DEVELOPMENT OF ADJUVANT THERAPY TO TREAT BIOFILM-RELATED STAPHYLOCOCCUS AUREUS PROSTHETIC JOINT INFECTION

Thesis submitted by

Narayan Pant (BSc, MSc)

James Cook University

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This thesis is dedicated to my parents……..

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DECLARATION OF ETHICS AND BIOSAFETY APPROVALS

The research in this thesis was conducted as per the NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics. The research protocol was approved by the James Cook University Animal Ethics Committee (AEC2486), the Mater Hospital Health Services North Queensland Human Research Ethics Committee (MHS20170808-01), and Biosafety Approval Committee (JCUIBC-170418-013).

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STATEMENT OF CONTRIBUTION

Supervision and Intellectual Support: My thesis research project was supervised by Professor Damon Eisen (Primary supervisor), A/Prof Jeffrey Warner (Supervisor Mentor), and A/Prof Catherine Rush (Secondary Supervisor). I prepared the first draft of all chapters in the thesis. Critical comments and English writing supports were provided by all of my supervisors. Dr Peter Mulvey supervised me during the early stage of my PHD.

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ABSTRACT

Arthroplasty is a life enhancing surgery as it relieves pain and restores mobility. With an aging world population, arthroplasty surgery is becoming more common. More than one million total hip and knee replacement surgeries were performed in 2010 and by then seven million people were living with a hip or knee replacement in the United States alone. The numbers were predicted to increase significantly over time. Prosthetic joint infection (PJI) may lead to the devastating complication of an arthroplasty surgery. The incidence of bacterial PJI is 2.18% for primary arthroplasty and 4.4% for revision arthroplasty, with *Staphylococcus aureus* being the most common causative agent. This incidence has been estimated to increase over time. Up to 50% of the *S. aureus* involved in causing PJI are methicillin resistant. Bacterial biofilms are intrinsic to PJI pathogenesis and are recalcitrant to antibiotic treatment. Currently available PJI treatments are costly and traumatic with significant failure rates and side effects. Adjuvant therapy using ticagrelor and antivirulence molecule savirin may enhance the success rate of existing treatments for biofilm-related *S. aureus* PJI.

Ticagrelor, a P2Y12 receptor inhibitor antiplatelet drug used to prevent thrombotic events in atherosclerotic cardiovascular disease patients, is known to have antibacterial and antibiofilm activity against *S. aureus* both *in-vivo* and *in-vitro*. Similarly, savirin has been shown to both prevent and treat biofilm-related *S. aureus* infection in animal models. However, the efficacy of

ticagrelor or savirin to treat biofilm-related *S. aureus* PJI and their molecular mechanisms for antibiofilm activity are yet to be evaluated.

Synovial fluid has been known to have antibacterial activity. It may be that synovial fluid has intrinsic actions in preventing bacterial growth including methicillin resistant *S. aureus* (MRSA) and possibly biofilm formation. The efficacy of synovial fluid including that containing cefazolin, from arthroplasty patients, to prevent the *in-vitro* MRSA growth and biofilm formation is unknown.

The first aim of this study was to find appropriate reference genes to study the effect of ticagrelor or savirin treatment on the expression of some key biofilm-related genes (*icaA, icaD, fib, ebps, eno, agr*) in *S. aureus*. Suitable reference genes were identified by testing 16 different candidate reference genes by relative quantification method using quantitative reverse transcriptase polymerase chain reaction (qRTPCR) and bestkeeper algorithm. As per this algorithm a suitable reference gene must have standard deviation (SD) less than one and a gene having the lowest SD and coefficient of variance (CV) is regarded as most stable.

The most appropriate reference gene for savirin treatment experiment was *fema* followed by *gapdh* and *16s*, while that for ticagrelor treatment experiment was *gmk* followed by *rpoB* and *rpoD*.

The second aim of this study was to determine the efficacy of ticagrelor, alone and in combination with cefazolin, to treat *S. aureus* PJI in a mouse model. The treatments were

assessed using both *in-vitro* and *in-vivo* method. The *in-vitro* component of the study assessed the antibacterial and antibiofilm activity of ticagrelor, alone and with antibiotics (cefazolin, rifampicin, and vancomycin), with microdilution and crystal violet staining method respectively. The effect of ticagrelor treatment on the expression of biofilm-related genes in *S. aureus* was determined by relative quantification method using qRTPCR. To study the *in-vivo* effect of ticagrelor in the treatment of *S. aureus* PJI a clinically relevant mouse model of PJI was used. Biofilm was established on the knee implants by inoculating *S. aureus* onto the implant's cut end protruding into the knee joint space. Six to ten week-old C57BL/6 female mice were randomised into five groups (n=8/group): (1) infected implants treated with ticagrelor; (2) infected implants treated with cefazolin; (3) infected implants treated with ticagrelor and cefazolin; (4) infected implants treated with phosphate buffer solution (PBS) (Positive control); (5) sterile implants (Negative control). Ticagrelor was administered orally from day four to day seven post-surgery, while cefazolin was injected intravenously on day seven. On day fourteen post-surgery, mice were euthanised using carbon dioxide, and K-wires and periprosthetic tissues were harvested aseptically for microbiological and histological analysis.

Ticagrelor showed the *in-vitro* antibacterial and antibiofilm activity against *S. aureus* including methicillin resistant *S. aureus* (MRSA), and potentiated the antibacterial and antibiofilm activity of the antibiotics except the antibacterial activity of rifampicin. In molecular tests, ticagrelor treatment showed the strain-specific downregulation of biofilm-related genes - *fib*, *icaD*, *ebps*, and *eno*. In the animal model, ticagrelor alone reduced bacterial counts on both implants and

periprosthetic tissues significantly compared with the PBS-treated positive control, while ticagrelor with cefazolin reduced bacterial counts only on implants. The analysis of tissue histology showed the presence of similar concentrations of Gram-positive cocci and neutrophils in the periprosthetic tissue of all the infected groups.

The third aim was to determine the efficacy of savirin, alone and with cefazolin, to treat *S. aureus* PJI in an animal model. The *in-vitro* antibacterial and antibiofilm activity of savirin, alone and with antibiotics (cefazolin, rifampicin, and vancomycin), its effect on the expression of key biofilm-related *S. aureus* genes, and the efficacy of savirin, alone and with cefazolin, to treat biofilm-related *S. aureus* PJI in a mouse model were studied using the same methods as in aim two. In the *in-vivo* component mice were randomised into five groups (n=8/group): 1) infected K-wire treated with savirin, 2) infected K-wire treated with cefazolin, 3) infected K-wire treated with savirin plus cefazolin, 4) infected K-wire treated with savirin diluent containing sterile PBS (positive control), 5) sterile K-wire (negative control). A single subcutaneous dose of savirin was injected immediately after surgery, while a single dose of intravenous cefazolin was given on day seven.

Savirin showed the *in-vitro* antibacterial and antibiofilm activity against *S. aureus* including MRSA, enhanced the inhibitory activity of the antibiotics against planktonic and biofilm growths, and downregulated the expression of all the key *S. aureus* biofilm-related genes, *icaA, icaD*, *eno*, *fib*, *ebps* and *agr*, studied. In the animal study, savirin reduced bacterial counts on the implants compared with the positive control, while savirin plus cefazolin reduced bacterial

counts on both implants and surrounding tissues. Tissue histology showed the signs of *S. aureus* infection (Gram-positive cocci and neutrophils) but there was no visual indication of reduction in the infection due to treatment.

The fourth aim was to determine the antibacterial and antibiofilm activity of synovial fluid, alone and when that contains cefazolin, from arthroplasty patients against *S. aureus* including MRSA. The minimum inhibitory concentrations (MICs) of cefazolin against the planktonic and biofilm form of *S. aureus* in glucose Luria Bertani (GLB) broth and synovial fluid were determined by broth microdilution and crystal violet staining method.

Synovial fluid alone inhibited the planktonic and biofilm form of both methicillin susceptible *S. aureus* (MSSA) and MRSA. Cefazolin-containing synovial fluid from arthroplasty patients had greater inhibitory activity against the planktonic and biofilm growth of *S. aureus* compared with the same cefazolin concentration in GLB.

In summary, this thesis describes the effect of antiplatelet drug ticagrelor and antivirulence molecule savirin to treat biofilm-related *S. aureus* PJI in a mouse model. Both ticagrelor and savirin showed the *in-vitro* antibacterial and antibiofilm activity against *S. aureus* including MRSA and also potentiated the *in-vitro* activity of selected antibiotics, cefazolin, rifampicin, and vancomycin. In the animal study, ticagrelor showed the *in-vivo* antibacterial and antibiofilm activity but it did not enhance the *in-vivo* activity of cefazolin. The antibiofilm activity of ticagrelor was related with the downregulation of biofilm-related genes: *fib*, *icaD*, *ebps*, and

eno. Similarly, savirin showed the *in-vivo* antibiofilm activity but no *in-vivo* antibacterial effect. However, savirin potentiated the *in-vivo* antibiofilm and antibacterial effect of cefazolin. The molecular mechanism for savirin's antibiofilm effect was through the downregulation of key biofilm-related genes: *icaA, icaD*, *eno*, *fib*, *ebps* and *agr*. Therefore, it can be concluded that adjuvant therapies with ticagrelor or savirin may enhance the efficacy of currently available treatments for *S. aureus* PJI. Ticagrelor and savirin should be considered for the development of adjuvant therapy for the treatment of *S. aureus* PJI after further study. Synovial fluid, including when it contains cefazolin, has *in-vitro* antibiofilm and anti-MRSA activity. In light of this information, it may be useful to assess any apparent MRSA preventive success of cefazolin in future studies of preoperative antimicrobial prophylaxis for PJI as being due to intrinsic antibiofilm activity of synovial fluid.

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LIST OF ABBREVIATIONS

GENERAL

agr	accessory gene regulatory
AIP	auto-inducing peptides
AMP	antimicrobial peptides
ANOVA	analysis of variance
ATCC	American type culture collection
Atl	autolysin
AUD	Australian dollars
Bap	biofilm-associated protein
CcpA	Catabolite control protein A
cfu	colony forming unit
Clf	clumping factor
Coa	coagulase
Ct	threshold cycle

CV	coefficient of variance
DAIR	debridement antibiotics and implant retention
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DPX	dibutylphthalate polystyrene xylene
eDNA	extracellular DNA
Emp	extracellular matrix binding protein
EPS	extracellular polymeric substance
FDA	Food and Drug Administration
FIC	fractional inhibitory concentration
FnBP	fibronectin-binding protein
Fur	ferric uptake regulator
GLB	glucose Luria Bertani broth
HE	Haematoxylin and eosin
i.e.	that is

ica	intercellular adhesion
ip	intraperitoneal
iv	intravenous
K-wire	Kirschner wire
LB	Luria-Bertani broth
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
MRM	multiple reaction monitoring
MRSA	methicillin resistant *S. aureus*
MSA	Mannitol salt agar
MSCRAMM	microbial surface components recognizing adhesive matrix molecule
MSSA	methicillin susceptible *S. aureus*
OD	optical density
PBS	phosphate buffer solution
PCR	polymerase chain reaction

pH	potential of hydrogen
PIA	polysaccharide intercellular adhesin
PJI	prosthetic joint infection
PMMA	polymethylmethacrylate
PNAG	poly-β(1-6)-N-acetylglucosamine
PSM	phenol soluble modulin
qRTPCR	quantitative reverse transcriptase polymerase chain reaction
QS	quorum sensing
RAP	RNA III activating protein
RIP	RNAIII-inhibiting peptide
sc	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulfate
TAE	Tris-acetate-EDTA
TE	Tris-EDTA

TRAP	Target of RAP
TSB	Tryptone Soy Broth
US	United States
USA	United States of America
USD	US dollars
VRE	vancomycin resistant *Enterococcus*

UNITS OF TIME

hr	hour
min	minute
sec	second

UNITS OF MEASUREMENTS

µl	microlitre
µm	micrometre

bp	base pair
G	gauge
gm	gram
Kg	kilogram
khz	kilohertz
M	molar
mg	milligram
ml	millilitre
mm	millimetre
ng	nanogram
nm	nanometre
nM	nanomole
^{O}C	degree centigrade
psi	pound per square inch
V	volt

µg	microgram

SYMBOLS

$	dollar
%	percentage
+	plus
<	less than
>	greater than
≤	less than or equal to

CHAPTER 1: GENERAL INTRODUCTION

Arthroplasty surgery enhances the quality of the life of patient with severe arthritis as it relieves pain and restores mobility. With an aging world population the number of arthroplasty surgery performed is on the rise. In 2010, more than one million total hip and knee replacement surgeries were performed in the United States (US) alone and the numbers were estimated to increase by four-fold by 2030 (1, 2). By 2010 seven million people were living with a hip or knee replacement in the US alone (3). A significant number of prosthetic joints fail due to biofilm-related bacterial infection (4). The incidence of bacterial infection-related prosthetic joint failure in the US was 2.18% with a high possibility of further increase over time (5). Prosthetic joint infection (PJI) complicates approximately 4.4% of revision arthroplasty surgeries (6). The predicted increase in bacterial PJI is perhaps related with increase in the number of multidrug resistant bacterial infection and debilitated patients requiring arthroplasty surgery. Up to 57% of total PJI are caused by *S. aureus* and 50% of the cases are caused by MRSA (4). The infection may be caused by inoculation during surgery or dissemination through haematogenous route (7).

Currently available PJI treatments involve major surgeries for debridement with prosthesis retention or the replacement of prosthesis, both followed by long term antibiotic therapy (8). However, these procedures are costly, traumatic, and have significant failure rates of up to 15% to 25% (9, 10). A recent study has reported a failure rate of up to 56% when debridement

antibiotics and implant retention (DAIR) was used (11). In cases where multiple revision surgeries fail, amputation may be required as a last resort (8). The cost for bacterial infection-related revision arthroplasty is 3.6- and 2.5- times that for primary and infection unrelated revision arthroplasty respectively (12). An Australian study has reported the cost for debridement and retention of a prosthetic joint to be $69,414 Australian dollars (AUD) versus $22,085 for primary arthroplasty in 2008 (13). The average total cost per case of total knee revision arthroplasty was $116,383 USD versus $28,249 USD for an uncomplicated primary total knee arthroplasty (14). The economic burden of PJI treatment on the health care system is substantial. The US health care system spent $566 million in 2009 on this corrective procedure, with an estimated expenditure of annual $1.62 billion predicted for 2020 (5). Apart from the high costs, these traumatic procedures may not be suitable for some debilitated patients (8), and prolonged aggressive antibiotic therapy may induce drug resistance among bacteria and disturbance in the composition of normal flora (15, 16). Additionally, currently available treatment cannot control the increasing drug resistant infection and antibiotics alone cannot eradicate biofilm-related infections in most cases (7). Antibiotics are medicines that are used to treat or prevent bacterial infections. Novel therapies that are less traumatic, cheaper, and with better clinical outcomes than the currently available treatment options are needed.

Adjuvant therapy when used in combination with a primary therapy (the first standard treatment given for a disease) may enhance the overall efficacy of a treatment. Adjuvant therapy with antivirulence molecules may be a good option to mitigate the shortcomings of the

existing PJI treatments. Antivirulence molecules are compounds that weaken bacteria by disarming them of their virulence factors rather than direct inhibition, making bacteria more vulnerable to clearance by antibiotics or the human immune system. These molecules, such as anti-quorum-sensing drugs, do not directly inhibit bacteria and therefore exert low selection pressure preventing or slowing the development of drug resistance (17). Ticagrelor or the antivirulence molecule savirin have previously been used for the treatment of biofilm-related *S. aureus* infection in animal models (18, 19).

Ticagrelor is a P2Y12 receptor inhibitor antiplatelet drug used to prevent thrombotic events in atherosclerotic cardiovascular disease patients (20). In a post-hoc analysis of large cardiovascular disease prevention studies, acute coronary syndrome and pneumonia patients treated with ticagrelor had the lower risk of infection related death and showed improved lung function (21-23). This molecule has been shown to have *in-vitro* and *in-vivo* antibacterial and antibiofilm activity against *S. aureus* including MRSA (19). In a pre-contaminated subcutaneous foreign body *S. aureus* infection mouse model, ticagrelor inhibited *S. aureus* biofilm growth and bacterial dissemination to surrounding tissues (19). This molecule had superior anti-MRSA activity to vancomycin and similar to daptomycin, and showed the *in-vitro* synergistic effect with rifampicin, ciprofloxacin, and vancomycin (19).

Similarly, savirin is a low molecular weight, lipophilic synthetic novel molecule known to inhibit and treat biofilm-related *S. aureus* skin and subcutaneous tissue infection in mouse models (18). This molecule is known to disrupt the *agr* quorum-sensing system by the inhibition of AgrA

attachment to promoter regions leading to the suppression of key virulence factors expression in *S. aureus* (18). This inhibition makes *S. aureus* less competent to survive inside host cells leading to their easy clearance (18). However, the direct antibacterial activity (ability to kill or prevent bacterial growth) of savirin has also been reported (24). The efficacies of ticagrelor or savirin to treat biofilm-related *S. aureus* PJI and the molecular mechanisms underlying their antibiofilm activity (ability to prevent or disperse biofilm) are unknown.

Synovial fluid itself is known to have antibacterial activity (25), therefore it may be worth investigating the effectiveness of synovial fluid to prevent the planktonic and biofilm growth of *S. aureus* including MRSA. This study may explain the MRSA preventive success of cefazolin in arthroplasty surgery. Cefazolin is a first-generation cephalosporin that prevents peptidoglycan synthesis by binding penicillin-binding proteins and inhibits cell wall biosynthesis. The main aims of this study are:

1. To identify appropriate reference genes to study the effect of ticagrelor or savirin treatment on the expression of key biofilm-related genes in *S. aureus* (*ica, fib, ebps, eno, agr*) (Chapter 4).
2. To determine the efficacy of ticagrelor, alone and with cefazolin, to treat *S. aureus* PJI in an animal model (Chapter 5).
3. To determine the efficacy of savirin, alone and with cefazolin, to treat *S. aureus* PJI in an animal model (Chapter 6).

4. To determine the antibacterial and antibiofilm activity of synovial fluid against *S. aureus* including MRSA (Chapter 7).

CHAPTER 2: LITERATURE REVIEW: NON-ANTIMICROBIAL ADJUVANT STRATEGIES TO TACKLE BIOFILM-RELATED *STAPHYLOCOCCUS AUREUS* PROSTHETIC JOINT INFECTIONS

2.1. *Staphylococcus aureus* biofilms; slow growing organisms highly resistant to drugs

Approximately, 20% and 60% of healthy adults are persistent and intermittent *S. aureus* nasal carriers respectively (26). *S. aureus* carriers are at high risk of endogenous infection. It has been shown that 80% of the cases of severe invasive infections in *S. aureus* carriers are caused by strains colonizing their anterior nares (27). Planktonic bacterial forms cause acute infections, while sessile forms are associated with biofilm formation - which characterises chronic infections (28). The sessile forms of bacteria are metabolically less active than planktonic forms and are protected by a filmy layer of "slime" referred to as extracellular polymeric substance (EPS) (29). These properties of the sessile forms of bacteria, particularly when they form biofilms, make them recalcitrant to antibiotic treatment (29, 30). Biofilm represents a mode of bacterial growth that acts as a multicellular structure, where each bacterial cell works in coordination to keep the structure alive and safe from adverse conditions (31).

2.2. Stages of biofilm formation in *S. aureus*

Bacterial biofilm formation occurs in three sequential stages: 1. Attachment, 2. Maturation, and 3. Dispersal. Free floating planktonic cells attach to surfaces and multiply to form

microcolonies. During maturation, these microcolonies produce extracellular matrix and form solid three dimensional biofilm structures. After the full maturation of biofilm, extracellular matrix degrades and releases bacteria to establish a new biofilm at another location (Figure 2.1).

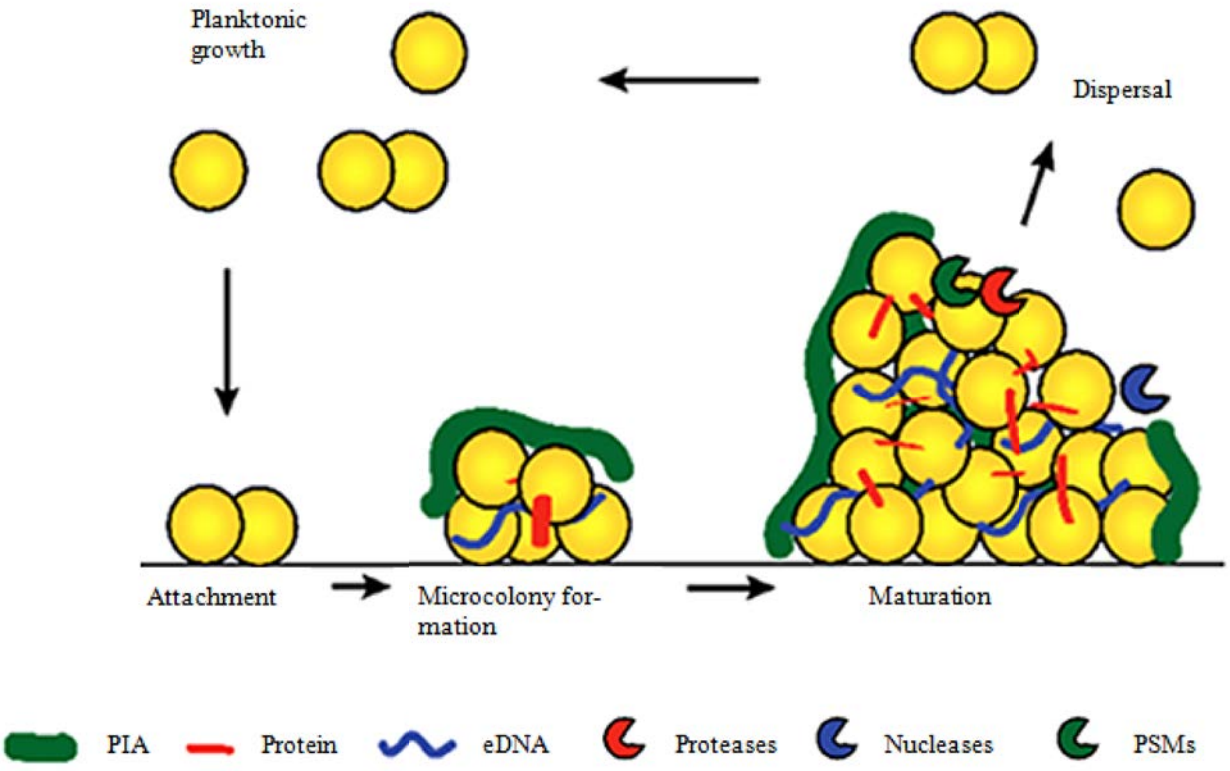


Figure 2. 1: Biofilm growth cycle. Briefly, planktonic cells attach to surfaces and multiply to form microcolonies. Microcolonies then produce extracellular matrix and mature into a solid three dimensional biofilm structure. After full maturation, extracellular matrix degrades and releases bacterial cells to establish a new biofilm at another location. (Adapted from https://www.frontiersin.org/files/Articles/123319/fcimb-04-00178-HTML/image_m/fcimb-04-

00178-g001.jpg by Lister JL, Horswill AR, *Staphylococcus aureus* biofilms: recent developments in biofilm dispersal, Frontiers in cellular and infection microbiology, 2014, by permission of copyright holder Lister and Horswill, 2014, under creative commons license http://creativecommons.org/licenses/by/4.0/) (26).

2.2.1. The first step: attachment of *S. aureus* to surfaces

Planktonic cells come into contact with surfaces with the help of gravitational forces and Brownian movement (32). Attracting and repelling forces arising from physicochemical and electrostatic interaction between bacterial cells and inanimate surfaces cause initial and reversible bacterial attachment (33). Negatively charged extracellular DNA (eDNA) helps to develop electrostatic interaction (26). In *S. aureus*, microbial surface components, such as fibronectin-binding proteins (FnBPA and FnBPB), clumping factors, and Protein A, referred to collectively as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) play an important role in initial attachment to surfaces (34-37). *S. aureus* expresses up to 24 different cell wall anchored proteins, including MSCRAMMs (FnBPs, ClfB, and SdrC proteins) and other proteins, like SasG, Bap and SasC (Table 2.1) (38). These intrinsic matrix molecules attach to *S. aureus* cell wall after being cleaved by a membrane-associated protein Sortase A (39), and interact with host matrix components, such as fibronectin, fibrinogen, collagen, and cytokeratin (40). Loss of the Sortase and mutations in the *fnbA* and *fnbB* genes encoding FnBPA and FnBPB respectively, reduce biofilm formation in methicillin resistant *S. aureus* (MRSA) (35). Similarly, the mutants of *S. aureus* Newman strain defective in

Clumping factor A, adhere poorly to fibrinogen coated polymethylmethacrylate (PMMA) coverslips and do not form clumps in soluble fibrinogen (36). Additionally, C1q receptor on the platelets in wounds has been identified as a novel binding site for staphylococcal Protein A (37).

MSCRAMMs play a lesser role in attachment to abiotic surfaces where electrostatic and hydrophobic interactions predominate in initial attachment (41). Apart from MSCRAMMs, teichoic acid, a negatively charged component of *S. aureus* cell wall, is also responsible for initial relatively loose attachment of planktonic cells (42). Therefore, while there are multiple mechanisms contributing to initial attachment, the process is dynamic one and bacteria may detach in response to repulsive forces and limited nutrient availability in biofilms including iron (33, 43).

2.2.2. Maturation of *S. aureus* biofilm

After initial attachment and in the presence of sufficient nutrients, bacteria begin to form microcolonies (44). Concurrently, changes in gene expression are triggered in response to surface contact leading to the upregulation of factors favouring transformation into sessile forms (45). As these microcolonies grow, they produce EPS to form a mature biofilm (46). Biofilm maturation is characterised by intercellular aggregation and three dimensional structure formation (47). In *S. aureus*, polysaccharide intercellular adhesin/poly-β(1-6)-N-acetylglucosamine (PIA/PNAG), is responsible for intercellular aggregation (48). The deletion of the intercellular adhesion (*ica*) locus, causes loss in ability to produce PIA/PNAG and biofilm

formation (48). PIA/PNAG in combination with teichoic acid and proteins forms the extracellular matrix of staphylococcal biofilm (47).

The expression of cell wall anchored proteins vary among strains and growth conditions, as some proteins are expressed only in iron-deficient conditions while other are preferentially expressed in exponential or stationary growth phase (38). These proteins facilitate intercellular binding and therefore the accumulation of bacterial cells (38). For example, *S. aureus* strains expressing biofilm-associated protein (Bap) were highly adherent, strong biofilm producers that caused persistent infection in a mouse infection model, in contrast to *bap* mutants that showed weak intercellular and surface adherence and significantly reduced biofilm formation (49). Similarly, the addition of Protein A to growth media induced biofilm formation, which was completely inhibited after the addition of anti-Protein A-specific antibodies (50). In a murine model of subcutaneous catheter infection, the number of wild-type bacteria recovered was significantly higher than Protein A-deficient bacteria, when the medical implant was co-infected with the both strains (50).

S. aureus also uses cytoplasmic proteins, such as enolase and GAPDH, as matrix components (51). These cytoplasmic proteins, probably released through autolysis, attach to cell surfaces and eDNA at low pH and help in the formation of a stable three dimensional biofilm structure (51-53). However, a *S. aureus* biofilm formation model in which the bacteria do not use dedicated biofilm matrix proteins but recycle cytoplasmic proteins released in stationary phase has been proposed (51). Other mechanisms for cytoplasmic proteins release may be secretion,

vesicle formation, and bacteriophage related cell lysis (54). Extracellular proteins such as phenol soluble modulins (PSMs) (55), and nucleoid-associated proteins also help in biofilm stabilisation by binding with eDNA (56), an important structural component of mature staphylococcal biofilm (57).

2.2.3. Triggering of biofilm dispersal response

Following biofilm maturation, bacterial cells disperse to start a new cycle of biofilm formation at distant sites (58). In *S. aureus* biofilms, early dispersal may begin after six hours through the nuclease dependent degradation of eDNA (59). This early dispersal is known as 'exodus', and helps in biofilm reorganisation (59). Exodus involves a subpopulation of biofilm cells that secret nuclease (59).

