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Synergistic effects of Non Contact Induction Heating & Antibiotics on Staphylococcus aureus Biofilm

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Abstract

Staphylococcus aureus is a major cause of prosthetic joint infection (PJI) in which it forms adherent biofilms, thick aggregates of extracellular polymeric substances (EPS) produced by the bacteria. Biofilm associated infections are difficult to treat as they have increased resistance to various antimicrobial agents, which means infected implants often require multiple procedures and prolonged antibiotic therapy. However, a new and emerging method of treatment of PJI is non-contact induction heating (NCIH) of metal implants. We sought to investigate the feasibility and effectiveness of NCIH along with synergistic effects of antibiotics (Vancomycin) in reducing bacterial load within surface associated biofilms *in vitro* on stainless steel and titanium washers. Our preliminary results support the hypothesis that NCIH of metal implants is effective in reducing bacterial load of *S. aureus* within a biofilm *in vitro*. In our study, the synergistical use of the dual treatment strategy (heat and antibiotics) resulted in a ~1000-fold total decrease in CFUs/ml (~3 log reduction). This suggests the potential synergistic effect between the heat and antibiotic treatment against biofilms. These results can be further explored as a new treatment modality for PJI and infections of orthopedic implants. Future work in this study will investigate if NCIH can be used synergistically with antibiotics to more effectively eliminate biofilm associated infections.

Keywords - Prosthetic joint infection (PJI), Total Joint Replacement, Induction heating, Biofilm, Non-contact induction heating.

Summary for Lay Audience

One of the most challenging complications after total joint replacement surgery is periprosthetic joint infections (PJI). *Staphylococcus aureus* is the bacteria responsible for the majority of these infections. One of the reasons that these infections are challenging to treat either with antibiotics or surgically with the present standard of care practices is that the residing bacteria form a "slime" like layer, called biofilms on the surfaces of the implants and the adjacent periprosthetic tissue. Biofilm associated infections are difficult to treat as they have increased resistance to various antimicrobial agents, which means infected implants often require multiple surgical procedures and prolonged antibiotic therapy. However, a new and emerging method of treatment of PJI is non-contact induction heating (NCIH) of metal implants. We sought to investigate the feasibility and effectiveness of NCIH of metal implants along with antibiotics in reducing bacterial load within surface associated biofilms *in vitro*. These preliminary results support the hypothesis that NCIH of metal implants is effective in reducing bacterial load of *S. aureus* within a biofilm *in vitro*. These results can be further explored as a new treatment modality for PJI and infections of orthopedic implants. Future work in this study will investigate if NCIH can be used synergistically with antibiotics to more effectively eliminate biofilm associated infections completely.

Dedication- I would like to dedicate this thesis to my wife Shubreet Randhawa Sidhu, who provided me with unconditional support during our time in London, ON. A special mention to our soon to be born baby girl, Saige Kaur Sidhu. Lastly, I would also like to mention the support of my parents and whole family, who despite the distance were always present. This COVID-19 time has truly been an unprecedented time, globally for all mankind. I hope we make it out victorious and stronger.

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List of Abbreviations

Abbreviation	Full Form
Agr	Accessory gene regulator
CFUs	Colony forming units
CRP	C-reactive protein
DAIR	Debridement, antibiotics, implant retention
ESR	Erythrocyte sedimentation rate
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin susceptible *Staphylococcus aureus*
NCIH	Non contact induction heating
PEMF	Pulsed electromagnetic field
PMN	Polymorphonuclear neutrophils
PJI	Periprosthetic joint infection
PSM	Phenol soluble modulin
THA	Total hip arthroplasty
TKA	Total knee arthroplasty
TJA	Total joint arthroplasty
TSB	Tryptic soy broth

Chapter 1 – Introduction

1.1 Introduction to Staphylococcus aureus biofilms and infection

A biofilm is defined as a sessile microbial community in which cells are attached to a surface or to other cells and embedded in a protective extracellular polymeric matrix (1). Biofilms act as environmental reservoirs for pathogens, and growth within a biofilm may provide organisms with survival advantages in natural environments and increase their virulence. *Staphylococcus aureus* is a gram-positive commensal bacterium that persistently colonizes the anterior nares of approximately 20– 25% of the healthy adult population and is the second most frequent cause of nosocomial blood infections. Approximately 80% of nosocomial infections caused by *S. aureus* are due to colonization by the same strain (2). Ellis *et al.*(3) report that that nasal colonization does not appear to be the only requirement for community-associated *S. aureus* related infection and other factors such as different anatomic sites, person-to-person spread, and fomites are likely important factors as well.

S. aureus is known to cause a diverse range of acute and chronic infections. For instance, acute bacteremia and skin abscesses, are caused by planktonic cells through the production of secreted toxins and exoenzymes (4). Chronic infections are linked with a biofilm mode of growth where *S. aureus* attaches and persist on host tissues, such as bone and heart valves, to cause osteomyelitis and endocarditis respectively or on medical and surgical implants, such as catheters, prosthetic joints, and pace makers (1).

1.1.1 MRSA and CA- MRSA (USA 300)

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant cause of morbidity and mortality in hospitals globally. MRSA is endemic in North America and has also been involved in epidemiologically unassociated outbreaks of skin and soft tissue infections in healthy individuals in at least 21 US states, Canada and Europe (8). Methicillin resistance in *S. aureus* is defined as an oxacillin minimum inhibitory concentration (MIC) of greater than or equal to 4 micrograms/mL (5). Healthcare-associated MRSA infections occur in individuals with predisposing risk factors for diseases, whereas community-associated MRSA (CA-MRSA) infections often occur in healthy individuals. CA-MRSA infections are known to be epidemic in some countries and are more virulent and transmissible than traditional hospital-associated MRSA strains (6). In addition to enhanced virulence, some CA-MRSA strains such as USA300 have the ability to spread readily (6). Ellis *et al. (3)* reported that USA300 MRSA colonization with MRSA associated skin and soft tissue infection is relatively uncommon, but when it does occur, it appears to be playing a role in pathogenesis.

1.2 Clinical significance

Biofilm associated infection is one of the most common causes for failure of orthopedic implants and cause extensive morbidity, high cost of care, and tremendous socioeconomic burden (7). The infection burden associated with total hip and knee arthroplasty is low, with estimates ranging from around 1% to 2% (8). The 5-year survival rate associated with PJI (87.3%) is worse than that of three of the most common cancers: prostate (99%), breast (89%), and melanoma(91%)

(9). All the materials used in orthopedic implants are vulnerable to attachment of biofilm forming bacteria, which places these implants at risk for surgical site infections (SSIs). Pathogenesis of biofilm associated implant-related infections develops after pathogens attach to the protein conditioned surface, which is known to occur intraoperatively, post operatively, and on a delayed basis. Present standard of care strategies include antibiotic suppression, debridement with retention of prosthesis (DAIR) (10, 11), excision arthroplasty including single-stage revision (11), or two-stage revision (12), multiple- stage revision; partial revision (13) and arthrodesis. The reported overall infection-free survivorship for two-stage revision TKA was 85% at 5 years and 78% at 10 years (14). More than 25% of revision surgeries are attributed to PJI and in many cases, multiple revision surgeries for failed eradication of PJI result in amputation or death. (7)

1.2.1 Diagnostic and therapeutic challenges

Most common signs and symptoms of periprosthetic joint infection (PJI) include local or diffused pain, joint swelling or effusion, erythema or warmth around the joint, fever, drainage, implant loosening or the presence of a sinus tract communicating with the joint , but a few cases can also present with few symptoms or signs (7, 15). With currently available diagnostic tests, clinical diagnosis can be challenging unless the dispersed microorganism is virulent enough to incite a host response. Biofilm bacteria are challenging to diagnose because, they often do not grow by routine culture resulting in a high false-negative rate, absence of definitive biofilm biomarkers and absence of specific imaging modality that can directly detect biofilms (16). Sensitivity of conventional microbiologic culture methods can be low, due to the inability of microorganisms to propagate in the sessile phenotype. Inability to isolate and identify the causative pathogen is not only associated with challenges in choosing the correct antimicrobials but also can

lead to continuation of the infection and higher chances of persistence of infection following revision surgery.

1.2.2 PJI - Periprosthetic Joint Infections

Joint replacement arthroplasty is a highly effective intervention that 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 (17). There is an increasing volume of primary joint arthroplasties, with a projected annual volume of more than 4 million cases by 2030 in the United States. The economic burden of treating infected revisions is estimated to be$1.62 billion in the United States by2020 (18). Despite global efforts to reduce postoperative infection, infection burden has actually increased worldwide, based on publicly reported data from 6 arthroplasty registries (8).

PJI associated with biofilms in particular, prove difficult to treat and the optimal surgical and antibiotic treatment of PJI is unclear (19, 20). One of the reasons that these infections are so difficult to treat by a single round of antibiotic therapy alone is that the residing bacteria form biofilms on the surfaces of the implants and the adjacent periprosthetic tissue (21).

1.2.3 Biofilm –Pathogens forming biofilm

Bacterial biofilms can be formed by gram-positive or gram-negative, motile or nonmotile, rapid or slow growing, and aerobic, facultative, or anaerobic species (22). All of the common pathogens associated with PJI have been shown to form biofilm by *in vitro* experiments or by *ex vivo* examination of retrieved components. These include the *Enterococcus faecium,*

Staphylococcus aureus, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species* as well as low-virulence pathogens such as *Cutibacterium* (formerly *Propionibacterium*) acnes and coagulase-negative staphylococci such as *Staphylococcus epidermidis*. Apart from bacteria, fungal pathogens such as *Candida* can also form biofilms (23).

1.3 Biofilm - Stages of development

The life cycle of biofilm is a complex continuum progressing through four stages (21): (1) Attachment -interaction between bacteria and the implant; (2) Accumulation -interactions between bacterial cells ,where cells on the surface begin to transition from the planktonic (free floating) to the biofilm phenotype where they begin to produce extracellular polymeric substance (EPS) and undergo cell division to produce small aggregates and clusters; (3) Maturation -formation of a viable 3D structure in which the structures are large enough to develop distinct microenvironments; (4) Dispersion –bacteria detachment from the biofilm. The timeline of biofilm development is variable depending on the organism involved (Figure 1).

