's approach is not to dictate specific animal models per se to support regulatory filings, but to rely on the peer reviewed literature to describe the current state of the art in preclinical testing. Of most interest would be published animal models that have been used to support the FDA clearance or approval of a similar device, however there are relatively few of these for technologies to address musculoskeletal infection.

Back to top

Publications:

Following the Second International Consensus Meeting (ICM) on orthopedic infections held in 2018, Bargon et al. detailed their summary of available models of periprosthetic joint infection (PJI). In their words, "…no ideal single animal model exists, to address implant-associated osteomyelitis. Therefore, we propose that researchers and clinicians should ask indication- and disease-specific questions and build on established appropriate animal models capable of answering their questions and enabling translations to the clinical situation".[5] This recommendation is in line with the FDA's approach.

The American Society for Testing and Materials (ASTM) hosted a symposium in August of 2019 on Antimicrobial Combination Devices. Selected technical papers from this symposium were published in 2020, including a chapter which highlighted the lack of published standards for preclinical evaluation of antimicrobial implants, stating "there is an urgent need for the community to work together toward developing standard methods that can better assess antimicrobial and antibiofilm technologies specifically for medical device applications."[6]

Several reviews have been published presenting best practices in preclinical in vivo testing of antimicrobial implant technologies[7], or reviews of in vivo bone infection models.[8,9,10,11,12] Multiple research groups have published detailed models of implant related infection for specific device types or disease states which are too numerous to list here.

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QUESTION 52

Are there best practice guidelines for pivotal preclinical studies supporting regulatory submissions for drugs, devices, and drug-device combination products?

David Armbruster, Robert Harten

Response/Recommendation: Yes

Although FDA has not published guidance on best practices specific to preclinical studies of musculoskeletal infection, it has published general guidance on animal studies for drugs and medical devices which should be followed in this case. Several groups have published expert opinion pieces in recent years describing best practices for preclinical in vivo studies in orthopedics or of orthopedic device related infection in particular. FDA's practice is to rely on the published preclinical literature to define current best practices in preclinical animal models of musculoskeletal infection, and to evaluate each sponsor's preclinical testing plan individually.

Strength of Recommendation: Moderate

Rationale

A search of the PubMed database using the terms "guidelines" and "preclinical studies" identified several systematic reviews that had been previously published of guidelines for *in vivo* animal experiments. Rather than repeat their methodology we will refer to these reviews for detailed descriptions of terms and database results.

In addition, a search was performed of the FDA web site for additional resources specific to the question of best practices for *in vivo* studies to support regulatory submissions. The results for the drug, medical device, and combination product pathways are summarized.

Review of published preclinical literature:

In 2013 Henderson et al. published "Threats to validity in the design and conduct of preclinical efficacy studies: a systematic review of guidelines for in vivo animal experiments". This review included a systematic literature review of the Medline and Google Scholar databases with a broad set of keywords around preclinical research guidelines. These searches resulted in over 2000 citations, which were reduced to 26 relevant published articles. Many of these were published guidelines for the conduct of preclinical studies for specific diseases (multiple sclerosis, renal failure, etc.), however none were specific to orthopedics in general or musculoskeletal infection in particular.[1]

More recently in 2020, Vollert et al. published their review titled "Systematic review of guidelines for internal validity in the design, conduct and analysis of preclinical biomedical experiments involving laboratory animals." Of over 13,000 identified publications, 676 were screened for full text, and 60 met the full criteria.[2] Of these references one was specific to orthopedic research, a 2007 publication by Auer et al. which documented the output of a consensus workshop, convened from a group of musculoskeletal researchers, veterinarians, legal experts, and ethicists on the appropriate use of animals in musculoskeletal research. The workshop identified "a list of 10 golden rules and

Back to top

requirements for conduction of animal experiments in musculoskeletal research."[3]

In 2019 a large group of researchers published "Recommendations for design and conduct of preclinical *in vivo* studies of orthopedic device related infection." The purpose of this opinion article was "to discuss best practices in preclinical *in vivo* testing of antimicrobial interventions targeting ORDI" (orthopedic device related infection), to enable both fundamental research and regulatory studies.[4] This summary is the most comprehensive recent overview of best practices for conducting preclinical research in musculoskeletal infection.