The later stages of *S. aureus* biofilm dispersal are orchestrated by the *agr* quorum-sensing (QS) system (Figure 2.2) (60). Quorum-sensing, is a coordinated cell to cell communication induced by chemical signals (61). In *S. aureus* these signals are short cyclical peptides known as auto-inducing peptides (AIPs) (62). In *S. aureus, agr* system consists of four genes (*agrA*, *agrB*, *agrC*, *agrD*), among which *agrD* and *agrB* synthesise and export AIPs to external environment, while *agrC* and *agrA* form a signal transduction system (62, 63). On the accumulation of extracellular AIPs to threshold level, they bind to and activate histidine kinase AgrC, which then phosphorylates AgrA and in turn AgrA binds to promoters P2 and P3, and finally regulatory molecules RNA II and RNA III respectively are expressed (62, 64). RNA II encodes the

components of *agr* system, i.e. AgrB, AgrD, AgrC and AgrA, (62) while RNA III encodes several other *S. aureus* virulence factors (65, 66). In addition, P3 activation increases protease activity through extracellular proteases production, which contributes to the degradation of protein based biofilm matrix (67).

Alternatively, *agr*-dependent dispersal may also occur through the production of PSMs, which have surfactant properties and cause biofilm dispersal by interacting with biofilm matrix (68, 69). These modulins are produced when phosphorylated AgrA binds to the *psm* operon promoter region (69). However, PSM aggregates can also stabilise biofilm structures through insoluble amyloid fibres production (70). Formation of these amyloid fibres is promoted by the presence of eDNA (55). Hence, the role of PSM in biofilms depends upon the state it is produced.

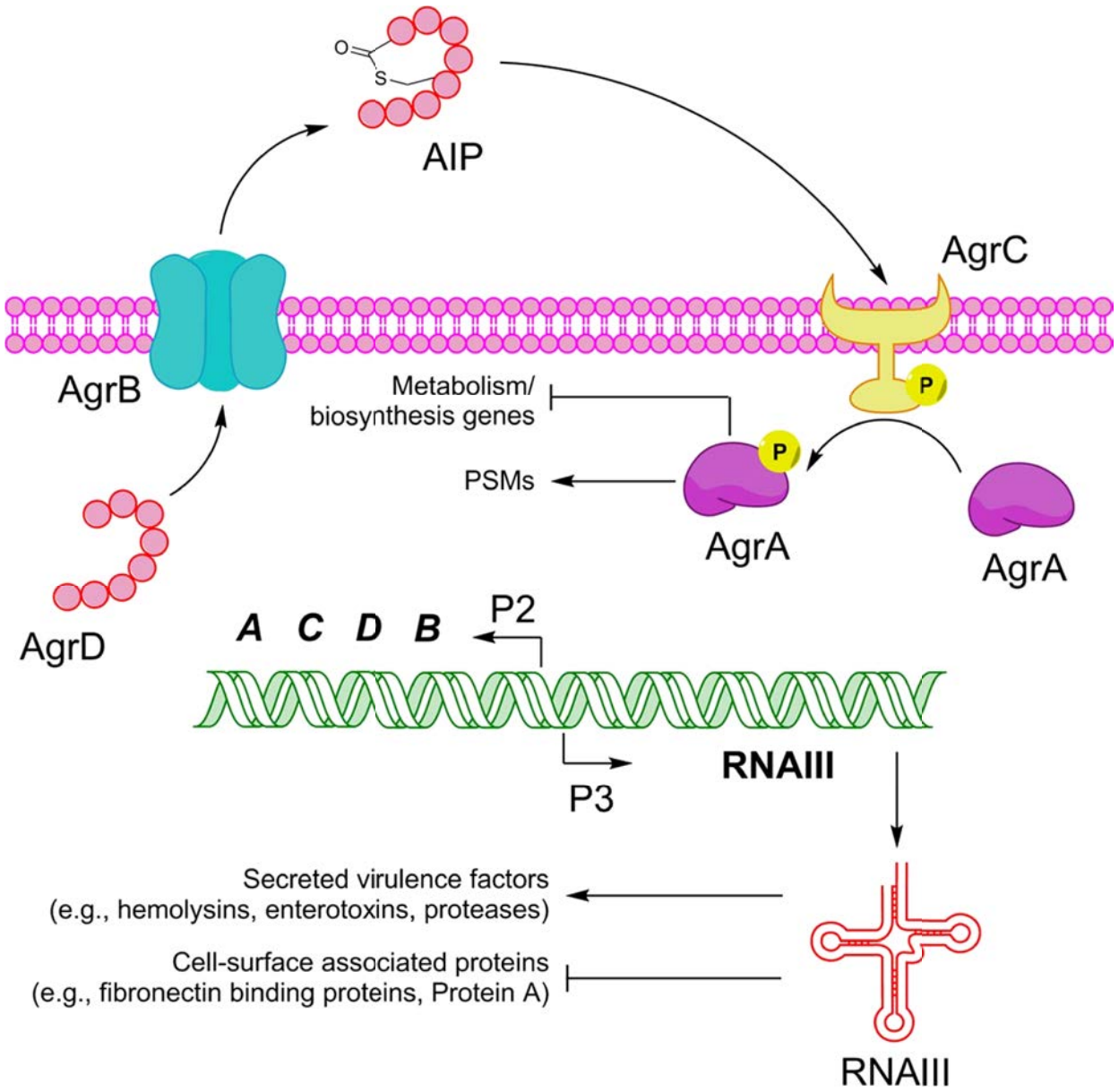


Figure 2. 2: *S. aureus* accessory gene regulatory (*agr*) system. Briefly, AgrD and AgrB synthesise and export AIPs to external environment. On the accumulation of extracellular AIPs to threshold level, they bind to and activate histidine kinase AgrC, which then phosphorylates AgrA. AgrA then binds to different promoter regions driving the expression of *agr* system

components, *S. aureus* virulence factors, and PSMs. (Reproduced from https://msphere.asm.org/content/msph/3/1/e00500-17/F1.large.jpg by Salam AM, Quave CL, Targeting virulence in *Staphylococcus aureus* by chemical inhibition of the accessory gene regulator system *in-vivo*, mSphere, 2018, by permission from copyright holder Salam and Quave, 2018, under creative commons license https://creativecommons.org/licenses/by/4.0/) (71).

Biofilm/cell components	Biofilm stages	Functions	References
eDNA	Attachment	Development of electrostatic interaction for initial attachment	(26)
	Maturation	Biofilm matrix formation and biofilm stabilisation	(53)
Cell wall anchored proteins	Attachment	Initial attachment	(34-38)
	Maturation	Intercellular binding and bacterial cell accumulation	(38)
Sortase A	Attachment	Cleavage of cell wall anchored proteins to catalyse initial attachment	(39)
Teichoic acid	Attachment	Initial attachment	(42)
	Maturation	Biofilm matrix formation	(47)
Cytoplasmic proteins	Maturation	Biofilm matrix formation and biofilm stabilisation by binding with eDNA	(51)
PSMs	Maturation	Biofilm stabilisation by forming insoluble amyloid fibres and binding with eDNA	(55, 70)

	Dispersal	Biofilm dispersal by interacting with biofilm matrix	(68, 69)
Nucleoid-associated proteins	Maturation	Biofilm stabilisation by binding with eDNA	(56)
Nucleases	Dispersal	Biofilm dispersal through degradation of eDNA	(59)
Proteases	Dispersal	Biofilm dispersal through degradation of protein component of biofilm	(67)
AIPs	Dispersal	Biofilm dispersal through activation of *agr* quorum sensing system	(62, 64)

Table 2. 1: *S. aureus* biofilm components and their functions

2.3. Composition and structure of *S. aureus* biofilm

Biofilms consist of 10–25% bacterial cells and 75–90% EPS matrix (72). An EPS matrix is highly hydrated (98% water) (73), and is composed of proteins, polysaccharide, and eDNA (Figure 2.1) (26). However, the contribution of each of these components to form biofilm depends upon strain characteristics and environmental conditions (74). In the presence of calcium chelators, Clumping factor B (clfB) associated biofilm is enhanced in some *S. aureus* strains (74).

Staphylococcal biofilms consist of glycocalyx, which was thought to be made up of teichoic acids (80%), and various staphylococcal and host proteins (75). However, later polysaccharide intercellular adhesin (PIA) was identified as a specific glycocalyx polysaccharide antigen that form the major part of *S. aureus* biofilm structure (76). The antigen consists of major cationic (polysaccharide I >80%) and minor anionic (polysaccharide II <20%) polysaccharides (77).

Polysaccharide I is a homoglycan consisting of 80 to 85% N-acetyl-D-glucosaminyl residues and non-N-acetylated, positively charged D-glucosaminyl residues (77). Polysaccharide II is structurally very similar to polysaccharide I but contains fewer non-N-acetylated glucosaminyl residues and the small amount of phosphate and ester-linked succinyl residues (77). *S. aureus* surface protein SasG and its homologous protein Pls are expressed predominantly in biofilms (78).

Polysaccharides along with carbohydrate binding proteins, and eDNA are responsible for the scaffolding of bacterial cells together, forming mushroom shaped three dimensional multilayered structure (46, 79, 80). Within biofilms, open water channels are responsible for the exchange of nutrients and waste products with the help of infusion fluids (81).

2.4. Biofilm formation through PIA/PNAG dependent mechanism

The production of PIA/PNAG is controlled by the *ica* operon which is upregulated in anaerobic conditions, such as inside biofilms (Figure 2.3) (48, 82). Under anaerobic conditions, SrrAB, a staphylococcal respiratory response regulator, induces PIA/PNAG production by the binding of phosphorylated SrrA to *ica* operon promoter region (83). However, the production of PIA/PNAG may also be induced by other adverse environmental conditions, such as excess glucose, subinhibitory antibiotic concentrations, high osmolarity, and high temperature (84). The stress response in *S. aureus* is regulated by Spx which downregulates biofilm formation by modulating IcaR, a negative regulator of *icaADBC* (85). However Rbf, a regulator of biofilm formation,

represses *icaR* and enhances biofilm formation by increasing *ica* expression and PIA production (86). TcaR, a teicoplanin associated locus transcriptional regulator, can also repress PIA synthesis (87). However, TcaR is a weaker negative regulator than IcaR, because *icaR* expression can mask the phenotypic effect of *tcaR* deletion (87). Therefore, it can be concluded that most of *ica* regulator rely on IcaR to regulate PIA/PNAG dependent biofilm formation. Catabolite control protein A (CcpA), a product of the *ccpA* gene, contributes mainly to bacterial accumulation and intercellular aggregation via the upregulation of *cidA* expression, *icaA* expression, and PIA production (88). The *cidA* gene contributes to eDNA production by releasing genomic DNA through cell lysis (57). The *gdpS* gene upregulates the *ica* operon and exopolysaccharide biosynthesis (89).

Insertion sequence (*IS256*) (90), and a two-component *ica* repressor system *arLRS* are other *ica* operon regulators (91). The insertion of *IS256*, inactivated *icaC* gene and converted a biofilm positive *S. aureus* strain to a biofilm negative phase variant by reducing bacterial adherence to surfaces, a preliminary step for biofilm formation (90). In contrast, initial attachment and PIA/PNAG accumulation are enhanced in *arlRS* mutant, and biofilm formation is not affected by the deletion of *icaADBC* operon (91). This indicates the presence of alternative mechanism of biofilm formation.

2.5. Biofilm formation through PIA independent mechanisms

The *ica* locus deletion had no effect on biofilm formation by MRSA strain BH1CC, while other mutant strains lost biofilm producing ability (92). PIA independent biofilms consist of eDNA and a long list of proteins those include surface adhesins, secreted proteins, and intracellular proteins released during cell lysis (Figure 2.3) (26, 93). In the absence of PIA, protein A (SpA) is an essential component of *S. aureus* biofilm (50). Surface adhesin FnBp also contributes to biofilm formation with the help of major autolysin (Atl) and *sigB* regulation (94), while secreted proteins, Eap and beta toxin (Hlb) help in mature biofilm establishment (95, 96). Eap is the most abundant protein detected in the biofilm matrix of *S. aureus* MR23 (96).

Hlb and DNABII family of proteins after binding with eDNA form insoluble component that helps to give a three-dimensional structure to biofilm (56, 95). Extracellular matrix binding protein (Emp), and Eap play important roles in *S. aureus* biofilm formation in iron-restricted growth condition, representative of the *in-vivo* environment (97). Under iron deficient conditions, these proteins are regulated by iron regulator Fur (ferric uptake regulator) (97). In addition, the *sae*, *agr,* and *ica* genes are essential for the expression of Eap and Emp, while *sarA* has a less significant role (97). However, the iron regulation of these secreted proteins is Fur independent (97).

Extracellular DNA helps in the maturation of biofilms and the initial establishment of Atl/FnBP-dependent biofilm (98). Earlier eDNA in biofilms was thought to be excreted through

membrane vesicles rather than cell death (99). However, later genomic DNA release was demonstrated to occur via *cidA* controlled cell lysis (57). The *cidA* mutant exhibited less adherent and moderately DNase I sensitive biofilm with more dead cells accumulated, indicative of reduced cell lysis, and five-fold less genomic DNA in comparison with parental strain's (UAMS-1) highly DNase I sensitive biofilm (57). The *cid* operon upregulates *atl* and *lytM*, leading to the production of murein hydrolases, which are responsible for bacterial autolysis (100). This autolysis is induced at certain biofilm microenvironment such as hypoxic condition (101). The *cidA* controlled cell lysis is downregulated through the activation of *lrgAB* operon by LytSR, a two-component regulatory system (98, 102). The *lytS* mutant formed more adherent biofilm containing higher amount of matrix-associated eDNA relative to parent strain (102). The *cid* and *lrg* operons work in a way close to bacteriophage holins and anti-holins (100). Holins and anti-holins are membrane proteins that regulate bacteriophage induced bacterial death and lysis (103). Phages have been detected in biofilm culture with the help of electron microscope (104). Activation of phage genes may also help in the release of *S. aureus* DNA through cell lysis leading to phage-release (104). However, these studies suggest that the mechanism of eDNA release is strain specific as different modes of eDNA release were found in different *S. aureus* strains.

Amyloid and fibrin are other types of PIA independent *S. aureus* biofilms (70, 105). Amyloid biofilm consist of amyloid fibres formed from PSM aggregates, (70) and the formation of these fibres is promoted by the presence of eDNA (55). Fibrin biofilm is formed on plasma coated

surfaces where coagulase (Coa) from *S. aureus* converts fibrinogen to fibrin (105). Fibrin thus formed makes scaffold for *S. aureus* biofilm (106). The *saeRS* system regulates *coa* expression thus taking part in the formation of fibrin-mediated biofilm (107).

The mechanism of biofilm formation and the development of different stages of biofilm cycle may significantly differ between different *S. aureus* strains. This difference is mainly caused by variation in the characteristics of colonised environments and colonising strains. Consequently, the effectiveness of an individual antibiofilm treatment, that targets a particular stage and biofilm formation mechanism, may differ largely between different *S. aureus* strains.

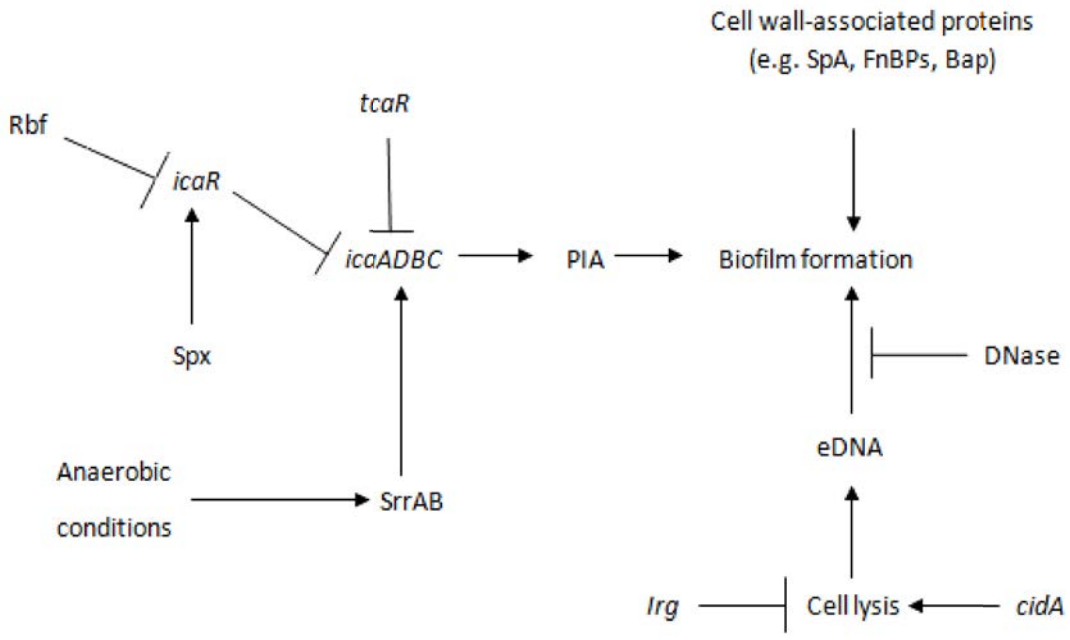


Figure 2. 3: PIA dependent and independent *S. aureus* biofilm formation. Briefly, Rbf downregulates *icaR*, while Spx upregulates it. The *icaR* and *tcaR* downregulate *ica*. Under

anaerobic condition, regulator SrrAB upregulates *ica* leading to PIA production and PIA dependent biofilm formation. Cell wall-associated proteins and eDNA released through *cidA* and *lrg* regulated cell lysis form PIA independent biofilms. The *lrg* downregulates cell lysis while *cidA* upregulates it. EDNA biofilm formation is prevented by DNase. (Adapted from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3322633/figure/F5/?report=objectonly by Archer NK, Mazaitis MJ, Costerton JW et al., *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease, Virulence, 2011, by permission from copyright holder Taylor & Francis, 2011, under creative commons license https://creativecommons.org/licenses/by-nc/3.0/) (108).

2.6. Regulation of *S. aureus* biofilm formation: the master controllers and their targets

Biofilm formation in *S. aureus* is under the control of *sarA*, *agr*, *sigB*, and *sae* regulons (67, 109-111) (Figure 2.4). The *sarA* and *agr* regulate a two-component virulence regulator system, *arlS-arlR* (112). This system downregulates the production of virulence factors, such as alpha-toxin (Hla), beta-haemolysin, lipase, coagulase, serine protease (Ssp) and Spa (112). Mutations in either *arlR* or *arlS* enhance the secretion of these proteins (112).

2.6.1. SarA

The *sarA* upregulates *ica* leading to PIA/PNAG production and consequently increased biofilm formation (113). Additionally, *sarA* downregulates the expression of a protein that either degrades PIA/PNAG or represses its production; *sigB* upregulates the protein synthesis (113).

Transcriptional profiling suggested that *fnbA* and *fnbB* are expressed in a *sarA* dependent manner (114). The *sarA* gene mutants showed reduced biofilm formation in six out of eight *S. aureus* strains tested (115), which can be recovered by *nuc* gene deletion or/and protease inhibition (116). In *sarA* mutants, there is increased production of extracellular nucleases and proteases that degrade biofilm components (116).

2.6.2. Agr

Presence of the *sarA* gene is required for optimal *agr* expression (117). In *sarA* mutants the level of *agr* regulatory molecule RNAIII is significantly reduced or absent, which was partially restored when an intact *sarA* gene was re-inserted (117). However, the regulatory role of *sarA* for biofilm formation is independent of *agr*, and *sarA* mutants showed reduced biofilm formation despite of the functional status of the *agr* gene (118). Inactive *agr* quorum-sensing system is required for *S. aureus* biofilm formation and the activation of this system by addition of AIP or glucose depletion in mature biofilm leads to dispersal (67). Depending upon strains and growth conditions, the role of *agr* quorum-sensing system may vary as disruption of the *agr* inhibits, enhances, or has no effect on biofilm formation (119, 120). Another *S. aureus* quorum-sensing system *luxS* is known to reduce biofilm formation by decreasing cell to cell adhesion through the downregulation of exopolysaccharide expression (121).

2.6.3. SigB

The *sigB*, another regulator of *S. aureus* biofilm formation, positively affects the expression of two microbial surface proteins, FnbA and ClfA, which are responsible for the initiation of biofilm formation (122). The *sigB* gene mutant strain BB1591 had two-fold lower capacity to be internalised by osteoblasts in comparison to its parent strain LS-1 (122). In addition, based on the level of *sigB* expressed, individual *S. aureus* strain had differing capacity to be internalised (122). It was suggested that *sigB* might increase the expression of MSCRAMMs, such as FnBPs, which play an important role during the internalisation of *S. aureus* by osteoblast (122). Additionally, *sigB* suppresses *agr* and inhibits biofilm dispersal (123). In *sigB* mutant strain USA300 LAC, *agr* RNAIII level was elevated, which is responsible for biofilm dispersal through the elevation of extracellular protease level (123). Similarly in the COL strain, thermonuclease, an enzyme that promotes biofilm dispersal through the degradation of eDNA, was more prominent in *sigB* mutant than in parent strain (98, 124). This higher production of thermonuclease suggests an inhibitory role of *sigB* on either production or excretion of the protein thus favouring biofilm development (124). However, the role of *sigB* on thermonuclease production is strain specific, as there was no difference in thermonuclease production between *sigB* mutant and wild type in strains Newman and 8325 (124).

There was no effect of *sigB* deletion in PIA/PNAG dependent biofilm formation (113), suggesting *sigB* is directly involved in the regulation of PIA/PNAG independent biofilm. However, in *sarA*-*sigB* double mutant, *ica* expression decreased but PIA/PNAG production and

biofilm formation increased in comparison with *sarA* single mutant (113). This means some indirect role of *sigB* in PIA/PNAG dependent biofilm formation may exist. Some researchers have reported the loss of PIA/PNAG production and biofilm formation in *sigB* mutant under osmotic stress suggesting the role of *sigB* in *S. aureus* biofilm regulation under environmental stress such as heat shock, alkaline shock, high salt, and stationary phase growth in complex media like Luria-Bertani (125, 126).

In the stationary phase of strains LAC, Newman, and 8325, *sigB* mutants showed increased lipase production in comparison with wild type, suggesting the inhibitory role of *sigB* on lipase, effecting biofilm formation negatively (124). Lipase-coding gene mutants produce weak biofilm in comparison with wild type strain (127), and biofilm formation can be inhibited by the addition of anti-lipase serum (128). In addition, the intraperitoneal injection of mice with a lipase mutant produced defective peritoneal abscess with the lower concentration of bacteria in different organ in comparison with wild type (127). More importantly, the immunization of mice with recombinant lipase saved them from lethal *S. aureus* infection (127). The *sigB* gene is regulated by positive regulator RsbU, negative regulator RsbW, and anti-RsbW regulator RsbV (126).

2.6.4. SaeRS

The *saeRS*, a two-component *S. aureus* regulatory system, inhibits biofilm formation by producing a heat-stable inhibitory protein that affects attachment step (111). In a *sae*

constitutively expressed *S. aureus* Newman strain, a weak biofilm producer, deletion of *saeRS*, resulted in the production of a robust biofilm (111). The *saeRS* consists of SaeS protein, a histidine kinase, which is responsible for the phosphorylation of the response regulator SaeR (111). The *sae* upregulates *atlR*, and bacteriophage genes; *atlR* encodes a repressor of *atlA* thus reducing autolysis and DNA release (94, 111).

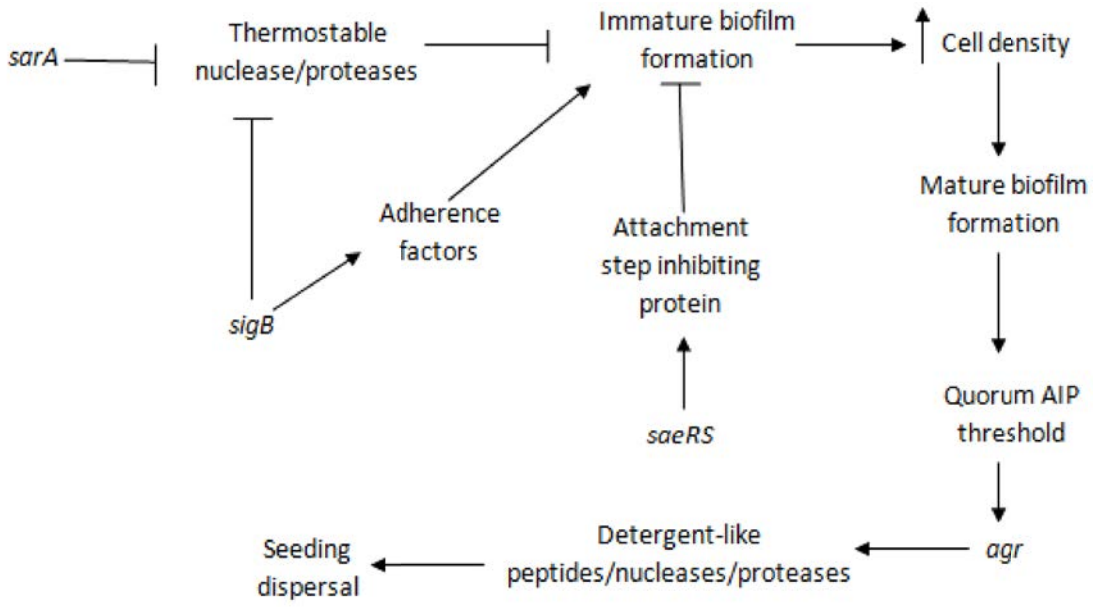


Figure 2. 4: The regulation of *S. aureus* biofilm formation. Briefly, *sarA* and *sigB* inhibit the expression of thermostable nucleases and proteases. The *saeRS* upregulates the expression of attachment step inhibiting protein. These enzymes and protein inhibit immature biofilm formation. The *sigB* upregulates the expression of adherence factors that promote immature biofilm formation leading to increased cell density and mature biofilm formation. When AIP accumulation reaches threshold level *agr* is activated which expresses detergent-like peptides,

nucleases, and proteases, leading to biofilm dispersal. (Adapted from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3322633/figure/F5/?report=objectonly by Archer NK, Mazaitis MJ, Costerton JW et al., *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease, Virulence, 2011, by permission from copyright holder Taylor & Francis under creative commons license https://creativecommons.org/licenses/by-nc/3.0/) (108).

2.7. Recalcitrance of biofilm-related *S. aureus* prosthetic joint infection to treatments

Bacterial biofilms show the higher degree of antibiotic resistance and human immune system tolerance in comparison with their planktonic counterparts (129, 130). The sessile cells within biofilm are protected by exo-polymeric matrix (131). These polymeric substances create a physical barrier for the components of immune systems (130, 132), antibiotics (131), and antimicrobial peptides (AMPs) (133, 134). Additionally, the negatively charged components of biofilm matrix either prevent the diffusion of positively charged antibiotics by binding with them (135, 136), or chelate cations activating an operon responsible for cationic AMPs and aminoglycosides resistance (137).

In the deeper layers of biofilms there is scarcity of oxygen and nutrients, and cells presenting in those locations have slow growth rate (138). These slow growing or inert cells, also known as persister cells, can survive antibiotic killing (139), as the antimicrobial properties of most of the antibiotics, such as beta-lactams, are growth dependent (140). Additionally, biofilms may also

contain antibiotic inactivating enzymes, such as beta-lactamases (141). The close proximity of bacterial cells, from one or more species, in biofilm makes the transfer of drug resistant determinants easier (142).

2.8. Clinical context of biofilm-related *S. aureus* prosthetic joint infections: failure of life-enhancing prosthetic joints

Medical devices, such as prosthetic joints, provide surface for bacterial proliferation and biofilm formation (143). Every year about two million total knee and hip arthroplasties are carried out worldwide (3). Arthroplasty surgeries have life changing benefits, as these surgeries relieve pain and restore function (144). However, a significant number of prosthetic joints fail due to biofilm-related bacterial infections that are difficult to treat. The incidence of prosthetic joint infection in the US was 2.18% of the total number of hip and knee arthroplasties performed in 2009 and has been estimated to increase over time (5). While the infections of orthopaedic devices carry low attributable mortality rate the economic burden of treatment is substantial (145).

S. aureus is the most common cause of prosthetic joint infections, studies reporting the involvement of this bacterium in up to 57% of total prosthetic joint infections (4). The mode of infection may be direct inoculation during surgery or haematogenous route (146). If the bacterium is inoculated during surgery, it causes acute infection within 3 months, however the infection may also occur at any time after surgery through haematogenous route (7). A low

number of bacteria such as <50cfu of *S. aureus* are enough to establish prosthetic joint infection in comparison with 10^4cfu in the absence of implant (147).