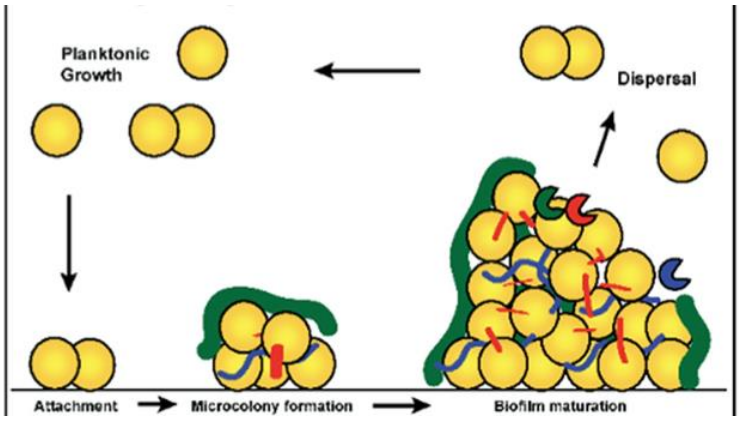


Figure 1 – S.aureus biofilm growth cycle(24)

1 - Initial Attachment - During initial attachment, an individual planktonic cell will reversibly associate with a surface, and if the cell does not dissociate, it will bind irreversibly to the surface. Attachment is mediated through surface proteins, referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (25). The fibronectin-binding protein A and B (FnBPA, FnBPB), the collagen-binding protein Cna and the fibrinogen-binding proteins, clumping factor A and B (ClfA and ClfB), belong to this family (26, 27). During infection, these proteins play major roles in attachment to host factors such as fibrinogen, fibronectin, and collagen. Biofilms in PJIs can form on all types of orthopedic components, as well as accessory materials such as sutures and bone cements, regardless of the construction material (28).

2- Accumulation and early biofilm formation - Once bacteria are attached, they produce an extracellular polymeric substance (EPS), which can vary from species to species and even between strains. On the surface begin to transition from the planktonic (free floating) to the biofilm phenotype where they begin to produce EPS and undergo cell division to produce small aggregates and clusters (23). Cells in the nascent biofilm can also undergo autolysis, releasing extracellular DNA (eDNA) which has been shown to play a structural role in biofilm EPS. Furthermore, these polymers can interact through polymer entanglement, electrostatic interactions, and cross-linking to form complex polymer networks.

3- Maturation - As cells divide and EPS is formed, the biofilm structures become larger and develop a three-dimensional architecture which leads to the development of microenvironments, combined with structural stability. *Ex vivo* microscopic examination of clinical specimens from orthopedic infections reveals that biofilms usually appear as aggregates with hemispherical-like structure, which are heterogeneously distributed on surfaces (29). Tracer

studies have shown that although there can be fluid flow in channels around the biofilm structure within the EPS matrix itself, there is no advective flow and mass transfer is limited by diffusion. As the biofilms become thicker, this diffusion limitation results in sharp gradients building up within biofilms as nutrients (i.e., glucose and oxygen) are consumed by respiring bacteria on the periphery of the aggregates faster than they can diffuse inward. Similarly, metabolites such as cell signalling molecules used to coordinate behavior between individual cells in a population and waste products, such as acids from fermentation, produced by cells in the interior of the biofilm can build up. Thus, the periphery of the biofilm might be in a normal physiological range while bacteria within a 100 μm may be in an anaerobic and acidic environment (29).

4. Dispersal – Dispersal is the last stage in the lifecycle of the biofilm. When biofilms get starved, they can initiate dispersal through cell signaling pathways to use different mechanisms such as the production of hydrolases that degrade EPS polysaccharides in the case of *P. aeruginosa* or the production of surfactants (PSM) in the case of *S. aureus* (30, 31). Most of the time, biofilms remain localized to an area and generally do not tend to spread; however, dispersal events may explain periodic acute episodes of sepsis. Biofilm can either be found adherent to a surface or as floating aggregates over a time period that ranges from minutes to hours *in vitro*, and days to weeks or longer in vivo (32).

Biofilms can mature before they present diagnosable findings, because it is the host response to bacteria outside of biofilms that leads to clinical symptoms, physical findings, and positive diagnostic tests. This limits the opportunity to intervene before the biofilm is established. *In vitro* experiments and in vivo animal studies report that progression of biofilms is mediated by the interplay of a number of microbial, host, and environmental factors (21), and the timeline for biofilm formation may not correlate with the onset of infection symptoms.

1.3.2 Biofilm – Structure Matrix

The *S. aureus* biofilm matrix is a complex glue that encases all of the cells in the mature structure, and it is thought to be composed of host factors, secreted and lysis-derived proteins, polysaccharide, and eDNA. A major constituent of the biofilm matrix is polysaccharide intercellular adhesin (PIA), also known as poly beta-1,6-N-acetyl-D-glucosamine (PNAG) (33). The PIA polymer plays an important role in the structural integrity of biofilms *in vitro* and in vivo. PIA is an important component in both *S. aureus* and *S. epidermidis* biofilms and is produced by enzymes encoded in the *icaADBC* locus. It is composed of β-1,6-linked N-acetylglucosamine polymer, and the proteins encoded in the *ica* locus are responsible for the synthesis, export, and modification of PIA. Numerous studies have identified *S. aureus* strains capable of forming *ica*-independent biofilms made up of proteins and eDNA, which act as intercellular adhesins in the absence of PIA (34-36). Due to the negative charge of the DNA polymer, eDNA potentially acts as an electrostatic polymer that anchors cells to a surface, to host factors, and to each other. Early biofilms are most sensitive to DNase treatment, suggesting that eDNA may be important during attachment (37).

1.3.3 Quorum sensing (Role of glucose in vitro and agr)

Intercellular signaling, often referred to as quorum sensing, has been shown to be involved in biofilm development by several bacteria (38). The *S. aureus* quorum-sensing system is encoded by the accessory gene regulator (*agr*) locus and the communication molecule that it produces and senses is called an autoinducing peptide (AIP) (39). Yarwood *et al*.(38) reported

that the *agr* quorum-sensing system is involved in biofilm detachment. This study demonstrated that bacteria dispersing from biofilms displayed high levels of *agr* activity, while cells in a biofilm had predominantly repressed *agr* systems forming more robust biofilms compared to wild types strains (40). Furthermore, Boles *et al*.(41) suggested a role for the *agr* system in *S. aureus* biofilm development, as *agr* mutants exhibit a high propensity to form biofilms and that repression of *agr* is necessary to form a biofilm and that reactivation of *agr* in established biofilms through AIP addition or glucose depletion triggers detachment. Additionally, Regassa *et al*. also reported that growth on rich media containing glucose represses the *agr* system through the no maintained generation of low pH (42).

1.3.4 Surface Properties for Biofilm formation

The physicochemical properties of implants that are known to affect the time required and robustness of the established biofilms include surface chemistry, surface charge, hydrophilicity/hydrophobicity, micro/nano-topography, and porosity (43, 44). *In vitro* experiments and in vivo animal models have found that modification of implant surface can decrease bacterial adherence, and thus decrease biofilm formation leading investigators to seek physico-chemical surface modifications and coatings to inhibiting bacterial adhesion to theoretically decrease the risk of infection without limiting osseointegration (45). However, biofilms can form on almost all prosthetic and biological surfaces (21).

1.4 S. aureus Biofilm-Related Diseases

S. aureus has been known to infect and form a chronic biofilm infection most often on orthopedic implants including prosthetic joints, wires, pins, external fixators, plates, screws and nails (46). Other medical devices that are prone to biofilm infection include stents, ventilators, intravenous catheters, invasive blood pressure units, infusion pumps, cardiac defibrillators, mechanical heart valves, aspirators, pace makers and cosmetic surgical implants. During cases of implant infection, the infection can occur during implantation, subsequent trauma or hematogenous seeding, as the surface of implant becomes coated with host derived extracellular matrix proteins, providing a rich surface for bacterial attachment (46).

1.5 Diagnosis of a biofilm

Commonly used tests or techniques available to diagnose PJI including either the direct method or the indirect method. The direct method involves laboratory isolation of the pathogen responsible for the PJI through either aspirate or tissue samples. Biofilm bacteria can be difficult to culture because of poor isolation and poor growth characteristics due to relatively dormant persisters and small colony variants (47). Some of the possible solutions for improving the isolation of these bacteria include the use of sonication of retrieved implants (48), as well as use of methods such as polymerase chain reaction (PCR), which help in tackling poor growth characteristics without depending on pathogen growth or culture.(49)

The indirect method includes some of the tests related to the host response to infection without the isolation of the actual pathogens. These indirect tests include the following: erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (17), synovial cell count and

neutrophil percentage (50), leukocyte esterase (51), alpha-defensin(52), d-dimer (53) and histology (neutrophils per high-powered field). Unfortunately, there is no single accepted set of diagnostic criteria as yet for PJI and in certain low-grade infections eg. *Cutibacterium acnes*, several of these criteria may not be routinely met despite the presence of PJI (38).

1.6.1 Management strategies for S. aureus biofilm infections

There are many management strategies and protocols to treat PJI, which need to be individualized to each patient to maximize success. This can be in the form of antibiotic suppression; debridement with retention of prosthesis (DAIR);(10, 11) or excision arthroplasty including single-stage revision (11), two-stage revision(12), multiple- stage revision, partial revision, and even arthrodesis or amputation (13). Use of cement or calcium sulfate beads has shown some efficacy in preventing infection via inclusion of an antimicrobial agent at the site of infection, which provide a rapid release of high concentrations of antibiotics at the wound site (54). Calcium sulfate beads also dissolve and so do not require surgical removal. The antibiotic selected for inclusion in beads must be active against the causative microbes & available as a powder that will harden properly and able to maintain activity despite the heat generated during the polymerization process (55). The majority of the agent is released in a biphasic manner, in the first few hours to days after implantation, with the remainder leaching out slowly over a matter of weeks, months or in some cases, years (56). Elution rates depend on conditions such as eluent solvent and pH. The elution characteristics of specific antibiotics vary depending on cement type, although Palacos cement provides more complete elution of most agents, including the commonly used aminoglycosides (57).

