Investigating the pathogenesis of Staphylococcus aureus in periprosthetic joint infections

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Graduate Program in Surgery
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ABSTRACT

The most challenging complication after total joint replacement is the periprosthetic joint infection (PJI). *Staphylococcus aureus* is a commensal bacterium and a human pathogen. It is responsible for more than half of the PJI. We demonstrated that synovial fluid has antimicrobial properties against *S. aureus* and the iron restriction is one of the mechanisms used for the host to prevent bacterial growth, and is even able to increase killing of *S. aureus*. We have shown a clear role of transferrin as a restrictive factor in synovial fluid that functions by iron starving the bacteria and sensitizing them to killing by some other unknown factor, such as antimicrobial peptides. The development of animal models would help to improve our understanding of the pathogenesis of PJI. We also successfully established an animal model of PJI, which allows the temporal observation of disease progression and gives the ability to track different bacterial mutants.

SUMMARY FOR LAY AUDIENCE

The most challenging complication after total joint replacement is the periprosthetic joint infections (PJI). *Staphylococcus aureus* is the bacteria responsible for more than half of the infections. We demonstrated that synovial fluid has antimicrobial properties against *S. aureus*. The iron is the most important nutrient used for bacteria and we found that the iron restriction is one of the mechanisms used for the host to prevent bacterial growth, and is even able to increase killing of *S. aureus*. We have shown a clear role of one of the proteins in synovial fluid (transferrin) as a restrictive factor in synovial fluid that functions sensitizing the bacteria for killing by some other unknown factor, such as antimicrobial peptides. The development of animal models would help to improve our understanding of the PJI. We successfully established an animal model of PJI, which allows the temporal observation of disease progression.

KEYWORDS: *Staphylococcus, aureus*, periprosthetic, infection, synovial fluid, animal model.
DEDICATION

I would like to dedicate this thesis to my wife Mariana Ferrero Pepicelli, who provided me with unconditional support during my stay in Canada. Her patience and love was the strength of my days at this stage of my professional career as orthopedic surgeon. A special mention to my newborn daughter Irina, who was the best thing that happened in my life. Lastly, I would also like to mention the support of my whole family, who despite the distance were always present.
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I would also like to extend a special thanks to David Watson for his great help during my time in the lab. David's help was invaluable in developing this work, he not only taught me all the techniques that were used throughout this work, he also helped me interpret and understand the results of my experiments in the lab.

I would especially like to thank Mariya Goncheva and Emily Truscott for their assistance in performing the mouse work. Their contributions and knowledge allowed me understand and develop an animal model for first time in my career.

Lastly, I would also like to extend my thanks to all of the members of the arthroplasty team at University Hospital for their contributions to my learning process.
TABLE OF CONTENTS

ABSTRACT .................................................................................................................. ii
SUMMARY FOR LAY AUDIENCE .............................................................................. i
DEDICATION .............................................................................................................. iii
ACKNOWLEDGEMENTS ............................................................................................. iv
TABLE OF CONTENTS ............................................................................................... v
LIST OF TABLES ......................................................................................................... viii
LIST OF FIGURES ...................................................................................................... ix
LIST OF APPENDICES ............................................................................................... x
LIST OF ABBREVIATIONS ........................................................................................ x

CHAPTER 1 - BACKGROUND .................................................................................... 1
  1.1 Introduction to immune response ........................................................................ 1
  1.2 *Staphylococcus aureus* as a pathogen .............................................................. 2
    1.2.1 Epidemiology ............................................................................................... 2
    1.2.2 Resistance mechanism ............................................................................... 3
  1.3 Periprosthetic joint infections .............................................................................. 6
    1.3.1 Prevention ................................................................................................... 6
    1.3.2 Diagnosis and Classification ....................................................................... 8
    1.3.3 Treatment ................................................................................................... 11
      1.3.3.1 Debridement, antibiotics, implant retention (DAIR) ......................... 11
      1.3.3.2 One stage exchange .............................................................................. 13
      1.3.3.3 Two stage exchange ........................................................................... 15
  1.3.4 MRSA Periprosthetic Joint Infections ........................................................... 16
  1.4 Synovial Fluid: Role in Periprosthetic Joint Infections ....................................... 17
  1.5 Animal Model for study of Periprosthetic Joint Infections .................................. 18
    1.5.1 In vivo molecular imaging in animal models ............................................. 19
      1.5.1.1 Optical Imaging .................................................................................... 20
  1.6 Project rationale ................................................................................................. 21
  1.7 Research objectives ........................................................................................... 22
    1.7.1 General objectives ...................................................................................... 22
    1.7.2 Specific objectives ...................................................................................... 22

CHAPTER 2 - MATERIALS AND METHODS .............................................................. 23
3.1.2 Iron limitation contributes to the ability of synovial fluid to restrict the growth of *S. aureus* .................................................................32

3.1.3 Iron restriction in synovial fluid increases killing of *S. aureus UAMS-1* .................................................................33

3.1.4 Synovial fluid can effectively kill multiple strains of *S. aureus*...34

3.1.5 Transferrin is responsible for sequestering iron in synovial fluid.................................................................36

3.1.6 Synovial fluid containing high molecular weight proteins reduce the bacterial growth more than filtrate synovial fluid.................................37

3.1.7 The absence of GraS makes *S. aureus* more susceptible in in synovial fluid.................................................................38

2.2 ANIMAL MODEL OF *S. AUREUS* PERIPROSTHETIC JOINT INFECTION (*pilot study*).............................................................40

2.2.1 *In vivo* bioluminescence imaging is able to monitor an animal model of periprosthetic infection.................................................................40

2.2.2 Bacterial burden was found in periprosthetic bone tissues and implants.............................................................................41

2.2.3 Correlation of CFU/mL to RLU and construct stability ............42

CHAPTER 4 - DISCUSSION AND FUTURES DIRECTIONS..............44

REFERENCES......................................................................................48

APPENDICES......................................................................................58

CURRICULUM VITAE.............................................................................64
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Host risk factors classification</td>
<td>7</td>
</tr>
<tr>
<td>Table 2</td>
<td>Musculoskeletal Infection Society (MSIS) criteria for a definite diagnosis of PJI</td>
<td>9</td>
</tr>
<tr>
<td>Table 3</td>
<td>PJIs classification (Time)</td>
<td>10</td>
</tr>
<tr>
<td>Table 4</td>
<td>Bacteria strains used in Synovial fluid assays</td>
<td>25</td>
</tr>
<tr>
<td>Table 5</td>
<td>Bacteria strains used in animal models</td>
<td>28</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 1</td>
<td>Model of <em>Staphylococcus aureus</em> biofilms development</td>
<td>4</td>
</tr>
<tr>
<td>Figure 2</td>
<td>The projected number of primary and revision arthroplasties in the United States from 2005 to 2030</td>
<td>6</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Diagram of the necessary prerequisites to fulfil a one-stage exchange revision joint replacement</td>
<td>13</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Mouse surgical procedures</td>
<td>29</td>
</tr>
<tr>
<td>Figure 5</td>
<td><em>In-vitro</em> growth analysis in synovial fluid</td>
<td>32</td>
</tr>
<tr>
<td>Figure 6</td>
<td><em>UAMS-1</em> growth in treated synovial fluid</td>
<td>33</td>
</tr>
<tr>
<td>Figure 7</td>
<td><em>In-vitro</em> <em>UAMS-1</em> viability assay in synovial fluid</td>
<td>34</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Different strains viability assays in synovial fluid</td>
<td>35-36</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)</td>
<td>37</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Centricon filtered synovial fluid growth assay</td>
<td>38</td>
</tr>
<tr>
<td>Figure 11</td>
<td>The absent of GraS render <em>S. aureus</em> more susceptible</td>
<td>39</td>
</tr>
<tr>
<td>Figure 12</td>
<td><em>In vivo</em> bioluminescence imaging in animal model of PJI</td>
<td>40-41</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Relative light units emitted from mouse knee over the time</td>
<td>41</td>
</tr>
<tr>
<td>Figure 14</td>
<td><em>In vitro</em> bacterial growth analysis with bone tissues and implants recovered from the animals.</td>
<td>42</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Correlation of CFU/mL to RLU</td>
<td>43</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>APPENDIX 1</td>
<td>Synovial fluid study: Ethics approval and consent to participate</td>
<td>59</td>
</tr>
<tr>
<td>APPENDIX 2</td>
<td>Animal model: Ethics approval</td>
<td>60</td>
</tr>
<tr>
<td>APPENDIX 3</td>
<td>Permission Figure 1</td>
<td>61</td>
</tr>
<tr>
<td>APPENDIX 4</td>
<td>Permission Figure 2</td>
<td>62</td>
</tr>
<tr>
<td>APPENDIX 5</td>
<td>Permission Figure 3</td>
<td>63</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
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<tr>
<td>AAOS</td>
<td>American Academy of Orthopedic Surgeons</td>
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<tr>
<td>Agr</td>
<td>Accessory gene regulator</td>
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<tr>
<td>ALC</td>
<td>Antibiotic loaded cement</td>
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<td>ASA</td>
<td>America Society of Anesthesiologists</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BLI</td>
<td>Bioluminescence</td>
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<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
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<tr>
<td>CFUs</td>
<td>Colony forming units</td>
<td></td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
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<td>DAIR</td>
<td>Debridement, antibiotics, implant retention</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
<td></td>
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<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
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<td>Fhu</td>
<td>Ferric hydroxamate uptake</td>
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<tr>
<td>FLI</td>
<td>Fluorescence imaging</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>HA</td>
<td>Hialuronic acid</td>
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<tr>
<td>HI</td>
<td>Heat inactivated</td>
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<tr>
<td>K-wire</td>
<td>Kirschner wire</td>
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<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
<td></td>
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<tr>
<td>MALDI-MS</td>
<td>Matrix associated laser desorption ionization-mass spectrometry</td>
<td></td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>MSSA</td>
<td>Methicillin susceptible <em>Staphylococcus aureus</em></td>
<td></td>
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<tr>
<td>NAM</td>
<td>N-acetylmuramic acid</td>
<td></td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared range</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
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<td>OSSA</td>
<td>Oxacillin susceptible <em>Staphylococcus aureus</em></td>
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<tr>
<td>PBP</td>
<td>Penicillin binding proteins</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>Abbreviation</td>
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<td>--------------</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophils</td>
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<tr>
<td>PJI</td>
<td>Periprosthetic joint infection</td>
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<tr>
<td>PurR</td>
<td>Regulator of purine biosynthetic operon</td>
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<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
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<tr>
<td>RLU</td>
<td>Relative light units</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute 1640 medium</td>
<td></td>
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<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
<td></td>
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<tr>
<td>SSIS</td>
<td>Surgical site infection</td>
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<tr>
<td>THA</td>
<td>Total hip arthroplasty</td>
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<tr>
<td>TJA</td>
<td>Total joint arthroplasty</td>
<td></td>
</tr>
<tr>
<td>TKA</td>
<td>Total knee arthroplasty</td>
<td></td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
<td></td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
<td></td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
<td></td>
</tr>
<tr>
<td>WTA</td>
<td>Wall teichoic acid</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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BACKGROUND