Implanted devices become coated with host proteins, such as fibrinogen, fibronectin and laminin, which provide a rich environment for staphylococcal attachment (148). During the early stage of biofilm development, innate inflammatory response effectors (e.g., PMNs) attempt to clear biofilm through non-specific mechanism (149). Importantly though the presence of a foreign body, such as a prosthetic joint, activates granulocytes leading to the production of human neutrophil peptides (defensins) (149). These defensins deactivate subsequent neutrophils through the impairment of neutrophil oxygen radical production (149).

Surgical interventions for prosthetic joint infections are debridement with polypropylene liner exchange and one or two stage re-implantation operations; all followed by prolonged antimicrobial therapy (146). While success rates are of the order up to 85-90% there are a proportion of patients who are either not suitable for surgery or in whom these costly procedures fail (146, 150, 151). Additionally, all surgical procedures have considerable morbidity (146). Novel adjuvant treatments which may be able to eradicate prosthetic joint infections would be highly beneficial.

2.9. Possible adjuvant treatments for biofilm-related *S. aureus* prosthetic joint infections – the search for a novel approach to an intractable problem

This review focuses discussion on treatment strategies such as quorum-sensing (QS) inhibitors that target biofilm regulators and have already been used successfully to treat biofilm-related infection in animal models (Table 2.2).

2.9.1. Quorum-sensing and quorum-sensing inhibitors: stopping the bacterial communication

Quorum-sensing includes a series of events, such as signal production, signal detection, and gene activation/inactivation (17), and results in group behaviours such as biofilm formation and the expression of other virulence factors (152). The interruption of any steps of QS leads to failure in quorum-sensing and have detrimental effect on bacterial pathogenicity (17). QS inhibiters do not directly kill bacteria, rather they repress signal generation, block signal receptors, and disrupt QS signal (17). Therefore, there is less selection pressure and low rate of resistance development, however the possibility of resistant mutants emergence still exists (17, 153). There is no data on dosage, route of administration, bioavailability, pharmacodynamic/pharmacokinetic profile, and toxicity of QS inhibitors particularly in relation to their use in the treatment of prosthetic joint infection. So, further studies are needed. Non-peptide small molecules, peptides, and proteins are three main classes of QS inhibitors (17).

2.9.1.1. RNAIII-inhibiting peptide

RNAIII-inhibiting peptide (RIP) alone or in combination with antibiotics and antimicrobial peptides inhibits *S. aureus* biofilm, including biofilm formation by MRSA and glycopeptide-intermediate strains, and also disperses established biofilm (154-157). This peptide was efficient in the treatment of central venous catheter-associated infection, polymethylmethacrylate subcutaneous implant infection, and graft infection in animal models (154-157). Synthetic RIP analogues and RIP derivatives have also similar activity as RIP (158, 159). However, not all RIP derivatives that inhibit RNAIII *in-vitro* show efficacy for the inhibition of *in-vivo* infection but only that containing lysine and isoleucine at positions 2 and 4, respectively (158). This indicates that the activity of RIP derivatives depend upon the positioning of the amino acids, that gives special spatial structure and property to the derived molecules making them active even inside living beings. Additionally, even for closely related molecules those show similar *in-vitro* activity, it is not guaranteed that they will also show similar *in-vivo* activity.

RIP also inhibits *S. aureus* pathogenesis through the inhibition of both *agr* transcripts, RNAII and RNAIII (158). Due to structural homology RIP competes with RNAIII activating protein (RAP), a protein responsible for RNAIII synthesis, and prevents the phosphorylation of its target (TRAP) (160). Vaccination using RAP was effective in the prevention of *S. aureus* infection in a cutaneous infection mouse model, as antibodies to RAP block the activation of RNAIII (159). However, RIP/RAP/TRAP system analysis showed no evidence for its involvement in virulence

determinant regulation challenging the related findings (161). Since, the efficacy of RIP for the treatment of *S. aureus* infections is already established in animal models, further studies are needed to confirm the mechanism of action (154-157). The RIP concentrations used in the animal studies were extremely high in comparison with effective native inhibitory AIP concentrations (162). Additionally, RIP has been shown to reduce *S. aureus* adherence through *agr* independent gene regulation (163). Given the antibacterial effect of RIP has never been tested it may be that RIP has direct non-specific inhibitory effect on *S. aureus* (162). Although RIP, RIP derivatives, and RAP have been effective in the treatment and prevention of biofilm-related *S. aureus* infection in some other animal models, they are yet to be tested in prosthetic joint infection animal study.

2.9.1.2. Hamamelitannin

Hamamelitannin, a non-peptide analogue of quorum-sensing inhibitor RNAIII-inhibiting peptide (RIP), prevents the *in-vitro* as well as *in-vivo* biofilm formation in *S. aureus* including MRSA, by inhibiting attachment (164). Hamamelitannin prevented infection in subcutaneous graft rat model (164). This plant derived compound, when used in combination with vancomycin or clindamycin, shows synergistic effect to remove biofilm and increases host survival (165). Hamamelitannin inhibits *traP* QS system by interfering with its receptor and increases the susceptibility of *S. aureus* biofilm to vancomycin (166). At molecular level, hamamelitannin alters the expression of genes involved in cell wall synthesis and eDNA release such that increase in cell wall thickness and eDNA release induced by vancomycin treatment is inhibited

(166). Hamamelitannin and its analogues are good antibiotic potentiators having been used successfully to treat a mouse model of *S. aureus* mastitis (167). However, hamamelitannin and its analogues are yet to be tested in *S. aureus* prosthetic joint infection animal model.

2.9.1.3. Auto-inducing peptides

Auto-inducing peptides (AIPs) are able to inhibit *agr* in multiple strains making these molecules good candidates for the development of anti-quorum-sensing strategy. The *agr* gene expression of group I *S. aureus* is inhibited by group IV *S. aureus* supernatant but not vice versa (168). However, the synthetic AIPs of *agr* group I and group IV inhibited the *agr* expression of each other (169). This discrepancy in the results might be due to difference in the purity of AIPs used in the two studies. In addition, the inhibitory role of synthetic AgrDII peptide on subcutaneous abscesses caused by group I *S. aureus* strains has already been reported (170). The thiolactone moiety gives cyclic structure to AIP and is required for both biological activities, self-activation and cross-group inhibition, of AIP (169, 170) – synthesised linear peptides (*agr* group II and III peptides, and RIP) which lack the thiolactone moiety are inactive (169). However, modification of AIP tail inhibits *agr* activation but not cross-group inhibition implying the existence of different mechanisms for activation and inhibition (170). Therefore, AIP or AIP analogues modified by tail removal or switching the position of rings and tails can be used as *agr* inhibitors (169, 171, 172). Group II and I thiolactone peptides without tail repress all four groups of *S. aureus agr* (169, 171). Alanine-modified AIP group I and II, AIP group II lactone, and lactone analogues are QS inhibitors that do not act as activators for any *agr* groups (169, 170). An auto-

inducing peptide mimetic significantly inhibited MRSA skin infection in a murine dermonecrosis abscess model (173).

Immunogenic challenge with cyclic peptide or analog carried on a macromolecule can activate humoral response against native AIPs (174). Antibodies produced thus, such as antibody against AIP-IV, quench QS system (175). Antibodies against AIP-IV inhibited abscess formation in a *S. aureus* subcutaneous infection model and protected mice from lethal intraperitoneal *S. aureus* challenge (175). In conclusion, *agr* quorum sensing can be inhibited either by preventing the accumulation of AIP or using the cross-group inhibition property of AIP, however by neutralizing its self-group activation activity. Synthetic AgrDII peptides and antibodies against AIP-IV are yet to be tested in prosthetic joint infection animal study. Other AIP related molecules described under this topic that have shown the *in-vitro* effectiveness are yet to be tested in animal model.

2.9.1.4. Savirin

Savirin is a small synthetic molecule that, when injected subcutaneously, can both inhibit and treat *S. aureus* skin and subcutaneous infections in mouse models (18). This molecule inhibits the attachment of AgrA to promoter regions, subsequently inhibiting *agr* quorum-sensing system and key virulence factors (18). Thus savirin disarms *S. aureus* making them less competent to survive inside host which is subsequently cleared by immune system (18). Due to savirin's low molecular weight (368), lipophilicity, and lack of reported animal model toxicity, this molecule meets the criteria of a candidate for drug development (18). Additionally, as

savirin is a synthetic molecule, it could be synthesised in large quantities in pure form. The study of biological activity could include structural modifications to improve savirin's efficacy. Since, the mode of action of savirin involves disarming bacterial virulence factors rather than direct inhibition, *S. aureus* does not appear to develop resistance to savirin as readily as it does against antibiotics (18).

Savirin may also be effective in the treatment of prosthetic joint infection as similar immune defense mechanisms relying on macrophages that are present in the skin also exist in joints (176). However, higher doses or different sites of injection that ensures higher bio-availability at the site of infection may be needed, as it is hard for drugs to penetrate through bones or joints (177). In addition, the pharmacokinetics and pharmacodynamics of this molecule are not known. So, further study is required to optimise the route of administration and dosage for the treatment of prosthetic joint infection.

2.9.2. Drug repurposing: can old become new again?

Drug repurposing relates to the use of existing or abandoned drugs for the treatment of diseases for which they were not originally developed (178). Cheaper and faster clinical translation along with known safety profiles and the pharmacology of existing drugs are the main advantages of drug repurposing (179). Here, this review discusses the drugs that have already shown efficacy in the treatment of biofilm-related *S. aureus* infection in animal models

(Table 2.2). This review also includes drugs with significant *in-vivo* antibiofilm activities, whose mode of actions are yet to be known.

2.9.2.1. Auranofin

Auranofin, an antiarthritis drug, and its derivative MH05 showed positive effect in the treatment of biofilm-related methicillin resistant *S. aureus* (MRSA) infection in an intraperitoneal polypropylene mesh implant infection mouse model and MRSA abscess in an intramuscular infection mouse model (180). Auranofin's antibacterial effect is through the inhibition of multiple key pathways responsible for synthesis of important cell components, such as cell wall, DNA, and proteins (181). Auranofin and MH05 did not eradicate the infection and monotherapy with them may not be sufficient to treat biofilm-related infections mainly in immunocompromised patients (180). Auranofin has been reported to show significant synergistic effect with antibiotics linezolid and fosfomycin, for the treatment of MRSA and MSSA cutaneous abscesses in mouse models (182). So, adjuvant therapies using auranofin in combination with antibiotics may be beneficial to eradicate biofilm-related infection. Additionally, the emergence of auranofin resistant *S. aureus* mutants is uncommon (183). This drug may also be effective in the treatment of prosthetic joint infection, however dose optimisation would be required. Additionally, the mode of action of this drug for the treatment of biofilm-related infection is not known and requires further study.

2.9.2.2. Aspirin

Aspirin is among the most widely used drugs for its preventive effect on cardiovascular disease. In a catheter-induced *S. aureus* endocarditis rabbit model, aspirin treatment reduced bacterial biofilm, bacteremia, and consequently embolism (184). Similarly, haemodialysis patients with tunneled catheters treated with aspirin are less likely to get *S. aureus* blood infection (185). Aspirin activates *sigB*, a stress induced operon, and inhibits the expression of the α-hemolysin (*hla*) and fibronectin (*fnbA*) genes (186). The *sigB* activation represses *sarA* and *agr* (186). However salicylic acid, the active component of aspirin, has also been reported to induce PIA-dependent *S. aureus* biofilm formation in a nasal colonisation murine model using Newman strain (187). Environmental stresses such as the acidic pH and salt content of nasal secretion may also contribute to increased biofilm formation (188). Salicylic acid stabilises the *in-vitro S. aureus* biofilm through *agr* quorum-sensing system inhibition (189). These results indicate that the *S. aureus* biofilm-related activity of aspirin is either strain dependent or biofilm type dependent. Due to differences in the composition of colonising materials in anterior nares and heart valves the mechanisms of biofilm formation and the types of biofilm formed may be quite different at the two locations. Heart valves are coated with plasma and may favour fibrin biofilm formation (105).

2.9.2.3. Ticagrelor

Ticagrelor is an antiplatelet drug that is used for the prevention of thrombotic events in atherosclerotic cardiovascular disease patients. It is a platelet adenosine diphosphate P2Y12 receptor inhibitor (20). Post-hoc analysis of large cardiovascular disease prevention studies showed that in acute coronary syndrome and pneumonia patients treated with ticagrelor lower risks of infection-related death and improved lung function were present (21-23). Subsequent investigation of this unexpected effect showed that ticagrelor inhibited *S. aureus* biofilm growth and bacterial dissemination to surrounding tissue in a pre-contaminated subcutaneous foreign body *S. aureus* infection mouse model (19). However, the mode of action for inhibition of biofilm formation is yet to be studied but it can be hypothesised that ticagrelor downregulates key biofilm-related genes. Additionally, its antibacterial effect may have contributed to inhibition in biofilm formation, as this requires bacterial concentration to reach a threshold level (190). The *in-vitro* experiments using ticagrelor showed a synergistic effect with rifampicin, ciprofloxacin, and vancomycin (19). The anti-MRSA antibacterial activity of ticagrelor alone was superior to vancomycin but similar to daptomycin (19). Antiplatelet drugs, such as ticagrelor and aspirin, have been known to reduce the effect of platelet antibacterial peptides against *S. aureus in-vitro*, when used alone or in combination (191). However, platelet-mediated clearance of *S. aureus* bacteremia, including that caused by MRSA, induced by ticagrelor has been reported *in-vivo* (192, 193).

2.9.2.4. Simvastatin

The lipid lowering statin class of drugs have been tested for their antibacterial activities, with simvastatin shown to have activity against *S. aureus* (194). Simvastatin also inhibits biofilm formation by *S. aureus* and is more potent than linezolid or vancomycin in the disruption of established *in-vitro S. aureus* biofilms (194, 195). Simvastatin reduced bacterial burden in a murine MRSA skin infection model significantly (195). This drug is known to inhibit adhesion, reduce cell viability, and reduce extracellular polysaccharide production (195). However, the molecular mechanism for the inhibition of *S. aureus* biofilm is yet to be studied (195). Additionally, there is no data on the activity of simvastatin for the treatment of prosthetic joint infection animal model.

2.9.2.5. Thioridazine

Thioridazine, an antipsychotic drug, inhibited the dissemination of epicutaneously inoculated MSSA to spleen and kidney, and reduced abscess size produced by intradermally injected MSSA and MRSA (196). This drug at its sub-inhibitory concentration enhanced the *in-vitro* bactericidal effect of β-lactam antibiotics to MRSA (197). However, the enhancement was not seen in a cutaneous abscess mouse model (196). The thioridazine concentration required to reverse the methicillin resistance of MRSA used in the latter study might be too high to attain in animal model (196). Thioridazine downregulates biofilm pathway genes, such as genes related to cell membrane and cell wall component synthesis and their transport, that are induced by *saeRS*

and disturbs peptidoglycan biosynthesis (197). This drug is yet to be tested in *S. aureus* prosthetic joint infection animal model. However, since thioridazine has significant toxicity, development of less toxic derivatives or significantly lower doses using adjuvant therapies would be beneficial.

Name of molecules	Advantages	Disadvantages
RNAIII-inhibiting peptide	Effective in animal models	High molecular weight
Hamamelitannin	Plant derived compound effective in animal models	Limited availability and expensive to purify
Auto-inducing peptides	Active against multiple *S. aureus* strains	Difficult to purify and no data on animal model
Savirin	Small lipophilic synthetic molecule effective in animal models, easy to synthesise in pure form	Pharmacokinetics, pharmacodynamics, and detailed animal toxicity not known, no data on device-related infection in animal model
Auranofin	An approved antiarthritis drug effective in animal models, easy clinical translation	Mechanism of antibiofilm activity against *S. aureus* not known, expensive

Aspirin	An approved antiplatelet, analgesic, antipyretic, and anti-inflammatory drug effective in animal models, easy clinical translation	Aspirin has also been reported to induce biofilm formation in *S. aureus*
Ticagrelor	An approved antiplatelet drug effective in animal model, easy clinical translation	Mode of antibiofilm activity against *S. aureus* not known
Simvastatin	An approved lipid lowering drug effective in animal model, easy clinical translation	Mechanism of antibiofilm activity against *S. aureus* not known, no data on device-related infection in animal model
Thioridazine	An approved antipsychotic drug effective in animal model, easy clinical translation	High toxicity, no data on device-related infection in animal model

Table 2. 2: Different molecules for the development of novel anti-biofilm strategy to treat prosthetic joint infection caused by *S. aureus*

2.10. Conclusions and future perspective

Biofilm-related *S. aureus* prosthetic joint infections cause significant morbidity, and as treatment relying on surgical debridement and antibiotics is not universally effective, there is growing interest in the development of novel therapies. This review highlights both novel molecules and repurposed drugs which have shown efficacy in the treatment of biofilm-related *S. aureus* infections in pre-clinical studies. In most cases, biofilms occurring in the animal models of prosthetic joint infection have not been studied.

While repurposed drugs have defined pharmacokinetics, pharmacodynamics, and toxicity profiles, these are not available for the novel molecules described here. Additionally, the mode of action of biofilm disruption of many of the described novel molecules and drugs are still unknown and require further investigation. As the world's population ages there is an increasingly reliance on the use of prosthetic joints. Arthroplasty surgery is among the most life enhancing of all modern medical treatments. Failure of prosthetic joints due to infection requires broad consideration of novel treatment strategies.

Given the limited time and resources for a PHD project, it was not possible to test all potential drugs. Therefore, on the basis of advantages and disadvantages analysis of different molecules reviewed (Table 2.2), savirin (among novel molecules) and ticagrelor (among Food and Drug Administration approved drugs) were chosen for further study for the development of adjuvant therapy for the treatment of *S. aureus* prosthetic joint infection.

2.11. Summary and research synopsis of the thesis

Chapter 2 discussed on *S. aureus* biofilms and described the stages of their formation. It particularly emphasised genetic and biochemical processes that might be vulnerable to novel treatment approaches. Against this background this chapter discussed treatment strategies which have been successful in the animal models of *S. aureus* biofilm-related infection, and considered their possible use for the prevention and eradication of biofilm-related *S. aureus* prosthetic joint infection.

This PHD thesis provides a comprehensive research on the development of adjuvant therapy using ticagrelor or savirin for the treatment of biofilm-related *S. aureus* prosthetic joint infection in an animal model.

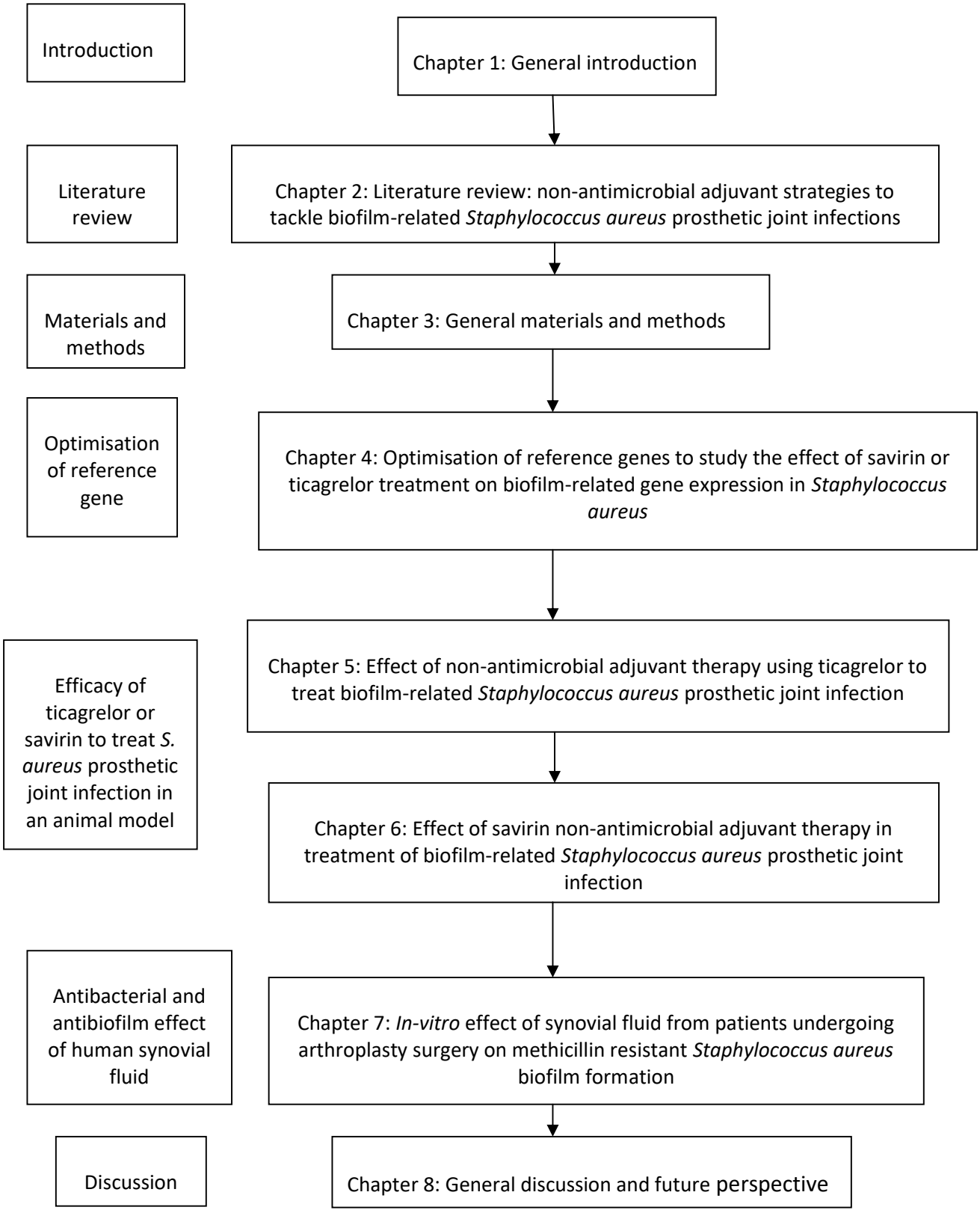
In **chapter 4** sixteen different candidate reference genes were investigated for their suitability to use as reference genes to study the effect of ticagrelor or savirin treatment in the expression of biofilm-related gene expression in *S. aureus*.

Chapter 5 investigated the efficacy of ticagrelor, alone and with cefazolin, to treat biofilm-related *S. aureus* prosthetic joint infection in a mouse model. This chapter also details the *in-vitro* antibacterial and antibiofilm activity of ticagrelor, alone and in combination with antibiotics (cefazolin, rifampicin, and vancomycin), and the molecular mechanism underlying the antibiofilm activity.

In **chapter 6** savirin was studied for its effect, alone and with cefazolin, for the treatment of biofilm-related *S. aureus* prosthetic joint infection in an animal model. This chapter also presents data on the *in-vitro* antibacterial and antibiofilm activity of savirin, alone and in combination with some selected antibiotics (cefazolin, rifampicin, and vancomycin), and the associated molecular mechanism for the antibiofilm activity.

Chapter 7 presents the *in-vitro* antibacterial and antibiofilm activity of synovial fluid from elective arthroplasty patients against *S. aureus* including methicillin resistant *S. aureus* (MRSA).

This thesis contributes significantly toward the development of an adjuvant therapy for the treatment of biofilm-related *S. aureus* prosthetic joint infection.


Introduction
Chapter 1: General introduction
Literature review
Chapter 2: Literature review: non-antimicrobial adjuvant strategies to tackle biofilm-related *Staphylococcus aureus* prosthetic joint infections
Materials and methods
Chapter 3: General materials and methods
Optimisation of reference gene
Chapter 4: Optimisation of reference genes to study the effect of savirin or ticagrelor treatment on biofilm-related gene expression in *Staphylococcus aureus*
Efficacy of ticagrelor or savirin to treat *S. aureus* prosthetic joint infection in an animal model
Chapter 5: Effect of non-antimicrobial adjuvant therapy using ticagrelor to treat biofilm-related *Staphylococcus aureus* prosthetic joint infection
Chapter 6: Effect of savirin non-antimicrobial adjuvant therapy in treatment of biofilm-related *Staphylococcus aureus* prosthetic joint infection
Antibacterial and antibiofilm effect of human synovial fluid
Chapter 7: *In-vitro* effect of synovial fluid from patients undergoing arthroplasty surgery on methicillin resistant *Staphylococcus aureus* biofilm formation
Discussion
Chapter 8: General discussion and future perspective

CHAPTER 3: GENERAL MATERIALS AND METHODS

3.1. Ethics and biosafety approvals

The animal studies were performed as per Australian code for the care and use of animals for scientific purposes 8th edition 2013. The animal ethics approval was obtained from the James Cook University Animal Ethics Committee (AEC2486), and the use of human samples was approved by the Mater Hospital Health Services North Queensland Human Research Ethics Committee (MHS20170808-01). Bio-safety approval was obtained for all bacteria used in this project (JCUIBC-170418-013) (Table 3.1).

3.2. Bacterial strains used in this project

The methicillin susceptible (TUHMSSA01, TUHMSSA03) and methicillin resistant (TUHMRSA02, TUHMRSA04) *S. aureus* clinical strains used in this study were isolated from patients attending the Townsville University Hospital, while 1698 MRSA was an ATCC strain (Table 3.1). These strains were used because they produced luxuriant biofilm as measured by optical density (OD) > 4 × (negative control mean OD + 3 standard deviation) (198). *S. aureus* strain with a cefoxitin (30µg) inhibition zone diameter of ≤21mm was confirmed as MRSA (199).

Bacterial strains	Chapters	Source of the isolates
TUHMSSA01	Chapter 4, Chapter 5, Chapter 6, Chapter 7	Urinary tract
TUHMRSA02	Chapter 4, Chapter 5, Chapter 6, Chapter 7	Wound

TUHMSSA03	Chapter 4	Wound
TUHMRSA04	Chapter 4	Wound
ATCC MRSA 1698	Chapter 7	ATCC strain

Table 3. 1: Bacterial strains used in this project

3.3. Bacterial culture preparation

S. aureus strains preserved at -80^{O}C were cultured in Luria-Bertani (LB) broth at 37^{O}C for 48hrs. The broth was discarded and the bacteria attached on the wall of culture tube were scraped and subcultured in 0.5% glucose containing LB (GLB) broth at 37^{O}C for 24hrs.

3.4. Antibacterial and antibiofilm activity testing of ticagrelor and savirin

The *in-vitro* antibacterial and antibiofilm activity of ticagrelor and savirin were studied by broth microdilution and crystal violet staining method respectively. The drugs were serially double-diluted in 50µl volume in triplicate in a flat bottom microtiter plate, using GLB. Then an equal volume of *S. aureus* broth containing 10^5cfu (2×10^6cfu/ml) was added to each well to make a final volume of 100µl and the microtiter plate was incubated at 37^{O}C for 24hrs. Antibacterial activity was determined spectrophotometrically by measuring optical density (OD) value at 600nm.

To study antibiofilm activity, the culture supernatant was discarded and the residual biofilm formed was fixed with 2% sodium acetate for at least 10min followed by overnight staining with

1% crystal violet. The microtiter plates were then rinsed with tap water, air dried overnight, and the crystal violet retained was reconstituted using absolute ethanol. Then the biofilm biomass was determined by measuring OD value spectrophotometrically at 570nm. *S. aureus* growth in drug diluents, and sterile drug diluents were used as positive and negative controls respectively.

3.5. Combined antibacterial and antibiofilm activity of ticagrelor or savirin molecule and antibiotics

The combined antibacterial and antibiofilm effect of ticagrelor or savirin molecule and antibiotics (cefazolin, rifampicin, and vancomycin) were investigated using checkerboard assay (200, 201). These antibiotics were selected because they are the most commonly used antibiotics in orthopaedic treatment (8). Cefazolin and vancomycin inhibit bacterial cell wall synthesis, while rifampicin inhibits bacterial RNA synthesis. A single intravenous prophylactic dose of cefazolin (2gm) or vancomycin (15mg/kg) is given, 30-60min prior to surgery, to prevent methicillin susceptible *S. aureus* (MSSA) or methicillin resistant *S. aureus* (MRSA) infection respectively. Intravenous cefazolin (1-2g, 8 hourly) and vancomycin (15mg/kg, 12 hourly) are also used for up to six weeks as the post surgery therapy for the treatment of MSSA and MRSA prosthetic joint infection respectively. Oral rifampicin (300–450mg, 12 hourly) is used as a companion drug for cefazolin and vancomycin for the treatment of PJI caused by rifampicin susceptible *S. aureus*. After the combination therapy of cefazolin/vancomycin plus rifampicin,

oral rifampicin is used in combination with another oral companion drug for up to additional 6 months.