The majority of present treatment strategies are maximally invasive therefore, impossible in patients with high comorbidities. Furthermore, the increasing antibiotic resistance of bacteria raises concern and limits the choices of antibiotics (58, 59). Therefore, it is vital that novel, non-invasive management options such as non-contact induction heating of metallic implants for the prevention and treatment of biofilm infections in implants are developed to reduce the burden of more invasive and extensive revision surgeries and possibly improve effectiveness of present standard of care practices.

1.6.2 Antibiotic resistance and tolerance

Antibiotic resistance generally means an increase in the minimum inhibitory concentration (MIC) value of an antibiotic due to a permanent change in the bacteria. Antibiotic tolerance, on the otherhand, is the ability of cells to survive the effect of an antibiotic due to a reversible phenotypic state (60). The use of these definitions is well suited for planktonic cells, but for biofilms, it is used in a different context. In biofilms, antimicrobial tolerance is related to the mode of growth of the biofilm. This is different from bacteria growing in planktonic culture, which, usually, will show susceptibility to antimicrobials (61). Biofilm formation is the natural state for the vast majority of bacteria in vivo. Therefore, antibiotic tolerance is a natural state of biofilms. Also, antibiotic resistance is a natural phenomenon, since bacteria have evolved to resist the action of natural antibacterial products for billions of years in the absence of human activity (62).

Antibiotic tolerance has been reported for a diverse number of bacterial species for a wide range of classes of antibiotics. Often the concentration required to achieve even a 3-log reduction

is well above the therapeutic window of what can be achieved systemically (23). Main factors promoting biofilm-specific antibiotic tolerance/ resistance include:

1 -Bacterial dormancy- limitation of nutrients inside biofilm, can lead the bacteria to go into a slow growing or dormant phenotype and thus are not engaging in cellular processes (i.e., cell wall synthesis, protein synthesis, DNA replication) which are interrupted by conventional antibiotics which are otherwise effective against rapidly growing cells.

2- Biofilm Thickness – In order to penetrate the cells within the biofilm, antibiotics have to penetrate the EPS slime matrix. The time to diffuse into the biofilm is proportional to the square of the distance traveled, which depends on the thickness of the biofilm. In addition to diffusion, limitation cationic antibiotics such as tobramycin and vancomycin (two commonly used antibiotics added to bone cement) have been shown to bind with anionic components (polysaccharides and eDNA) in the EPS further hindering transport into *P. aeruginosa* and *S. epidermidis* biofilms. In these studies, and other studies, subminimum inhibitory concentrations (sub-MIC) of antibiotics have been shown to stimulate the production of biofilm or EPS components such as eDNA, presumably as a defense mechanism (16, 63).

3- Persister cells – Bacterial cells may escape the effects of antibiotics without undergoing genetic change; these cells are known as persisters. Unlike resistant cells that grow in the presence of antibiotics, persister cells do not grow in the presence of antibiotics. These persister cells are a small fraction of exponentially growing cells (due to carryover from the inoculum) but become a significant fraction in the stationary phase and in biofilms (up to 1%). Critically, persister cells may be a major cause of chronic infections (47). These persister cells arise due to a state of dormancy, as a state in which cells are metabolically inactive. Persister

cells enter a dormant state regardless of nutrient availability and appear to be responsible for the resistant nature of chronic infections, since antibiotics kill the majority of cells. However, persisters remain viable and repopulate biofilms when the level of antibiotics drops (47).

Quorum-sensing (Q-S) regulates several factors that contribute to biofilm formation and persistence. Q-S is involved in the production of eDNA, which inhibits the penetration of some antibiotics into the Biofilm. Since there is no indication that Q-S promotes antibiotic tolerance in planktonic cells, this mechanism maybe biofilm-specific (61).

4- Sub-minimum inhibitory antibiotic concentration -Sub-minimum inhibitory concentrations of a variety of antibiotics were found to induce biofilm formation in a number of phylogenetically diverse Gram-positive and Gram-negative bacteria *in vitro* (64). It is likely that some bacterial cells in biofilm are exposed to sub-MIC levels of antibiotics during antimicrobial chemotherapy due to falling concentrations by dilution or diffusion gradients for antibiotics in biofilm. Sub-MICs of antibiotics can induce mutagenesis, which confers resistance to other antibiotics (65).

1.6.3 Present management options for biofilm related periprosthetic joint infections

There are many therapeutic strategies to treat PJI, which need to be tailored to each patient to maximize success. It is essential to have a multidisciplinary approach between microbiologists, surgeons, and infectious disease departments to yield the best outcome in these challenging situations (16). There are many management strategies and protocols to treat PJI, which need to be individualized to each patient to maximize success. This can be in the form of antibiotic suppression; debridement with retention of prosthesis (DAIR) (10, 11), or excision arthroplasty

including single-stage revision (11), or two-stage revision (12), multiple- stage revision, partial revision (13) and even arthrodesis and, if necessary, amputation. The majority of present treatment strategies are maximally invasive therefore, impossible in patients with high comorbidities along with the increasing antibiotic resistance of bacteria, raises concern and limits the choices of effective antibiotics (58, 59). Therefore, it is paramount that alternative modalities of biofilm treatment and eradications of infections in orthopaedic implants are developed.

1.7. – Need for novel alternatives treatment options for biofilm management

This subject was assigned to the Biofilm Workgroup during the second International Consensus Meeting on Musculoskeletal Infection held in Philadelphia, USA (ICM 2018) with representations of experts from around the world. The aim of the meeting was to identify the best practices & develop research studies, dedicated to advancing our understanding of biofilms and their role in human implant-related infections. The consensus document suggested biomedical research funding agencies and the pharmaceutical industry should recognize these areas as a priority, for urgent development of better diagnosis and eradication strategies (21).

1.7.1- Effects of heat on biofilm

In food preservation studies, heating has been reported to be an effective way to reduce the bacterial load of *S. aureus* (66). The effect of heat treatment on three physical properties of the biofilms: the bacterial cell morphology and viability, the polymeric properties of the extracellular polymeric substance (EPS) and the rheological (study of the deformation and flow of matter)

properties of the bulk biofilm have been studied & observed to have an order of magnitude reduction in the biofilm yield stress after 60 °C temperature treatment. No such difference was found for treatment at 45 °C. From these results, it has been established that the yield stress of bacterial biofilms is temperature-sensitive, and that this sensitivity is correlated with cell viability. The yield stress is of particular interest, as it is a measure of how much force must be applied to an apparently solid material to get it to flow and show liquid-like behavior (67).

The observed significant decrease in yield stress with temperature suggests a means to weaken the mechanical integrity of *S. epidermidis* biofilms with applications in areas such as the treatment of biofilm-infected medical devices (67).

1.7.2 Non-contact electromagnetic induction heating for eradicating biofilm

Heating is an effective way to reduce the bacterial load in food preparation, and studies on hyperthermia treatment for cancer have shown that it is possible to heat metal objects with pulsed electromagnetic fields selectively (PEMF). Non-contact induction heating of a titanium disk is effective in reducing bacterial load *in vitro* (68). Log reduction of bacterial load were calculated using the following equation: log10(A/B), where A is the number of viable micro-organisms before the experiment in CFU/ml and B is the number of viable micro-organisms after the experiment in CFU/ml. These promising results can be further explored as a new treatment modality for infections of metal orthopedic implants (68).

1.7.3 PEMF

Non-contact induction heating (NCIH) uses the principles of pulsed electromagnetic fields (PEMF) to induce ‘eddy currents’ within metal objects which causes them to heat up. This heat

can be used to cause thermal damage to bacterial biofilms on the metal implant, hence, killing the bacteria and weakening the biofilm (68, 69). In the field of hyperthermia cancer treatment, several studies have shown the feasibility of induction heating of "thermal seeds" and nanoparticles (70). In the field of fracture healing with shape memory devices. Müller *et al*. (71) have also shown the feasibility and safety of contact-free electromagnetic induction heating of Nickel Titanium alloy (NiTi) implants in a rat model.

Hence this novel application of heat can be used as an adjuvant to chronic suppressive antibiotic therapy or severely immunocompromised patients, who are not fit for surgery. The metal implant fixed to the bone can be heated noninvasively with care taken to avoid excessive heating to areas that are in close contact with important anatomical structures. Alternatively, NCIH can be used during surgery to increase the effectiveness of the surgical procedure such as in Debridement, Antibiotics and Implant Retention (DAIR), allowing heating of parts of the implant that cannot be reached and mechanically cleaned. During surgery, soft tissue can be protected by keeping it away from the heated part of the implant.

1.7.4 Antibiotic synergy

Since contemporary treatment methods such as surgical debridement with pulse lavage and antibiotics may not be effective once biofilm formation has reached a certain bioburden threshold, induction heating may prove to be a valuable addition to these treatments (72). Furthermore, the increasing resistance of bacteria to antibiotics raises concern and limits the choice of antibiotics (73). Hajdu *et al*.(74) have shown that the antibacterial activity of antimicrobial agents is significantly enhanced by increasing the ambient temperature. Pijls *et al*.(75) report that in induction heating, the heat originates at the biofilm-implant interface and travels into the biofilm,

whereas the cocktail of antibiotics diffuses into the biofilm starting at the outer border of the biofilm and ultimately ending at the biofilm–implant interface. This bi directional attack may be a mechanism for the observed synergistic effect that leads to total eradication (75). Thus, the PEMF may work synergistically with thermal damage and antibiotics. These synergistic effects require further investigations, since the future application of non-contact induction heating of metal implants, will likely be applied in a clinical setting where antibiotics are part of the treatment strategy (68).