1.1 Introduction to immune response

Microbes are ubiquitous throughout the gastrointestinal tract and on the human skin, yet these bacteria rarely cause infection. Epithelial cells lining the skin, respiratory, gastrointestinal, urogenital tracts provide a physical barrier, which microbes are unable to cross. In addition to the physical barrier these environments also produce several chemical barriers, such as the low pH of the upper gastrointestinal tract. Moreover, human skin has a high amount of salt and fatty acids, which prevent the growth of many bacteria (1, 2). However, when these barriers are breached by some means, pathogens will be able to bypass the physical and chemical barriers allowing them to invade into the host tissues.

If pathogens are able to circumvent the initial barriers, they may then be faced with the humoral component of innate immunity including the complement system. The complement system consists of small soluble proteins. When activated the complement system allow a proteolytic cascade resulting in the formation of the membrane attack complex (MAC). The MAC forms transmembrane channels leading to osmotic lysis of the host cells (3).

Another mechanism of defense of many host tissues to infection is known as colonization resistance, whereby commensal bacteria colonizing the host compete with pathogens for nutrients and attachment sites. Iron is an essential micronutrient for virtually all microorganisms owing to its wide range of redox potentials. It serves as the catalytic center of enzymes involved in critical cellular processes such as DNA synthesis and electron transport. However, the amount of free iron in biological systems is low due
to its tendency to form insoluble oxyhydroxides under aerobic conditions at a neutral pH. The amount of iron available in the host environment is further reduced by sequestration into proteins such as transferrin and lactoferrin, virtually no free iron exists in living organisms (4, 5, 6).

1.2 *Staphylococcus aureus* as a pathogen

*Staphylococcus aureus* is both a commensal bacterium and human pathogen. Many people are colonized with *S. aureus* and it is often present asymptotically on parts of the human body such as skin, skin glands, and mucous membranes, including nares and guts of healthy individuals. It is responsible for many different infections in the human population as bacteremia, infective endocarditis, skin and soft tissue infections, pleuropulmonary, as well as osteoarticular and device-related infections (7, 8).

*S. aureus*, a member of the family *Micrococcaceae*, is a Gram-positive coccus whose cells tend to occur either singly or if dividing cells do not separate, form pairs, tetrads and distinctive irregular “grape-like” structures (8, 9).

These organisms were first described and classified as *Staphylococcus* in 1882 by the Scottish surgeon Sir Alexander Ogston (8, 10). Two years later a German physician, Friedrich J. Rosenbach described 2 pigmented colonies of *Staphylococcus* and proposed the nomenclature *S. albus* (Latin for “white”) and *S. aureus* (Latin for aurum: “gold”) (11).

1.2.1 Epidemiology

*S. aureus* is one of the first described pathogens and it still is one of the most common causes of infections in humans. It is important due to its ability to cause infections as well as its capacity to adapt to diverse environmental conditions. It can affect the bloodstream, skin and soft tissues, and lower respiratory tract and can cause infections related to medical instrumentation (8).
One of the main issues associated with *S. aureus* is its ability to acquire resistance against multiple antibiotics, complicating treatment. Resistance in *S. aureus* emerged 2 years after the introduction of penicillin. The semisynthetic methicillin antibiotic was developed in the late 1950s, and the resistant strain (*MRSA*) was clinically identified in 1960 (8, 12).

The expansion of *MRSA* began in the late 1970s and this has turned into a nonstop evolutionary journey. *MRSA* resistant to gentamicin emerged in Europe and the United States (US), continued by Australia and Latin America until in 2003, this microorganism was isolated in nearly 60% in US intensive care units (12, 13).

In Canada, *MRSA* was first documented in 1964 and the first outbreak occurred in 1978 at the Royal Victoria Hospital in Montreal. From the time of its emergence until the 1980s, *MRSA* was essentially a hospital-acquired pathogen. Today, these isolates of *MRSA* are called health care–associated *MRSA* (HA-MRSA) and are highly resistant to most oral antibiotics (14).

Infections due to methicillin-resistant strains of *S. aureus* are associated with higher mortality rates than infections caused by methicillin-susceptible strains. In addition, they result in increased lengths of hospital stays as well as associated health care costs. The prevalence and the epidemiology of *MRSA* are constantly changing, with novel *MRSA* clones appearing in different geographical regions. Therefore, continuous vigilance for *MRSA* through monitoring the characteristics, host specificity, and modes of transmission is required (8).

*S. aureus* is one of the major causes of hospital and community-acquired infections, resulting in serious consequences. Although many actions to reduce healthcare-associated infections such as better antibiotic selection, isolation of infected patients, skin and nose decontamination treatment prior to surgery, and improved hand hygiene helped to reduce *MRSA* infections, its prevalence is still increasing (12).

### 1.2.2 Resistance mechanism

The success of *S. aureus* as a human pathogen is attributable to its ability to adapt. It has been able to evolve, acquiring resistance to nearly all
antibiotics used to treat it (12, 15). *S. aureus* has wall glycopolymers, including wall teichoic acid (WTA), peptidoglycan, lipoteichoic acid, and capsular polysaccharides. These bacterial surface glycopolymers are recognized by serum antibodies and a variety of pattern recognition molecules leading to complement activation and host defense responses (16).

Survival of bacteria depends on the biofilm formation (17). The biofilm is a polymeric matrix that adheres to prosthetic material. It acts as a sanctuary site where *S. aureus* is relatively protected from antimicrobial agents and the host immune response. In addition, organisms within a biofilm generally enter a stationary phase of growth and are thus much more resistant to antimicrobial killing (7).

Understanding the development of *staphylococcal* biofilms is essential to generate new treatment strategies for these infections. A known basic model consists of three stages: 1) attachment, 2) accumulation/maturation, and 3) detachment/dispersal. The use of new technologies (e.g. BioFlux1000 system) in recent studies allowed researchers to observe the development of the *S. aureus* biofilm in real time. This consists of 5 stages: attachment, multiplication, exodus, maturation, and dispersal (18).

![Figure 1: Model of Staphylococcus aureus biofilms development (18).](image-url)
During growth and division, bacteria biosynthesize cell wall, which is a polymer made up of the peptidoglycan as the principle building unit. The peptidoglycan is composed of repeats of the disaccharide \(N\)-acetylglucosamine (NAG)-\(N\)-acetylmuramic acid (NAM) with peptide stems on the NAM unit. The peptide stems of neighboring peptidoglycan strands are the points of crosslinking to give the mature cell wall. Synthesis of the peptidoglycan backbone is carried out by the catalytic function of transglycosylases, with transpeptidases - also referred to as penicillin binding proteins (PBPs) - performing the crosslinking reaction. Coordination of these reactions is an elaborate process, which ultimately results in the cell-wall structure (17). The final step of the biofilm life cycle involves the dispersal of cells with the ability to travel to distal sites to disseminate infection. The mechanism by which \textit{S. aureus} regulates this step is largely mediated by the accessory gene regulator (agr) quorum-sensing system. The agr system responds to cell density through the accumulation of signal molecules, allowing for dispersal to occur once a threshold density is reached, leading to bacteremia (18).

The penicillin resistance was reported in 1942, and the mechanism was described in the mid-1940s based on an inducible beta-lactamase. Methicillin was introduced in 1959 and two years later its resistance was reported, beginning the “era” MRSA (12, 19).

The methicillin resistance is not due to beta-lactamase production but due to the expression of an additional penicillin-binding protein (PBP2a) with low affinity for beta-lactams (8, 9). PBP2a is encoded by chromosomal gene \textit{mecA}, located in a mobile genomic element known as the \textit{staphylococcal} cassette chromosome (SCC), in this case \textit{SCCmec}. The \textit{mecA} gene is widely distributed among \textit{S. aureus} as well as \textit{coagulase-negative staphylococci}. Therefore, it has been speculated that the methicillin resistance determinant (\textit{mec} determinant) is freely transmissible among \textit{staphylococcal} species (8, 20).
1.3 Periprosthetic Joint Infections (PJIs)

Joint replacement is a highly effective intervention that significantly improves the quality of life of the patient, relieves symptoms, restores joint function, and improves mobility and independence in patients with severe osteoarthritis, inflammatory arthritis, post-traumatic arthritis and many conditions that affect the articular surfaces (21).