The ticagrelor or savirin molecule and antibiotics were serially double diluted. Then, 50µl each of ticagrelor or savirin molecule, antibiotic, and bacterial broth were added to microtiter plate wells to make a final volume of 150µl. Further processing for the determination of antibacterial and antibiofilm activity was done as described in section 3.3. Fractional inhibitory concentration (FIC) index value was determined and the interaction of ticagrelor or savirin molecule and antibiotics was categorised as synergy (FIC<0.5), antagonism (FIC>4), additive or no effect (FIC=0.5-4). The combined activity of subinhibitory concentrations of ticagrelor or savirin molecule and antibiotics was also compared with the activity while using each compound alone.

3.6. Genomic DNA extraction

Genomic DNA was extracted by ethanol precipitation method. LB suspension was prepared in an Eppendorf's tube from a 24hr *S. aureus* culture on LB agar. Then, 0.1 volume of 3M sodium acetate (pH=5.2) was added followed by the addition of 2-3 volume of absolute ethanol. The solution was then incubated for 2hrs at -80^{O}C followed by centrifugation for 30min at > 14000g at 4^{O}C. The supernatant was discarded and the DNA pellet was rinsed with 70% ethanol followed by re-centrifugation for 15min at > 14000g at 4^{O}C. The supernatant was discarded again and the DNA pellet was air dried for 20min. Then the pellet was dissolved in sterile TE

(Tris-EDTA) buffer and stored at -20^{O}C. The quality and quantity of the DNA were checked using electrophoresis, Nanodrop and Qubit.

3.7. RNA extraction

RNA was extracted from 8hr *S. aureus* culture test samples treated with ticagrelor (12.5µg/ml) or savirin (10µg/ml) using Qiagen RNeasy mini kit. At 8hrs all the biofilm-related genes, *icaA*, *icaD*, *eno*, *fib*, *ebps*, and *agr*, studied were expressed. For controls, RNA was extracted from *S. aureus* culture treated with ticagrelor diluent (1% dimethylformamide) or savirin diluent (0.02% dimethylsulphoxide). The quality and quantity of the RNA extracted was determined by using nanodrop.

3.8. Polymerase chain reaction

Polymerase chain reaction was performed by using Qiagen Multiplex Polymerase Chain Reaction (PCR) plus kits. The reaction volume for each gene's PCR was 10µl and contained 0.2µl genomic DNA template, 1×PCR master mix, and 200nM of each primer. PCR parameters used were: initial denaturation (95^{O}C, 5min), followed by the 35 cycles of [denaturation (95^{O}C, 30sec), annealing (56^{O}C, 1.5min), elongation (72^{O}C, 30sec)] and final extension (68^{O}C, 10min). PCR products were analysed by electrophoresis at 90V for 1.5hrs in 2% gel red stained agarose gel and visualised under ultraviolet light.

3.9. Quantitative reverse transcriptase polymerase chain reaction (qRTPCR)

Bio-Rad iTaq universal SYBR green one-step kits were used for qRTPCR. Relative quantification method was used to test the effect of anti-virulence molecules on the expression of biofilm-related genes, *icaA*, *icaD*, *eno*, *fib*, *ebps*, and *agr*, in *S. aureus*. These genes are among the most important biofilm-related genes those are involved at the different stages of biofilm formation. The level of the effect was measured by comparative C_t ($\Delta\Delta C_t$) method (202). The results were presented as fold change ± standard deviation compared with the control. The reference genes used were *gmk* and *rpoB* for ticagrelor treatment and *fema* for savirin treatment. The qRTPCR reaction was carried out in 10µl volume consisting of 5µl 2× iTaq universal SYBR green reaction mix, 0.125µl iScript reverse transcriptase, 0.8ng RNA template in 1µl volume, 1nM of primer mix in 1µl volume, and 2.9µl nuclease free water. The thermo-cycler parameters used were: reverse transcription (50^{O}C, 10min), polymerase activation and DNA denaturation (95^{O}C, 1min), 40 cycles of denaturation at 95^{O}C for 10sec and annealing/extension + plate read at 60^{O}C for 30sec.

3.10. Animal experiments

3.10.1. Animal husbandry

Six to ten week old C57BL/6 female mice (Animal Resources Centre, Western Australia) were acclimatised for one week and ear marked. The mice were housed in a standard animal facility (maximum 5 mice per cage) with controlled temperature, humidity, and light system (18-22^{O}C,

70-75% relative humidity, and 12hrs light/dark cycle), using saw dust bedding. The mice were checked on a regular basis to ensure that they had adequate food and water. The mouse cages, bedding, and water bottles were changed every week before surgery but every two days after surgery to prevent infection.

3.10.2. Experimental design

Sample size was calculated using G* Power. Female mice are less aggressive and therefore easier to handle. Mice were randomised into 5 experimental groups (8 mice/group): 1) infected Kirschner (K)-wire ticagrelor or savirin molecule treated group, 2) infected K-wire cefazolin treated group, 3) infected K-wire ticagrelor or savirin molecule plus cefazolin treated group, 4) infected K-wire PBS/savirin diluent in PBS treated group (positive control), and 5) sterile untreated K-wire group (negative control).

3.10.3. Prosthetic joint infection mouse model

Surgery was performed as described previously (203). Ketamine/xylazine (90mg/kg/10mg/kg, ip) and buprenorphine (0.2mg/kg, sc) were used as pre-surgery anaesthesia and analgesia respectively. Fur from the right thigh of mouse was shaved and the area was disinfected with povidone iodine. A skin incision was made just above the knee and the kneecap was displaced to expose the femoral bone tip. A hole was then manually reamed through the femoral intramedullary canal using a 26G needle and a precut orthopaedic-grade stainless steel K-wire (diameter 0.6mm) was inserted leaving a 1mm protrusion into the knee joint space. The k-wire

and joint space were contaminated with a 2µl *S. aureus* (TUHMSSA01) normal saline inoculum (500cfu). The kneecap was reduced back to its original position and the surgical site was closed with a 5-0 absorbable suture. A combination of subcutaneous (0.2mg/kg) and oral (2.5ml/160ml drinking water) buprenorphine was given for 72hrs for post-surgical analgesia. Isoflurane (2%) was used to re-anaesthetise the mice that awoke during surgery.

Since MSSA PJI is significantly more frequent than MRSA PJI (204) and TUHMRSA02 is cefazolin resistant, we tested the efficacy of ticagrelor or savirin, alone and in combination with cefazolin, in an animal model using TUHMSSA01 only. Additionally, MSSA infection is easier to treat compared with MRSA infection because fewer treatment options are available for MRSA. So, in this project we started with the bacterium that is more frequently associated with PJI but relatively easier to treat than MRSA. Based on the data generated, future study using MRSA strain can be planned.

3.10.4. Bacteriological and histological analysis of K-wire and periprosthetic tissue

K-wires were extracted from the surgical sites of mice and washed with cold sterile PBS to remove planktonic cells, and then placed in 5ml of cold LB broth. The biofilm was disrupted by sonication at 44khz for 5min using a waterbath sonicator. Similarly, tissues were collected in 800µl of ice-cold PBS to minimize the bacterial multiplication and homogenised using a Navy Lysis Kit (BioTools, Australia). The bacteria in the sonication fluids and the tissue homogenates were quantified by drop dilution method whereby they were serially ten-fold diluted and

cultured on LB agar and Mannitol salt agar (MSA) at 37^{O}C for 48hrs. Bacterial concentrations were calculated and presented as log10cfu/ml. For histology, the tissues were fixed with 10% formalin for 24hrs. The tissues containing bones were then decalcified for up to 5 days, processed, and imbedded in paraffin wax followed by 5µm section preparation. The tissue sections were then stained with Gram's and haematoxylin eosin stain.

3.11. Statistical analysis

The data distribution was normal as determined by normality tests in GraphPad. One-way ANOVA was performed using GraphPad version 8.2.0 (GraphPad Software, San Diego, California, USA) followed by Tukey post-hoc test. P-value < 0.05 indicated statistical significance.

CHAPTER 4: OPTIMISATION OF REFERENCE GENES TO STUDY THE EFFECT OF SAVIRIN OR TICAGRELOR TREATMENT ON BIOFILM-RELATED GENE EXPRESSION IN *STAPHYLOCOCCUS AUREUS*

4.1. Introduction

Quantitative real time PCR (qRTPCR) was first established in 1992 and since then it has been routinely used for gene expression analysis (205). Errors may get introduced during the RNA extraction, reverse transcription, and amplification stage of qRTPCR experiment (206). Therefore, normalisation of qRTPCR data to compensate for errors is essential for the generation of reliable results (206). The most commonly used normalisation technique is by using an internal control reference gene (207). Reference genes are constitutively expressed genes that are responsible for basic cellular function maintenance required for cell survival. The expression of a reference gene is expected to remain unaffected by experimental conditions (208). However, there are no universal reference genes and they need to be validated for a given experimental condition (209). The use of an inappropriate reference gene can give significantly different and even error results (210). Although single reference gene is used regularly in many studies, using two or more genes can give more reliable results if small changes in gene expression are to be detected (211).

Staphylococcus aureus is one of the most common bacterial human pathogens responsible for causing a large number of infections including biofilm-related infections, such as prosthetic

joint infection (4, 212). Biofilm-related genes, *icaA, icaD, ebps, fib, eno,* and *agr* in *S. aureus* are responsible for the establishment of a difficult to treat biofilm-associated infection (213). Expression of these genes is bacterial growth stage and strain specific (213). Understanding the mechanism of how new treatments can influence the expression of the biofilm-related genes can open an avenue to search for new treatment options.

Different reference genes have been evaluated and used to study gene expression in *S. aureus* (214, 215). Savirin and ticagrelor are known to have antibiofilm activity against *S. aureus* (18, 19). This study tested the effect of savirin or ticagrelor treatment on the expression of sixteen *S. aureus* candidate reference genes. To study the effect of these treatments on biofilm-related genes, it is necessary to identify the bacterial growth stage time point at which the gene expression is most prominent. Therefore, this study also investigated the expression of these key biofilm-related genes in different *S. aureus* strains at different time points.

The specific aims investigated in this chapter were:

1. To investigate the expression of key biofilm-related genes (*ica*, *fib*, *ebps*, *eno*, *agr*) in different *S. aureus* strains at different time points.
2. To identify the most stably expressed suitable reference genes to study the effect of ticagrelor or savirin treatment on the expression of the biofilm-related genes in *S. aureus*.

4.2. Materials and methods

Two methicillin sensitive *S. aureus* (MSSA) strains (TUHMSSA01, TUHMSSA03), and two methicillin resistant *S. aureus* (MRSA) strains (TUHMRSA02, TUHMRSA04) isolated from patients attending the Townsville University Hospital were used. These strains produced luxuriant biofilms [optical density (OD) > 4 × (negative control mean OD + 3 standard deviation)] (198). The MRSA strains were detected by cefoxitin (30µg) disc diffusion assay, where the strains showing a zone of inhibition diameter of ≤21mm were regarded as MRSA (199). *S. aureus* strains stored at -80^{O}C were cultured in Luria-Bertani (LB) broth at 37^{O}C for 48hrs followed by sub-culturing in 0.5% glucose LB (GLB) broth for 24hrs.

4.2.1. RNA extraction

RNA was extracted from 3hr, 6hr, 8hr, 12hr, and 24hr *S. aureus* cultures in GLB using Qiagen RNeasy mini kit. Similarly, RNA was also extracted from ticagrelor (12.5µg/ml) treated, ticagrelor diluent (1% DMF) treated, savirin (10µg/ml) treated, and savirin diluent (0.02% DMSO) treated 8hr *S. aureus* cultures. Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, USA) was used to check the quality and quantity of RNA.

4.2.2. Qualitative detection of the expression of *S. aureus* biofilm-related genes at different time points

One-step RTPCR was performed using Veriti 96-well thermal-cycler (Applied Bio-systems) in 10µl reaction volumes. The reaction mixture consisted of RT-PCR buffer 2µl, dNTP mix 0.4µl,

RTPCR enzyme mix 0.4µl, water, 600nM each primer, and 1µl RNA template. The PCR cycling conditions used were: reverse transcription (50^{O}C, 30min), initial PCR activation (95^{O}C, 15min), 35 cycles of [denaturation (94^{O}C, 1min), annealing (56^{O}C, 1min), extension (72^{O}C, 1min)] followed by final 10min extension at 72^{O}C. The PCR products were analysed using electrophoresis. The primers used for qualitative RTPCR are listed in Table 4.1.

Primers name	Oligonucleotide sequence (5' ⟶ 3')	Product size (bp)	References
icaA (F)	ACACTTGCTGGCGCAGTCAA	188	(216)
icaA (R)	TCTGGAACCAACATCCAACA		
icaD (F)	ATGGTCAAGCCCAGACAGAG	198	(216)
icaD (R)	AGTATTTTCAATGTTTAAAGCAA		
eno (F)	ACGTGCAGCAGCTGACT	301	(217)
eno (R)	CAACAGCATTCTTCAGTACCTTC		
ebps (F)	CATCCAGAACCAATCGAAGAC	180	(217)
ebps (R)	CTTAACAGTTACATCATCATGTTTATCTTTG		
fib (F)	CTACAACTACAATTGCCGTCAACAG	405	(217)
fib (R)	GCTCTTGTAAGACCATTTTCTTCAC		
agr (F)	AATTTGTTCACTGTGTCGATAAT	135	(218)
agr (R)	TGGAAAATAGTTGATGAGTTGTT		

Table 4. 1: The primers used for qualitative RTPCR

4.2.3. Stability testing of candidate reference genes using qRTPCR

Bio-Rad iTaq universal SYBR green one-step kit and comparative $C_t(\Delta\Delta C_t)$ method were used to test the effect of ticagrelor or savirin treatment in sixteen different candidate reference genes (202). These are commonly used reference genes in different gene expression studies in *S. aureus.* The primers used are listed in Table 4.2. The qRTPCR reaction volume (10µl) consisted of 5µl 2× iTaq universal SYBR green reaction mix, 0.125µl iScript reverse transcriptase, 0.8ng RNA template in 1µl volume, 1nM primer mix in 1µl volume, and 2.9µl nuclease free water. The thermo-cycler parameters used were: reverse transcription (50^{O}C, 10min), polymerase activation and DNA denaturation (95^{O}C, 1min), 40 cycles of denaturation at 95^{O}C for 10sec and annealing/extension + plate read at 60^{O}C for 30sec.

Primers name	Forward primer	Reverse primer	References
glyA	CTACAAACTCACAGCCAC	GTATCGGAAGCGGTTATG	(215)
gmk	CCATCTGGAGTAGGTAAAGG	CTACGCCATCAACTTCAC	
gyrA	GTGTTATCGTTGCTCGTG	CGGTGTCATACCTTGTTC	
proC	GGCAGGTATTCCGATTGA	CCAGTAACAGAGTGTCCAAC	
pyk	GCATCTGTACTCTTACGTCC	GGTGACTCCAAGTGAAGA	
fabD	CCTTTAGCAGTATCTGGACC	GAAACTTAGCATCACGCC	
recF	AGTTATAGACACGGCACG	GCGTCGTCTTATTTGAGG	
rho	GGAAGATACGACGTTCAGAC	GAAGCGGGTGGAAGTTTA	
rpoD	CACGAGTGATTGCTTGTC	GATACGTAGGTCGTGGTATG	
gyrB	GGTGCTGGGCAAATACAAGT	TGGGATACCACGTCCGTTAT	(219)
spa	AGCACCAAAAGAGGAAGACAA	GTTTAACGACATGTACTCCGT	(220)
fema	TGCCTTTACAGATAGCATGCCA	AGTAAGTAAGCAAGCTGCAATGACC	(221)
pta	AGAAGCAATCATTGATGGCGA	ACCTGGCGCTTTTTTCTCAG	(222)
gapdh	TGACACTATGCAAGGTCGTTTCAC	TCAGAACCGTCTAACTCTTGGTGG	(223)
rpoB	CAGCTGACGAAGAAGATAGCTATGT	ACTTCATCATCCATGAAACGACCAT	(213)
16s	AGAGATAGAGCCTTCCCCTT	TTAACCCAACATCTCACGACA	(218)

Table 4. 2: The primers for candidate reference genes used in qRTPCR

4.2.4. Stability evaluation of the candidate reference genes

Bestkeeper algorithm was used for the identification of appropriate reference genes. This algorithm evaluates the stability of candidate reference genes on the basis of standard deviation (SD) and coefficient of variance (CV) of threshold cycle (Ct) value (224). The candidate reference genes with the Ct SD value of less than one and the lowest SD and CV value were regarded as most stable (224, 225).

4.3. Results

4.3.1. Qualitative detection of the expression of biofilm-related genes in *S. aureus*

All four *S. aureus* strains expressed the biofilm-related genes, *icaA, icaD, ebps, fib, eno,* and *agr*, at different time points as shown in Table 4.3. Three *S. aureus* strains (TUHMRSA02, TUHMSSA03, TUHMRSA04) expressed almost all the biofilm-related genes at all the time points. TUHMSSA01 strain expressed most of the genes at 8hrs and 12hrs. The *S. aureus* strains used in this study for the evaluation of stability of the candidate reference genes were TUHMSSA01 and TUHMRSA02 because these strains produced more luxuriant biofilm than other two strains. For this evaluation 8hr old *S. aureus* culture was used because both the TUHMSSA01 and TUHMRSA02 strains were found to express all of the biofilm-related genes when confirmed by qRTPCR method.

	icaA					icaD					ebps					fib					eno					agr				
hrs	3	6	8	12	24	3	6	8	12	24	3	6	8	12	24	3	6	8	12	24	3	6	8	12	24	3	6	8	12	24
TUHMSSA01	-	-	+	+	-	-	+	-	+	-	-	-	-	+	-	-	-	+	+	-	-	-	+	-	+	+	+	+	+	+
TUHMSSA03	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TUHMRSA02	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+
TUHMRSA04	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	-

Table 4. 3: Expression of biofilm-related genes in different *S. aureus* strains

4.3.2. Stability evaluation of the candidate reference genes

The PCR efficiency for the primers used ranged from 1.81 to 2 and the correlation coefficient was above 0.99. Sixteen candidate reference genes were included in the study. The specific PCR product was confirmed by the single product of expected size and single melting curve peak.

4.3.3. Stability evaluation of the candidate reference genes for savirin treatment experiment

Threshold cycle (Ct) values for the candidate reference genes for savirin treatment ranged from 17 to 36 (Figure 4.1). The *fema* and *gapdh* genes had the lowest SD and CV, and therefore were the most appropriate reference gene for this experimental condition in TUHMSSA01 (Table 4.4 and Figure 4.2). The *16s* gene was also a suitable reference gene with SD < 1. The most unstable reference genes were *proC, fabD*, and *pta*. However, for TUHMRSA02 strain SD values for all the

candidate reference genes exceeded 1 and therefore no suitable reference gene was identified. Savirin (10µg/ml) slowed down the growth of TUHMRSA02 strain (Figure 4.3).

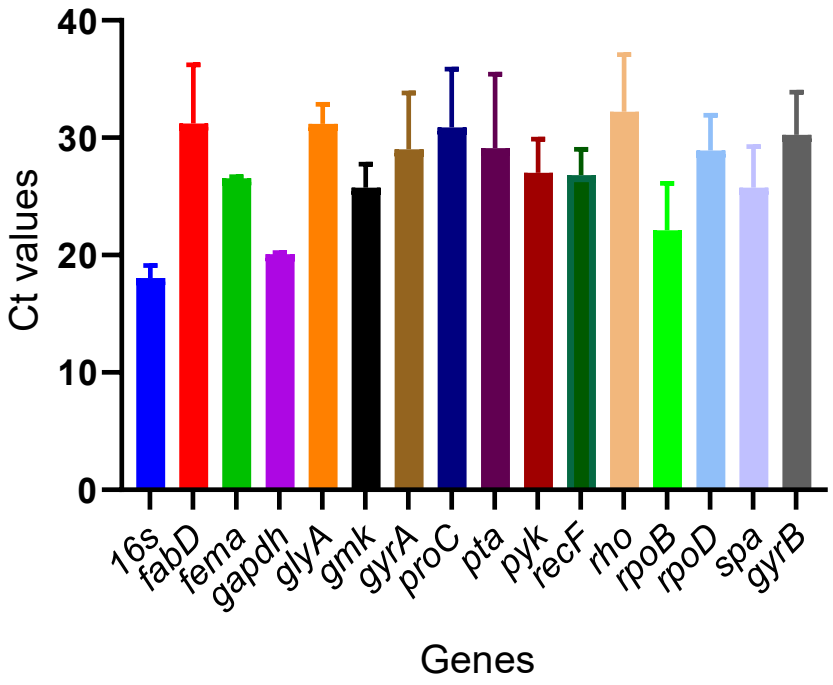


Figure 4. 1: Ct values for different candidate reference genes for savirin treatment. The experiment was performed in triplicates and the data are presented as mean ± SD. Error bars indicate SD.

	16s	fabD	fema	gapdh	glyA	gmk	gyrA	proC	pta	pyk	recF	rho	rpoB	rpoD	spa	gyrB
Geometric mean	18.04	31.00	26.56	20.08	31.17	25.69	28.82	30.67	28.76	26.93	26.76	32.01	21.93	28.85	25.63	30.15
Arithmetic Mean	18.06	31.20	26.56	20.08	31.20	25.74	29.02	30.87	29.10	27.01	26.81	32.20	22.11	28.93	25.75	30.26
SD	0.75	3.54	0.09	0.10	1.17	1.44	3.38	3.52	4.46	2.03	1.56	3.45	2.83	2.13	2.48	2.56
CV	4.13	11.35	0.34	0.50	3.73	5.58	11.65	11.40	15.33	7.52	5.80	10.71	12.80	7.35	9.61	8.46

Table 4. 4: Different parameters for Ct values of candidate reference genes for savirin treatment

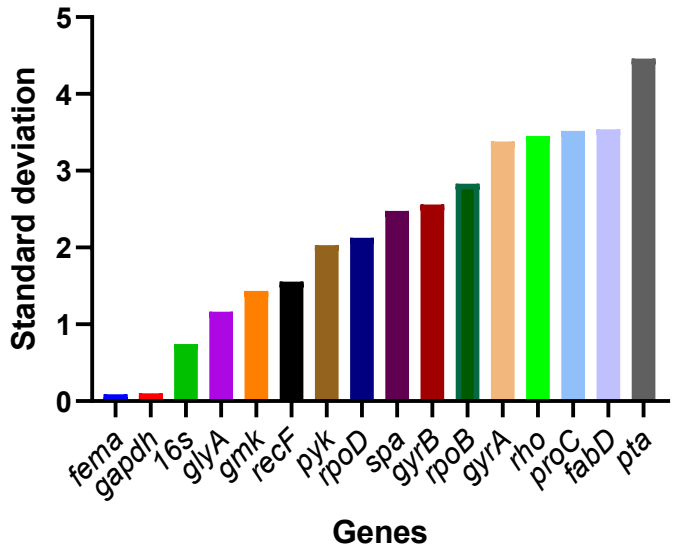


Figure 4. 2: Standard deviation (SD) of the Ct values of different candidate reference genes for savirin treatment experiment. The gene with less than one and the lowest standard deviation is most stable.

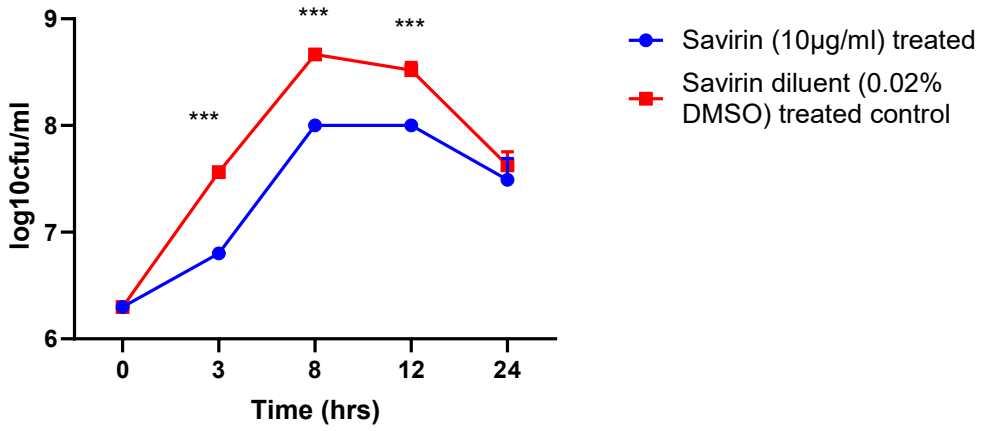


Figure 4. 3: Slowing down the growth of TUHMRSA02 strain by savirin (10µg/ml) treatment compared with savirin diluent (0.02% DMSO) treated control (***p<0.001). The experiment was performed in triplicate (N=3) and the data were presented as mean ± standard deviation (SD), where error bars indicate SD.

4.3.4. Stability evaluation of candidate reference genes for ticagrelor treatment experiment

Ct values for the candidate reference genes for ticagrelor treatment experiment ranged from 13 to 31 (Figure 4.3). The *gmk* followed *rpoB and rpoD* genes had the lowest SD and CV and were the most suitable reference genes for ticagrelor treatment experiment in both

TUHMSSA01 and TUHMRSA02 strains (Table 4.5, Figure 4.4). However, the *spa, gyrA, gapdh, recF, 16s, proC, fema,* and *gyrB* genes also met the criteria for an appropriate reference gene (SD<1). The most unstable reference genes were *fabD*, *glyA,* and *pta*.

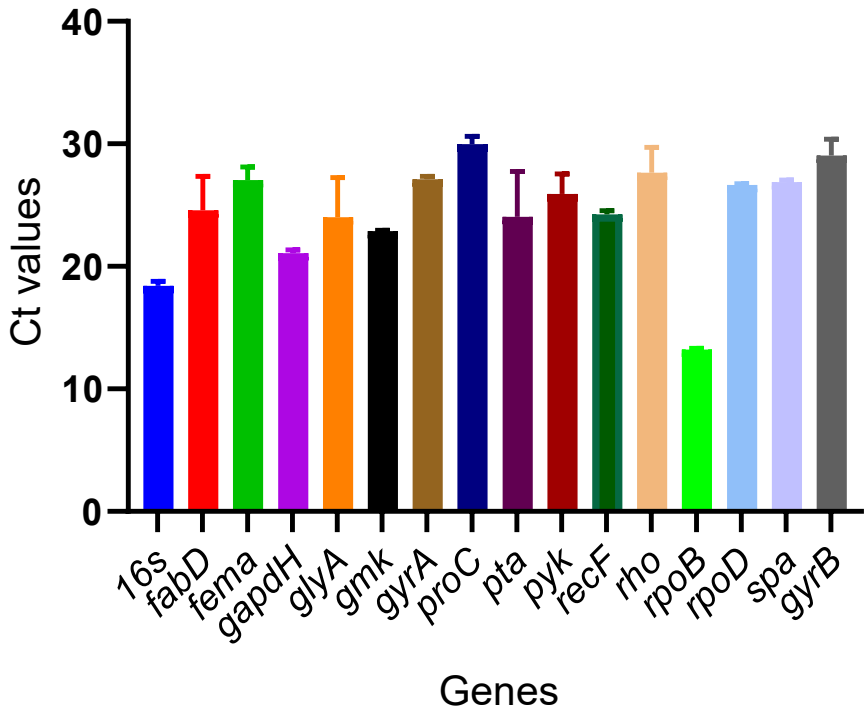


Figure 4. 4: Ct values for different candidate reference genes for ticagrelor treatment experiment. The experiment was performed in triplicates and the data are presented as mean ± SD. Error bars indicate SD.