1.7.5 Concerns - Temperature elevation & effect on soft tissues

There may be concerns for potential tissue necrosis with induction heating due to relatively high temperatures at the prosthesis-bone interface. Temperatures greater than 50°C (122°F) occur routinely during the process of intramedullary reaming as well as stimulation of revascularization by the breaking-up of intramedullary scar tissue (76, 77).There are also animal experiments that confirm the lack of necrosis after induction heating up to 60°C (71). Samara *et al*. (78) have shown that bone cement, reaches temperatures of 80°C for more than 10 min during the curing process. Additionally, special heating techniques such as segmental induction heating can be used to apply localized heating to a segment of an implant, using the remainder of the implant as a heat sink (79). The surrounding tissue is heated to some extent by thermal conduction from the heated metal, but if the tissue is well perfused by arterial and venous blood flow, the heat will very likely be significantly reduced as in coagulation procedures (80, 81). Since cemented prostheses have an excellent long-term track record in several national joint registries, the concern of necrosis due to temperatures of around 60°C from curing cement remain theoretical. Studies with bone cement and drilling in bone have shown that curing temperatures of bone cement and drilling in cortical

bone readily exceed 60°C, hence induction heating at this 60°C temperature range should be safe which was found to be the most effective temperature in the present study (82, 83).

1.8 Project Rationale

Periprosthetic joint infection (PJI) is a devastating complication of surgery, often requiring multiple rounds of antibiotic therapy and surgeries to treat. Furthermore, the increasing resistance of bacteria to antibiotics raises concern and limits the choice of antibiotics (73). Contemporary treatment methods such as surgical debridement with pulse lavage and antibiotics may not be effective once biofilm formation has reached a certain bioburden threshold. This multidisciplinary study between orthopaedics, microbiology and medical biophysics, will serve as a foundation to better understand biofilm related implant infections and develop novel strategies for its clinical management.

Staphylococcus aureus is a major cause of prosthetic joint infection (PJI) in which it forms adherent biofilms. Biofilm associated infections are difficult to treat as they have increased resistance to various antimicrobial agents, which means infected implants often require multiple procedures and prolonged antibiotic therapy. Bacterial biofilm infections can be difficult to eradicate with antimicrobials (84). Furthermore, increasing antibiotic resistance of bacteria raises concern and limits choices of antibiotics (84). However, knowledge gaps exist in the potential role of physical methods such as heat, for biofilm treatment. Therefore, it is vital that novel treatment options such as NCIH are explored that improve outcome, reduce patient morbidity and mortality and can be effectively used in the treatment of biofilm infections in implants.

Studies dedicated to advancing our understanding of biofilms and implant-related infections are required for development of better diagnosis and eradication strategies. A new and

emerging method of treatment of PJI is non-contact induction heating (NCIH) of metal implants. Hence we sought to investigate the feasibility and effectiveness of NCIH of metal implants in reducing bacterial load within surface associated biofilms *in vitro* (68). It is currently unknown whether NCIH can reduce or even eradicate *S. aureus* from the biofilm on stainless steel and titanium alloys, which are common metals used in orthopedic trauma and arthroplasty implants. It is important to evaluate the possible synergistic effect of NCIH and antibiotics, because NCIH will very likely be applied to a scenario in a clinical setting where antibiotics are part of the established treatment protocol and heat has been shown to enhance the antibacterial activity of antimicrobial agents against staphylococcal biofilm (74).

This multidisciplinary collaboration study will help provide a better understanding of the environment of the biofilm and could lead to development of novel treatment strategies, which in the future can be applied for further validation via animal testing.

1.9 Research objectives

The purpose of this study was (1) to study the potential role of NCIH as a method for reducing bacterial load in a biofilm in-vitro model and (2) to investigate its synergistical use with antibiotics, to increase the effectiveness of antimicrobial therapy.

Chapter 2 - Material and methods

In order to advance our understanding of the role of biofilms in PJI, it is vital that novel treatment options such as NCIH and its possible synergistic effects with antibiotics are explored that can potentially improve treatment outcomes and reduce patient morbidity and mortality.

2.1 Purpose

The purpose of the current study was (1) to design an *in vitro* model to test the effect of heating duration and temperature of non-contact induction heating stainless steel and titanium washers inoculated with *S. aureus* based mature biofilm to study the potential role of physical methods (heat) as a non-invasive method for the eradication of biofilm. (2) To determine the possible synergistic effects of non-contact heating with antibiotics (vancomycin).

2.2 In Vitro Biofilm Growth

2.2.1 Bacteria Strain and culture conditions

S. aureus strain USA300 was used throughout this study due to its association with implant-based infections. CA-MRSA strains such as USA300 have the ability to spread readily (6). It has been involved in epidemiologically unassociated outbreaks of skin and soft tissue infections in healthy individuals in at least 21 US states, Canada and Europe (85). These micro-organisms were chosen as representatives of gram-positive bacteria, associated with infections of orthopedic implants, as *S. aureus* is a major cause of hospital acquired & implant associated infections (86).

The bacteria were grown overnight in 5mL cultures of tryptic soy broth (TSB) and 600µL was pelleted by centrifuging and then normalized to an OD_{600} of 1.0 in 1mL of sterile saline. Stainless steel and titanium washers (DePuy Synthes 13mm Spherical Washers, dimensions -

13mm x 1.5 mm x6.6mm, used with 4.5 - 7.3 mm diameter screws and manufactured from stainless steel and titanium), were sterilized by autoclaving and placed into the wells of a 12 well tissue culture plate (Figure 2). The wells containing washers were then filled with TSB and inoculated with *S. aureus* at an OD600 of 0.01 and 0.4% glucose was added to the growth media (TSB-G) to stimulate biofilm formation as per earlier work done by Boles *et al*.(41). The plates were then incubated statically at 37ºC for 24 hours to allow the biofilms to grow.

2.3.2 Non-Contact Heating

The stainless steel and titanium washers were exposed to a PEMF from an induction cooker (Master Chef Induction Hot Plate ,1800W) after contamination. An induction cooker was chosen because several studies have indicated that the PEMF generated by induction cookers, on the order of 20 kHz to 30 kHz, is safe for humans for non-contact temperature measurement (87). The cooktop had been modified in order to automatically activate and deactivate to maintain the heating of the washers at a specified target temperature. Multiple trials were done to check the maximum temperature attained by the washers when heated to the maximum capacity of the induction heater. Due to physical limitations such as the size and shape of the washers/ induction heater, the maximum temperature that could be reliably maintained was 60ºC. This temperature was chosen because it has been shown to have a 6-log reduction when used alone , as well shown complete eradication of biofilm when used with a cocktail of antibiotics (68, 75). This temperature has been reported to be within the clinically safe range as several studies with bone cement and drilling in bone have shown that curing temperatures of bone cement and drilling in cortical bone readily exceed 60°C (82)

After biofilms had grown on the washers, they were gently rinsed with 300µL sterile saline before being exposed to a pulsed electromagnetic field (PEMF) with a maximum of 1800 watts at 27 kHz to evaluate the relationship between alternating magnetic field (AMF) exposure and bacterial survival. An identical control washer that did not have a surface associated biofilm, was used for each the stainless steel and titanium washers as a control to monitor the temperature during heating. All discs were heated up to and then maintained at 60ºC for the specific times indicated for each experiment.

Figure 2 - Sterilized washers placed into the wells of a 12 well tissue culture plate.

2.3.3. Bacterial Enumeration

Washers containing biofilms that had either been heated using the PEMF, or non-heated control samples were placed into 13mL snap cap tubes with 3mL of sterile saline. These samples were then sonicated using a probe sonicator (Misonix XL2020) to disrupt the biofilms but did not affect bacterial viability (Figure 3).

Sonication is the application of ultrasonic energy to a sample immersed in a fluid (the sonicate fluid) to dislodge biofilm embedded bacteria. Through this process, the sensitivity of cultured samples is improved. The efficiency of sonication to achieve dislodgment of bacteria from biofilm on titanium or stainless steel implants has been shown to be superior to scraping with a surgical blade (88).

These samples were then serially diluted and 10µL drops were plated in duplicate on tryptic soy agar (TSA) plates, which were incubated at 37ºC overnight to determine the number of colony-forming units (CFU) for each of the samples.

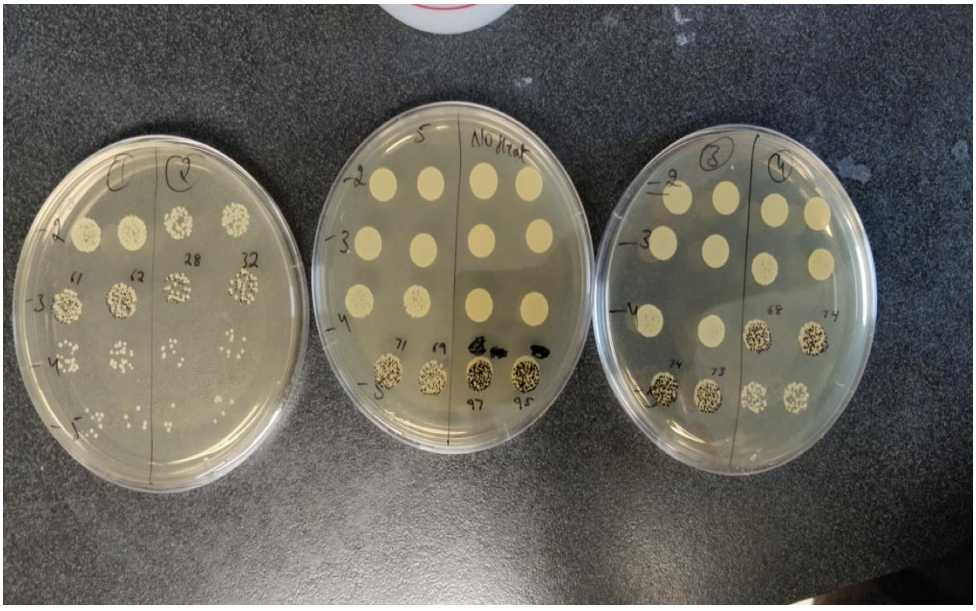


Figure 3- Sonication and plating on TSA plates

2.3.4 Temperature Measurement

Washers were placed the wells of a sterile plastic dish that was designed such that each well would receive identical levels of heat from the induction cooktop. A control washer of the same material as those being heated was placed into one of the wells and a calibrated thermometer (thermocouple) was used in order to constantly measure the temperature of the control washer. The control temperature readings were used as feedback to deactivate heating when the washers reached the target temperature and then reactive heating as soon as the washer dropped below the target temperature in order to keep the temperature constant. Figure 4 shows the arrangement of the induction system and images of a heated washers. Our study was limited by the physical characteristics of the heater and washers and as such, the maximum temperature that could be reliably reached and maintained for the implants was 60°C. Notably, this temperature has previously been shown to have inhibitory effects in a planktonic heating mode. Pijls et al. (70) grew a *S. aureus* biofilm model on titanium alloy cylinders and exposed to incremental target temperatures (35°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C) for up to 3.5 minutes with NCIH and reported that at 60°C and higher there was a 6-log reduction (70).