The number of arthroplasty procedures performed every year is increasing, with a projected annual volume of more than 4 million cases by 2030 in the United States (22). As a result of the increasing volume of primary procedures, there is an anticipated increase in revision arthroplasty. The leading cause of revision surgery is a periprosthetic joint infection (PJI), which is one of the most challenging complications after total joint arthroplasty (23, 24). Recent studies estimate risk of infection to be approximately 1-2% for both hip and knee arthroplasties (25).

Figure 2: The projected number of: a) Primary THA and TKA procedures in the United States from 2005 to 2030. b) Revision THA and TKA procedures in the United States from 2005 to 2030 (22).

1.3.1 Prevention

The evolution of total joint arthroplasty in recent times in terms of design and fixation of implants, has led to improvements in the control of periprosthetic infection (26). The rate of this complication has been declining
over the last two to three decades, mostly due to operating room environments and operative techniques (27). The use of prophylactic antibiotics and other precautions has helped reduce the prevalence of clinically recognized periprosthetic infection from nearly 10% in the early years in which arthroplasty was performed to less 1% in some series (26).

Despite this decline, periprosthetic infection is associated with immense physiological, psychological, and financial costs (26). Numerous challenges can be associated with the development of a PJI that may include the need for multiple operations, a long period of disability for the patient, and sometimes, a suboptimal result. The economic and psychological burden of this complication leads to an ongoing effort to develop strategies to help prevent and reduce its incidence to a minimum (28).

Pulido et al (28), in their study confirmed that some previously implicated risk factors, such as wound drainage, hematoma formation, a history of rheumatoid arthritis, prolonged operative time and urinary tract infection, are still important predisposing factors for development of a PJI. Other risk factors that were obtained from this analysis were prolonged hospital stays, blood transfusions and obesity.

Table 1: Host risk factors classification.

<table>
<thead>
<tr>
<th>Host risk factors of PJIs</th>
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<tbody>
<tr>
<td><strong>Modifiable host risk factors</strong></td>
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<tr>
<td><strong>Non-modifiable host risk factors</strong></td>
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Intra-articular steroid injections are widely used for the symptomatic relief of osteoarthritis of the knee before elective total knee arthroplasty. Cancienne et al (29) concluded that patients who received an intra-articular injection between 0 and 3 months before surgery had an increased risk of infection 3 months after the operation, compared to patients who had
received intra-articular injections more than 3 months ago. Schairer et al (30), obtained similar results about intra-articular corticosteroid injections in the hips.

Diabetes mellitus has been considered a well-established risk factor for surgical site infections (SSIs) following many surgical procedures. However, it remains a controversial issue regarding the development of PJIs after primary total hip and knee replacement. In a high quality study utilizing the Mayo Clinic Total Joint Registry (31), diabetes was reported not to be a risk factor for PJIs when the variables were adjusted for age, gender, BMI, type of surgery, ASA score and operative time. Martinez-Huedo et al (32) obtained similar conclusions, demonstrating that the diabetes mellitus was not associated with increased PJIs in patients undergoing THAs. Despite this, The American Diabetes Association (33) supports the control of glucose in all patients with diabetes mellitus and in those who receive therapies associated with hyperglycemia, based on the evidence that the prevalence of hyperglycemia in hospitalized patients is high and the Hospitalization period offers the opportunity to diagnose unrecognized diabetes mellitus.

The screening of MRSA colonization in all patients before an elective total joint arthroplasty (TJA) is a relevant topic in relationship to prevention of PJIs. Several studies determined that high-level nasal carriage of S. aureus was a significant independent factor with a relative risk of developing a SSI and the quick, inexpensive, and simple nasal decolonization of the carriers on admission is an effective technique to reduce the PJIs (34, 35).

1.3.2 Diagnosis and Classification

In order to diagnose a disease with certainty and determine its prevalence, it is important to define the criteria for its diagnosis (26). The different guidelines recommend that clinicians should suspect a PJI in patients who have undergone a total joint arthroplasty (TJA) when they present with any of the following signs and symptoms:

- Persistent wound drainage over a joint prosthesis.
- Acute onset of a painful prosthesis.
• Chronic painful prosthesis at any time after prosthesis implantation, particularly in the absence of pain-free interval in the first years following implantation (36).

Table 2: Musculoskeletal Infection Society (MSIS) criteria for a definite diagnosis of PJI (36).

| 1. A sinus tract communicating with the prosthesis; or |
| 2. A pathogen isolated by culture from two separate tissue or fluid samples obtained from the affected prosthetic joint; or |
| 3. Four of the following six criteria exist: |
|     a. Elevated serum erythrocyte sedimentation rate (ESR) or serum C-reactive protein (CRP) concentration. |
|     b. Elevated synovial white blood cell (WBC) count. |
|     c. Elevated synovial neutrophil percentage (PMN%). |
|     d. Presence of purulence in the affected joint. |
|     e. Isolation of a microorganism in one culture of periprosthetic tissue or fluid. |
|     f. Greater than five neutrophils per high-power field in five high-power fields observed from histologic analysis of periprosthetic tissue at 400 times magnification. |

However, it should be noted that PJI may be present even if fewer than four of these criteria are met.

The evaluation of patients with a painful TJA begins with a thorough history, physical examination, and joint specific radiographic imaging. Multiple surgeries on the same joint, history of prior PJI, history of surgical site infections of the same joint, comorbidities resulting in an immunocompromised state (i.e. diabetes mellitus, inflammatory arthropathy, etc.) or patients with increased risks of skin barrier penetrations (i.e. intravenous drug abuse, skin ulceration, chronic venous stasis, etc.) should be considered at higher risk for PJIs. Physical exam findings suggestive of PJIs include joint erythema, warmth, or large atraumatic effusion (37).

After the physical examination, the evaluation of the radiographic studies is very important. There are a few non-specific changes suggestive of chronic infection that may be apparent on plain radiographs. These include periosteal reaction, osteolysis, or generalized bone resorption in
absence of implant wear. The main role is to rule out other conditions such as implants wear or fractures (26).

No single laboratory test has perfect sensitivity and specificity for diagnosis infection. Screening test results that may suggest the possibility of infection include elevation of the erythrocyte sedimentation rate (ESR) and serum C-reactive protein (CRP) level more than three months after an arthroplasty (26, 37, 38).

One of the most important tests in the evaluation for potential periprosthetic infection is culture of the fluid aspirated from the joint (26). Synovial fluid analysis should include a total cell count and differential leukocyte count, as well as culture for aerobic and anaerobic organisms (21). The American Academy of Orthopaedic Surgeons (AAOS) clinical practice guidelines (39) suggest patients be off antibiotics for a minimum of 2 weeks prior to obtaining intra-articular culture.

PJIs are typically classified according to the timing of symptom development and the mechanism of infection as acute, chronic and hematogenous infection (37).

Table 3: PJIs classification (Time).

<table>
<thead>
<tr>
<th>PJIs Classification (Time)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>Within the first 4 weeks</td>
</tr>
<tr>
<td>Chronic</td>
<td>More than 4 weeks</td>
</tr>
<tr>
<td>Acute hematogenous</td>
<td>Secondary to another infection</td>
</tr>
</tbody>
</table>

Acute postoperative infections are a result of organisms that gained access to the joint during the operation, or soon after it, from the overlying skin or a draining wound. Infections of this type generally become symptomatic within a few days or weeks after the arthroplasty. Late chronic infections may result from organisms introduced during the operation, either from the air, from surgical instruments, or from the implant itself. The lag period is the time needed for the organisms to proliferate and induce symptoms of the infection. Hematogenous infections are the result of the

INVESTIGATING THE PATHOGENESIS OF S. AUREUS IN THE PERIPROSTHETIC JOINT INFECTIONS
seeding of an arthroplasty site by organisms carried by the bloodstream from a different site (26).

1.3.3 Treatment

Prosthetic joint infection remains a serious problem, despite modern operative technologies, new implants and rigorous perioperative antibiotic prophylaxis. The therapeutic goal must remain prevention, but the treatment of established PJI aims for complete eradication of the infection with maintenance of the joint function (40).

Once the diagnosis of periprosthetic joint infection is confirmed, the characteristics of the infection are important. These include the duration of symptoms, patient immune status and overall health characteristics, history of periprosthetic joint infection in the current joint and all other joints, status of any joint wound, joint function expectations, and characteristics of the infecting organism. These data will dictate the surgical treatment selection (26).

An essential component of the care of patients with PJI is strong collaboration between all involved medical and surgical specialists (21). Treatment algorithms are typically dependent on the classification (37).

1.3.3.1 Debridement, antibiotics, implant retention (DAIR)

Open debridement, antibiotics, irrigation and implant retention is an option less invasive and that decreases morbidity and could be the solution in patients with an early infection, within four to twelve weeks of surgery. Recent studies demonstrated a rate overall proportion of success 70% associated with DAIR in the treatment of infected THAs (41).

DAIR should be considered an urgent procedure, as the time period from the onset of symptoms until the operation has been reported to be an important factor affecting the success of the procedure. Type of infecting organism, duration of symptoms before intervention, type and duration of antibiotic therapy, age, presence of underlying inflammatory conditions, exchange of modular components, and the presence of preoperative
comorbidities are known factors that affect the outcomes of DAIR (42, 43). Kunutsor et al (44), in their results showed that a longer duration between onset of symptoms to DAIR (≥ 21 days) is associated with lower infection control compared with a shorter duration (< 21 days).

Patient medical comorbidities are one consideration prior to DAIR; however, the effect of individual medical comorbidities on failure of DAIR is not clear (35). Obesity has been associated with the risk of PJI, however, no direct correlation has been found between the failure of DAIR treatment and high BMI when this is taken as a continuous variable (45).

Compromised host immunity may lead to inferior outcomes. Diseases such as diabetes mellitus and inflammatory arthritis increase the risk of primary PJI; however, their association with failure of DAIR is less clear. Although an optimized host maximizes the chances of success for DAIR; individual comorbidities by themselves do not appear to provide a contraindication to the procedure (44, 45).