	16s	fabD	fema	gapdh	glyA	gmk	gyrA	proC	pta	pyk	recF	rho	rpoB	rpoD	spa	gyrB
Geometric mean	18.42	24.50	27.02	21.08	23.90	22.89	27.10	29.98	23.90	25.88	24.24	27.63	13.22	26.65	26.87	29.05
Arithmetic Mean	18.42	24.58	27.04	21.08	24.01	22.89	27.11	29.98	24.05	25.91	24.25	27.67	13.23	26.66	26.88	29.07
SD	0.25	1.97	0.75	0.19	2.29	0.05	0.16	0.45	2.62	1.17	0.21	1.46	0.08	0.08	0.14	0.92
CV	1.36	8.00	2.79	0.90	9.52	0.22	0.61	1.50	10.88	4.50	0.85	5.26	0.57	0.28	0.50	3.18

Table 4. 5: Different parameters for the Ct values of candidate reference genes for ticagrelor treatment

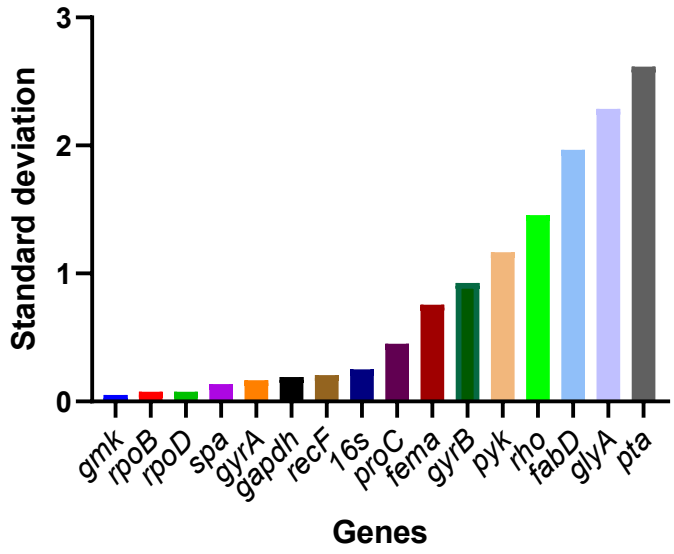


Figure 4. 5: Standard deviation (SD) of the Ct values of different candidate reference genes for ticagrelor treatment experiment. The gene with less than one and the lowest SD is most stable.

4.4. Discussion

The suitability of sixteen *S. aureus* genes for their use as reference genes to study the effect of savirin or ticagrelor treatment on the expression of biofilm-related genes in *S. aureus* was tested. The expression of biofilm-related genes in different *S. aureus* strains at different time point was also studied and was found to be strain specific. This strain specific expression of biofilm-related genes is in accordance to the findings of an earlier study (213). However, since the method used in this study was qualitative PCR, the exact level of gene expression was not known. The main aim of this study was to identify the most stable reference genes, whose expression was independent of savirin or ticagrelor treatment. The concentrations of savirin (10µg/ml) or ticagrelor (12.5µg/ml) used for treatment were sufficient to prevent biofilm formation without significant planktonic growth inhibition. Several algorithms, such as genorm, normfinder, and bestkeeper are available to analyse the stability of potential reference genes. However, these algorithms show comparable results on the choice of reference genes (226, 227). In this study, bestkeeper algorithm was used to identify the most stable reference gene. This algorithm identifies the most stably expressed candidate reference gene through evaluation of the standard deviation (SD) and coefficient of variance (CV) of Ct values (224). A suitable reference gene must have a SD of less than one and a gene having the lowest SD and CV are regarded as the most stable (224).

In this study, for savirin treatment experiment the most stable reference gene was *fema* followed by *gapdh* and *16s*, while for ticagrelor treatment the most suitable reference gene

was *gmk* followed by *rpoB* and *rpoD*. The most unstable genes for both the experiments were *fabD* and *pta*.

This is the first study to investigate the suitability of several candidate reference genes for the normalisation of gene expression to study the effect of savirin or ticagrelor treatment on biofilm-related genes. Ticagrelor and antivirulence molecule savirin inhibit *S. aureus* biofilm formation and can improve the treatment outcome of biofilm-related infection (18, 19). The genes tested in this study are the commonly used reference genes in different gene expression studies in *S. aureus*. Additionally, the genes belonging to different bacterial biochemical pathways were chosen to minimise the inclusion of the co-regulated genes that might be affected by same experimental condition (215).

A previous study that investigated the effect of 5µg/ml savirin on the expression of *agr* gene used *16s* as a reference gene (18). Additionally, the same study used microarray analysis and showed no effect of savirin (5µg/ml) treatment in the expression of most of the candidate reference genes studied in this project (18). The discrepancy between results of the previous and this study might be due the higher savirin (10µg/ml) concentration used in this study. The previous study incubated *S. aureus* with 5µg/ml of savirin for 5hrs in Tryptone Soy Broth with AIP1, while in this study the bacteria were incubated with 10µg/ml of savirin for 8hrs in glucose containing Luria-Bertani broth. While the previous study did not report any antibacterial activity of savirin (5µg/ml) against *S. aureus*, higher concentration of savirin is known to be antibacterial (24). This study also showed antibacterial activity of savirin at higher concentration (20µg/ml)

(Chapter 6). Savirin concentration 10µg/ml, but not 5µg/ml, showed significant antibiofilm activity (Chapter 6). In the previous study, 5µg/ml savirin downregulated *agr* and some other *agr* dependent genes (18). The *agr* gene being responsible for *S. aureus* biofilm dispersal, 5µg/ml savirin would have been expected to enhance biofilm formation in *S. aureus* (60). Activation of the *agr* quorum-sensing system causes biofilm dispersal in *S. aureus* and disruption of this system promotes robust biofilm formation. However, this study did not show increased biofilm formation when *S. aureus* was treated with 5µg/ml savirin. This difference in results between the previous and this study might be due to difference between the growth conditions and *S. aureus* strains used in the two studies.

To our knowledge, the effect of ticagrelor treatment on *S. aureus* genes has never been studied before. Apart from the *gmk*, *rpoB*, and *rpoD* genes, eight other genes also met the criteria for a suitable reference gene for ticagrelor treatment experiment (SD<1). The *fema*, *gapdh*, and *16s* genes whose expression were not affected by savirin treatment were also found to be suitable for using as reference genes for ticagrelor treatment. The *pta* gene was the most unstable for both savirin and ticagrelor treatment experiment. In this study the *16s* gene, which has already been used as reference gene in a savirin treatment experiment, was found to be stably expressed when treated with savirin or ticagrelor (18). However while the *16s* gene has high target copy numbers, its transcripts do not represent the overall *S. aureus* mRNA and therefore might not be an ideal internal control (215).

The *fema* gene*,* which was found to be most stably expressed in savirin treatment study, is involved in peptidoglycan biosynthesis and has been used as a molecular marker for *S. aureus* identification (228). Similarly, *gapdh* plays an important role in glycolysis and is used frequently as reference gene in different experimental conditions, including to study the resistance mechanism of *S. aureus* against amoxicillin (223, 229, 230). For the genes most stably expressed in ticagrelor treatment experiment, *gmk* is involved in nucleotide metabolism, while *rpoB* and *rpoD* contribute in transcription (215). The *gmk* gene has been found suitable to be used as reference gene for a gene expression study under photodynamic treatment (230), while *rpo* in an experiment involving the treatment of *S. aureus* by manuka honey (213, 231). However, *gmk* has been found to be the most unstable gene in *S. aureus* crystal violet treatment experiment (215). In this study, *tpiA* was the most unstable gene in both savirin and ticagrelor treatment experiment. This gene is involved in gluconeogenesis and has also been known to be the most unstable gene in *S. aureus* crystal violet treatment experiment (215). However, *tpiA* is an appropriate reference gene for ethidium treatment experiment (215). It can be concluded that genes that are involved in mechanisms required for cell survival are appropriate internal controls and there are no universal reference genes. An appropriate reference gene should be confirmed for each experimental condition.

For savirin treatment experiment the most stably expressed genes were *fema, gapdh*, and *16s*. These genes also met the criteria to be used as a reference gene in ticagrelor treatment experiment. However, the most stably expressed genes for ticagrelor treatment experiment

were *gmk, rpoB*, and *rpoD*. This study provides a foundation for other researches to choose an appropriate reference gene to study the effect of savirin or ticagrelor treatment on *S. aureus* genes.

4.5. Chapter 4 summary

- The main aim of this study was to identify the most stably expressed reference genes in *S. aureus* when treated with ticagrelor or savirin.
- Relative quantification method, using qRTPCR and bestkeeper algorithm, was used to identify the most stable reference genes from a list of 16 different candidate reference genes.
- The most appropriate reference gene for savirin treatment experiment was *fema* followed by *gapdh* and *16s*, while that for ticagrelor treatment experiment was *gmk* followed by *rpoB* and *rpoD*.

CHAPTER 5: EFFECT OF NON-ANTIMICROBIAL ADJUVANT THERAPY USING TICAGRELOR TO TREAT BIOFILM-RELATED *STAPHYLOCOCCUS AUREUS* PROSTHETIC JOINT INFECTION

5.1. Introduction

Arthroplasty surgery is one of the most commonly performed orthopaedic procedures. However, 2.0 to 2.4% of these life-enhancing surgeries fail because of biofilm-related bacterial infections that are difficult to treat (5). *Staphylococcus aureus,* a part of normal human flora, is the most common cause of prosthetic joint infection (PJI), being involved in up to 57% of infections (4). Bio-inert medical implants coated with host proteins, such as fibrinogen, provide a rich environment for *S. aureus* attachment and biofilm proliferation (148). Consequently, a very low number of bacteria (<50cfu) are enough to establish joint infection in the presence of a prosthesis compared with 10^4cfu in its absence (147). Physical barriers and the presence of metabolically inert cells in biofilm make its eradication through antibacterial therapy alone difficult (131, 139). As a result, surgical intervention to replace or debride a prosthesis followed by long term antibiotic therapy is the current treatment of choice (232). However, these procedures are traumatic and expensive with failure rates of up to 25% (10).

Ticagrelor, a P2Y12 receptor inhibitor antiplatelet drug used to prevent thrombotic events in atherosclerotic cardiovascular disease patients, protected acute coronary syndrome patients from infection by Gram positive bacteria such as *S. aureus* (233-235). This molecule has also

been shown to inhibit biofilm-related *S. aureus* infection in a subcutaneous prosthesis infection animal model (19). Ticagrelor has also shown synergistic effect with antibiotics, rifampicin, ciprofloxacin, and vancomycin, for the *in-vitro* inhibition of methicillin resistant *S. aureus* (MRSA) (19).

Ticagrelor's molecular mechanism of *S. aureus* biofilm inhibition and its efficacy to treat *S. aureus* PJI have not been defined up until now. In chapter 4, it was shown that the *gmk*, *rpoB*, and *rpoD* genes are the most stably expressed reference genes in *S. aureus* to study the effect of ticagrelor treatment in biofilm-related genes. This study tested the *in-vitro* antibacterial activity of ticagrelor, alone and with antibiotics, as well as its effect in biofilm-related gene regulation. This project also studied the efficacy of ticagrelor in the treatment of biofilm-related *S. aureus* infection in a PJI mouse model. It was reasoned that early reintroduction of ticagrelor post-operatively may improve arthroplasty outcomes by preventing PJI.

The specific aims studied in this chapter are:

1. To determine the *in-vitro* antibacterial and antibiofilm activity of ticagrelor, alone and with antibiotics (cefazolin, vancomycin, and rifampicin), against *S. aureus*.
2. To determine the effect of ticagrelor treatment in the expression of key biofilm-related genes (*ica, agr, fib, eno, ebps*) in *S. aureus*.
3. To determine the effect of ticagrelor, alone and with cefazolin, to treat *S. aureus* PJI in a mouse model.

5.2. Materials and methods

Two *S. aureus* clinical strains, TUHMSSA01 (methicillin susceptible), and TUHMRSA02 (methicillin resistant), isolated from patients treated at the Townsville University Hospital, Queensland, Australia were used in this study. These strains were chosen from among nineteen different *S. aureus* strains including an ATCC 25923 available for use in this study. The two *S. aureus* strains were chosen because they produced the most luxuriant biofilms [as measured by optical density (OD) > 4 × (negative control mean OD + 3 standard deviations)] (198). Biofilm production was induced in *S. aureus* strains by culturing in Luria-Bertani (LB) broth at 37^{O}C for 48hrs without shaking followed by sub-culturing in 0.5% glucose containing LB (GLB) broth for 24hrs.

5.2.1. *In-vitro* antibacterial and antibiofilm activity of ticagrelor

Fifty µl of bacterial broth containing 10^{5}cfu was added to microtiter plate wells containing eight serially double diluted ticagrelor concentrations to make a final volume 100µl and final ticagrelor concentrations 50µg/ml to 0.75µg/ml followed by incubation for 24hrs at 37^{O}C. Antibacterial activity was measured by determining OD at 600nm. Minimum bactericidal concentration (MBC) was determined by quantifying bacteria from wells with no visible growth following the drop dilution method (236). The minimum ticagrelor concentration that reduced bacterial concentration by more than 99.9% was taken as MBC. To determine the antibiofilm activity of ticagrelor, this study used previously reported biofilm assay procedures with a slight

modification (237). The culture supernatant was discarded and the residual biofilm was fixed with 2% sodium acetate for 10min. Then the biofilm was stained overnight with crystal violet followed by rinsing with tap water and air drying. The crystal violet retained was then reconstituted with absolute ethanol and OD values were measured at 570nm. The experiments were performed in triplicates. *S. aureus* growth in ticagrelor diluent dimethylformamide (DMF) (4.15%) was used as positive control while the sterile DMF was used as negative control.

5.2.2. *In-vitro* combined antibacterial and antibiofilm effect of ticagrelor and antibiotics (cefazolin, rifampicin and vancomycin)

The combined effect of ticagrelor (50µg/ml to 0.8µg/ml), with cefazolin (0.5µg/ml to 0.007µg/ml), vancomycin (2.5µg/ml to 0.03µg/ml), and rifampicin (0.015µg/ml to 0.0002µg/ml) was tested as described above except the final volume used was 150µl (50µl each of ticagrelor, antibiotic, and bacterial suspension). The fractional inhibitory concentration (FIC) index value was determined by using checkerboard assay. The combined effects of sub-inhibitory concentrations of ticagrelor and antibiotics compared with those of each alone were also tested. The highest sub-inhibitory concentrations of the antibiotics that showed either no or minimal antibacterial and antibiofilm activity were chosen.

5.2.3. Analysis of the effect of ticagrelor treatment on *S. aureus* biofilm-related gene expression

5.2.3.1. Polymerase chain reaction (PCR)

Ethanol precipitation method was used to extract genomic DNA. Primers for *icaA*, *icaD*, *eno*, *fib*, *ebps*, and *agr* genes were used to detect the *S. aureus* biofilm pathway genes (Table 5.1). Qiagen Multiplex PCR plus kit (Qiagen, Hilden, Germany) was used for polymerase chain reaction (PCR). PCR reaction volume used was 10µl and contained 0.2µl of genomic DNA template, 1×PCR master mix, and 200nM of each primer. PCR parameters used were: initial denaturation (95^{O}C, 5min), followed by 35 cycles of [denaturation (95^{O}C, 30sec), annealing (56^{O}C, 1.5min), elongation (72^{O}C, 30sec)], and final extension (68^{O}C, 10 min). PCR products were analysed by gel electrophoresis.

Primers name	Oligonucleotide sequence (5' ⟶3')	Product size (bp)	References
PCR primers			
icaA (F)	ACACTTGCTGGCGCAGTCAA	188	(216)
icaA (R)	TCTGGAACCAACATCCAACA		
icaD (F)	ATGGTCAAGCCCAGACAGAG	198	(216)
icaD (R)	AGTATTTTCAATGTTTAAAGCAA		
eno (F)	ACGTGCAGCAGCTGACT	301	(217)
eno (R)	CAACAGCATTCTTCAGTACCTTC		

ebps (F)	CATCCAGAACCAATCGAAGAC	180	(217)
ebps (R)	CTTAACAGTTACATCATCATGTTTATCTTTG		
fib (F)	CTACAACTACAATTGCCGTCAACAG	405	(217)
fib (R)	GCTCTTGTAAGACCATTTTCTTCAC		
agr (F)	AATTTGTTCACTGTGTCGATAAT	135	(218)
agr (R)	TGGAAAATAGTTGATGAGTTGTT		
qRTPCR primers		Function of the related genes (217, 238-241)	
icaA (F)	CAATACTATTTCGGGTGTCTTCACTCT	Slime production	(213)
icaA (R)	CAAGAAACTGCAATATCTTCGGTAATCAT		
icaD (F)	TCAAGCCCAGACAGAGGGAATA	Slime production	(213)
icaD (R)	ACACGATATAGCGATAAGTGCTGTTT		
eno (F)	AAACTGCCGTAGGTGACGAA	Encode cell surface associated proteins	(213)
eno (R)	TGTTTCAACAGCATCTTCAGTACCTT		
ebps (F)	ACATTCAAATGACGCTCAAAACAAAAGT	Encode cell surface associated proteins	(213)
ebps (R)	CTTATCTTGAGACGCTTTATCCTCAGT		
fib (F)	GAATATGGTGCACGTCCACAATT	Encode cell surface associated proteins	(213)
fib (R)	AAGATTTTGAGCTTGAATCAATTTTTGTTCTTTTT		
agr (F)	AATTTGTTCACTGTGTCGATAAT	Biofilm dispersal	(218)
agr (R)	TGGAAAATAGTTGATGAGTTGTT		
rpoB (F)	CAGCTGACGAAGAAGATAGCTATGT		(213)

rpoB (R)	ACTTCATCATCCATGAAACGACCAT		
gmk (F)	CCATCTGGAGTAGGTAAAGG		(215)
gmk (R)	CTACGCCATCAACTTCAC		

Table 5. 1: Primers used for PCR and qRTPCR

5.2.3.2. RNA extraction for quantitative reverse transcriptase polymerase chain reaction (qRTPCR)

RNA was extracted from 8hr *S. aureus* test and positive control cultures treated with 12.5µg/ml ticagrelor and 1% DMF respectively, using Qiagen RNeasy mini kit. Ticagrelor 12.5µg/ml showed significant antibiofilm activity without inhibiting planktonic growth. Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, USA) was used to measure RNA quality and quantity.

5.2.3.3. Measurement of gene expression

Bio-Rad iTaq universal SYBR green one-step kit (Bio-Rad, United States) was used for qRTPCR. The effect of ticagrelor on the expression of biofilm-related *S. aureus* genes, *icaA*, *icaD*, *eno*, *fib*, *ebps*, and *agr*, was tested in triplicate by relative quantification method (Table 5.1). The level of the effect was measured by comparative C_t ($\Delta\Delta C_t$) method (202). The results were presented as fold change ± standard deviation in comparison with the positive control. Reference genes used were *rpoB* and *gmk* because their expression was treatment independent. These genes were

selected from among 16 different candidate reference genes because they were most stably expressed in the experimental condition used (chapter 4). The qRTPCR reaction was carried out in 10µl volume that contained 5µl 2× iTaq universal SYBR green reaction mix, 0.125µl iScript reverse transcriptase, 0.8ng RNA template in 1µl volume, 1nM primer mix in 1µl volume, and 2.9µl nuclease-free water. Thermo-cycler parameters used were: reverse transcription (50^{O}C, 10min), polymerase activation and DNA denaturation (95^{O}C, 1min), 40 cycles of denaturation at 95^{O}C for 10sec and annealing/extension + plate read at 60^{O}C for 30sec.

5.2.4. Animal studies

Ethical approval to conduct the animal study was granted by the James Cook University Animal Ethics Committee (AEC2486). Sample size was calculated using G* Power. Female mice are less aggressive and therefore easier to handle. Six to ten week-old C57BL/6 female mice (Animal Resources Centre, Western Australia) were randomised into five groups (n=8/group): (1) infected implants treated with ticagrelor alone; (2) infected implants treated with cefazolin alone; (3) infected implants treated with ticagrelor and cefazolin; (4) infected implants treated with phosphate buffer solution (PBS) (positive control); and (5) sterile implants untreated (negative control).

5.2.4.1. Surgical technique

The animal model used to emulate prosthesis-related joint infection was described by Bernthal et al. (203). Buprenorphine (0.2mg/kg, sc) was administered 30min pre-surgery, while

ketamine/xylazine (90mg/kg/10mg/kg, ip) was used just before surgery. Hair was removed from the right thigh and the skin was disinfected with povidone iodine. An incision was made above the right knee to displace the knee cap and to access the femoral intercondylar notch. Then the femoral intramedullary canal was reamed manually with a 26G needle and an orthopaedic-grade stainless steel Kirschner (K)-wire (diameter 0.6mm) was inserted to leave its 1mm cut end protruding into the knee joint space. The K-wire was contaminated with 500cfu of *S. aureus* in 2µl of a PBS bacterial suspension pipetted into the joint space. The knee cap was replaced and the skin was closed with a 5-0 absorbable suture. Combined subcutaneous (0.2mg/kg) and oral (2.5ml/160ml drinking water) buprenorphine was given as an analgesic for 72hrs.

5.2.4.2. Treatment administered

The ticagrelor alone treatment group was treated with ticagrelor (3mg/kg loading dose followed by 1.5mg/kg twice daily in 100µl volume) orally from day 4 to day 7 post-surgery (19). This is the dose/weight equivalent to human treatment (19). This drug intervention timing was used because it mimics the time of reintroduction of an antiplatelet drug in human arthroplasty surgery to avoid drug-related bleeding from fresh wounds. Similarly, the cefazolin alone treatment group was injected with a single intravenous dose of cefazolin (2.5mg/kg) on day 7 post-surgery. To test whether biofilm dispersed by ticagrelor would be killed by an antibiotic, the ticagrelor plus cefazolin treatment group was administered ticagrelor from day 4 to day 7 followed by a single cefazolin (2.5mg/kg) dose on day 7 post-surgery. The concentration and

the route of administration of cefazolin in mice were defined with the help of a previous pilot study that suggested the no effect of intravenously injected cefazolin (2.5mg/kg) on biofilm formed. The cefazolin dose given was not intended to eradicate the biofilm infection, rather it was designed to measure whether any combined effect was present with ticagrelor. Clinical parameters such as weight, eating, drinking, mobility, and pain indicators were recorded daily. The mice were kept alive for sufficient time after the ticagrelor and cefazolin treatments ended to let the infection develop again if not eradicated. On day 14 post-surgery, mice were culled using carbon dioxide, and implants and surrounding tissues were collected for bacteriological and histological analysis.

5.2.4.3. Bacterial culture of k-wires and tissues, and histological analysis

The extracted K-wires collected were rinsed with sterile cold PBS to wash off planktonic cells. The K-wires were then sonicated at 44khz in 5ml cold LB for 5min using a water bath sonicator to disrupt the biofilm and remove the attached cells. Periprosthetic tissues were collected in 800µl ice-cold PBS to slow down bacterial multiplication, and then homogenised using Navy Lysis Kit (BioTools, Australia). Sonication fluids and homogenised tissues were serially ten-fold diluted and cultured on LB and mannitol salt (MSA) agar for 48hrs at 37^{O}C. Bacterial colony counts were presented as log10cfu/ml. For histology, the formalin-fixed tissues were decalcified, processed, and imbedded in paraffin wax. A tissue section of 5µm was prepared and stained with Gram's and haematoxylin eosin stain. The section was then screened using a light microscope.

5.2.2. Statistical analysis

The data were normally distributed. Graphpad version 8.2.0 (GraphPad Software, San Diego, California, USA) was used for performing one way ANOVA followed by Tukey multiple comparison test. P-value <0.05 was taken as statistically significant.

5.3. Results

5.3.1. Antibacterial and antibiofilm activity of ticagrelor

Ticagrelor did not show strain specific activity judged by identical results for experiments using TUHMSSA01 and TUHMRSA02 isolates. The minimum bactericidal concentration (MBC) of ticagrelor for both strains was 50µg/ml. Ticagrelor also exhibited significant antibiofilm activity (Figure 5.1).

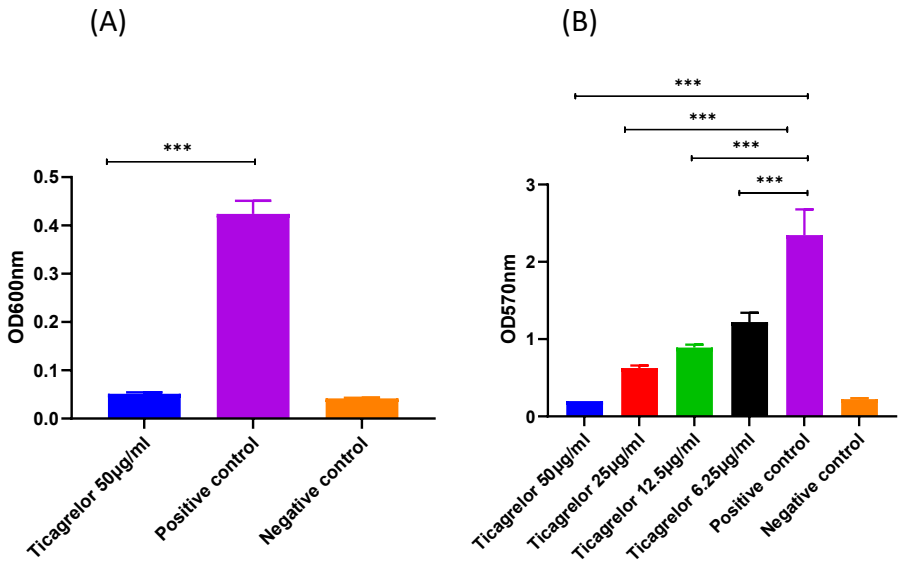


Figure 5. 1: *S. aureus* planktonic (A) and biofilm (B) growth in the presence of ticagrelor. Experiments were performed in triplicate (N=3) and data were presented as mean ± standard deviation (SD) with the error bars indicating SD (***<0.001). Ticagrelor showed antibacterial and antibiofilm activity against *S. aureus*.

5.3.2. Antibacterial and antibiofilm activity of ticagrelor in combination with antibiotics

Different ticagrelor and antibiotic (cefazolin, rifampicin, vancomycin) concentrations were tested for their combined effect on the planktonic and biofilm growth of *S. aureus*. TUHMRSA02 being resistant to cefazolin, ticagrelor and cefazolin combination was not tested in this strain. However, since ticagrelor showed antibacterial and antibiofilm activity against the cefazolin resistant strain, it is worth investigating if ticagrelor could sensitise this strain towards cefazolin in future studies. Sub-inhibitory concentrations of ticagrelor and antibiotic in combination showed higher antibacterial and antibiofilm activity compared with using each one (Figure 5.2). However, ticagrelor in the presence of rifampicin did not show enhanced antibacterial activity. Therefore, the FIC index values of 0.75 to 2 for all three ticagrelor and antibiotic combinations implied an additive (cefazolin, and vancomycin) or no effect (rifampicin). The sub-inhibitory concentrations of ticagrelor and antibiotic alone presented in Figure 5.2 had either no or minimal antibacterial and antibiofilm activity.

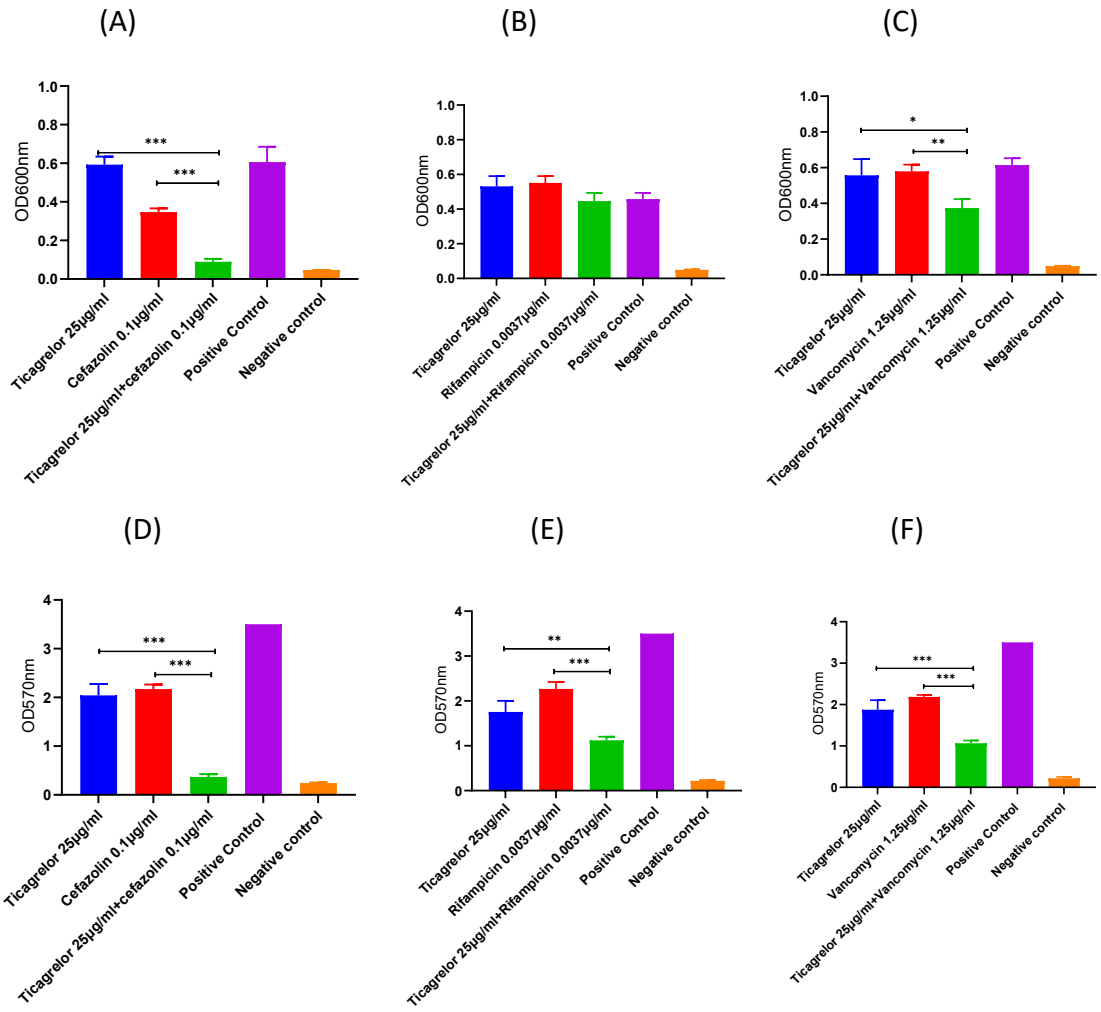


Figure 5. 2: Combined antibacterial (A, B, C) and antibiofilm (D, E, F) activity of sub-inhibitory concentrations of ticagrelor and antibiotics (cefazolin, rifampicin, vancomycin) in comparison with each alone. Sub-inhibitory ticagrelor concentration was used in this experiment. Ticagrelor in combination with antibiotics had higher activity compared with ticagrelor alone except for antibacterial activity of ticagrelor plus rifampicin. Data are presented as mean (N=3) ± standard deviation (SD) and error bars indicate SD (***=p<0.001, **=<0.01, *=<0.05).