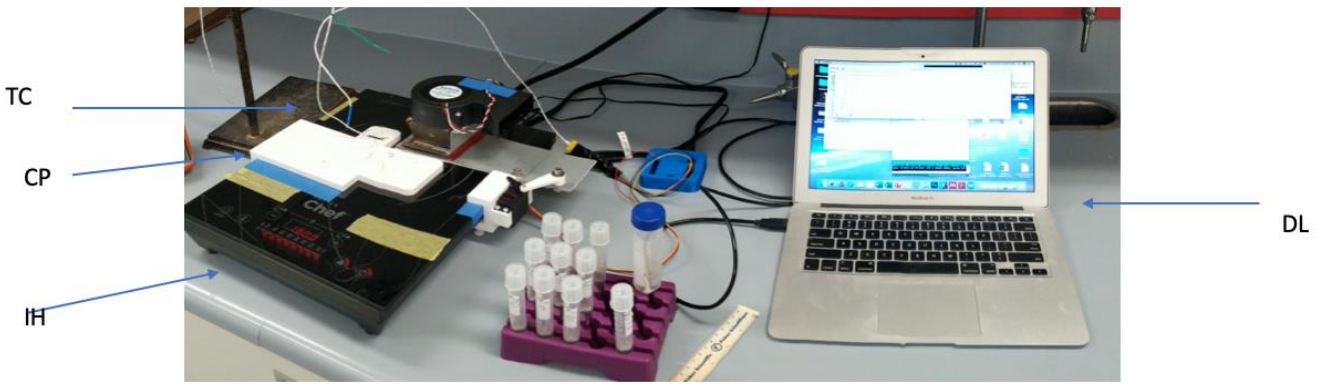


Fig. 4 Photograph of the arrangement of the induction systems, washers on a modified platform; with temperature data logging with laptop; IH- Inductiotion heater ; TC – Thermocouple Temperature sensor ; DL – Data logger (Laptop) , CP- Custom petridish

2.3.5 Effect of Temperature and Duration

The washers were initially exposed to maximum target temperature of 60°C for 3.5, 10 and 15 minutes. The initial duration of 3.5 minutes was chosen from results in published studies with food products which revealed that 3.5 minutes caused at least a 3-log reduction of bacteria (66). Pijls *et al*. (68) showed an effect of a more than 6-log reduction for 2.5, 3.0 and 3.5 minutes at 60°C, additionally total eradication of the biofilm has been reported with induction heating at 60°C at 3.5 min with subsequently exposure to cocktails of vancomycin, rifampicin and NAC (75). Furthermore, to test the effect of longer heat treatments, washers were maintained at 60ºC for 10 and 15 minutes before plating to enumerate any viable bacteria that remained. Washers that were heated for the various times were compared to control washers that were not exposed to the PEMF.

2.3.6 Effect of Vancomycin

In a clinical setting, intravenous vancomycin is the drug of choice for most MRSA infections seen in hospitalized patients. It can be used both as empiric and definitive therapy as most MRSA infections are susceptible to vancomycin. The dosage depends upon the type and severity of the infection. Vancomycin trough is obtained just before the fourth dose to ascertain a therapeutic level. The goal trough range typically is between 10 and 20 ug/mL. For complicated infections, the goal is between 15 and 20 ug/mL. Vancomycin and daptomycin are considered adequate empiric therapy according to the Infectious Diseases Society of America guidelines of 2011. MRSA isolates in the bloodstream with vancomycin MIC greater than or equal to 2 ug /mL

may not respond adequately to vancomycin. Therefore, in these cases, daptomycin is a better option (5).

Hence in order to determine if heating the biofilm resulted in increased sensitivity to vancomycin, we conducted the following series of experiments.(Table 1) Washers that had been heated for 10 minutes as previously described were then placed back into new 12 well plates containing sterile TSB with vancomycin added at 2 or 20ug/mL. These concentrations were chosen as they are sufficient to kill planktonic *S. aureus*, *in vitro* and fall within the normal concentration range of intravenous vancomycin therapy for infected patients *in vivo* (5).

After a 24-hour incubation with vancomycin, the washers were sonicated and plated for CFUs as described above. These washers were compared to both control washers that were heated and then put into fresh media with no vancomycin, and control washers that were not heated but were exposed to the same concentration of vancomycin to determine if there was any synergistic effect of heat and vancomycin compared to either treatment alone.

	Time Duration			**Vancomycin** (Conct.)	
	3.5	10	15	(Low)2ug/mL	(High)20ug/mL
S.S washers	+	+	+	+	*(Pending)
Titanium	+	+	+	*(Pending)	+

Table 1 – Flow chart of various conditions tested (Temperature, Duration, Antibiotic concentration)

2.3.7 In Vitro Growth Analysis

Data was graphed and analyzed using GraphPad Prism V7.0 (GraphPad Software, La Jolla, CA). Data points are presented as the mean +/- standard deviation for at least 3 biological replicates from a minimum of 2 independent experiments. Statistical significance determined by a one-way ANOVA with Dunnetts multiple comparison * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Chapter 3- Results

3.1 Biofilm grown on washers

To initially explore the effects of heat and antibiotics for the treatment of biofilm-based implant infections, the first requirement was establishing the ability to grow biofilms on metal implants. To do this, we used an *S. aureus, in vitro* biofilm model using strain USA300 grown in the presence of stainless-steel & titanium washers (N= 3 in each group over 2 independent experiments). Bacteria were grown on the washers within sterile culture plates using TSB (tryptic soy broth) growth media with or without the addition of 0.4 % glucose. This method has previously been demonstrated by Lim *et al*., to stimulate biofilm formation (89). After growth the washers were rinsed with sterile saline to remove any non-adhered bacteria before sonicating and plating. It was found that the control specimens that did not get glucose had approximately 12-fold fewer bacteria adhered to the surface of the washers than those grown in the presence of glucose. This confirmed that the addition of 0.4% glucose to TSB is an effective method for establishing adherent biofilms on metal washers (Figure 5).

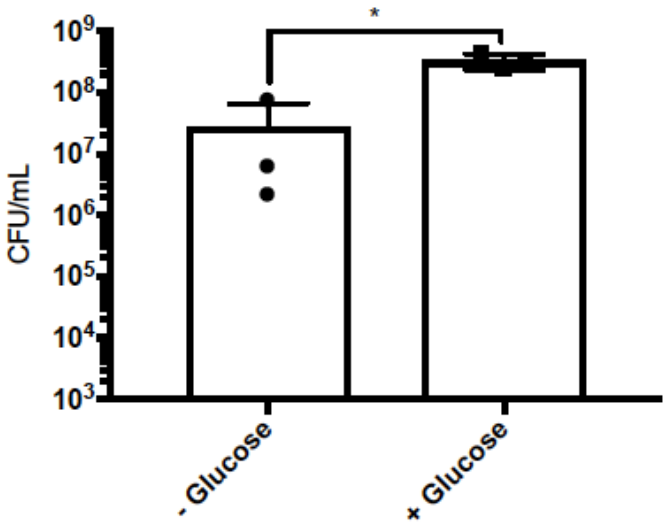


*Figure 5 – The ability of S. aureus to form an adherent biofilm on stainless-steel washers. S. aureus USA300 grown in the presence of washers with or without glucose supplementation. Samples were incubated statically for 24 hours before being washed then sonicated and plated to determine the number of adherent CFUs. Data shown as the mean +/- SD and statistical significance was determined by an unpaired T-test where * P<0.05. N=3*

3.2 Tested effects of Vancomycin treatment on pre-formed biofilm

The next objective was to confirm that biofilm formation leads to increased antibiotic resistance. To this end, vancomycin, which is typically used clinically to treat MRSA infections, was added to the culture media after the biofilms had been grown on the washers. Vancomycin was added at 2µg/mL, double the concentration previously shown to inhibit the growth of planktonic *S. aureus* USA300, and incubated for another 24 hours before plating, as done before. (N=4 for no Vancomycin and N=6 for addition of Vancomycin over 2 independent experiments) Twenty-four hours incubation was chosen as it is the accepted standard for *S. aureus* growth experiments and MIC testing is done at 24 hour time intervals (90). Addition of vancomycin lead to a higher CFU/mL, which could possibly be due to some contamination, dilution error or use of

sub-MIC vancomycin use. This confirmed that the biofilms grown on the washers are resistant to vancomycin at a concentration known to be effective against planktonic bacteria. This resistance to antibiotics necessitates the study of novel methods for treating biofilm-based implant infections (Figure 6).

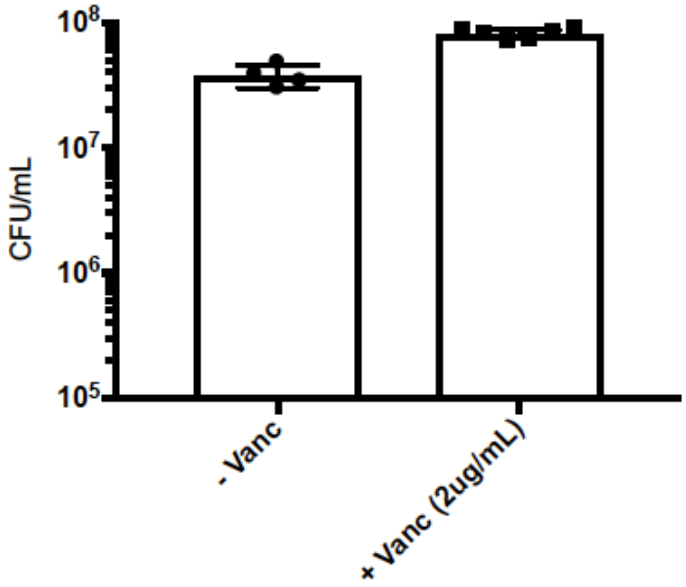


Figure 6 – *S. aureus* biofilms are resistant to vancomycin treatment. *S. aureus* USA300 biofilms were grown on stainless-steel washers as before, after growth the washers were placed in fresh media either with or without 2µg/mL vancomycin and incubated for 24 hours before plating. Data are presented as the mean +/- SD with and N=4 for -Vanc and N=6 for +Vanc over 2 independent experiments.