Fink et al. (46), had successful outcome in 71.6% of patients overall, with 82.1% for early postoperative infections and 57.1% for acute hematogenous infections.

Regarding of analyses by specific infecting organism, the evidence suggests similar infection control for Gram negative and Gram positive bacteria. Further analysis confirmed higher infection control for streptococcal infection (89.5%) compared with staphylococcal species (75%) (44). Triantafyllopoulos et al. (47), concluded that infection by Staphylococcus aureus, particularly methicillin-resistant Staphylococcus aureus (MRSA), shown to be associated with high failure rates after DAIR. According that, DAIR should be discouraged when the chance of failure without removing the implant is very high. Therefore, chronic PJIs should be considered an absolute contraindication of this procedure, as a fully developed mature biofilm with the presence of "persistor cells" excludes the possibility for cure without removal implant (48).
1.3.3.2 One stage exchange

One stage exchange arthroplasty was originally described by Buchholz et al. (49) and is used in several centers today, particularly in Europe. One-stage exchange arthroplasty is similar to the two-stage procedure but the interval between removal of the arthroplasty and reimplantation of a new one is only a few minutes. The benefits are only one anesthetic procedure, shorter hospitalization, less cost and earlier recovery (50, 51).

In addition, strict preoperative, intraoperative and postoperative criteria must be considered to indicate this management. The correct patient selection, identification of bacteria preoperatively, aggressive soft tissue debridement, meticulous removal of the prior cement material and implants, specific postoperative antibiotic regimens are important for success of this procedure. It is essential to have an experienced surgical team and an infectious disease specialist (microbiologist) (51).

![Diagram of the necessary prerequisites to fulfill a one-stage exchange revision joint replacement (52).](image-url)
There are several contraindications of a 1-stage exchange, including, but not limited to (40, 52):

- Unidentified bacteria preoperatively;
- Failure of >2 previous 1-staged procedures;
- Infection involving the vascular/neurovascular bundle;
- Lack of appropriate antibiotics;
- Infection with a highly virulent organism.

Generally, the surgical technique consists of open aggressive debridement with removal of all components and cement, during which multiple samples are sent to microbiology before administration of antibiotics and the joint is irrigated with hydrogen peroxide and an antiseptic solution, and pulsatile lavage. The wound is then soaked in aqueous antiseptic solution and the wound edges are approximated. The patient is then redraped, the surgical team rescrubs, and new instruments are used. After a further lavage, implantation of a new prosthesis is performed using antibiotic-loaded cement (ALC) according to known sensitivities at a volume of 5% of the total weight of cement powder. Postoperatively, patients continue antibiotic therapy tailored to the sensitivities of intraoperative cultures for at least 6 weeks until inflammatory markers (CRP, ESR) and nutritional markers such as plasma albumin concentration return to stable limits. The change from intravenous to oral therapy is affected as soon as a full organism sensitivity profile is obtained and after consultation with infectious diseases team (52, 53).

Infection control using a single-stage strategy in selected patients is achieved in 67% to 95% of patients (53). The patients must be conscientious of the possible risks associated with one-stage exchange, for example: risk of recurrent infection rate 10-15%; reoperation for hematoma, wound debridement or persistent infection; and risk of intra and post-operative fracture (40).
1.3.3.3 Two stage exchange

Two-stage reimplantation was first described by Insall et al. (54). Currently, it is the gold standard for the treatment of a chronic PJI. This involves a minimum of 2 surgeries for the patient, the first to remove the infected prosthesis, extensive debridement of nonviable tissues, irrigation and implantation of a temporary antibiotic-impregnated cement spacer; and once the infection is eradicated, and a second surgery to place a new prosthesis. Antibiotics are administrated based on the sensitivities of the infecting organisms (55, 56).

There are two relevant topics about the two-stage exchange, first is antibiotics-impregnated static cement spacer vs. articulating cement spacer; and second is the duration of therapy systematic antibiotics and the timing of reimplantation.

There is no clear consensus on the ideal type of spacer for management of PJIs of the hip and knee. The goal of the spacer is to preserve the joint space and reduce the soft tissue contracture while delivering high doses of antibiotics. Static spacers are generally recommended for cases with massive bone loss, lack of functional collateral ligaments, extensor mechanism disruption and the need for soft tissue reconstruction, while general functional improvement is expected with articulating spacers. The results for infection eradication are similar in individual studies (55). Park et al. (57) concluded that the articulating spacers have been shown to be associated with improved range of motion, better function, and also with the ability to facilitate ease of dissection at the second stage. However, few data are available on the incidence and type of spacer-related problems. Struelens et al. (58) found that there was a 57% incidence of spacer-specific problems, the most frequent being spacer tilting and medial lateral translation. Lanting et al. (59) found a spacer complication rate of 8.4%, but they did not include lateral and posterior subluxation cases due to these complications were common, probably secondary to inadequate soft tissue tensioning and incorrect position of the spacers. In addition, articulating spacers typically come in a limited number of sizes and have less constraint than the primary knee arthroplasty removed.
Regarding the duration of therapy of systematic antibiotics and the timing of reimplantation, Insall et al. (54) suggest a protocol prolonged of intravenous antibiotics between the two stages of surgery (6 weeks). Several recent studies have questioned the standard, prolonged antibiotic therapy used in two-stage revision procedures for PJI. Infection and surgical trauma can cause the blood supply to the periarticular tissue to be attenuated, which prevents antibiotics administered systemically from reaching the desired site of action (60). Antibiotic-loaded cement spacers can maintain levels of antibiotics that are effective against infection for up to 4 months following resection arthroplasty (61). Furthermore, a shorter course of intravenous antibiotic therapy decreases the likelihood of systemic toxicity and may reduce the emergence of drug-resistant organisms (62). The short period of antibiotic treatment has a successful eradication rate similar to that reported with longer regimens and is associated with less morbidity (61, 62).

1.3.4 MRSA Periprosthetic Joint Infections

The management of infection after total joint replacement has been compounded by the emergence of organisms with resistance to some antibiotics (63).

In recent years, there has been a worrisome increase in number of PJIs caused by MRSA. According to recent studies, more than one half of PJIs treated in some institutions are caused by methicillin-resistant organisms. Approximately 47% of *S. aureus* clinical isolates in the United States are methicillin-resistant (64).

Patients with PJIs is associated with a higher number of readmissions, lengthier of hospitalization, an increased number of clinic visits and poor clinical outcomes (65). The impact of these factors imply that the costs of treating PJI caused by these organisms are higher compared to infections caused by methicillin-sensitive organisms (64).

Treatment of prosthetic joint infection creates an economic burden for the patient and healthcare facility with estimated costs higher than a $100,000 per episode (65, 66). Parvizi et al. (64) found that the
management of *MRSA* PJI has a higher cost due mainly to the length hospital stay. Similarly, the cost of treating intensive care unit admissions was also found to be higher compared to infections caused by methicillin-sensitive organisms.

*MRSA* has become a prominent cause of infection in hip replacement, typically presenting early, reflecting its high virulence. Treatment is often prolonged, with a high rate of infection of the implant (63). Retention of the total hip or knee components, regardless of whether the components were well-fixed, generally was not successful when the infecting organisms were *S. aureus* or *S. epidermidis* that were resistant to currently available intravenous antibiotic therapies. The most successful treatment protocols include excision of all components, repeated surgical debridement, removal of all components and cement, and waiting for longer periods before reimplantation (a minimum of 5 weeks for knee arthroplasties, and 8 to 12 weeks for hip replacements). In addition, the use of intravenous vancomycin and oral rifampin is indicated (62).

Given the increasing number of THA and TKA required for an aging population, infection with *MRSA* has implications for the future success of joint replacement surgery, the quality of life and of mortality (63).

### 1.4 Synovial Fluid: Role in Periprosthetic Joint Infections

Osteoarthritis (OA) is a common disease that affects joints by causing a breakdown of cartilage and synovial fluid that results in increased wear, pain, inflammation and reduce mobility (67, 68).

The synovial fluid is the body’s natural synovial joint lubricant and it has been established which are its changes in OA (67, 68). Protein concentration, protein constituent fractions, osmolality, thermal stability, HA concentration plays an important role in OA (68). However, its role in the development of periprosthetic joint infections it is not well known.

Innate immunity against bacterial pathogens is present in many body fluids, including saliva, pleural, and peritoneal fluids (69). Although dental procedures are not risk factors for subsequent total hip or knee infection (71), occult bacteremia is estimated to occur in patients as often as 80% of
the time following routine dental or gastrointestinal manipulations (70, 71). The relatively infrequent occurrence of septic arthritis in this setting suggests that although synovial joints frequently may be exposed to transient bacteremia events, synovial fluid likely possesses antimicrobial properties sufficient to the joints are able suppress these episodes and prevent progression to a clinically detectable septic arthritis.

The evidence suggests that in addition to the adaptive defense of the body’s cell-mediated immune response, the knee joint possesses inherent defense mechanisms against infection. The synovial fluid may contribute to a joint’s innate defense against certain microbial infections. However, the specific immunologic properties and protective mechanisms of synovial fluid against common gram-positive bacterial pathogens that are encountered clinically remain unknown (72).

1.5 Animal Model for study of Periprosthetic Joint Infections

The increase in the number of periprosthetic infections in recent years and their consequent devastating results with an increase in morbidity and mortality, as well as high patient dissatisfaction and poor quality of life, has led to the development of animal models for the study of periprosthetic infections. Once established, such models are likely to transform our understanding of the pathogenesis of PJI and lead to novel treatments (73).