5.3.3. Effect of ticagrelor treatment on biofilm-related *S. aureus* genes expression

All the biofilm-related genes, *icaA*, *icaD*, *eno*, *fib*, *ebps*, and *agr*, tested were detected in both TUHMSSA01 and TUHMRSA02 strains. Ticagrelor showed strain specific downregulation of these biofilm-related genes. The *fib* and *icaD* genes were downregulated in TUHMSSA01 while *ebps*, *eno*, *fib*, and *icaD* were downregulated in TUHMRSA02 (Figure 5.3).

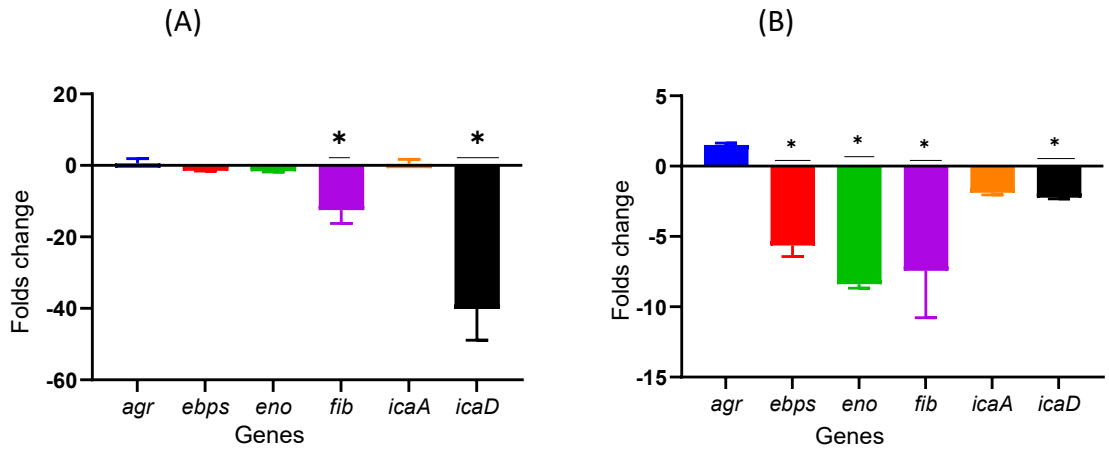


Figure 5. 3: Downregulation of biofilm-related genes in TUHMSSA01 (A) and TUHMRSA02 (B) strains after 8hrs of growth in the presence of ticagrelor (12.5ug/ml). The reference genes used were *gmk* and *rpoB*. Effect of ticagrelor treatment on gene expression was determined by comparative C_t ($\Delta\Delta C_t$) method. Data are presented as mean fold changes (N=3) ± standard deviation (SD) compared with ticagrelor diluent (1% dimethylformamide) treated control and error bars indicate SD (*=down regulated by > 2 folds)

5.3.4. Effect of ticagrelor treatment alone and with cefazolin to reduce bacterial concentration on K-wire implants and periprosthetic joint tissues

This project proceeded to use cefazolin in animal study because this is the most commonly used antibiotic in arthoplasty surgery and showed better *in-vitro* combined effect with ticagrelor compared with the other antibiotics tested. This study tested the effect of ticagrelor, alone and in combination with cefazolin, on TUHMSSA01 infected K-wire implants and periprosthetic tissues in a mouse model. Ticagrelor alone and in combination with cefazolin significantly reduced bacterial concentration on the implants extracted from experimentally infected mice knees in comparison with the PBS treated control (log10cfu/ml, 0.8 versus 3.2, $p<0.001$ and 1.6 versus 3.2, $p<0.05$) (Figure 5.4). Ticagrelor reduced bacterial dissemination into periprosthetic tissues compared with the positive control (log10cfu/ml, 3.6 versus 7.1, $p<0.001$). There was a non-significant increase in bacterial concentrations on implants and periprosthetic tissues from mice administered cefazolin in addition to ticagrelor compared with that from mice administered ticagrelor alone. However when compared with the PBS treated positive control, the inhibitory activity of ticagrelor alone was statistically more significant with p-value <0.001 than that of ticagrelor plus cefazolin where p-value was <0.05. Tissue histology showed the presence of similar concentrations (counts/oil immersion field) of Gram positive cocci and neutrophils in periprosthetic tissue of all the infected groups.

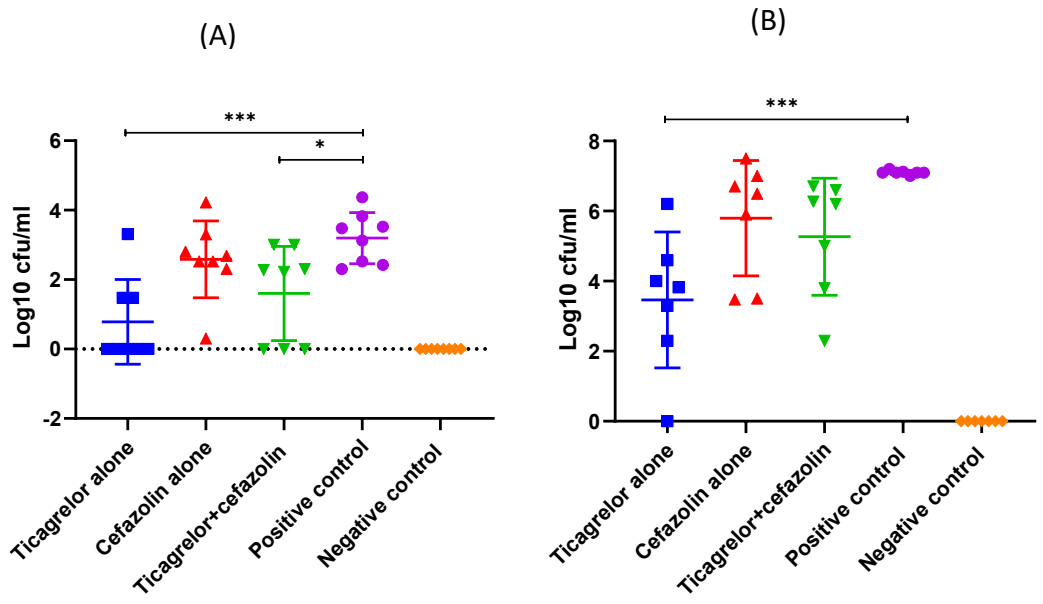


Figure 5. 4: Bacterial concentration (N=8) on k-wire (A) and peri-prosthetic tissue (B) from different treatment and control group on day 14 post-surgery (***p<0.001, *p<0.05). Ticagrelor alone reduced bacterial concentration in both implant and peri-prosthetic tissue, while ticagrelor and cefazolin combination reduced bacterial concentration only on implant. The data were presented as mean log bacterial concentration ± standard deviation (SD).

5.4. Discussion

Ticagrelor, an antiplatelet drug, shows *in-vivo* as well as *in-vitro* antibacterial and antibiofilm activity against *S. aureus* (19). The use of ticagrelor for the treatment of prosthesis-related *S. aureus* joint infection and the underlying molecular mechanisms of its antibiofilm activity have not been investigated. This study investigated the efficacy of ticagrelor used as non-antimicrobial adjuvant therapy to treat biofilm-related *S. aureus* infection in a prosthetic joint infection (PJI) mouse model and the associated molecular mechanism.

Ticagrelor showed the *in-vivo* antibacterial and antibiofilm activity such that it reduced PJI due to TUHMSSA01 strain in an animal model. The antibiofilm activity is attributed to the inhibition of critical biofilm pathway genes. Ticagrelor also exhibited the *in-vitro S. aureus* planktonic and biofilm growth inhibition, and additive effects with cefazolin and vancomycin in both TUHMSSA01 and TUHMRSA02 strains. However, ticagrelor showed enhanced antibiofilm activity but no additive antibacterial effect when combined with rifampicin.

Only one previous study that used a pre-contaminated subcutaneous foreign body *S. aureus* infection mouse model has reported the *in-vitro* and *in-vivo* antibacterial and antibiofilm activity of ticagrelor against *S. aureus* including MRSA (19). Mice were subcutaneously implanted with polyurethane disks contaminated with *S. aureus*, and bioluminescent imaging was used to determine the efficacy of ticagrelor treatment (19). While both the previous and this study reported ticagrelor's *in-vivo* and *in-vitro* antibacterial and antibiofilm activity including the enhanced activity of antibiotics, there were some key differences. The magnitude of antibacterial activity shown was higher in the previous study (minimum bactericidal concentration = 20μg/ml) (19), and this study did not report the enhanced antibacterial activity of rifampicin. The discrepancy in results on ticagrelor's antibacterial activity between the previous and this study might be due to different bacterial strains or the methods used. For instance, this study used broth microdilution method followed by the drop dilution method for viable count, while the former study used time-kill assay and disk diffusion assay (19). Another study has reported the minimum inhibitory concentration (MIC) of ticagrelor to be 33μg/ml,

and its *in-vitro* additive effect with cefazolin and ertapenem against methicillin susceptible *S. aureus* (MSSA) (193).

For the first time, this study has explored the genetic mechanism of antibiofilm effect of ticagrelor in both TUHMSSA01 and TUHMRSA02 strains. In chapter 4, *gmk*, *rpoB*, and *rpoD* were found to be the most stably expressed reference genes for studying the effect of ticagrelor treatment in the biofilm-related genes. Using *gmk*, and *rpoB* as reference genes, this study has demonstrated the strain specific downregulation of some key biofilm-related genes: *fib, icaD*, *ebps*, and *eno*. In general, biofilm formation involves quorum sensing. Consequently, biofilm inhibition involves the combination of lowering of bacterial concentration and the regulation of different biofilm-related genes. The *eno*, *ebps*, and *fib* genes initiate biofilm formation through the expression of cell wall associated proteins that promote *S. aureus* attachment and colonisation (217, 238-240); *icaA* and *icaD* produce slime and help in biofilm maturation (241). So, downregulation of all or any one of these genes affects biofilm production negatively. Strain specific expressions of different biofilm-related genes in weak and strong biofilm producer *S. aureus* have already been reported (213). Although both the strains used in this study were strong biofilm producers, TUHMSSA01 produced more luxuriant biofilm than TUHMRSA02. The *agr* gene helps in *S. aureus* biofilm dispersal (60). However, amyloid fiber, a product of *agr* quorum sensing, is known to stabilise biofilm (70). So, the role of *agr* in *S. aureus* biofilm may be strain specific (60), and this study did not notice any effect of ticagrelor treatment on this gene.

Since TUHMRSA02 is cefazolin resistant, this study proceeded to test the efficacy of ticagrelor, alone and in combination with cefazolin, in an animal model using TUHMSSA01 only. Additionally, PJI is more frequently caused by MSSA than MRSA (204). Ticagrelor demonstrated the *in-vivo* antibacterial and antibiofilm activity against TUHMSSA01 strain used. In the animal prosthetic joint infection model, ticagrelor reduced bacterial concentration on K-wire and periprosthetic tissue. However, none of the treatments used in this study sterilised the infection. In a clinical context, for a successful cure of a PJI it is necessary to sterilise the infection. Since ticagrelor alone showed *in-vitro* sterilisation of *S. aureus* growth it might also be possible to attain this *in-vivo*, through the variation of drug dosing and timing. These factors could be investigated with more animal studies. Reduction in biofilm formation and bacterial dissemination to surrounding tissue due to ticagrelor treatment, with the same dosages as in our study, in a pre-contaminated subcutaneous disc *S. aureus* infection mouse model has been reported (19).

The pathogenesis for PJI is complex and the animal model used in this study was chosen for its simplicity but it is not an ideal representation of human infections. Studies using large animal prosthetic joint infection models that use the same materials and techniques as used in modern arthroplasty could better represent human PJI pathogenesis. This study lays a foundation for research in this direction.

When antiplatelet drugs are recommenced as early as possible after arthroplasty surgery in patients there is no increase in bleeding risk (242). The earliest possible use of ticagrelor post-

surgery may prevent biofilm-related infection effectively improving outcomes for an arthroplasty surgery. As the procedure performed in this study emulated high-bleeding-risk orthopaedic surgery, to minimise bleeding risk we waited for 3 days until sufficient haemostasis and wound healing were achieved, and then commenced ticagrelor treatment. Consequently, this study did not encounter complications associated with ticagrelor-related bleeding.

Ticagrelor in combination with cefazolin has never been used before in the treatment of bacterial infections in an animal model. The combination of ticagrelor and cefazolin showed greater *in-vitro* antibacterial and antibiofilm activity than ticagrelor alone. In this study the reverse pattern of inhibition was seen *in-vivo* as ticagrelor alone showed better antibacterial and antibiofilm activity compared with the ticagrelor and cefazolin combination. Thus, the reduction in *S. aureus* infection seen in our PJI mouse model appears to be mainly due to ticagrelor. Ticagrelor and cefazolin could have opposite effects on the *in-vivo* expression of biofilm-related genes. Combining these two drugs in gene expression analysis might give better insight into the *in-vivo* antagonistic effect of ticagrelor and cefazolin. There is also possibility that cefazolin might alter the pharmacokinetics (absorption, distribution, metabolism, elimination) of ticagrelor leading to reduced activity of the combination therapy and further animal studies are needed to understand this better.

Platelets mediate *S. aureus* clearance, while *S. aureus* α-toxin induces thrombocytopenia (192, 243). At physiological concentration ticagrelor had protective effect on platelets against *S. aureus* α-toxin and enhanced platelet mediated MRSA and MSSA killing (192, 193). Given the

maximum achievable systemic ticagrelor concentration (1.2μg/ml), with standard dosages for acute coronary syndrome, is significantly lower than the direct inhibitory supraphysiologic concentrations (20μg/ml to 50μg/ml) (193), the reduction in infection seen in this study might not be related to the direct antibacterial effect of ticagrelor. Alternatively, biofilm-related *S. aureus* infection involves interaction between bacterial clumping factor A, GPIIb/IIIa platelet receptor, and fibrinogen (244, 245). Platelet inactivation by ticagrelor might prevent *S. aureus* attachment to platelet and consequently to host tissue, leading to infection clearance.

S. aureus does not develop resistance to ticagrelor as easily as it does to conventional antibiotics (19). When either MSSA or MRSA strains were serially treated with sub-inhibitory concentrations of ofloxacin or rifampicin or ticagrelor, development of resistance with the antibiotics was observed but not with ticagrelor (19). Ticagrelor's *in-vitro* anti-MRSA and anti-VRE activity indicates that ticagrelor's mode of antibacterial action is not same as that of cefazolin and vancomycin. Ticagrelor has been speculated to cause leakage of cellular components (246). Cefazolin prevents peptidoglycan synthesis and vancomycin stops transpeptidation both leading to the inhibition of bacterial cell wall synthesis.

Biofilm dispersal using adjuvant, non-antimicrobial therapy with ticagrelor may improve the success rate of PJI treatment. Further animal model and human observational data indicating a benefit of ticagrelor for the prevention of biofilm-related infections may support intervention trials in humans. Repurposing this Food and Drug Administration approved antiplatelet drug for the treatment of PJI due to *S. aureus* could be cheap and rapid.

5.5. Chapter 5 summary

- Prosthetic joint infection (PJI), frequently caused by *Staphylococcus aureus*, leads to the significant arthroplasty failure rates. Surgical and antibiotic therapy could be combined with non-antibacterial adjuvants to improve overall treatment success.
- Ticagrelor, a Food and Drug Administration approved P2Y12 receptor inhibitor antiplatelet drug, is known to have anti-staphylococcal antibacterial and antibiofilm activity. However, the molecular mechanism for ticagrelor's antibiofilm activity and its efficacy in the treatment of *S. aureus* PJI are unknown.
- The main aim of this study was to determine the efficacy of ticagrelor, alone and with cefazolin, in the treatment of PJI in an animal model.
- The *in-vitro* antibacterial and antibiofilm activity of ticagrelor were determined by broth microdilution and crystal violet staining method, the combined effect of ticagrelor with antibiotics (cefazolin, rifampicin, and vancomycin) by checkerboard assay, the effect of ticagrelor on the expression of *S. aureus* biofilm genes (*icaA*, *icaD*, *ebps*, *fib*, *eno*, and *agr*) by relative quantification method, and the *in-vivo* effect of ticagrelor in the treatment of *S. aureus* PJI by using a clinically relevant mouse model.
- Ticagrelor, alone and with selected antibiotics, showed the *in-vitro* antibacterial and antibiofilm activity against *S. aureus*. The strain specific downregulation of biofilm-related genes, *fib*, *icaD*, *ebps*, and *eno*, was shown.

- In an animal model of biofilm-related *S. aureus* PJI, ticagrelor alone and combined with cefazolin reduced the bacterial infection. Therefore, ticagrelor should be considered for the development of an adjuvant therapy for the treatment of *S. aureus* PJI.

CHAPTER 6: EFFECT OF SAVIRIN NON-ANTIMICROBIAL ADJUVANT THERAPY IN THE TREATMENT OF BIOFILM-RELATED *STAPHYLOCOCCUS AUREUS* PROSTHETIC JOINT INFECTION

6.1. Introduction

Indwelling medical devices, including prosthetic joints, create a favourable environment for biofilm-related bacterial infection (247). Consequently infection-related arthroplasty failure, mainly due to *S. aureus* infection, is common (4). Current treatments include major surgery either to replace or debride infected prostheses, both followed by long term antibiotic use (146). However, these procedures have significant drawbacks - they are costly, potentially traumatic and have failure rates ranging from 15% to 25% (9, 10). Biofilms, a key *S. aureus* virulence factor that contributes to prosthetic joint infection (PJI) pathogenesis, are recalcitrant to antibiotic treatment (248). Therefore, antimicrobial therapy alone is not sufficient to treat most prosthetic joint infections (7).

Savirin, (*Staphylcoccus aureus* virulence inhibitor), is a low molecular weight, lipophilic, synthetic novel molecule suitable for drug development (18). This molecule prevents AgrA attachment to promoter regions. It inhibits activation of the *agr* quorum-sensing system, an operon responsible for controlling many important *S. aureus* virulence factors, resulting in increased host-mediated bacterial killing (18). Savirin has been shown to prevent as well as treat biofilm-related *S. aureus* infections in rodent skin and subcutaneous infection models (18).

Savirin might also be active against mature biofilm, as it was able to reduce infection even when administered 24 to 48hrs post infection establishment (18). Savirin was not toxic in doses used in the two animal models (18). It appears that *S. aureus* is less likely to develop resistance to savirin than to conventional antibiotics as multiple *in-vivo* or *in-vitro* passages did not induce resistance in *S. aureus* to *agr* inhibition by savirin, while this did induce resistance to clindamycin (18).

In chapter 4, *fema*, *gapdh*, and *16s* were found to be the most stable reference genes to study the effect of savirin treatment on biofilm-related genes in *S. aureus*. Chapter 5 details the efficacy of a Food and Drug Administration (FDA) approved antiplatelet drug ticagrelor for the treatment of biofilm-related *S. aureus* PJI in a mouse model. There are limited data those characterise the antibiofilm efficacy of a novel molecule savirin and no previous studies has investigated its use in the treatment of prosthesis-related infection caused by *S. aureus*. The objective of the current study was to investigate the effect of savirin, alone and in combination with antibiotics, on *S. aureus in-vitro* planktonic and biofilm growth and to determine the molecular mechanisms underlying biofilm inhibition. Further, this study tested savirin's effect as a non-antimicrobial adjuvant treatment in a mouse model of PJI.

The specific aims investigated in this chapter were:

1. To determine the *in-vitro* antibacterial and antibiofilm activity of savirin, alone and with antibiotics (cefazolin, vancomycin, and rifampicin), against *S. aureus*.

2. To determine the effect of savirin treatment in the expression of key biofilm-related genes (*ica, agr, fib, eno, ebps*) in *S. aureus*.
3. To determine the effect of savirin, alone and with cefazolin, to treat *S. aureus* PJI in a mouse model.

6.2. Materials and methods

Two clinical strains of *S. aureus* were used in this study: a methicillin susceptible *S. aureus,* TUHMSSA01 strain and a methicillin resistant *S. aureus*, TUHMRSA02 strain, isolated from patients attending the Townsville University Hospital, Queensland, Australia. The strains were selected from among nineteen different *S. aureus* strains including an ATCC 25923, because of their ability to produce the most robust biofilm [as measured in microtiter plate assay as optical density (OD) > 4 × (negative control mean OD + 3 standard deviations)] (198). Cefoxitin resistance was tested as per CLSI guidelines - a cefoxitin (30µg) inhibition zone of ≤21mm diameter confirmed MRSA (199).

6.2.1. Bacterial broth culture

S. aureus isolates were cultured in Luria-Bertani (LB) broth at 37^{O}C for 48hrs without shaking. Subculturing in 0.5% glucose containing LB (GLB) broth for 24hrs induced ample biofilm production.

6.2.2. *In-vitro* antimicrobial and antibiofilm activity of savirin

Broth microdilution and crystal violet staining methods were performed in triplicates using microtiter plates. Fifty microlitres of bacterial broth containing 10^5cfu of *S. aureus* was added to eight serial two-fold dilutions of savirin (80µg/ml to 0.62µg/ml) in 50µl volumes and incubated for 24hrs at 37^OC. This resulted in a concentration range of savirin from 40µg/ml to 0.31µg/ml. Antibacterial activity was determined by measuring optical density (OD) at 600nm. The minimum bactericidal concentration (MBC) of savirin was determined by plating microtiter plate wells showing no bacterial growth. Microtiter plate biofilm assay procedures were adapted from a previous study (237). Bacterial broth in microtiter plate wells after 24hrs of growth at 37^OC was discarded and the biofilm formed was fixed with 2% sodium acetate for 10min followed by overnight staining with 1% crystal violet. Crystal violet retained was reconstituted with absolute ethanol and OD values were measured at 570nm. *S. aureus* growth in savirin diluent (0.08% DMSO) was used as positive control while the sterile DMSO was used as negative control. The efficacy of savirin to disperse mature biofilm was also investigated. For this 48hr-old preformed biofilm was treated with savirin.

6.2.3. Combined inhibitory effect of savirin and antibiotics (cefazolin, rifampicin, and vancomycin) on planktonic and biofilm growth

Savirin (26.67µg/ml to 0.42µg/ml) in combination with cefazolin (0.5µg/ml to 0.007µg/ml), vancomycin (2.5µg/ml to 0.03µg/ml), and rifampicin (0.015µg/ml to 0.0002µg/ml) was used as

described above, except with a total well volume of 150µl (50µl each of savirin, antibiotic, and bacterial broth culture suspension). The combined effect was tested by checkerboard assay and the fractional inhibitory concentration (FIC) index value was determined. The inhibitory effects of combined subinhibitory concentrations of savirin and antibiotics were also compared with use of each alone. The highest sub-inhibitory concentrations of the antibiotics that showed either no or minimal antibacterial and antibiofilm activity were chosen.

6.2.4. The effect of savirin on the expression of key biofilm-related genes in *S. aureus*

6.2.4.1. RNA extraction

RNA was extracted from 8hr test- and control-culture samples treated with 10µg/ml savirin and 0.02% DMSO respectively, using the Qiagen RNeasy mini kit. Ten µg/ml savirin was used as this concentration reduced biofilm formation without inhibiting planktonic growth. The quality and quantity of RNA was determined using Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, USA).

6.2.4.2. Gene expression quantification

Quantitative reverse transcriptase polymerase chain reaction (qRTPCR) was performed in triplicate for each gene by using Bio-Rad iTaq universal SYBR green one-step kit (Table 6.1). The reference gene *fema* was chosen from among sixteen different candidate reference genes, because this gene was most stably expressed when *S. aureus* was treated with savirin (chapter 4). The reaction mixture (10µl) consisted of 5µl of 2×iTaq universal SYBR green reaction mix,

0.125µl iScript reverse transcriptase, 0.8ng RNA template in 1µl volume, 1nm primer mix in 1µl volume, and 2.9µl nuclease free water. The cycling conditions used on the BioRad CFX96 Touch Real-Time PCR Detection System were: reverse transcription (50^{O}C, 10min), polymerase activation and DNA denaturation (95^{O}C, 1min) followed by 40 cycles of denaturation (95^{O}C, 10sec), and annealing/extension, and plate read (60^{O}C, 30sec). The effect of savirin treatment on the expression of key *S. aureus* biofilm pathway genes, *icaA*, *icaD*, *eno*, *fib*, *ebps*, and *agr* was determined by the comparative C_t ($\Delta\Delta C_t$) method using BioRad CFX Manager software (202). The results were expressed as fold changes ± standard deviation in comparison with control.

Primers	Oligonucleotide sequences (5' ⟶ 3')	Function of the related genes (217, 238-241)	References
icaA (F)	CAATACTATTTCGGGTGTCTTCACTCT	Slime production	(213)
icaA (R)	CAAGAAACTGCAATATCTTCGGTAATCAT		
icaD (F)	TCAAGCCCAGACAGAGGGAATA	Slime production	(213)
icaD (R)	ACACGATATAGCGATAAGTGCTGTTT		
eno (F)	AAACTGCCGTAGGTGACGAA	Encode cell surface associated proteins	(213)
eno (R)	TGTTTCAACAGCATCTTCAGTACCTT		
ebps (F)	ACATTCAAATGACGCTCAAAACAAAAGT	Encode cell surface associated proteins	(213)
ebps (R)	CTTATCTTGAGACGCTTTATCCTCAGT		
fib (F)	GAATATGGTGCACGTCCACAATT	Encode cell surface associated proteins	(213)

fib (R)	AAGATTTTGAGCTTGAATCAATTTTTGTTCTTTTT		
agr (F)	AATTTGTTCACTGTGTCGATAAT	Biofilm dispersal	(218)
agr (R)	TGGAAAATAGTTGATGAGTTGTT		
fema (F)	TGCCTTTACAGATAGCATGCCA		(249)
fema R)	AGTAAGTAAGCAAGCTGCAATGACC		

Table 6. 1: Primers used for qRTPCR

6.2.5. Animal experiments

Ethics approval was obtained from the James Cook University Animal Ethics Committee (AEC2486). Sample size was calculated using G* Power. Female mice are less aggressive and therefore easier to handle. Six to ten weeks old C57BL/6 female mice (Animal Resources Centre, Western Australia) were randomised into 5 experimental groups (8 mice/group): 1) infected K-wire savirin treated group, 2) infected K-wire cefazolin treated group, 3) infected K-wire savirin plus cefazolin treated group, 4) infected K-wire savirin diluent containing sterile PBS treated group (positive control), 5) sterile K-wire untreated group (negative control).

6.2.5.1. *S. aureus* prosthetic joint infection mouse model

Surgery was performed using previously described procedures (203). Mice were anaesthetised with ketamine/xylazine (90mg/kg/10mg/kg, ip) prior to surgery. Buprenorphine (0.2mg/kg, sc) was used as analgesic 30min pre-surgery. Fur from the right thigh region was removed followed

by disinfection with povidone iodine. The skin was incised just above the knee and the kneecap was displaced to expose the tip of femoral bone. A hole was then made through the femoral intramedullary canal using a 26G needle and a precut orthopaedic-grade stainless steel Kirschner (K)-wire (diameter 0.6mm) was inserted leaving a 1mm protrusion into the joint space. A 2µl *S. aureus* (TUHMSSA01) normal saline inoculum (500cfu) was pipetted into the joint space. The kneecap was returned to its original position and the surgical site was closed with a 5-0 absorbable suture. A combination of subcutaneous (0.2mg/kg) and oral (2.5ml/160ml drinking water) buprenorphine was given for 72hrs as post-surgical analgesia.