3.3 Tested effects of duration of heating 'Thermal shock' via induction heating on biofilm

After establishing an *in vitro* model of a biofilm with resistance to regular antibiotic (vancomycin) use , I sought to study NCIH as a novel method for treating biofilm-based implant infections, as recently reported by Pijls *et al* (68). To analyze the effects of duration of heat exposure on a mature biofilm on an orthopedic implant, each group of washers (stainless steel and

titanium), with surface-associated biofilms, were heated to and maintained at 60°C for different time intervals i.e 3.5, 10, 15 minutes (Stainless steel – N= 3 each , Titanium 5 each). These heated washers were then compared to washers that were not exposed to heat to determine the difference in bacterial burden. After heating, the stainless-steel washers showed a 24-, 25-, and 48-fold reduction (3.5, 10, and 15 minutes respectively) on average in CFU/mL compared to controls. However, the titanium washers showed a 5-, 20-, and 24-fold reduction on average compared to controls for the same conditions. I did an ANOVA and multiple comparison for the stainless-steel washers to compare the 3.5 minutes to 10 minutes and the 10 minutes to the 15 minutes. For the 3.5 vs 10minutes P=0.997, and for the 10 minute vs 15 minutes P=0.884, which are both non-significant. For the titanium washers the 3.5 vs 10 minutes P=0.128, the 10 vs 25 minutes P=0.997 and the 3.5 vs 15 minutes P=0.114. It is worth noting that although the titanium washers had smaller decreases in CFU/mL compared to the stainless-steel washers for the same conditions, the results were more statistically significant, which is likely due the larger number of replicates for the titanium washers. While the bacteria were not eliminated this has shown that biofilm grown on stainless steel & titanium washers heated to 60°C showed significant reduction in the number of bacteria adhered to the surface. Because the differences observed when heating for 10 or 15 minutes were comparable, the 25 fold vs 48 fold difference is not statistically significant because there are only 3 replicates and there is some variation between each of the replicates, 10 minutes was chosen as the standard heat treatment for further testing. (Figure 7).

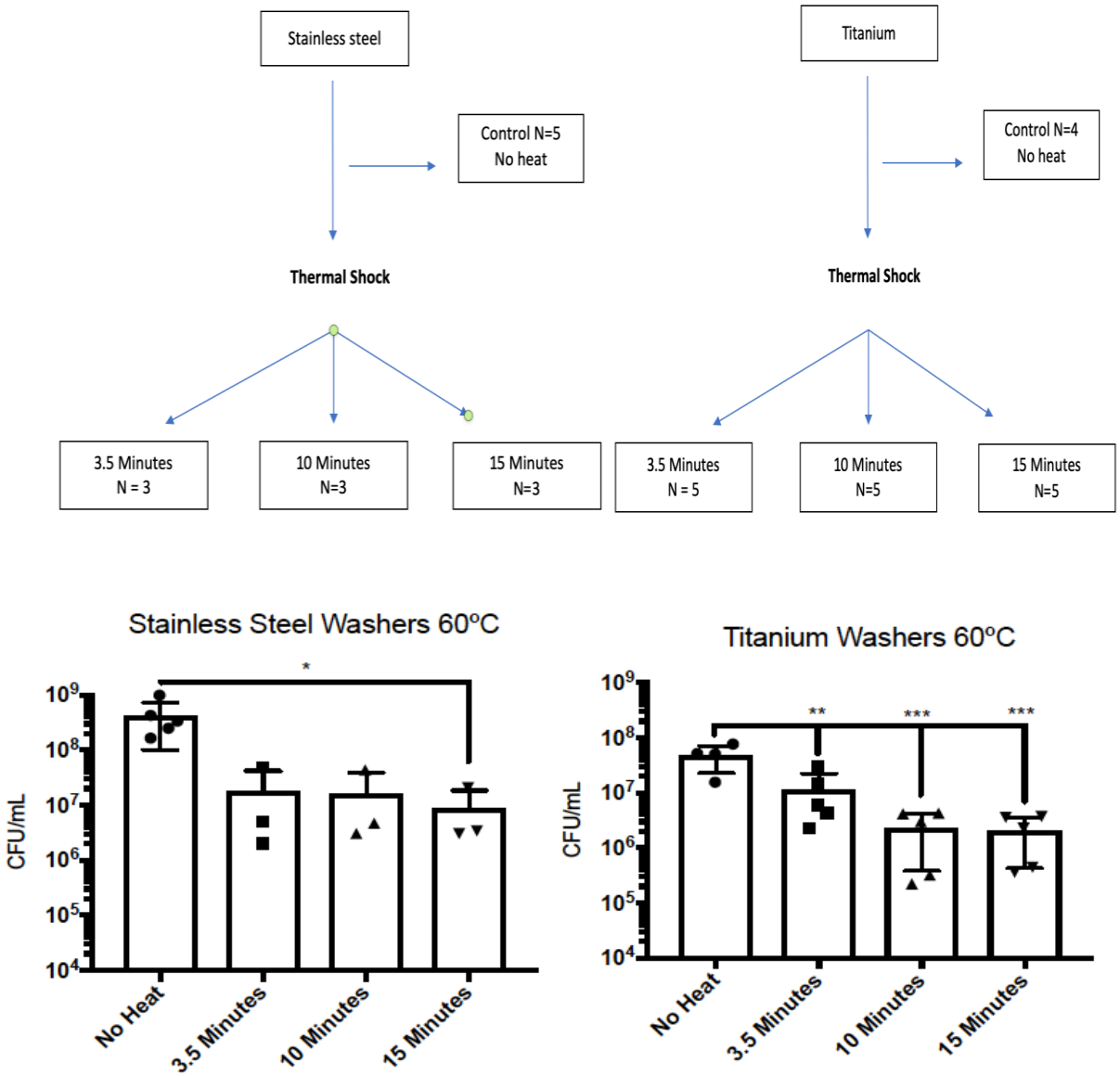
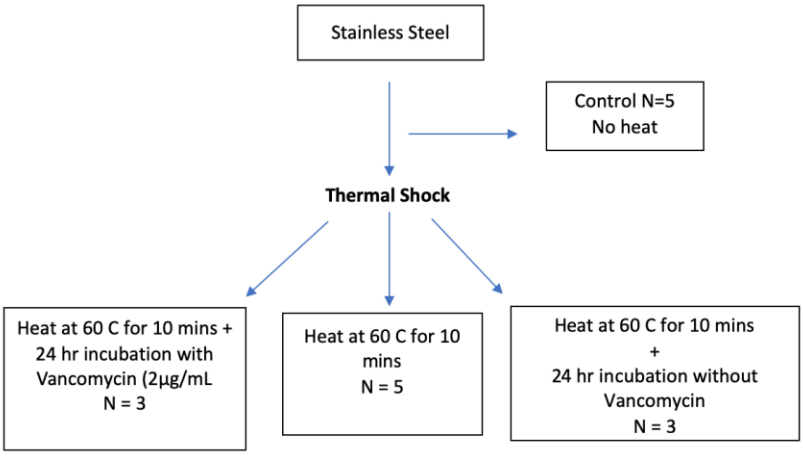


*Figure 7 – Induction heating of mature biofilms grown on stainless steel and titanium washers. USA300 based biofilm grown on stainless steel (A) or titanium (B) washers, either exposed to thermal shock of 60 ℃ for various times or left unheated. Data shown as the mean +/- SD, statistical significance determined by a one-way ANOVA with Dunnetts multiple comparison * P<0.05, ** P<0.01, *** P<0.001. In Stainless steel (A) N=5 for the control no heat, N=3 each for 3.5/10/15-minute intervals for both groups over 2 independent experiments, in (B) Titanium N=4 for the control and N=5 for all other samples over 2 independent experiments.*

3.4 Tested Synergistic effects of low dose Vancomycin after thermal Shock (Stainless steel washers)

The next objective was to determine whether there is a synergistic effect of vancomycin treatment after an initial thermal shock to the biofilm. As a control (N=5) to establish the starting number of CFUs in the biofilms, plating was done for a group of washers before heating, then the remaining washers with biofilms were heated at 60ºC for 10 minutes, as mentioned before. Again, stain less steel washers (N=5) were selected and plated to determine level of killing by thermal shock alone, while the remaining washers that were heated, were then put into fresh media with or without vancomycin (2µg/mL) and incubated for 24 hours before plating (N=5 washers for "no heat" and "heated" groups, N=3washers for "Heat+Vancomycin" and "Heat-Vancomycin, for all other samples over 2 independent experiments).This concentration was chosen to represent clinically relevant concentrations (91). It was found that the vancomycin (low dose) treatment after thermal shock still had no effect and the number of CFUs in the biofilm actually increased, matching the sample that had no vancomycin exposure. Therefore, while the heat causes significant levels of bacterial death it did not restore planktonic levels of antibiotic sensitivity and biofilm remained resistant to vancomycin (Figure 8).