Carli et al. (74), in their recent study proposed four standardized criteria that animal models should meet to be considered as clinically representative of PJI:

1. Musculoskeletal and immunological properties comparable to those in humans.
2. The implant utilized should be made of clinically relevant materials, bear load, and effectively reproduce the periprosthetic environment.
3. The model should utilize clinically relatable bacteria and demonstrate biofilm formation on the implant surface.
4. The model methodology should include quantitative measurements of bacteria, biofilm, and host immune response.
The use of animals in research, teaching, and testing is acceptable only if it promises to contribute to understanding of fundamental biological principles, or to the development of knowledge that can reasonably be expected to benefit humans or animals. A continuing sharing of knowledge, review of the literature, and adherence to the Russell-Burch "3R" tenet of "Replacement, Reduction and Refinement" are also requisites. Those using animals should employ the most humane methods on the smallest number of appropriate animals required to obtain valid information (75).

1.5.1 In vivo molecular imaging in animal models

In vivo molecular imaging in animals is an expanding discipline in biomedical research in the current post-genomic era. The advancement in molecular and cellular techniques, the increased numbers of animal models and the development of novel imaging systems have converted the non-invasive in vivo imaging in an advantage tool over conventional cytology/histology microscopy techniques, which involve chemical fixation of removed tissues from which it can be difficult to generate quantitative data (76).

It is currently possible to develop non-invasive in vivo imaging of specific molecular and cellular mechanisms; to simultaneously monitor multiple molecular events; to track specific molecular targets; to optimise drug and gene therapy; to visualise drug effects at the molecular and cellular level; and to assess disease progression (77).

In addition, obtaining in vivo longitudinal imaging of the same animal model at multiple time points can achieve more valuable information than would be obtained from multiple individual models, avoiding the sacrifice of a large number of experimental animals. In these systems, the animal acts as its own control and the dynamic data show the progressive biological changes and the therapeutic response (78).

In recent years, there has been considerable progress in the development of non-invasive small animal in vivo imaging technology. The most popular techniques utilised by researchers are Magnetic Resonance Imaging (MRI), Computed Tomography (CT), Positron Emission
Tomography (PET) and optical imaging (bioluminescence and fluorescence) (78).

1.5.1.1 Optical Imaging

This imaging modality produces excellent data from organs and structures close to the skin surface in small animals. This technique employs quantitative light emission i.e. photons to obtain measurements of relevant biological parameters, including proteins and nucleic acids within individual living cells. To detect low levels of light or photons, a very sensitive charge coupled device (CCD) detector is used. The CCD detector is capable of detecting light near-infrared range (NIR). There are two main forms of optical imaging, fluorescence imaging (FLI) and bioluminescence (BLI) (79).

In FLI, the visible light (395 to 600 nm) is used to excite fluorescence within the subject and a camera or fluorescent microscopy system detects the emitted light from the region of interest within the subject. The strategy is to fluorescently tag the cells, tissue or molecules under investigation with substances known to fluoresce. In the recent past, a popular choice has been the green fluorescent protein (GFP) which is derived from jellyfish Aequorea victoria. The wildtype GFP emits light at 509 nm, while its variant EGFP has a longer emitting wavelength and is 35-fold brighter. Another popular fluorescent protein is the red fluorescence protein (RFP) DsRed, a faster maturing and more soluble variant of reef coral protein from the Discosoma species which allows more rapid appearance of red fluorescence and reduce the aggregation. Currently, new fluorescent probes were developed from a genetic modification of RFP such as mBanana, td-Tomato, mTangerine, mStrawberry and mCherry with longer emission wavelength and greater stability (78).

In BLI, light emission does not require excitation of the reporter. Light emission originates from an exergonic catalysis reaction of the substrate which releases photons of visible light. One common approach used in animal models is transfection of one of the luciferase family of photo-proteins through tail vein injection just prior to BLI (79).
The luciferase can be isolated either from the sea-pansy (*Renilla reniformis*) or from the North American firefly (*Photimus pyralis*). Furthermore, its expression can be controlled so that it is expressed only when required. Cells expressing luciferase can be easily identified through their emission of light in the range from 400 to 620 nm (79).

The main advantage of BLI over FLI is its capability to detect very low levels of light signal. In addition, in FLI, the external light source required for fluorochrome excitation could also generate background autofluorescence from animal tissues. However, an advantage over BLI is that FLI does not require administration of a substrate for visualisation. This overcomes the potential issues of difficult intravenous access in small animals (78).

### 1.6 Project rationale

Periprosthetic infection is a leading cause of failure after total hip and knee arthroplasty. Infections remain a challenge for both patients and surgeons to treat and occur in approximately 1% of cases. It's still not clearly understood what the composition of the synovial fluid is of patients about to undergo these procedures. Owing to the increasing prevalence of resistant bacterial strains, and other factors, the likelihood of successful eradication of a chronic infection continues to decline, which is very challenging for the patient and surgeon and very expensive for a healthcare system with diminishing fiscal resources.

A better understanding of the environment in which the infection causing bacteria is growing could help lead to improved treatment strategies in the future.

This current study will serve as the foundation for a body of work, which is the result of a multidisciplinary collaboration with orthopaedics, microbiology and medical biophysics to work to solve the most challenging clinical problem facing arthroplasty patients and surgeons from the standpoint of treatment and clinical outcomes.
1.7 Research objectives

1.7.1 General objectives

1. Study the behavior of *S. aureus* in the synovial fluid for a better understanding of these infections in the joints and leading to novel strategies in the treatment of periprosthetic joint infections.

2. Develop a murine model of prosthetic joint infection that allows future studies for investigating the parameters involved in *S. aureus* infections after total joint replacements.

1.7.2 Specific objectives

1. Determine the effect of synovial fluid on *S. aureus* growth in-vitro.

2. Determine which specific factors serve to affect bacterial growth in synovial fluid.

3. Evaluate the behavior of three different strains of *S. aureus* into the mouse knee joint after surgical implanted of Kirschner wire.
2.1 SYNOVIAL FLUID

2.1.1 Ethics statements

Prior to patient enrollment for the synovial fluid collection, this research was approved Health Sciences Ethics Board of University Western Ontario and Lawson Research Institute. An informed consent was obtained from each patient prior to the surgery.

2.1.2 Synovial fluid collection

The synovial fluids samples were obtained from ten patients undergoing elective primary total knee replacement for treatment of symptomatic end-stage osteoarthritis. All samples were collected under sterile conditions by the same surgeon in the operating room at University Hospital. As per standard of care, to minimize the possibility of contamination with blood, a tourniquet was applied, and skin incision was carried out. Once the extensor mechanism and joint capsule were exposed, a sterile 18-gauge needle and 20 cc syringe were used. The needle was introduced into the joint anteromedially and fluid was withdrawn from the knee. The samples were then analyzed in the Heinrichs Lab at Siebens Drake Research Institute of University Western Ontario.

Patients with severe medical comorbidities, a diagnosis of inflammatory arthropathy (i.e. rheumatoid arthritis, gout) or a diagnosis of fibromyalgia were excluded.
The samples of synovial fluid collected were labelled as sample 1, 2, etc. No identifiable data were retained with the samples. After analysis was complete, the samples were destroyed.

Patients were monitored at their standard follow-up appointments with the surgeon for adverse events and complications according to the standard of care at our institution. There was no follow-up monitoring for this study.

2.1.3 Synovial fluid preparation

After sterile collection in the operating room at University Hospital, the synovial fluid samples were transported to Dr. Heinrichs Lab at the Siebens Drake Research Institute of University Western Ontario.

These were centrifuged at 5000 rpm for 10 minutes within 12 hours of collection to remove any possible blood cell contamination. The cell-free supernatant of synovial fluid was aspirated and transferred to cryovials using sterile technique. The samples then were transferred to a -80°C freezer for storage until final analysis.

The samples that did not centrifuge to a clear supernatant were discarded and excluded from analysis.

2.1.4 Bacteria strain and culture conditions

*S. aureus* strains; *UAMS-1* (osteomyelitis isolate) (80), *USA300* (plasmid cured) (81), *USA300 ΔgraS* (16), *SH1000* (82), *RN6390* (83), *Newman* (84), and *Newman ΔsfaΔsbn* (85) were used in this study (Table 4). Bacteria were routinely cultured at 37°C in tryptic soy broth (TSB) (Difco) with shaking or on TSB agar (1.5% w/v). When necessary bacteria were cultured in the presence of 50 μg/mL kanamycin, 4 μg/mL tetracycline, or 300 μg/mL spectinomycin.
Table 4: Bacteria strains used in Synovial fluid assays

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UAMS-1 WT</strong></td>
<td>UAMS-1; Osteomyelitis isolate</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td><strong>USA300 WT</strong></td>
<td>USA300 LAC, MRSA cured of antibiotic resistance plasmids</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>(\Delta)graS</td>
<td>Derivative of <em>S. aureus</em> USA300 with (\Delta)graS created by pKOR mutagenesis</td>
<td>Flannagan. 2018 (16)</td>
</tr>
<tr>
<td><strong>SH1000</strong></td>
<td>WT <em>S. aureus</em> strain derived from 8325-4</td>
<td>Horsburgh. 2002 (82)</td>
</tr>
<tr>
<td><strong>RN6390</strong></td>
<td>Prophage-cured wild-type strain</td>
<td>Peng. 1988 (83)</td>
</tr>
<tr>
<td><strong>Newman</strong></td>
<td>Wild-type clinical osteomyelitis isolate</td>
<td>Duthie.1952 (84)</td>
</tr>
<tr>
<td>(\Delta)sfa(\Delta)sbn</td>
<td>Derivate of <em>S. aureus</em> Newman with (\Delta)sfa(\Delta)sbn deletion</td>
<td>Beasley. 2009 (85)</td>
</tr>
</tbody>
</table>

2.1.5 In-vitro synovial fluid analysis

2.1.5.1 Synovial fluid growth assay

Synovial fluid samples from different donors were thawed and in-vitro growth analyses were performed using various *S. aureus* strains. Overnight cultures of each strain were grown in TSB and then diluted to and OD_{600} of 1.0 in saline. Tubes containing a series of 2 fold dilutions of the synovial fluid in RPMI medium were inoculated with 10\(\mu\)L of the OD=1 suspension for each strain to achieve a starting OD_{600} of 0.01 for each tube.