6.2.5.2. Savirin and antibiotic treatments

A single subcutaneous dose of savirin (40µg in 100µl) was administered immediately after surgery to the infected K-wire savirin treated group. This is the highest non-toxic dose previously tested in Vero cell line culture (cell survival ~100%). A single cefazolin (2.5µg/g, iv) dose in 100µl volume was administered on day 7 post-surgery to the infected K-wire cefazolin treated group. The infected K-wire savirin plus cefazolin treated group was administered a single dose of savirin (40µg in 100µl volume, sc) immediately after surgery followed by a single cefazolin dose (2.5µg/g in 100µl volume, iv) on day 7 post-surgery. The concentration and the route of administration of cefazolin in mice were determined with the help of a previous pilot study that suggested the no effect of intravenously injected cefazolin (2.5mg/kg) on biofilm. Cefazolin was used in this manner not to sterilise the biofilm but to assess for any increased effect where it was used with savirin. Mice were weighed and animal well-being parameters,

such as eating, drinking, mobility, interaction with other mice, and reaction to external stimuli were recorded daily. Mice were euthanised on day 14 post-surgery to check if the infection was eradicated. The intramedullary K-wires were removed *in-toto* and peri-prosthetic tissues were collected aseptically for bacteriological culture and histological analysis.

6.2.5.3. Bacteriological analysis of k-wires and peri-prosthetic tissues, and histological study

K-wires were collected in 5ml of cold LB broth after washing three times with cold sterile PBS to remove planktonic cells. Sonication at 44khz for 5min using a waterbath sonicator was performed to thoroughly disrupt the attached biofilms. Similarly, tissues were collected in 800µl of ice-cold PBS to minimize bacterial multiplication followed by homogenisation using a Navy Lysis Kit (BioTools, Australia). Bacterial quantification of sonication fluids and tissue homogenates was performed by the drop dilution method whereby they were serially diluted and cultured on LB agar and mannitol salt agar (MSA) at $37^{O}C$ for 48hrs. Bacterial concentrations were calculated and presented as log10cfu/ml. For histological analysis 5µm tissue sections were prepared and stained with Gram's and haematoxylin eosin stains.

6.2.6. Statistical analysis

The data were normally distributed. One-way ANOVA was performed using GraphPad version 8.2.0 (GraphPad Software, San Diego, California, USA) followed by Tukey post-hoc test. P-value < 0.05 indicated statistical significance.

6.3. Results

6.3.1. Antibacterial and antibiofilm activity of savirin

Savirin's minimum bactericidal (MBC) and minimum inhibitory (MIC) concentrations were 40µg/ml and 20µg/ml respectively in both TUHMSSA01 and TUHMRSA02 strains. Savirin at 20µg/ml and 10µg/ml inhibited biofilm formation significantly. The same savirin concentrations also dispersed mature biofilm (48hr), which corresponded with reduced planktonic growth (Figure 6.1). Savirin showed better *in-vitro* activity against immature *S. aureus* biofilm compared with that against mature biofilm. The data presented are for TUHMSSA01, because this was the strain used in the animal model and as we did not demonstrate strain specific effects.

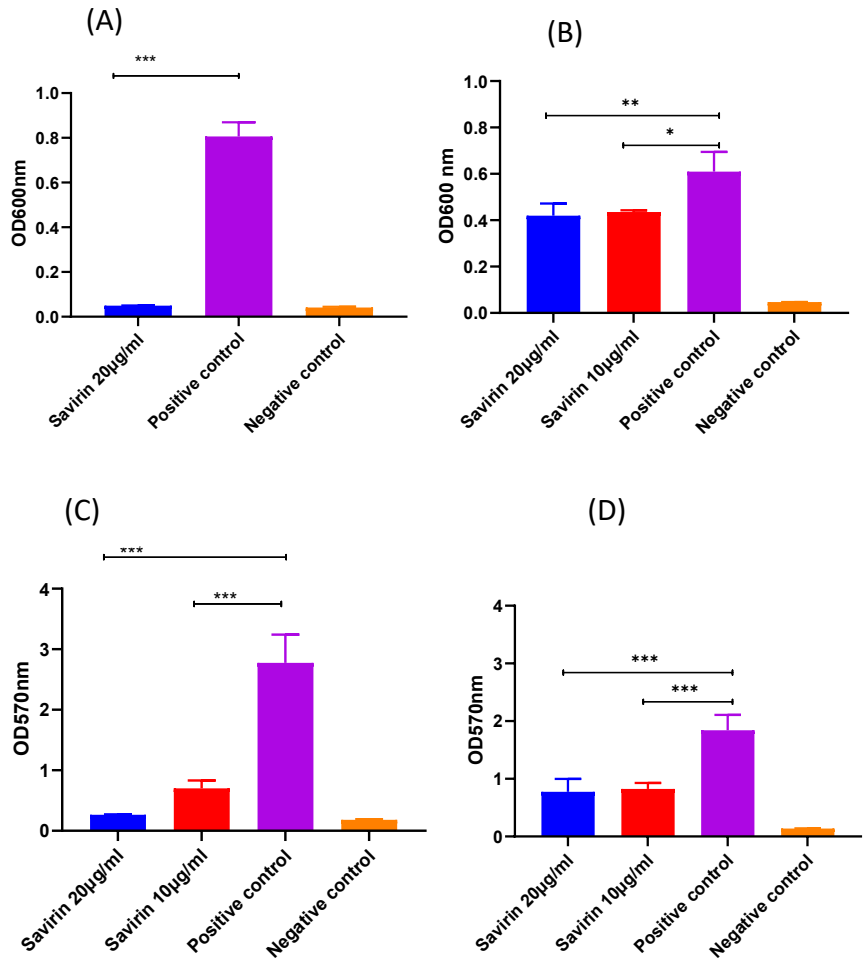


Figure 6. 1: Planktonic growth of 24hr (A) and 48hr (B) *S. aureus* cultures in the presence of savirin. Biofilm growth of 24hr (C) and 48hr (D) *S. aureus* cultures in the presence of savirin. Triplicate wells were used for each treatment (N=3) and experiments were repeated twice. Data are presented as mean ± standard deviation (SD) and the error bars indicate SD (***=p<0.001, **=<0.01, *=<0.05).

6.3.2. Combined antibacterial and antibiofilm activity of savirin and antibiotics (cefazolin, rifampicin, and vancomycin)

Since, MRSA is resistant to cefazolin the combination of cefazolin and savirin was not tested in TUHMRSA02 strain. However, since savirin showed antibacterial and antibiofilm activity against the MRSA strain, it is worth investigating if savirin could sensitise this strain towards cefazolin in future studies. Multiple savirin and antibiotic concentration combinations were tested for both TUHMSSA01 and TUHMRSA02 strains. As no difference was shown between results for the *S. aureus* strains tested, data presented in Figure 6.2 are for TUHMSSA01 alone. Combined sub-inhibitory concentrations of savirin and antibiotics showed significant enhanced antibacterial and antibiofilm activity compared with testing them alone (Figure 6.2). The fractional inhibitory concentration (FIC) index values for all three savirin and antibiotics combinations ranged from 0.75 to 2 indicating an additive effect. The sub-inhibitory concentrations of savirin and antibiotics were chosen because they have no/minimal inhibitory activity when used alone such that enhanced combined inhibitory activity could be observed.

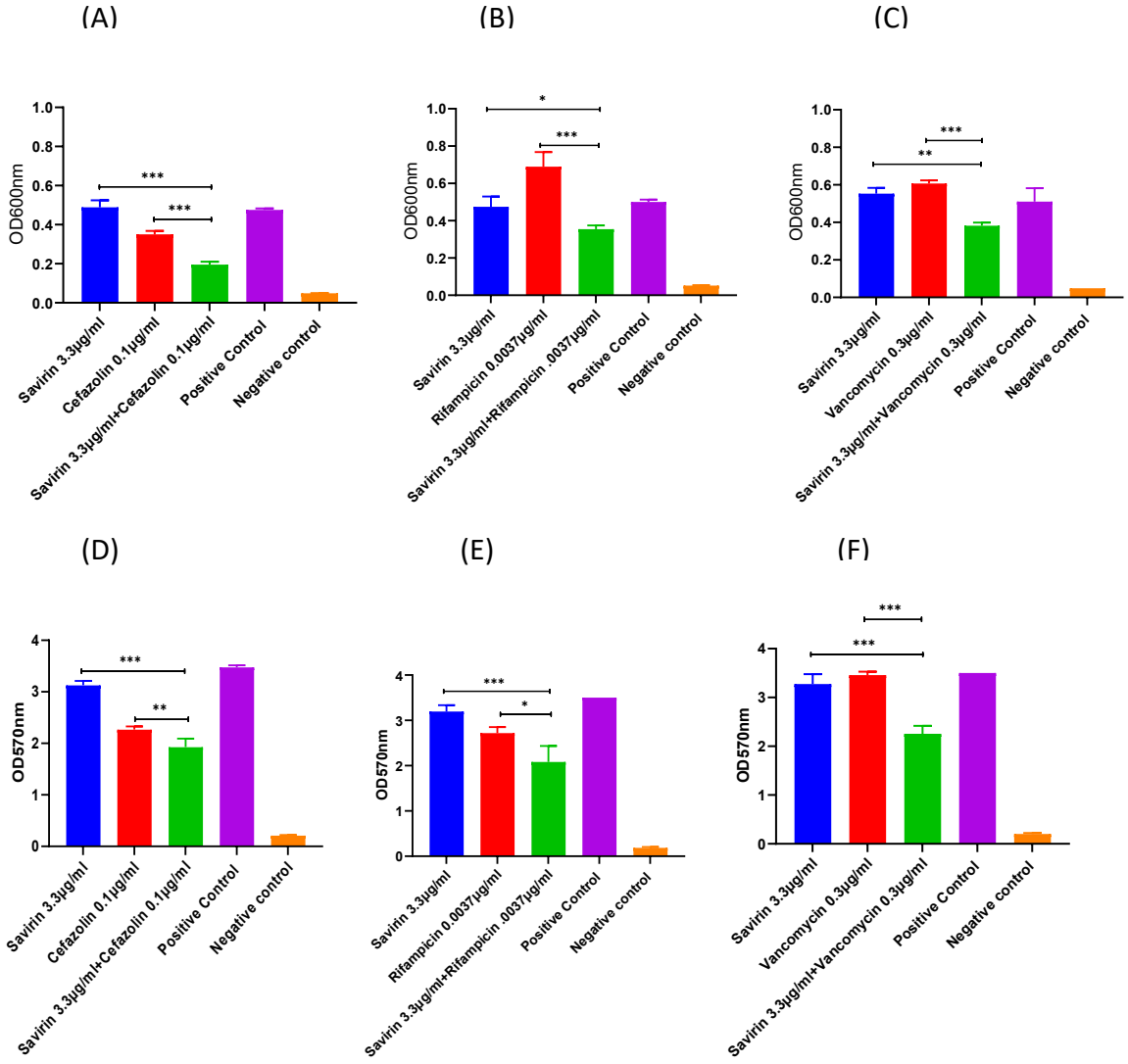


Figure 6. 2: Inhibition of planktonic (A, B, C) and biofilm (D, E, F) growth by combined savirin and antibiotics (cefazolin, rifampicin, and vancomycin) sub-inhibitory concentrations compared with savirin and antibiotics alone (***=p<0.001, **=<0.01, *=<0.05). Savirin concentration used in this experiment was sub-inhibitory. Savirin in combination with antibiotics showed enhanced antibacterial and antibiofilm activity against *S. aureus* compared with savirin alone.

Experiments were performed in triplicates (N=3) and data are presented as mean ± standard deviation (SD) with error bars indicating SD.

6.3.3. Effect of savirin on the expression of *S. aureus* biofilm-related genes

In TUHMSSA01 strain, savirin downregulated all the biofilm-related genes tested significantly (> 2-fold) in comparison with the untreated positive control (Figure 6.3).

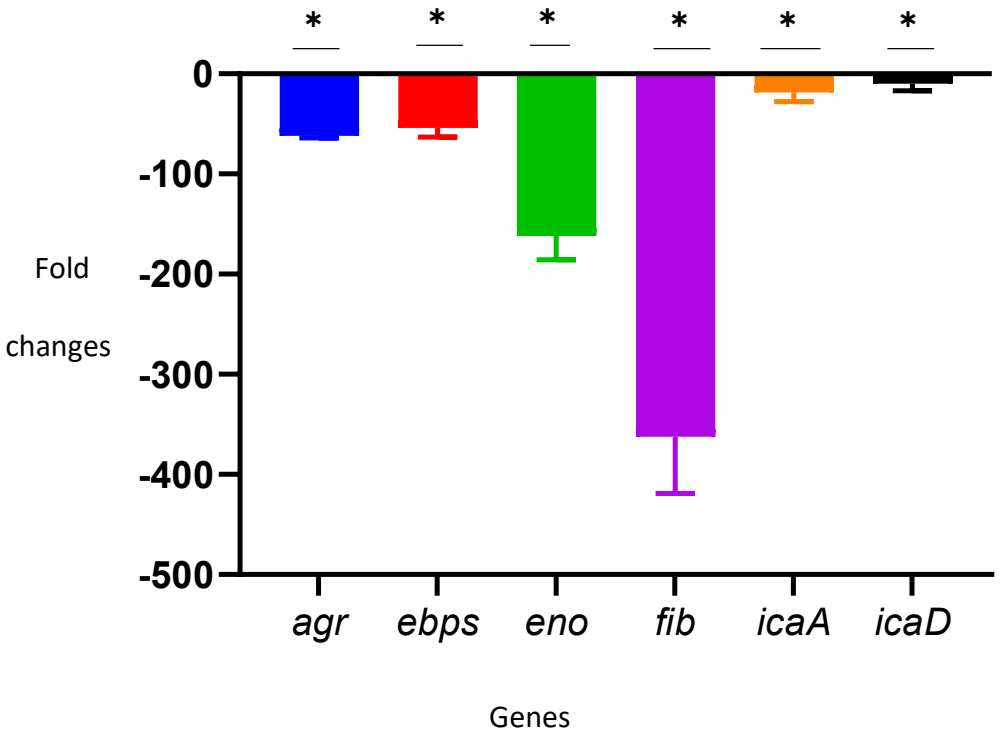


Figure 6. 3: Downregulation of biofilm pathway genes by savirin in TUHMSSA01 strain after 8hr of culture (*=downregulated > 2-fold). The reference gene used was *fema* and the experiment was performed in triplicate (N=3). To determine the effect of savirin (10ug/ml) treatment on

the *S. aureus* biofilm-related genes, comparative C_t ($\Delta\Delta C_t$) method was used and the results were expressed as mean fold changes ± standard deviation (SD) in comparison with savirin diluent (0.02% dimethylsulphoxide) treated control. The error bars indicate SD.

6.3.4. Effect of savirin and/or cefazolin treatment on bacterial concentrations on K-wire implants and peri-prosthetic joint tissues

Cefazolin was chosen to use in the animal experiment over other antibiotics tested *in-vitro* because this is the most commonly used antibiotic in arthroplasty surgery, and this antibiotic showed better *in-vitro* activity when combined with savirin. Savirin significantly reduced bacterial counts on K-wires removed from the femur of mice with experimentally-induced prosthesis associated septic arthritis in comparison with the PBS treated control (log10cfu/ml, 3.2 versus 1.6) ($p<0.05$). Similarly, savirin plus cefazolin reduced bacterial counts on both implants (log10cfu/ml, 3.2 versus 1) and peri-prosthetic tissues (log10cfu/ml, 7.1 versus 4.5) in comparison with PBS treated control ($p<0.01$) (Figure 6.4). The absence of an effect of cefazolin alone given at day 7 is keeping with persistence of *S. aureus* infection due to biofilm, indicating that this antibiotic failed to cure established biofilm. Tissue histology showed signs of *S. aureus* infection (Gram positive cocci and neutrophils) but there was no visual indication of reduction in the infection due to treatment as measured by cell/bacterial counts per microscopic oil immersion field.

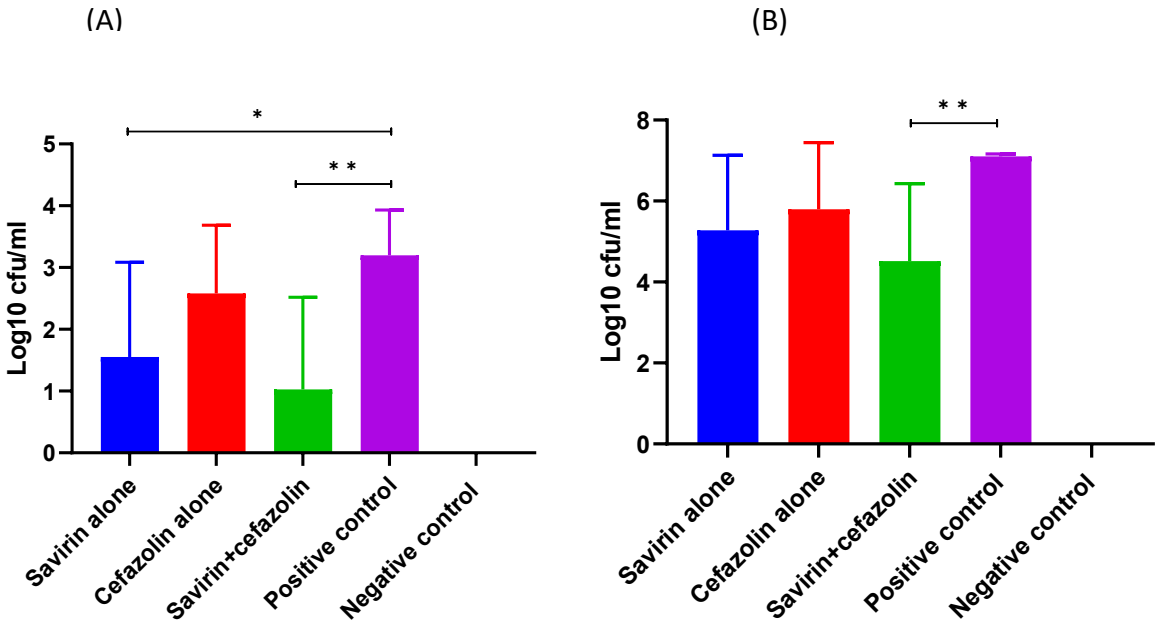


Figure 6. 4: Bacterial counts (N=8) on implant (A) and tissue (B) of different experimental and control groups on day 14 post-surgery (**p<0.01, *p<0.05). Savirin alone reduced bacterial concentration on K-wire, while savirin plus cefazolin reduced bacterial concentration on both K-wire and periprosthetic tissue. The data are presented as mean log10cfu/ml ± standard deviation (SD).

6.4. Discussion

Savirin is a small, lipophilic, novel synthetic molecule that may be suitable for drug development (18). This molecule inhibits *agr* quorum-sensing activation by preventing AgrA attachment to promoter regions and increases host mediated bacterial killing (18). The expression of many important *S. aureus* virulence factors are controlled by the *agr* quorum-

sensing system (18). Savirin has been reported to prevent as well as treat biofilm-related *S. aureus* infections in rodent skin and subcutaneous infection models (18). The efficacy of savirin to treat *S. aureus* prosthetic joint infection is unknown and its antibiofilm activity has not been fully characterised. Therefore, this study tested the antibacterial and antibiofilm *in-vitro* activity of savirin and its efficacy to treat *S. aureus* PJI in a mouse model.

Savirin showed *in-vitro* antibacterial and antibiofilm activity against both the *S. aureus* strains studied. This molecule also potentiated the *in-vitro* activity of selected antibiotics (cefazolin, rifampicin, and vancomycin) against both TUHMSSA01 and TUHMRSA02 strains used. Savirin downregulated all the key biofilm-related genes (*agr, ebps, eno, fib, icaA, icaD*) tested in the TUHMSSA01 strain. As shown in the chapter 4, *fema* was used as reference gene for the gene expression study. In the PJI mouse model, savirin reduced TUHMSSA01 bacterial infection. Only TUHMSSA01 strain was used in the animal study because this study aimed to test the efficacy of savirin in combination with cefazolin, the most commonly used antibiotic in arthroplasty surgery.

This study reported the MBC and MIC of savirin for *S. aureus* including MRSA to be 40μg/ml and 20μg/ml respectively. While Sully et al. did not demonstrate direct inhibitory activity of savirin against MRSA up to 2.33μg/ml another study reported the MIC of savirin against *S. aureus* to be 36.8μg/ml (18, 24). This indicates that savirin has direct antibacterial effect at higher concentration. However, the mode of direct *in-vitro* antibacterial activity of savirin is not known. This study also reported the dispersal of *in-vitro* mature biofilm (48hrs) and enhanced

antibacterial and antibiofilm activity of the antibiotics by savirin in both TUHMSSA01 and TUHMRSA02 strains. The *in-vitro* antibiofilm and bactericidal effect of savirin and its combined effect with antibiotics have not been previously studied.

The inhibitory role of savirin (5µg/ml) against the *agr* quorum-sensing system and a few other AgrA regulated genes, *hla*, *psm alpha*, *pvl* (*lukS*), have been reported previously (18). However, the same study showed no effect of savirin (5µg/ml) treatment in the expression of most of the biofilm-related genes by microarray analysis (18). Consequently inhibition of *agr*, a gene responsible for biofilm dispersal in *S. aureus,* by savirin (5µg/ml) would have been expected to enhance biofilm formation (60). However while 5µg/ml savirin had no effect, 10µg/ml showed significant antibiofilm activity in this study. This might be because *agr* has strain-specific roles in staphylococcal biofilm formation and dispersal, and *agr* disruption might have increased, decreased, or no effect in biofilm formation in different strains (119, 120). The discrepancy between the results of the previous and this study might also be related to the higher savirin (10µg/ml) concentration used in this study, and the different *S. aureus* strains and growth conditions used. For gene expression analysis, *S. aureus* was incubated with 5µg/ml of savirin for 5hrs in Tryptone Soy Broth (TSB) with AIP1 by shaking in the previous study (18), while the bacteria were cultured with 10µg/ml of savirin for 8hrs in GLB broth without shaking in this study.

The *ebps, eno,* and *fib* genes encode cell surface associated proteins and promote *S. aureus* adherence and colonisation (217, 238-240), while *icaA* and *icaD* induce bacterial slime

production (241). This study was not able to quantify gene expression in the TUHMRSA02 strain because savirin (10µg/ml) slowed down the growth of this strain (chapter 4). Hence, this study was unable to identify a stable and suitable reference gene for qRTPCR normalisation. However, this study concluded that the antibiofilm activity seen in the TUHMRSA02 strain due to savirin was probably associated with growth inhibition.

Chapter 5 showed the efficacy of an FDA approved drug ticagrelor to reduce the biofilm-related *S. aureus* PJI infection in an animal model. Similarly in the prosthetic joint infection mouse model, the novel molecule savirin significantly reduced bacterial counts on K-wires and savirin plus cefazolin reduced bacterial counts on both implants and peri-prosthetic tissues in comparison with the PBS treated control. This implies that savirin alone has *in-vivo* antibiofilm activity probably due to dispersal of the infection on the K-wire prosthesis but no antibacterial activity. Savirin instead disarmed the bacteria by inhibiting biofilm which was then cleared partially by cefazolin used on day 7. There was no significant reduction in the bacterial counts in both the implants and tissues due to savirin plus cefazolin treatment compared with savirin alone treatment. This is inconsistent with the *in-vitro* results, where savirin plus cefazolin had significantly enhanced activity compared with savirin alone. None of the treatments used in mice sterilised the implant or tissue infection even though the drugs showed complete *in-vitro* growth inhibition. This is probably due to the sub-inhibitory concentrations of savirin and cefazolin, to which *S. aureus* is being exposed to *in-vivo*. However, more animal studies to determine the concentrations of savirin and cefazolin in the blood or tissues are needed to

reach the definitive conclusions in this regard. Additionally, animal studies to establish that the *in-vitro* savirin inhibitory concentration for *S. aureus* growth can be safely used *in-vivo*, are needed. Savirin's pharmacodynamics and pharmacokinetics are not well understood as it is a novel molecule. It may be that the *in-vivo* diminished effect of savirin in this study relates to its rapid elimination before administering cefazolin to mice. Determination of savirin concentrations in the blood and knee joints of mice at different time points would provide insight into the drug concentration to which the *S. aureus* was exposed *in-vivo* and this could help to determine the optimal dose to sterilise infections in the mouse model.

There is only one other study that reported the influence of savirin on prevention and treatment of *S. aureus* skin and subcutaneous infections in a mouse model (18). In the previous study, savirin reduced infection even when administered 24 to 48hrs post infection establishment implying its effectiveness against established *S. aureus* mature biofilms (18). Savirin doses of 5μg and 10μg were used to prevent and treat skin and subcutaneous tissue infections induced with *S. aureus* infectious doses 2×10^7 to 4×10^7cfu (18). This study used a higher savirin dose (40μg) but a lower *S. aureus* infective dose (500cfu) than the previous study in PJI mouse model and confirmed that savirin reduced infection. The infective dose used in the PJI model was chosen to establish a chronic septic arthritis, while the savirin dose was chosen because of theoretical concerns of reduced penetration into joints or bones.

Since the detailed animal toxicity profile of savirin is not known, this study was unable to use higher savirin doses through different routes that might have sterilised the infection. Dose and

toxicity finding studies are needed to allow for further animal model experiments. Testing of savirin in large animal prosthetic joint infection models, using materials and techniques used in a modern arthroplasty surgery is recommended.

S. aureus did not develop resistance against low concentration of savirin (5µg/ml) in comparison with antibiotics (18). At 5µg/ml savirin does not directly inhibit bacteria but acts as a quorum-sensing (QS) inhibitor and disarms bacteria exerting low selection pressure (17). The possibility of quorum-sensing inhibitor resistance development among Gram negative bacteria has been postulated previously (250). Induction of dysfunctional *agr* has been reported, therefore the development of savirin (5µg/ml) resistance through the selection of *agr* dysfunctional mutants or stimulation of drug efflux requiring higher savirin concentration is possible (18, 251). Savirin's binding site to AgrA includes a known mutation in *agrA* in human isolates that have been tested (252). This mutation has been shown to occur in strains colonising the nose before the initiation of infection (252). These *S. aureus agrA* mutant strains have been shown to not be efficiently transmitted between patients (253). With this information in mind it may be that *agrA* mutant *S. aureus* strains would not be a serious problem particularly in relation to PJI. However, there may be the possibility of resistance development of *S. aureus* against the direct inhibitory higher concentration of savirin and this needs to be investigated.

From our results, it can be concluded that savirin should be considered for the development of an adjuvant therapy for the treatment of *S. aureus* PJI.

6.5. Chapter 6 summary

- Savirin, a lipophilic, small, novel synthetic molecule, has been used to prevent and treat *S. aureus* skin infections in animal models.
- The main aim of this study was to explore the application of savirin in a prosthetic joint infection (PJI) mouse model to determine its utility as a non-antimicrobial adjunct therapy to treat PJI.
- The *in-vitro* antibacterial and antibiofilm activity of savirin, with or without antibiotics (cefazolin, rifampicin, and vancomycin), against *S. aureus* were investigated by broth microdilution, and crystal violet staining method. The molecular mechanisms for *S. aureus* biofilm inhibition were studied using quantitative reverse transcriptase polymerase chain reaction. The *in-vivo* efficacy of savirin, alone and with cefazolin, was determined using a clinically relevant PJI mouse model.
- Savirin inhibited planktonic and biofilm *in-vitro* growth of *S. aureus,* showed enhanced inhibitory activity when combined with antibiotics, and downregulated the expression of key *S. aureus* biofilm-related genes (*icaA, icaD*, *eno*, *fib*, *ebps* and *agr*).
- Savirin, alone and combined with cefazolin, significantly reduced *S. aureus* PJI in a mouse model and therefore might enhance biofilm dispersal and efficacy of the currently available PJI treatments.