Resources from FDA:

For preclinical testing of technologies that will be regulated via the drug pathway, FDA guidance recommends following the International Conference on Harmonisation (ICH) guidance "GUIDANCE ON NONCLINICAL SAFETY STUDIES FOR THE CONDUCT OF HUMAN CLINICAL TRIALS AND MARKETING AUTHORIZATION FOR PHARMACEUTICALS M3(R2)". This document recommends international standards for the nonclinical safety studies recommended to support human clinical trials.[5] Although FDA has published specific guidelines for preclinical testing of some product types, there are no specific guidances for bone infection or implant related infection. FDA guidance also requires that pivotal preclinical studies supporting both drug and device submissions comply with Good Laboratory Practice (GLP). GLPs are codified as US law in the Code of Federal Regulations 21 CFR Part 58.[6]

FDA has published a draft guidance for preclinical testing of medical devices titled "General Considerations for Animal Studies for Medical Devices - Draft Guidance for Industry and Food and Drug Administration Staff". The guidance provides "…a reference of best practices for the approach to and conduct of animal studies, and the presentation of animal study data intended to demonstrate that the device under study is sufficiently safe for early human experience … or to demonstrate device safety in support of a marketing application."[7] While this guidance remains in draft form, it gives insight into FDA's thinking and expectations for animal studies submitted in support of an application.

Many technologies developed to address musculoskeletal infection fall into the category of drug-device combination products. FDA's guidance "Early Development Considerations for Innovative Combination Products" gives helpful guidance to navigate the testing requirements of this product type. It states that "When developing a combination product, it is likely that neither isolated approach would fully address the relevant preclinical development questions for both constituents as well as for the combination product as a whole. Instead, FDA recommends that developers consider the scientific and technical issues raised by the combination product and its constituents and propose an approach that appropriately addresses these issues without requiring duplicative or redundant studies."[8]

References*:*

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Back to top

QUESTION 53

Are there best practice guidelines for the duration of an antimicrobial effect from an orthopedic implant in vivo to prevent clinical infection?

Volker Alt, Thomas Schaer

Response/Recommendation: No

There is no set standard for antimicrobial activity on a given implant to prevent clinical infection in animal models. Studies vary tremendously and outcome variables are often not clinically relevant or defined. To date there is one study that examines the temporal dynamics between host-cell integration and bacterial colonization, concluding that after 7 days there was a direct relationship between host immune cell attachment and a decrease in bacterial colonization.

Strength of Recommendation: Strong

Rationale

The database PubMed was searched using the key words „animal model“, „infection“, „surface“ and „implant“. The considered time period was 1980 to October 2022. The search identified n=3 publications, whereby two were excluded as these did solely report *in vitro* data. Thus, only one publication could be included.[1]

The authors of the study used a bilateral intramedullary rat model. After 1, 3 and 7 days after implantation, the animals were infected with *Staphylococcus aureus*. Two weeks following inoculation, the host cell adherence was evaluated using flow cytometry and histological methods. The results indicated at time-dependent relationship between implant surgery and bacterial colonization showing that when bacteria were inoculated after 7 days, there were little to no bacteria on the implant despite the presence of bacteria within the bone tissue.

In conclusion, the reduction of bacterial colonization after 7 days seems to show a certain host cell coverage with reduction of bacterial colonization of the implant in the context of the “race for the surface”.

References

[1] Shiels SM, Mangum LH, Wenke JC. Revisiting the "race for the surface" in a pre-clinical model of implant infection. Eur Cell Mater. 2020 Jan 29;39:77-95. doi: 10.22203/eCM.v039a05. PMID: 31995226.

06. Rapid Fire

Back to top

QUESTION 54

Are there any effective host immune responses against biofilm bacteria?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 55

How does host immunity against a pathogen change based on the location of the MSKI?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 56

Are there any effective host immune responses against bacteria within the OLCN?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 57

Do bone cells mount an immune response during MSKI?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 58

What are the proven outcome measures of host immunity during MSKI?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 59

Is there host immunity against small colony variants and persister cells?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 60

What is known about antigen specific T-cells in MSKI?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 61

What are the important antigen presenting cells in MSKI?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 62

Is septic non-union associated with host immunity?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 63

Do different pathogens influence host immunity against each other in polymicrobial MSKI?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 64

Are monoclonal antibodies capable of eradicating biofilms in animal models of MSKI?

Response/Recommendation: Unknown

Strength of Recommendation: Low

QUESTION 65

What are the best models to study T-cell responses during MSKI?

Response/Recommendation: Unknown

Strength of Recommendation: Low

Back to top