Two common restriction mechanisms employed by the host are the production of antibacterial proteins and limitation of bacterial access to nutrients such as iron. By inactivating antibacterial proteins such as the complement system or by adding exogenous iron sources we can investigate whether these specific immune mechanisms are active in synovial fluid. For these reason, in certain experiments exogenous iron was added to the synovial fluid in the form of 25\(\mu\)M FeCl\(_3\); and in some cases the
synovial fluid was heated at 55°C for 1 hour to inactivate heat sensitive proteins prior to dilution in RPMI.

After 37°C incubation for 24 hrs, the bacteria were serially diluted and plated on tryptic soy agar (TSA) and incubated overnight at 37°C to determine the number of colony forming unit per ml (CFU/mL).

2.1.5.2 Bacterial viability assay

Bacterial viability assay was performed in saline solution containing synovial fluid with and without additional iron to determine if the synovial fluid can kill various strains mutants of S. aureus rather than simply preventing growth. Synovial fluid samples from different donors were thawed and tubes containing either 20% or 50% of synovial fluid in saline solution were prepared. Tubes containing saline without synovial fluid were included as a negative control and when necessary 25mM FeCl₃ was added to the synovial fluid samples. Overnight cultures of all of the strains tested were grown in TSB then diluted in saline and the samples were inoculated at an initial OD₆₀₀ of 0.01. All of the samples were plated on TSA at time 0 to confirm all samples started with an equivalent number of CFU/mL. The samples were then incubated at 37°C for 24 hours after which they were again plated on TSA to determine the number of CFU/mL remaining.

2.1.5.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) / Mass Spectrometry

Synovial fluid samples were thawed and diluted to an initial concentration of 20% in saline. Two-fold dilution series were then generated and mixed at a 1:1 ratio with Laemmli and the samples were boiled for 10 minutes. After cooling, 7μL of each dilution was loaded and ran on a 12% sodium dodecyl sulfate polyacrylamide gel in Tris-glycine buffer at 150V, and 500mA for 90 minutes. Upon completion the gel was removed from the dock and stained overnight with InstantBlue™. After staining the gel was taken to the department of biochemistry where an automated spot picker was used to isolate the individual bands from the gel. These samples were
then sent for digestion and matrix associated laser desorption ionization-mass spectrometry (MALDI-MS) by the Department of Biochemistry.

2.1.5.4 Centricon filtered synovial fluid growth assay

Synovial fluid samples from different donors were thawed and diluted in RPMI containing either 20% or 5%. The synovial fluid samples were filtered using an Amicon ultra centrifugal filter units with a 30 kDa MW cut-off for 20 minutes. The filtrate containing proteins below 30 kDa and the concentrate containing proteins above 30 kDa were re-suspended in their initial culture volume. The filtered samples along with unfiltered control were used to make a series of dilutions (20, 10, 5, 2.5, 1.25, 0.612 and 0 %) of the synovial fluid. These tubes were inoculated at an initial OD$_{600}$ equivalent 0.01 and after 24 hours incubation at 37°C the samples were plated on TSA to determine the number of CFU/mL.

2.1.5.5 Statistical Analysis

Data are presented as the mean ± SD. All statistical analyses and graph production were done through GraphPad Prism (GraphPad Software, La Jolla, CA). Data were analyzed using unpaired t-tests or a one-way analysis of variance with either Dunnett’s or Bonferroni’s multiple comparison test as indicated. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

2.2 ANIMAL MODEL OF S. AUREUS PERIPROSTHETIC JOINT INFECTION (pilot study)

2.2.1 Ethics statements

The animal protocol was reviewed and approved by the University of Western Ontario Animal Use Subcommittee, according the guidelines set out by the Canadian Council on Animal Care. The study was conducted in strict accordance with the recommendations in the Guide by the Canadian Council on Animal Care.
2.2.2 Mice

Three female C57BL/6 mice (Charles River Laboratories, Canada) (12 weeks old) were used for periprosthetic joint infections animal models.

2.2.3 Bacterial strains

Three different *S. aureus* subsp. *aureus* strain *USA300* were used in animal models: *USA300* wild-type, *fhuCBG::ermC* mutant strain (86) and *purR::ΦNΣ* mutant strain (87) (Table 5).

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<th>Description</th>
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<td><em>USA300 LAC, MRSA</em> cured of antibiotic resistance plasmids</td>
<td>Laboratory stock</td>
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<tr>
<td><em>fhuCBG::ermC</em></td>
<td>Derivate of <em>S. aureus Newman</em> with <em>fhuCBG</em> operon deletion derivative of <em>S. aureus</em></td>
<td>Speziali. 2006 (86)</td>
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<td><em>purR::ΦNΣ</em></td>
<td>Strain <em>USA300 LAC</em> containing a transposon insertion in the <em>purR</em> gene</td>
<td>Goncheva. 2019 (87)</td>
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</table>

2.2.4 Mouse surgical procedures

Mice were anesthetized with isoflurane in a laminar flow hood. The mice were placed on their back and the operative leg was maximally flexed at the knee. Using a #15 scalpel blade, an anterior longitudinal midline incision was made centered over the knee. A subsequent incision was made just medial to the patella and extensor mechanism. The extensor mechanism was subluxated laterally and the distal end of the femur was identified. A 25-gauge needle was used to open the femoral canal. A medical-grade stainless steel Kirschner wire (K-wire) (0.6 mm) was surgically placed into the femur in a retrograde fashion up to the greater trochanter and cut with 1
mm protruding into the knee joint space. Surgeries were performed by myself under sterile conditions.

*S. aureus* (2x10^5 CFU in 2 μL saline) were inoculated into the joint space using a Hamilton syringe. The patella was relocated and the surgical incision was closed with Vicryl 5-0 sutures.

A high-resolution X-ray was taken immediately after the surgery to ensure proper placement of the implant. Buprenorphine (0.1 mg/kg) was administered subcutaneously every 72 hours as an analgesic for the first 6 postoperative days.

In *vivo* bioluminescence imaging was performed with the IVIS imaging system at the Robarts Research Institute (London, ON, CA) at 7, 14, 21, 28, 35 and 42 postoperative days.

Mice were anesthetized with isoflurane. Data are presented on color scale overlaid on a grayscale photograph of mice and quantified as total flux (photons/s) within a circular region of interest with Living Image software.
2.2.6 In-vitro growth analysis

The mice were sacrificed at postoperative day 42. The implants were removed and the proximal aspect of the tibia, the distal aspect of the femur and implant were cut into small pieces and placed into sterile, 5-mL centrifuge tubes (Eppendorf) containing 2 mL of 4°C PBS + 0.1% Triton-X100 solution. The contents were homogenized for 5 minutes (Bullet Blender; Next Advance) at setting 16, diluted in 4°C TSB, and then plated by duplicate on TSA to determine the number of CFU/mL in the periprosthetic tissues and implant. These samples also were plated on TSA with chloramphenicol, which allows us to determine the bacteria recovered still carry the bioluminescence genes and the associated chloramphenicol resistance marker.
RESULTS

3.1 S. AUREUS GROWTH IN SYNOVIAL FLUID

3.1.1 S. aureus UAMS-1 grows poorer in synovial fluid than S. aureus USA300

To initially explore the effect of synovial fluid on the growth of S. aureus in-vitro growth assays were performed using two strains of S. aureus, USA300 and UAMS-1. The control specimen with no synovial fluid demonstrated exponential growth as expected. In contrast, bacterial counts in all of the synovial fluid specimens were decreased compared to controls. Although, USA300 strain demonstrated a significant decrease (**P<0.01, ***P<0.001, ****P<0.0001) of CFU/ml in all concentrations of synovial fluid, no bacterial death was demonstrated (Figure 5A). In contrast, the UAMS-1 strain demonstrated greater sensitivity to the synovial fluid. A synovial fluid concentration of 2.5% or higher was sufficient to significantly reduce growth compared to the control (**P<0.01). Moreover, concentrations of 5% or higher prevented visible growth and in some cases resulted in bacterial death (**P<0.001, ****P<0.0001) (Figure 5B). Taken together, these data indicate that synovial fluid has antimicrobial properties and S. aureus UAMS-1 is more sensitive than S. aureus USA300.
3.1.2 Iron limitation contributes to the ability of synovial fluid to restrict the growth of *S. aureus*

The next objective was to determine the mechanism of bacterial restriction in synovial fluid. To this end in-vitro growth curves were performed in RPMI with synovial fluid that had either been heat inactivated (H.I.) or with the addition of 25μM FeCl₃ (the latter serves to saturate the iron sequestering capacity of lactoferrin/transferrin). After 24 hours incubation at 37°C, we found the additional iron significantly increased bacterial growth in 10% and 20% synovial fluid samples compared to the control specimen (**P<0.01). No significant differences were found in 5% synovial fluid concentration or lower. The heat inactivation did not demonstrate significant variations in bacterial growth compared with the control (Figure 6). This together shows that iron restriction is one of mechanisms used by the host to prevent bacterial growth in synovial fluid.
Figure 6: **UAMS-1 growth in treated synovial fluid.** As before growth assays were performed with synovial fluid diluted in RPMI. The synovial fluid was either Heat inactivated at 55°C for 1 hour or exogenous iron was added in the form of 25µM FeCl₃ prior to inoculation with *S. aureus* UAMS-1. Tubes were incubated at 37°C for 24 hours and the CFU/ml was determined. Statistical significance was determined by Bonferroni’s multiple comparison test where *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 N=3. Dashed line indicates the CFU/mL inoculated into each tube.