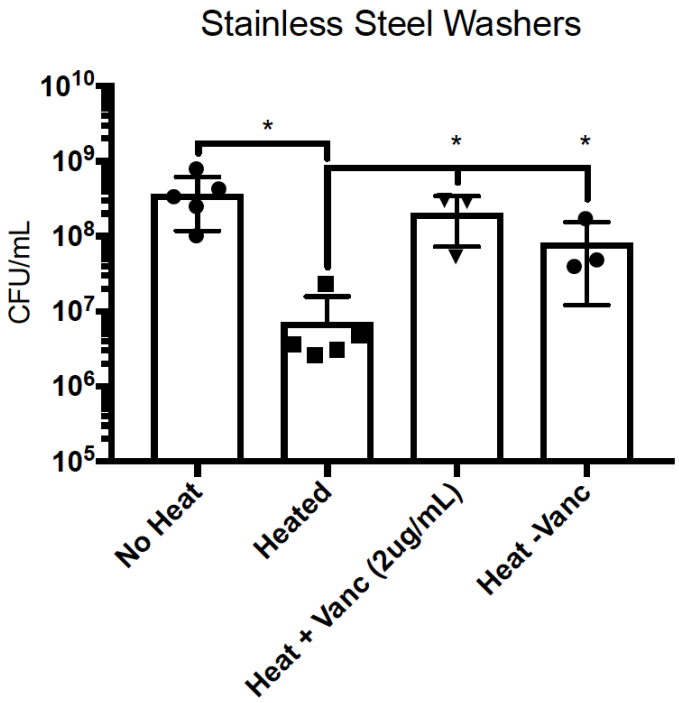
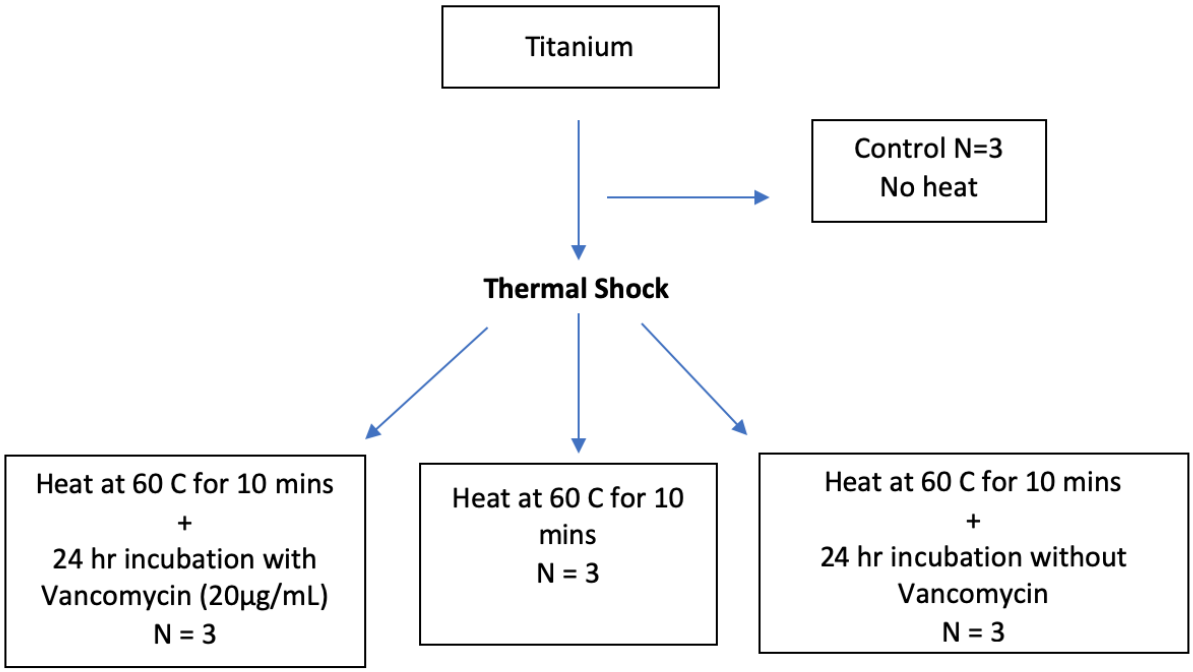


*Figure 8 –Vancomycin (2µg/mL) is not effective against biofilms on stainless steel washers even after heating - USA300 biofilms grown on stainless steel washers exposed either to thermal shock (60°C, 10minutes) alone, or in combination with a secondary 24-hour incubation with or without vancomycin (2µg/mL). Data shown as the mean +/- SD for N=5 for "no heat" and "heated", N=3 for "Heat+Vanc" and "Heat-Vanc" Statistical significance determined by a one-way ANOVA with a Dunnetts multiple comparison post-test where * P<0.05.*

3.5 Tested Synergistic effects of higher dose Vancomycin at (20µg/mL) after thermal shock (titanium washers)

Since induction heat with low dose of vancomycin concentration did not show significant decrease in CFU/ml , a similar set of experiments were repeated on titanium washers with a higher concentration(20µg/mL) of vancomycin (higher range of serum concentrations in IV therapy) making it a clinically relevant concentration. As before, washers were selected (N= 3 titanium washers in each group) before and after heating (60ºC, 10 minutes) to be plated as controls, and the remaining samples that were heated were incubated in media with or without vancomycin (20µg/mL) before plating.. There was a 20-fold decrease in CFU/ml after initial effect of thermal shock, but then another 50-fold decrease for the washers receiving both the heat and then the high dose vancomycin treatment for 24-hours. This dual treatment resulted in a ~1000-fold total decrease in CFUs compared to untreated controls. This suggests that although the bacteria were not eliminated there is a potential synergistic effect between the heat and antibiotic treatment against biofilms (Figure 9).



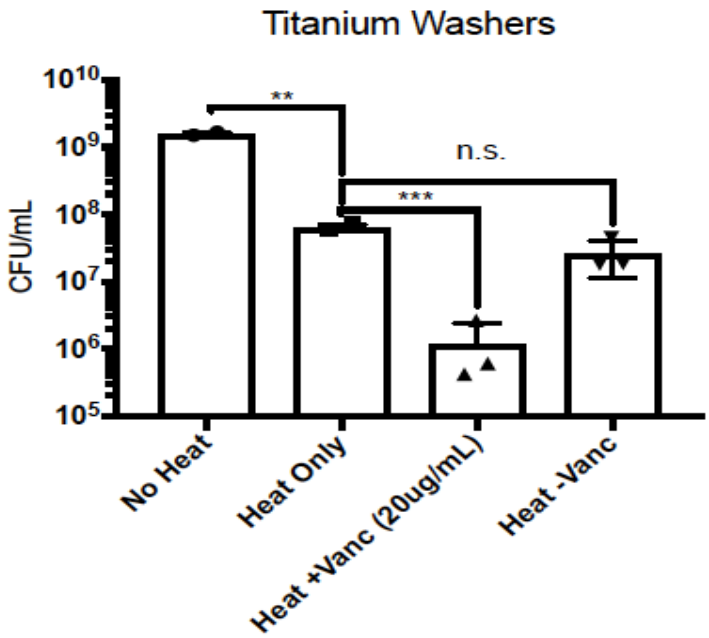


Figure 9-Vancomycin (20µg/mL) is effective against biofilms on titanium washers after heating. USA300 based biofilm grown on titanium washers plated before & after exposure to thermal shock of 60℃ for 10 minutes. After heating select washers were incubated with or without 20µg/mL vancomycin before plating. Data shown

*as mean +/- SD for N=3, statistical significance determined by a one-way ANOVA with a Dunnetts multiple comparison where * P<0.05, ** P<0.01, *** P<0.001.*

3.6 – Series of experiments pending due to interruptions caused by COVID-19

We had planned on the following experiments to better elucidate the relationship between heating and antibiotics on both stainless steel and titanium washers, but the lab was closed because of COVID-19. List of pending / possible future series of experiments include -

1. Testing of Synergistic effect on
    - Heat + (high concentration) Vancomycin on Stainless Steel washers
    - Heat + (low concentration) Vancomycin on Titanium washers
2. Independent effect of (high cont.) Vancomycin on biofilm grown on Stainless steel + Titanium washers, (non-heated – control group)
3. Synergistic use of antibiotic cocktail (eg. Vancomycin, Rifampicin / NAC)
4. Replicate series of experiments in different species (eg. *S. epidermidis*)

Chapter 4 -Discussion and future directions

The effects of antibiotics and antimicrobial agents on bacterial biofilm infections have received significant attention in the literature (86, 92). As strategies based on antibiotics typically have shown to fail in the treatment of infected implants, the physical and mechanical properties of biofilms, need to be researched as potential therapeutic targets. The physical methods that have received attention are magnetic fields, ultrasound, and pulsed electrical fields (67, 93, 94).

To establish the scope of potential role of physical methods, such as heat, we have investigated the impact of heat treatment and its synergistic effects with antibiotic therapy on stainless steel and titanium orthopedic implants (washers). Stainless steel and commercially pure titanium are widely used materials in orthopedic implants. Stainless steel is the most frequently used material in orthopedics because of its mechanical strength, low costs, and the possibility of bending and shaping the implant to create a custom fit in the operating room. However, major disadvantages of stainless steel are well-documented: surface corrosion phenomena and the high rate of locally and systemically released corrosion products (95), which led many authors to recommend the use of titanium for orthopedic implantation as an 'bio-inert' material (96). But neither of the materials has been termed the 'golden standard' material in general (97).

The results of our study show that non-contact induction heating of orthopedic implants appears feasible and was effective *in vitro*, in the reducing the bacterial load for *S. aureus* biofilm. There was a 20-fold decrease in CFU/ml after initial effect of thermal shock, and then another 50-fold decrease for the washers receiving both the heat and then the high dose vancomycin treatment for 24 hours. But complete eradication was not observed.

Periprosthetic joint infection (PJI) is the leading cause of revision surgery, and is the most challenging complication after total joint replacement (98). It is essential to have a multi-disciplinary approach between microbiologists, surgeons, and infectious disease departments to yield the best outcome in these challenging situations (16). There are many management strategies and protocols to treat PJI, which need to be individualized to each patient to maximize success. This can be in the form of antibiotic suppression; debridement with retention of prosthesis (DAIR) (10, 11), or excision arthroplasty including single-stage revision (11), two-stage revision;(12) multiple- stage revision, partial revision, and even arthrodesis or amputation.(13)

The majority of present treatment strategies are maximally invasive; therefore, challenging in patients with high comorbidities. The current gold stand of 2 stage revision surgery still cannot treat 15-20% infections at 5 years (99). Hirakawa *et al*. (100) reported that reimplantation was successful in 80.0% of knees with low-virulence organisms (coagulase-negative *Staphylococcus*, *Streptococcus*), 71.4% with polymicrobial organisms, and 66.7% with high-virulence organisms (methicillin-resistant *Staphylococcus aureus*) (100). Furthermore, the increasing antibiotic resistance of bacteria raises concern and limits the choices of antibiotics(58, 59). Therefore, it is vital that novel, non-invasive management options such as non-contact induction heating of metallic implants for the prevention and treatment of biofilm infections in implants are developed to reduce the burden of more invasive and extensive revision surgeries and possibly improve effectiveness of present standard of care practices.