### 3.1.3 Iron restriction in synovial fluid increases killing of *S. aureus* UAMS-1

Given that *S. aureus* UAMS-1 growth decreased due to iron restriction, the next step sought to determine whether the iron restriction simply prevents bacterial growth or if it can also contribute to the killing of UAMS-1. Bacterial viability assays were performed in tubes containing saline with 20% synovial fluid with and without the addition of exogenous iron. The synovial fluid resulted in an obvious killing of UAMS-1 compared to saline alone controls, but the addition of FeCl₃ did improve bacterial viability (Figure 7A and B). However, the addition of FeCl₃ to synovial fluid did not restore viability to the level of the saline alone controls. It is worth noting that when the samples were sorted by each individual donor the statistical analysis showed no significant differences in survival due to a low number of replicates for each donor (Figure 7A). Meanwhile, a combined analysis
demonstrated a statistically significant increase of bacterial survival in the samples with iron additional compared with the synovial fluid alone (****P<0.0001) (Figure 7B). This confirms that iron is restricted in synovial fluid and this restriction results in increased killing of *S. aureus* UAMS-1.

![A) % Survival - UAMS-1](image1)

**Figure 7: In-vitro UAMS-1 viability assay in synovial fluid.** In (A) Bacterial viability assays were performed with *UAMS-1* in saline solution containing 20% synovial fluid from different donors with and without the addition of 25µM FeCl₃. The CFU/mL of bacteria was determined at the time of inoculation and 24 hours later in order to determine the percent survival from the inoculum N=2. In (B) a combined analysis was performed with all synovial fluid donors included n=12. Statistical significance was determined by Dunnett’s multiple comparison test where *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Dashed lines separate the results of each donor.

### 3.1.4 Synovial fluid can effectively kill multiple strains of *S. aureus*

Because this work previously found that *USA300* was much more resistant to synovial fluid than *UAMS-1*, the next goal was to determine whether the synovial fluid is able to kill other strains of *S. aureus*. To test this notion, bacterial viability assays were performed with the following *S. aureus* strains: *SH1000, RN6390, Newman* and *Newman ΔsfaΔsbn*.

The synovial fluid in *SH1000* demonstrated a significant decrease in the CFU/ml compared to the control saline (***P<0.001). The addition of FeCl₃ did appear to improve bacterial viability in *SH1000* strain, compared to the synovial fluid samples without iron, although the increase was not statistically significant. (Figure 8A). *RN6390* was entirely eliminated in the
synovial fluid, yet the addition of iron restored viability to the level of saline alone control (Figure 8B). Furthermore, both Newman WT and the Newman \( \Delta \text{sfa} \Delta \text{sbn} \) mutant were killed in the synovial fluid and did not show improved viability with the addition of FeCl\(_3\). Interestingly the \( \Delta \text{sfa} \Delta \text{sbn} \) mutant which is defective for iron acquisition had \(~3\) fold reduced viability compared to the WT strain (Figure 8C-D). Taken together these data indicate that the synovial fluid can kill multiple different S. aureus strains and the ability to gather iron appears important for survival. However, it should be noted that unlike before, the presence of iron did not significantly improve the bacterial viability, likely attributable to a low number of replicates as most of the strains are trending towards significance.
INVESTIGATING THE PATHOGENESIS OF S. AUREUS IN THE PERIPROSTHETIC JOINT INFECTIONS

3.1.5 Transferrin is responsible for sequestering iron in synovial fluid

The amount of iron available in the host environment is reduced by sequestering proteins such as transferrin and lactoferrin (6). To determine if one or both of these proteins is responsible for limiting access to iron in the synovial fluid, we attempted to identify the presence of these proteins in synovial fluid. A 12% sodium dodecyl sulfate polyacrylamide gel was prepared and varying dilutions of synovial fluid were subjected to electrophoresis. The gel showed a significant amount of protein around 75 kDa, the approximate size of transferrin or lactoferrin, is present in synovial fluid (Figure 9). Either transferrin or lactoferrin could be responsible for competing with bacteria to access any available iron in synovial fluid. To determine the specific identity of the 75kDa protein samples were sent for digestion and matrix associated laser desorption ionization-mass spectrometry (MALDI-MS). This analysis confirmed that the 75kDa protein identified in the synovial fluid is in fact transferrin (data not shown).
Supporting the previous observation that bacterial access to iron is restricted in synovial fluid.

![Image](image-url)

**Figure 9: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)** Synovial fluid samples were diluted to a concentration range of 20%, 10%, 5% and 2.5% and 7µL of each sample was loaded onto a 12% sodium dodecyl sulfate polyacrylamide gel. The gel was run for 90 minutes at 150V and 500mA after which it was stained with InstantBlue to visualize the various proteins present in the synovial fluid. Samples were sent for Mass spectrometry at the department of biochemistry at the University of Western Ontario. Boxed are the 75kDa marker (right) and the synovial fluid bands identified at transferrin.

### 3.1.6 Synovial fluid containing high molecular weight proteins reduce the bacterial growth more than filtrate synovial fluid

The next objective was to confirm the role of transferrin in restricting bacterial growth in synovial fluid. In vitro growth experiments were performed using synovial fluid that had been separated into fractions containing proteins larger (with transferrin) and smaller (without transferrin) than 30kDa (concentrate and filtrate respectively). The growth of various strains of *S. aureus* was analyzed in the 2 fractions of filtered synovial fluid. It was found that the synovial fluid concentrate, which would contain transferrin, significantly reduced the growth of both *UAMS-1* and *RN6390* compared to the filtrate which lacks transferrin (Figure 10). Importantly the *Newman WT* and ΔsfaΔsbn strains did not have statistically significant differences between the
filtrate and concentrate due to a low number of replicates, but the trend is clear and the data are trending to significance. All together this demonstrates a clear role for transferrin as a restrictive factor in synovial fluid that functions by iron starving the bacteria sensitizing them to killing by some other unknown factor.

![Graphs showing growth assay results](image)

**Figure 10: Centricon filtered synovial fluid growth assay.** 30kDa molecular weight cut off filter to separate the fluid into fractions containing proteins above (concentrate) or below 30kDa (filtrate). Dilution series of the filtrate and concentrate were inoculated with the various strains and incubated for 24 hours after which the samples were plated to determine the CFU/mL. In (A) UAMS-1 and in (B), RN6390 showed decreased CFU/mL in the concentrate. In (C), Newman WT and in (D) Newman ΔsfaΔsbn there was no statically significant differences.*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 N=3. Dashed lines indicate the inoculum CFU/mL.

3.1.7 The absence of GraS makes *S. aureus* more susceptible in synovial fluid.

While the presence of transferrin in the synovial fluid explains the iron limitation, it does not directly kill bacteria, so there must be another factor(s) at
work causing the bacterial killing. To begin to investigate other factors that may be responsible for bacterial killing we sought to determine the effect of synovial fluid on a ΔgraS mutant of USA300. The ΔgraS is part of a two-component regulatory system that is required by S. aureus for resistance against antimicrobial peptides, meaning a ΔgraS mutant is highly sensitive to killing by antimicrobial peptides. To this end bacterial viability assays were performed with USA300 WT and ΔgraS. The WT USA300 showed no significant decrease in CFU/mL over 24 hours in synovial fluid compared to the same strain in saline alone (Figure 11). In contrast to WT, the ΔgraS mutant was much more sensitive to killing by the synovial fluid and had a 2-log decrease compared to the same strain in saline alone (**P<0.01 Figure 11). This finding suggests that there are likely antimicrobial peptides present in the synovial fluid that result in bacterial killing.

Figure 11: The absent of GraS render S. aureus more susceptible. Bacterial viability assays were performed for the ΔgraS mutant and strain USA300 WT in 50% synovial fluid compared with saline solution alone. The CFU/mL of bacteria was determined immediately after inoculating each strain and again after 24 hour incubation. Percent survival was determined by dividing the CFU/mL at 24 hours by the CFU/mL inoculated for the corresponding sample. **P<0.01 N=3.
3.2 ANIMAL MODEL OF *S. AUREUS* PERIPROSTHETIC JOINT INFECTION (pilot study)

3.2.1 *In vivo* bioluminescence imaging is able to monitor an animal model of periprosthetic infection.

*In vivo* bioluminescence images were made at 7, 14, 21, 28, 35, 42 postoperative days (Figure 12). The Relative Light Units (RLU) emitted by *S. aureus* from the mice knee joint were decreasing progressively over the days. Although the *WT* strain demonstrated better signal in the first three weeks, it decreased quickly after post-operative day 21. In contrast, the *purR*::ΦΝΣ and *fhuCBG*::ery strains kept relatively stable after post-operative day 21 (Figure 13).
Figure 12: *in vivo* bioluminescence imaging in animal model of PJI. (A) Postoperative day 7. (B) Postoperative day 14. (C) Postoperative day 21. (D) Postoperative day 28. (E) Postoperative day 35. (F) Postoperative day 42.

Figure 13: Relative light units emitted from mouse knee over the time.

### 3.2.2 Bacterial burden was found in periprosthetic soft tissues and implants.

We next sought to determine the bacterial burden in periprosthetic soft tissues and implants recovered. For this, *in vitro* bacterial growth analyses were performed in TSB and TSB with chloramphenicol. The growth on chloramphenicol allows us to determine the bacteria recovered still carry the
bioluminescence genes and the associated chloramphenicol resistance marker. After 24 hours incubation at 37°C, the number of CFU/mL were determined.

All bone tissue samples demonstrated the presence of bacterial burden. In contrast, the results obtained from the culture of the implants showed variability, with bacteria recovered in only two samples, and with no clear association with a particular bacterial strain.