In our study, non-contact induction heating for 10 minutes at 60°C, on contaminated stainless steel & titanium washers showed an average of 25- and 20-fold reduction in CFU/ml of bacteria yield, respectively. Notably titanium washers had smaller decreases in CFU/mL compared to the stainless-steel washers for the same conditions, the results were more statistically significant, which is likely due the larger number of replicates for the titanium washers. Titanium may demonstrate some bacteriostatic properties with a varying degree to different types of micro-organisms (101). These results were comparable with a similar in-vitro study done by Pijls *et al* which reported a 6-log reduction of bacterial load on titanium discs by using PEMF induction heating at 60°C for 3.5 minutes (68).

In our study, the synergistical use of the dual treatment strategy (heat and antibiotics) resulted in a ~1000-fold total decrease in CFUs/ml. Suggests the potential synergistic effect between the heat and antibiotic treatment against biofilms. Although the bacteria were not

completely eliminated , Hajdu *et al* (74). have also reported on the significant increased antibacterial activity of antimicrobials agents by increasing the ambient temperatures of heating and using high concentration of antibiotics. They reported that the moderate increase in the incubation temperature to 45°C resulted in a decrease in biofilm thickness with treatment with different antibiotics (daptomycin, tigecycline, fosfomycin, cefamandole and vancomycin) and showed significant reductions in bacterial growth with high antimicrobial concentrations. As compared to Hajdu *et al*.(74) , we did not incubate with antibiotics at higher temperatures, since we heated the washers and then put them in antibiotics at 37°C. Pickering *et al*.(102) reported that PEMF may work synergistically with thermal shock and increased the effectiveness of gentamicin against the five-day biofilms of *S. epidermidis*, hence it may be of value in the treatment of biofilm-associated implant-related infections. Since our planned set of experiments were delayed due to restriction of COVID-19, we could not test for different cocktails of antibiotics or different strains of *Staphylococcus*.

In our series of experiments, to observe the effects of Sub MIC dose of vancomycin on pre-formed biofilm, addition of vancomycin at 2µg/mL lead to a higher CFU/mL. This could possibly be due to some contamination or dilution error. This 'close to the mic' concentration may have an effect on the bacteria, which in response to this lower concentration of vancomycin, could change their surface structure and actually cause more biofilm to form. Another possible reason that could have led to higher CFU/ml rather than contamination could be that sub-MIC vancomycin can induce biofilm formation. Sub MIC vancomycin has been reported to cause biofilm formation (103). Sub-MIC (2ug/mL) which is 2x Planktonic MIC but is below the MIC for biofilms, was added to pregrown biofilms, so it was sub-biofilm-MIC vancomycin and may have made more adherence than the samples with no vancomycin. This was to be empirically tested but due to

interruptions caused due to COVID-19, further testing could not be done. We would need more experiments to prove that theory.

In our experiment, we were unable to show complete eradication of bacteria. Pijls *et al*. (75), reported a total eradication of biofilm on titanium implants at 60 °C, with a combination induction heating, cocktail of antibiotics (vancomycin , rifampicin) and NAC. The possible mechanism of this synergistical effect has been reported by them is that PEMF together with eddy currents may interfere with the transport of charged molecules within the bacteria, at the implant surface possibly making them more susceptible to thermal shock. Furthermore, with induction heating the heat originated on the biofilm-implant interface, travels into the biofilm, whereas the antibiotics diffuses into the biofilm starting at the outer border of the biofilm and ultimately ending at the biofilm implant interface. This attack from two directions may be a mechanism for the observed synergistic effect.

The major advantage of induction heating of metallic implants is that only the metallic implant is actively heated while induction heating has no direct heating effect on the surrounding tissue. The surrounding tissue is heated to some extent by thermal conduction from the heated metal, but if the tissue is well perfused by arterial and venous blood flow, the heat will very likely be significantly reduced as in coagulation procedures (81). Multiple concerns for potential tissue necrosis with induction heating due to relatively high temperatures at the prosthesis -bone interface may arise. However, animal model studies by Muller *et al*.(71) demonstrated evidence of a lack of thermal necrosis by heating a nickel-titanium shape intramedullary rod in the femur of rats at 40°C to 60°C using induction heating and demonstrated no necrosis of the surrounding bone and tissue. Furthermore ,clinically relevant studies using drilling in bone and bone cement, which achieves durable fixation for hip and knee implants, have shown that curing temperatures of cement and

drilling in cortical bone readily exceed 60°C (78, 82, 83). Additionally, Pijls *et al*.(79) reported heating techniques such as segmental induction heating, which can be used to apply localized heating to a segment of an implant, using the remainder of the implant as a heat sink. Hence the chosen temperature of 60°C is within the safe temperature range.

Various limitations exist regarding non-contact temperature control. The temperature sensor such as the one used in our experiments cannot be readily used in clinical situations because of the absence of direct contact, there is tissue and bone between the implant and the sensor. Cheng *et al*. (104) developed a non-invasive temperature safety system, using remote acoustic sensing, to detect sounds associated with boiling on the implant-tissue interface. Multiple studies regarding hip Implants with temperature measurement sensors have been reported (105). Future research to address these challenges might involve developing clinically relevant and safe implants with temperature sensors.

Furthermore, our experiments were *in vitro* and may not translate entirely to in vivo situations with possibility of more mature biofilms. Hyperthermia can have varying physiological and molecular effects. Localized hyperthermia has been shown to increase blood flow and vessel permeability, which could result in better availability of antibiotics, which could be helpful in relieving the infection (106).

The future clinical application of non-contact induction heating can be used in an operative or non-operative situation as part of a multi-modality treatment plan. The non-operative application of NCIH can be used in clinical situations as an adjuvant to chronic suppressive antibiotic therapy or severely immunocompromised patients, who are not fit for surgery. The metal implant fixed to the bone can be heated noninvasively with care taken to avoid excessive heating to areas that are in close contact with important anatomical structures. Since the current gold

standard therapy, i.e. revision 2 stage surgery cannot completely treat 15-20% infections (99), the noninvasive application of induction heating may be particularly beneficial in patients for whom surgical treatment is not possible and receive suppression antibiotic therapy.

Alternatively, NCIH can be used during surgery to increase the effectiveness of the surgical procedure such as in debridement, antibiotics and implant retention (DAIR). Allowing heating of parts of the implant that cannot be reached and mechanically cleaned. During surgery, soft tissue can be protected by keeping it away from the heated part of the implant. Additionally, surgery allows for direct temperature control by direct temperature measurement (e.g., thermocouple) or by IR thermal imaging.

Future work needs to be done to find the ideal conditions of heat antibiotic use and applications as well as potential for implant related modifications. Further directions for implants related modifications include, development of anti-biofilm agents, used as a coating to implants to counteract bacterial adhesion. Surface treatment by natural or modified polysaccharide polymers with anti-adhesive and bactericidal coatings has been reported to be a promising means to fight against implant-associated biofilm infections(107).

Preliminary results support the hypothesis that NCIH of stainless steel and titanium implants, is effective in reducing bacterial load of *S. aureus* within a biofilm *in vitro*. There was a 20-fold decrease in CFU/ml after initial effect of thermal shock, and another 50-fold decrease for the washers receiving both the heat and then the high dose vancomycin treatment for 24 hours.

This Dual treatment resulted in a ~1000-fold total decrease in CFUs compared to untreated controls. (~ 3 log reduction). Combined effect of heat and use of antibiotics was seen, but complete eradication was not observed. Since our planned set of experiments were delayed/interrupted due

to restriction imposed due to COVID-19, we could not further test for different combinations cocktails of antibiotics or different strains of *Staphylococcus*.

In conclusion non-contact induction heating of metallic (orthopedic) implants appears feasible and was effective *in vitro*, in the reducing the bacterial load for *S. aureus* biofilm Furthermore use of vancomycin, after the thermal shock showed a synergistic effect, leading to a further decrease in bacterial load. But complete eradication was not observed.

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- Schubert M, **Sidhu R**, Getgood A, Sherman S. Failures of Realignment Osteotomy. Operative techniques in sports medicine. Volume 28, Issue !, March 2020
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Conference Presentations

- **March 2019** Kooner P**, Sidhu R**

"The fragility of statistically significant findings from randomized controlled trials in a premier Orthopaedic journal "

AAOS Annual meeting , Las vegas, USA (**Podium Presentation)**

- **Sept 2017 ,** Kooner P, **Sidhu R**, Grewal R

"Complications of Distal Radius Fractures in the Elderly: A systematic review and meta-analysis."

San Francisco, USA (**Podium Presentation)**

- **April 2017 , Sidhu R** , Patil S , Ravi M

" Convertion Hip Arthroplasty for Failed Hemiarthroplasty : Preventing Dislocations
Indian Society of Hip and Knee Surgeons Annual National Conference ,

New Delhi , India **(Podium Presentation)**

- **Feb 2017 , Sidhu R** , Patil S , Ravi M

" Convertion Hip Arthroplasty for Failed Hemiarthroplasty : Preventing Dislocations

Karnataka Orthopedic Association Annual State Conference,

Hubali , Karanatak,India **(Podium Presentation)**

- **Aug , 2015** , Sidhu R. , Yammin M. , **Sidhu R**.

"Syndesmotic Injuries – Our experience (case series) "

Indian Society of Foot and Ankle Surgeons Annual National Conference

Ludhiana, **(Podium Presentation)**

- **Sept 2014** - Uppin R, **Sidhu R,** Patil S

"The Functional Outcome of Tibial Plateau Fractures (Schatzker Type V & VI) with Locking Compression Plate" - IOACON Indian Orthopedic Association conference

- Bengaluru, India **(Poster Presentation**)