Although these findings demonstrate the feasibility of the model, in both bioluminescent tracking and recovery of bacteria, the size of the pilot study does not allow for statistical differences to be calculated.

![Figure 14: In vitro bacterial growth analysis allowed to determine the presence of bacteria in bone tissues and implants recovered from the animals.](image)

### 3.2.3 Correlation of CFU/mL to RLU and construct stability

Our next goal was to determine whether there was a correlation between the RLU emitted from the mouse knee with the number of bacteria recovered from the bone tissues and implants. In WT strain and purR::ΦNΣ mutant strain there was a clear correlation, with the strength of the signal relating to the number of bacteria recovered. In contrast, fhuCBG::ermC mutant strain did not emit bioluminescence signal on day 42, therefore we
were not able to observe a correlation in this mutant even though there was bacterial growth in the recovered bone tissues samples.

Taken together, these findings demonstrate the successful establishment of an animal model of PJI, which allows the temporal observation of disease progression and gives the ability to track different bacterial mutants. This is a powerful tool that will allow us to further study the specific *S. aureus* factors involved in the establishment and development of PJI and inform the design of novel preventative techniques. Nevertheless, the findings demonstrated here were a pilot experiment, and further conclusions will require the use of a higher number of animals for the different conditions tested.

![Figure 15: Correlation of CFU/mL to RLU.](image-url)
DISCUSSION AND FUTURES DIRECTIONS

The number of arthroplasty procedures performed every year is increasing, as a result of this, there is an anticipated increase in revision arthroplasty (24). Periprosthetic joint infection (PJI) is the leading cause of revision surgery, and is the most challenging complication after total joint replacement (23). Infections remain a challenge for both patients and surgeons to treat and occur in approximately 1% of cases.

*S. aureus* is an opportunistic human bacterial pathogen responsible for nosocomial and community-associated infections (80). Its genetic plasticity has facilitated the evolution of many virulent and drug-resistant strains, presenting a major and constantly changing clinical challenge. Despite this, there has been relatively little research regarding the pathogenic mechanisms in synovial fluid. This study demonstrated that *S. aureus* has a high in vitro sensitivity in synovial fluid. Synovial fluid can not only reduce bacterial growth, it can also kill bacteria.

It's still not clearly understood what the composition of the synovial fluid is of patients about to undergo joint replacements (67). Recent studies suggest that the synovial fluid may contribute to a joint’s innate defense against certain microbial infections (68). Iron is an essential micronutrient used by bacteria for their growth. Supported by the observation that the additional iron significantly increased bacterial growth compared to the control specimen, we were able to confirm that iron restriction is one of the mechanisms used for the host to prevent bacterial growth in synovial fluid. In addition, in our in vitro assays, we could observe that limited access to iron not only prevented bacterial growth, it was even killing bacteria. These findings were present in different strains of *S. aureus*. 
As previously noted, iron is essential for basic bacterial physiologic processes such as electron transport or nucleotide synthesis (6). However, free iron is virtually unavailable in host environments. Human molecules, such as protoporphyrins (heme) or proteins (ferritin, transferrin, lactoferrin, hemoglobin, etc.) sequester most of the iron from the extracellular space, competing with the bacteria for it (88). These glycoproteins have a high affinity for free iron. *S. aureus* indirectly steals iron from lactoferrin or transferrin through the production of siderophores. Siderophores are secreted small molecules that have an extremely high affinity for iron and outcompete host iron-binding proteins. Siderophore-iron complexes are recognized by cognate receptors on the bacterial surface permitting the theft of iron from lactoferrin or transferrin (89). The synovial fluid composition is still not well known. Through our in-vitro experiments, we could confirm a significant amount of protein around 75 kDa, the approximate size of transferrin or lactoferrin, is present in synovial fluid. After matrix associated laser desorption ionization-mass spectrometry mass spectrometry (MALDI-MS), we confirmed that the 75kDa protein identified in the synovial fluid is in fact transferrin, which could be responsible for competing with bacteria to access any available iron in synovial fluid. Furthermore, after different assays using synovial fluid that had been separated into fractions containing proteins larger (with transferrin) and smaller (without transferrin) than 30kDa (concentrate and filtrate respectively) we were able to demonstrate that the synovial fluid concentrate significantly reduced the growth of different strains of *S. aureus*. This finding speaks to clear role of transferrin as a restrictive factor in synovial fluid that functions by either competing the bacteria for iron, or sensitizing them and killing by some other unknown factor.

While the presence of transferrin in the synovial fluid explains the iron limitation, it does not directly kill bacteria, so there must be another factor(s) at work causing the bacterial killing. To begin to investigate other factors that may be responsible for bacterial killing, we were able to determine the effect of synovial fluid on a $\Delta$GraS mutant of USA300. The GraS contributes to *S. aureus* survival in vivo in the acute stages of systemic infection and is part of a two-component regulatory system that is required by *S. aureus* for resistance against antimicrobial peptides, meaning a $\Delta$GraS mutant is highly
sensitive to killing by antimicrobial peptides (16). Consistent with our observations, there are likely antimicrobial peptides present in the synovial fluid that result in bacterial killing. However more work is needed as the specific peptide(s) in the synovial fluid have not been identified and there may still be other factors that contribute to the restriction and/or killing of \( \text{S. aureus} \) in synovial fluid.

In light of limited treatment successes, persistence of poor patient outcomes, and an increasing number of infections, we need to develop animal models to enhance our fundamental understanding and treatment of PJI. Once established, such models are likely to transform our understanding of the pathogenesis of PJI and lead to novel treatments (74). An important factor to consider when investigating mechanisms pertinent to human infection is whether animal models accurately reflect clinical features of human disease (90). Currently it is possible to develop non-invasive in vivo images of specific molecular and cellular mechanisms to optimize gene and drug therapy, visualize the effects of medications and assess the progression of the disease (76). Furthermore, obtaining in vivo longitudinal images of the same animal model at multiple time points would avoid the sacrifice of a large number of experimental animals; in these systems, the animal acts as its own control and the dynamic data show the progressive biological changes and the therapeutic response (78). Given the diversity of \( \text{S. aureus} \) strains and their propensity to cause community- or hospital-acquired infections, we performed a preliminary animal model with three different \( \text{S. aureus} \) strains (\( \text{USA300 wild-type, thuCBG::ermC} \) mutant strain, and \( \text{purR::ΦNΣ} \) mutant strain). Our findings allowed us to establish an animal model of PJI and observe the progression of the infection over the several weeks. Although this model represents only preliminary work, and a greater number of repetitions is needed to obtain statistically significant conclusions, we have managed to create a successful animal model of periprosthetic infection that allowed us to follow the infection in vivo and obtain a correlation between the number of recovered bacteria from the bone tissues and the relative light units emitted from the knee of the mouse. This model will serve as the foundation for future work with animal models of periprosthetic infection.
In summary, while the observations in this thesis are expected to generate significant interest, there is still a lot of work to be completed. It was demonstrated that the synovial fluid is able to prevent and even kill different *S. aureus* strains through the presence of transferrin which competes with the bacteria for the extracellular iron, but clearly more work is needed as there may still be other factors that contribute to the restriction and/or killing of *S. aureus* in synovial fluid. We found there are likely antimicrobial peptides present in the synovial fluid that result in bacterial killing, however, the specific peptide(s) in the synovial fluid have not been identified yet. Lastly, we created a successful animal model of periprosthetic joint that will serve as the basis of new models to solve the most challenging clinical problem facing arthroplasty patients and surgeons from the standpoint of treatment and clinical outcomes.
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APPENDICES

1. SYNOVIAL FLUID STUDY: ETHICS APPROVAL

LAWSON FINAL APPROVAL NOTICE

LAWSON APPROVAL NUMBER: R-18-698
PROJECT TITLE: Synovial Fluid Composition of Patients Undergoing Total Knee Arthroplasty for Severe Osteoarthritis

PRINCIPAL INVESTIGATOR: Dr. Edward Vasarhelyi

LAWSON APPROVAL DATE: Friday, 8 February 2019

ReDA ID: 5328

Overall Study Status: Active

Please be advised that the above project was reviewed by Lawson Administration and the project:

Please provide your Lawson Approval Number (R#) to the appropriate contact(s) in supporting departments (eg. Lab Services, Diagnostic Imaging, etc.) to inform them that your study is starting. The Lawson Approval Number must be provided each time services are requested.

Dr. David Hill
V.P. Research
Lawson Health Research Institute
Dear Dr. Edward Vasarhelyi,

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. All other required institutional approvals must also be obtained prior to the conduct of the study.

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB, except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP): Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,
Patricia Sargeant, Ethics Officer (ext. 85990) on behalf of Dr. Philip Jones, HSREB Vice-Chair
2. ANIMAL MODEL: ETHICS APPROVAL

Table of Contents
- Animal Use Protocol Overview
- Funding Source List
- Purpose of Animal Use
- Hazardous Materials
- Animal Movement Between Sites
- Animal Groups and Experimental Timelines Overview
- Mouse
  - Tissue Collection
  - Justification for Choice of Species
    - The 3Rs: Replace, Reduce, Refine
    - Genetic Selection
  - Animal Transfer
  - Environmental Enrichment
  - Animal Housing, Housing and Use Location Information
  - Hauling Beyond 12 hours
  - Acclimatization Period & Quarantine
  - Physical Restraint Devices List
  - Veterinary Drugs
  - Experimental Agents Information
- IACUC List
- Procedures Checklist for Reporting and Training
- Principles Narrative
- Procedural Consequences & Monitoring
- Endpoint Method Information
- Animal Numbers Requested
- Personnel List
- Protocol Attachments
- Amendment Request
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Gehrke T, Zahar A, Kendoff D. One-stage exchange: it all began here. Bone Joint J 2013; 95-B (11 Suppl A): 77-83. (Fig 1)

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