CHAPTER 7: *IN-VITRO* EFFECT OF SYNOVIAL FLUID FROM PATIENTS UNDERGOING ARTHROPLASTY SURGERY ON *STAPHYLOCOCCUS AUREUS* BIOFILM FORMATION INCLUDING THAT BY METHICILLIN RESISTANT STRAINS

7.1. Introduction

Prosthetic joint infections (PJI) complicate around 2% of arthroplasty surgeries (232). *Staphylococcus aureus* is the commonest cause of PJI being present in up to 57% of cases (4). Half of *S. aureus* PJI are caused by methicillin resistant *S. aureus* (MRSA) (4). Bacterial biofilms are intrinsic to the pathogenesis of PJI as they are recalcitrant to antibiotic treatment due to the presence of biofilm matrix protective barrier and metabolically inactive persister cells.

Pre-operative antibiotic administration including cefazolin reduces the incidence of PJI (254). Synovial fluid has been shown to have intrinsic antibacterial role and may also contribute to biofilm inhibition (25). In chapter 5 and chapter 6 the efficacies of ticagrelor and savirin, alone and in combination with cefazolin, to treat biofilm-related *S. aureus* PJI in mouse model were shown. These efficacies might also be related to how synovial fluid including that containing cefazolin affect the planktonic and biofilm growth of *S. aureus* including that of MRSA. Synovial fluid might also have contributed to the reduced infection due to ticagrelor and savirin adjuvant therapy. Additionally, there are no studies evaluating the anti-MRSA antibacterial and antibiofilm effect of synovial fluid including where it contains cefazolin. Therefore, in this study

the *in-vitro* anti-staphylococcal and antibiofilm activity of synovial fluid samples collected from arthroplasty patients administered cefazolin preoperatively were tested.

The aim of this study was:

1. To determine the *in-vitro* antibacterial and antibiofilm activity of synovial fluid including that containing cefazolin, from patients undergoing elective total knee arthroplasty surgery, against *S. aureus* including MRSA.

7.2. Materials and methods

7.2.1. Synovial fluid characterisation

Synovial fluid samples were collected from 26 patients undergoing elective total knee arthroplasty surgery (ethics approval number MHS20170808-01) (255). Intravenous cefazolin (2gm) was given prior to surgery as per Australian Guidelines (256). The synovial fluid samples were aspirated from the knee joint within 5min of the intravenous administration of cefazolin and just before surgical incision and tourniquet inflation. Up to 5ml of synovial fluid was collected from the study subjects. Total cefazolin concentrations were measured by ultra-high performance liquid chromatography-tandem mass spectrometry (257). Stationary phase Kinetex C8, 2.1×50mm analytical columns (Phenomenex, USA) and a mobile phase consisting of gradients of acetonitrile containing 0.1% formic acid were used. Cefazolin was detected through the monitoring of positive mode electrospray at multiple reaction monitoring (MRM) of 455.1→323.1. The mean total cefazolin concentration of the synovial fluid samples was

45.5±22.4μg/ml. One synovial fluid sample taken during the same project did not contain cefazolin as the percutaneous aspiration occurred prior to antibiotic being administered. The antibiofilm and antibacterial activity of the cefazolin-containing synovial fluid samples were compared with glucose Luria Bertani (GLB) broth containing the same concentration of cefazolin.

7.2.2. Planktonic and biofilm culture assays

Two clinical *S. aureus* strains, TUHMSSA01 (methicillin susceptible), TUHMRSA02 (methicillin resistant), and a type-MRSA strain (ATCC 1698) were used in this study. They were selected because they produced ample biofilm [optical density (OD) greater than four times the three standard deviations above the mean OD of the negative control] (198).

Experiments were performed in triplicate using cefazolin-containing synovial fluid samples from three different patients. Synovial fluid and GLB broth containing equal concentration of cefazolin were serially double diluted (46μg/ml to 0.04μg/ml) with GLB broth in microtiter plates. One synovial fluid sample with no cefazolin was also serially 2-fold diluted. The final test volumes of 100μl consisted of bacteria in concentration $1x10^6$cfu/ml added to synovial fluid or GLB broth. Higher bacterial inocula (1.5×10^8cfu/ml) were tested to assess for concentration dependent effects. Microtiter plates were then incubated for 24hrs at 37^OC. Planktonic culture cefazolin MICs were determined spectrophotometrically by measuring OD at 600nm. Culture supernatants were discarded, and residual biofilms were rinsed with water to remove any

remaining planktonic cells. Biofilms were fixed with 2% sodium acetate and stained with 1% crystal violet. Further washing in water, overnight drying, and the reconstitution of retained crystal violet with absolute ethanol was performed. Biofilm cefazolin MICs were determined by measuring OD at 570nm. The minimum concentrations of cefazolin that inhibited bacterial growth, indicated by no change in OD after 24hrs of incubation at $37^{O}C$, were taken as MICs.

The controls consisted of positive and negative GLB broth cultures along with a negative synovial fluid control. The dilutions of cefazolin-free synovial fluid that showed the complete inhibition of planktonic and biofilm growth were also determined.

The bactericidal effect of synovial fluids including that containing cefazolin was measured by plating wells showing growth inhibition. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test.

7.3. Results

The inhibitory effects of synovial fluid with and without cefazolin are shown in Figure 7.1. Synovial fluid completely inhibited the biofilm and planktonic growth of all three *S. aureus* strains at 16- to 32- fold dilutions. The complete inhibition of biofilm and planktonic growth of both MSSA and MRSA was shown in cefazolin-containing synovial fluid. The antibacterial effect of synovial fluids, at concentration where initial inhibition was seen, was bacteriostatic. At higher synovial fluid concentrations, bactericidal activity was shown with the reduction of initial bacterial concentration by ≥99.9% at the highest concentration. MICs for cefazolin in synovial fluid for planktonic and biofilm growth of all three strains was 0.7μg/ml compared with 1.4μg/ml for TUHMSSA01, and 23μg/ml for TUHMRSA02 and MRSA ATCC 1698 in glucose Luria Bertani (GLB) broth. This reduction in cefazolin MIC in synovial fluid, particularly for the MRSA strains, was due to the inhibitory role of synovial fluid alone. No bacterial-concentration specific effect was seen in all synovial fluid experiments, as the inhibition of *S. aureus* growth and biofilm formation including MRSA was equivalent in cultures using the bacterial concentrations of $1x10^6$cfu/ml and 1.5×10^8cfu/ml.

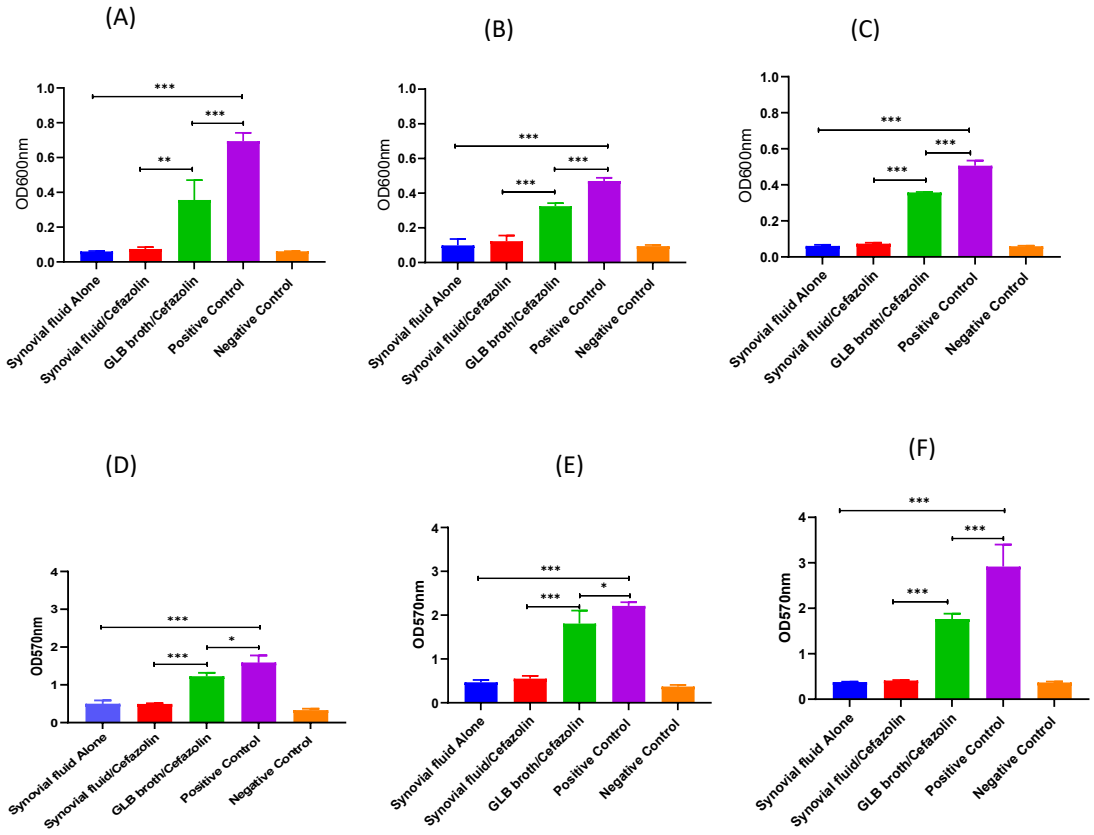


Figure 7. 1: Planktonic and biofilm growth of; TUHMSSA01 A and D, TUHMRSA02 B and E, and MRSA ATCC1698 C and F, in synovial fluid alone, synovial fluid containing cefazolin (0.7µg/ml), glucose Luria Bertani (GLB) broth containing cefazolin (0.7µg/ml), and GLB broth (positive control). The experiments were performed in triplicate and error bars indicate standard deviations, while the asterisks represent statistical significance (*=<0.05, **=<0.01, ***=<0.001). There was no measured difference between synovial fluid alone, synovial fluid containing cefazolin, and the negative control in any of these experiments.

7.4. Discussion

In this study, synovial fluid including that containing cefazolin inhibited the biofilm and planktonic growth of both MSSA and MRSA. It was demonstrated that synovial fluid alone was responsible for the antibacterial and antibiofilm activity. The measured cefazolin MIC in synovial fluid sample for one methicillin susceptible and two methicillin resistant *S. aureus* strains was 0.7µg/ml irrespective of whether the bacteria were in planktonic or biofilm state. The cefazolin MICs measured in GLB were higher for MSSA (1.4µg/ml) and MRSA (23µg/ml) strain.

Synovial fluid has previously been shown to have *in-vitro* anti-Gram positive (25), and anti-Gram negative effect (258). The antibacterial property of synovial fluid against *S. aureus, Staphylococcus epidermidis*, and *Streptococccus pyogenes* was shown in samples from non-infected joints (25). An earlier study of synovial fluid taken from patients with rheumatoid or degenerative arthritis demonstrated low level killing of *Escherichia coli* (258). There are no data on the antibiofilm activity of synovial fluid.

A study that reported on antibacterial efficacy in relation to antibacterial impregnated cement showed anti-MSSA but not MRSA killing in cefazolin-containing synovial fluid (259). The anti-MSSA activity was detected by method involving inhibition zones measured around discs impregnated with 35µl of synovial fluid (259). MSSA inhibition in synovial fluid was present for up to 32hrs after cefazolin administration (259). The more sensitive, microdilution method used

in this study may account for the result that shows MRSA inhibition by synovial fluid, including that containing cefazolin. In another study, bacterial clumping was suggested as the cause of low *S. aureus* counts in synovial fluid (260). However, this study demonstrated significantly reduced bacterial viable counts.

The direct antibacterial activity of synovial fluid may be due to its high concentration of hyaluronic acid (261). The intrinsic antibiofilm effect of synovial fluid could be a result of its prevention of bacterial attachment (262). Additional antibiofilm and antibacterial mechanism may be present in synovial fluid from inflamed joints. Bactericidal peptides released from immune-cells (neutrophils, monocytes, and macrophages) in the presence of inflammation act through the disruption of bacterial cytoplasmic membrane and membrane-associated physiological activity (25, 263). However, the isolation and study of the antibacterial and antibiofilm activity of the individual components of synovial fluid may give better understanding on its mechanism of activity. In this study the synovial fluid samples were taken from patients having elective arthroplasty surgery on non-inflamed joints.

The *in-vitro* data of this study suggest that synovial fluid has intrinsic properties that inhibit *S. aureus* biofilm growth including that due to MRSA. This may explain the suggestion from retrospective studies that routine preoperative cefazolin is effective in the prevention of MRSA PJI. At an institution with a high prevalence of MRSA infection, low rates of surgical site infection (1.06%) were maintained with cefazolin prophylaxis and strict infection control measures (264). Less than half (38/79) of *S. aureus* infections causing early (<90 days post-

surgery) PJI, were resistant to methicillin the others being susceptible to the cefazolin used pre-arthroplasty surgery (265). Another retrospective study of PJI rates pre- and post a change in the preoperative antibacterial regimen from cefazolin to vancomycin showed an overall reduction in PJI rates but no significant reduction of infections due to MRSA (266).

A planned, randomised controlled trial of preoperative cefazolin with and without vancomycin in joint arthroplasty surgery will provide more information in this regard (267). Considering the results of this study, it will be interesting to understand whether MRSA infections are significantly more common than MSSA PJI where cefazolin is used as the sole preoperative antibiotic. Benefits from MRSA PJI prevention with preoperative vancomycin may need to be balanced against an increased risk of infection due to vancomycin-resistant organisms (267). The current absence of evidence of MRSA prevention of human PJI by cefazolin may not represent evidence of absence of this effect.

On the basis of the results of this study, it may be worth investigating any apparent MRSA preventive success of preoperative cefazolin prophylaxis for PJI as being related to intrinsic antibiofilm activity of synovial fluid. Ticagrelor and savirin adjuvant therapies studied in chapter 5 and chapter 6 have been shown to reduce *S. aureus* prosthetic joint infection in animal model. Synovial fluid might also have contributed to this treatment success. However, studying the antibacterial and antibiofilm activity of synovial fluid with exogenously added ticagrelor or savirin might give better understanding in this regard.

7.5. Chapter 7 summary

- Bacterial biofilm is a key component in the pathogenesis of prosthetic joint infection (PJI). Synovial fluid has been shown to have inhibitory activity against planktonic bacteria. However, the contribution of synovial fluid in the inhibition of *Staphylcoccus aureus* including MRSA planktonic and biofilm form is unknown.
- The main aim of this study was to test the *in-vitro* antibacterial and antibiofilm activity of synovial fluid, including that containing cefazolin, against MSSA and MRSA.
- Antiplanktonic and antibiofilm activity of synovial fluid collected from patients given preoperative cefazolin while undergoing elective arthroplasty surgery were determined by broth microdilution and crystal violet staining method.
- Synovial fluid inhibited the planktonic and biofilm culture of MSSA and MRSA. Cefazolin-containing synovial fluid had greater antibacterial and antibiofilm activity than the same cefazolin concentration in glucose Luria Bertani (GLB) broth. This may explain the apparent effect of cefazolin in the prevention of MRSA PJI.

CHAPTER 8: GENERAL DISCUSSION AND FUTURE PERSPECTIVE

8.1. General discussion

While prosthetic joint infection (PJI) is an infrequent complication of an arthroplasty surgery it may lead to devastating outcomes. PJI results in pain and impaired mobility in an arthroplasty patient, and substantial economic burden on the health care system (145, 204). Currently, treatment for PJI consists of surgery with debridement and implant retention or prosthesis replacement being the main alternatives (146). Surgery is followed by the prolonged courses of antibiotics (146). Current treatments therefore are expensive, traumatic, and have significant failure rates of up to 56% (11). The involvement of biofilm producing bacteria, mainly *S. aureus*, in infection makes PJI treatment difficult (4). A number of different strategies including adjuvant therapies have been studied for their effect on the treatment of biofilm-related infections but none of them has reached the treatment developmental stage of clinical trials in human (268, 269). Ticagrelor and the antivirulence molecule savirin have been used successfully for the treatment of biofilm-related *S. aureus* infections in animal models (18, 19). However, the effectiveness of these molecules in the treatment of biofilm-related *S. aureus* prosthetic joint infection is not known. This study aimed to assess the efficacy of savirin or ticagrelor to treat *S. aureus* prosthetic joint infection, using a clinically relevant mouse model, and the molecular mechanisms underlying their antibiofilm activities. This study also investigated the *in-vitro* antibacterial and antibiofilm activity of cefazolin-containing synovial fluid from arthroplasty patients.

The animal model used in this project is a Kirschner (K)-wire prosthetic joint infection mouse model (203). This is a clinically relevant simplest animal model of prosthetic joint infection in which K-wire is inserted into the femoral intramedullary canal leaving 1mm protrusion into the knee joint space. This protrusion represents the prosthetic joint implant of a human arthroplasty, and when gets coated with host proteins, fibrinogen, fibronectin, and laminin, provides a rich environment for staphylococcal attachment and biofilm formation (148). The housing of mice and surgical procedures in them is relatively easy. Therefore the mouse model used in this study is a less expensive yet effective animal model for preliminary animal study before starting an expensive large animal model study.

Chapter 4 determined the most appropriate reference genes for studying the effects of savirin or ticagrelor treatment on biofilm-related gene expression in *S. aureus*. While there are no data on the effect of ticagrelor treatment in the expression of *S. aureus* genes, these have been reported for savirin treatment (18). Sully et al. used the *16s* reference gene to study the effect of 5µg/ml savirin on expression of the *agr* gene (18). In the microarray analysis, there was no effect of savirin (5µg/ml) treatment on most of *S. aureus* genes except *agr* and a few other AgrA regulated genes, such as *hla*, *psm alpha*, *pvl* (*lukS*) (18). In this study among sixteen candidate reference genes studied, only *fema* followed by *gapdh*, and *16s* were eligible reference genes for savirin (10µg/ml) treatment experiment, while *gmk* followed by *rpoB*, and *rpoD* were the most suitable reference genes for ticagrelor treatment. The *16s* gene also met the criteria (SD<1) for a reference gene in ticagrelor treatment experiment in *S. aureus*. The *16s* is a

frequently used reference gene in gene expression studies in *S. aureus* (214, 270). This gene is present in a high target copy number and regulates an essential bacterial biochemical activity that leads to the translation of 16s ribosomal rRNA subunit (215, 270). However, since the transcripts of 16s gene do not represent the overall *S. aureus* mRNA, this gene might not be an ideal internal control (215). The discrepancy between the results of this study and Sully et al. might be because of the higher savirin concentration (10µg/ml) being used in this study and the difference in bacterial strains and growth conditions used in the two studies.

Chapter 5 demonstrated the *in-vitro* antibacterial and antibiofilm activity of ticagrelor against *S. aureus* including methicillin resistant *S. aureus* (MRSA). Ticagrelor showed enhanced activity when combined with antibiotics (cefazolin, rifampicin, and vancomycin), that are commonly used to treat PJI. The *in-vitro* antibacterial and antibiofilm activity of ticagrelor against *S. aureus* has also been reported in a previous study (19). Different *S. aureus* strains and methods were used in this and the previous study. This study used broth microdilution method and checkerboard assay to study the *in-vitro* antibacterial and antibiofilm activity of ticagrelor against *S. aureus*. The previous study used disc diffusion method and time kill assay (19). These factors are the probable contributors to why the level of antibacterial activity of ticagrelor was lower in this study compared with the previous study and the enhanced antibacterial activity of rifampicin when combined with ticagrelor was not reported in this study. However, the combined antibiofilm activity of ticagrelor and antibiotic has not been reported previously.

Ticagrelor is also known to have antibacterial and antibiofilm activity against *S. epidermidis*, vancomycin resistant *Enterococcus* (VRE), and *Streptococcus agalactiae* but not against gram negative bacteria (19). Ticagrelor's antibacterial and antibiofilm activity against another Gram positive bacterium *Clostridium difficile* has also been reported (246). The leakage of cellular components has been identified as a possible mechanism for bactericidal activity (246). Although, this study did not report any strain specific antibacterial activity of ticagrelor against *S. aureus* (i.e. the level of antibacterial activity was same for both strains used), this has been reported in *Clostridium difficile* (246). Study using multiple *S. aureus* strains, including type strains such as ATCC25923 and USA300, is required to make a definitive conclusion about the possibility of strain specific activity of ticagrelor. There was no effect of antibiotic (cefazolin and vancomycin) resistance on ticagrelor's *in-vitro* activity because this molecule showed anti-MRSA and anti-VRE activity (19). This indicates that the mode of action of ticagrelor is not same as cefazolin and vancomycin.

In an animal model, the conventional oral antiplatelet ticagrelor dosages (3mg/kg loading dose then 1.5mg/kg twice daily from day four to day seven post-surgery) alone and in combination with cefazolin (single 2.5mg/kg on day seven) reduced the *S. aureus* prosthetic joint infection. Ticagrelor alone reduced bacterial concentration on both K-wire and periprosthetic tissue. As in this study, an earlier study using pre-contaminated subcutaneous disk showed the *in-vivo* antibacterial and antibiofilm activity of ticagrelor against *S. aureus* (19). Ticagrelor in combination with cefazolin reduced bacterial concentration only on K-wire. The efficacy of this

combination to clear the bacterial infection was inferior to ticagrelor alone indicating the presence of *in-vivo* antagonistic effect.

The earliest possible introduction of ticagrelor therapy after surgery might be effective to prevent biofilm-related infection in an arthroplasty surgery. No increase in bleeding events was reported when the antiplatelet drugs were resumed just after the hip or knee arthroplasty (242). However this observation may be unique to this particular study and the chances of bleeding-related complication when antiplatelet drugs are resumed immediately post-surgery still exist. To minimise the risk of bleeding, it is recommended to resume antiplatelet drugs after 72hrs (271). So, in this project ticagrelor treatment was started after 3 days when the sufficient haemostasis and wound healing were achieved. In this study, complications associated with ticagrelor-related bleeding were not encountered and the overall outcome of the arthroplasty surgery in the animal model improved.

Chapter 6 presented the *in-vitro* antibacterial and antibiofilm activity of savirin against *S. aureus* and enhanced activity of cefazolin, rifampicin, and vancomycin when used in combination with savirin. The direct antibacterial activity of savirin (36.8µg/ml) has already been reported (24). However, it is not known whether the inhibitory concentration of savirin is safely achievable *in-vivo*. Savirin's antibiofilm activity and its combined activity with antibiotics have not been reported before. Savirin (single 40mg dose just after surgery) alone and in combination with cefazolin (single 2.5mg/kg on day seven) reduced *S. aureus* infection in a prosthetic joint infection animal model. The savirin dose used in this study was found to be safe when tested in

Vero cell line culture. Building on the *in-vitro* results of savirin that showed better activity against immature biofilm than mature biofilm savirin was used immediately after surgery. Only one previous study reported the efficiency of savirin to prevent and treat *S. aureus* skin and subcutaneous infection in mouse models (18). This earlier study investigated the efficacy of savirin to treat acute infections and concluded that savirin downregulates the *agr* quorum-sensing system, an important virulence factor determinant in *S. aureus*, and promotes host immune cell mediated clearance of skin infection. This project studied the efficacy of savirin on PJI animal model, representative of chronic infection, and savirin alone reduced bacterial concentration in K-wire but not in periprosthetic tissue. This might be because savirin showed *in-vivo* antibiofilm activity but not *in-vivo* antibacterial effect. Savirin perhaps neither promoted host immune system mediated killing of *S. aureus* in the knee joint, a very different environment for bacterial survival than skin, nor the savirin dose used in this study had direct *in-vivo* antibacterial activity. Savirin instead disarmed the bacteria by inhibiting biofilm, which was then cleared partially by cefazolin used on day 7. Staquorsin, a savirin analogue, has also been known to be a potent *agr* quorum-sensing inhibitor and has been used successfully to treat *S. aureus* skin abscess in a murine model (24). As in savirin, *S. aureus* does not develop resistance to staquorsin (24).

The suboptimal cefazolin dose was used purposefully in the animal studies of this project to enable biofilm growth and was not able to sterilise the infection even when combined with savirin or ticagrelor. Therefore, it is recommended that future research build on the findings

presented here by assessing the doses required to induce the sterilization of *S. aureus* infection when used in combination with ticagrelor or savirin molecule. Ticagrelor or savirin molecule alone also sterilised the *in-vitro S. aureus* growth and therefore there might be the possibility of achieving sterilization of the *in-vivo* infection. The ticagrelor or savirin molecule doses used in this study perhaps were not sufficient to sterilise the *in-vivo* infection. Animal models with higher and extended cefazolin and ticagrelor or savirin doses are recommended. In a clinical context, for a successful cure of a PJI it is necessary to sterilise the infection. No earlier data on the treatment strategies used in this project was available for PJI. Therefore, in this study the non-toxic doses of ticagrelor or savirin molecule and the suboptimal dose of cefazolin were used. The suboptimal cefazolin dose used enable biofilm growth so as to see the combined effect. These treatment regimes reduced but did not sterilise the infection and provide the baseline data for future researches. Ticagrelor dosage used in this project relate to human, while for savirin highest possible non-toxic dose as confirmed by Vero cell-line culture was used. The concentration of the drugs to which *S. aureus* was exposed *in-vivo* might be low compared with *in-vitro*.

Chapter 5 and chapter 6 also presented the molecular mechanism underlying the antibiofilm activity of ticagrelor and savirin respectively. Ticagrelor showed strain specific downregulation of biofilm-related genes with the *fib,* and *icaD* genes being downregulated in TUHMSSA01 and *ebps*, *eno*, *fib*, and *icaD* in TUHMRSA02. Similarly, savirin downregulated all the biofilm-related genes, *icaA, icaD, fib, ebps, eno,* and *agr,* studied. While the effect of ticagrelor treatment on

biofilm-related genes in *S. aureus* has never been studied before, this has been investigated for savirin. A previous study using microarray analysis reported that savirin (5µg/ml) downregulates *agr* but does not affect other biofilm-related genes (18). This discrepancy between the previous and this study might be due to higher savirin concentration (10µg/ml) used in this study and the difference in bacterial strains and growth conditions used in the two studies. Genes, *eno*, *ebps*, and *fib* help in *S. aureus* attachment and colonisation through the expression of cell wall associated proteins (217, 238-240); *icaA* and *icaD* produce biofilm matrix (241). However, the expression of these biofilm-related genes might be strain specific (213). Additionally, the role of *agr* in biofilm formation might also be strain specific as *agr* helps in *S. aureus* biofilm dispersal, while amyloid fiber, a product of *agr* quorum-sensing, is known to stabilise biofilm (60, 70). Since biofilm formation is regulated by quorum-sensing, antibiofilm activity involves a combination of lowering of bacterial concentration and biofilm-related gene expression.

While both the adjuvant therapies using savirin or ticagrelor reduced prosthetic joint infection in this study, caution should be taken while interpreting the results. In this project, only two clinical *S. aureus* strains were used in the *in-vitro* and molecular experiments, and one in the *in-vivo* experiment. The *in-vivo* data generated in this project are very preliminary to make a conclusion about their clinical implication. More studies using different *S. aureus* strains in the same or different prosthetic joint infection animal model than those used in this project are recommended. However, from the results of this project it can be concluded that savirin and

ticagrelor are worth investigating for the development of adjuvant therapies for the prevention and/or treatment of *S. aureus* PJI. An added benefit is *S. aureus* does not develop resistance against savirin or ticagrelor as easily as it does with conventional antibiotics. Additionally, if the benefits of ticagrelor for the treatment of biofilm-related *S. aureus* PJI are confirmed, repurposing of this Food and Drug Administration approved drug might be very easy.

A range of other adjuvant therapies have been tested for their effectiveness in the treatment of *S. aureus* PJI in different animal models, however none of them has reached the human clinical trial stage. An adjuvant therapy using PGE_1 vasodilator in combination with ceftriaxone showed significant reduction in an orthopaedic implant-related infection in a diabetic mouse model (272). The efficacy of this vasodilator for the treatment of orthopaedic implant-related infection might be related to increased blood perfusion leading to easy antibiotic delivery to infection site and the inhibition of platelet aggregation that is needed for *S. aureus* biofilm formation (272).

Morris et al. used bacteriophage adjuvant therapy with no overall reduction in *S. aureus* prosthetic joint infection in a rat model (248). However, implants coated with both lytic phage and linezolid showed significant reduction in bacterial adherence without the development of drug resistance in a *S. aureus* PJI mouse model (273). Similarly, bacteriophage PlySs2 and vancomycin reduced bacterial concentration on periprosthetic tissues and implants significantly in a debridement, antibiotics, and implant retention (DAIR) mouse model (274). The difference between results of the different studies using bacteriophage might be due to different animal

models, bacterial strains, treatment regime, and bacteriophage used in different studies. There is a case report in which bacteriophage followed by DAIR improved final outcome, with significant clinical improvement, and restored loss of function in a relapsing *S. aureus* PJI (275). Similarly, in another case report an adjunctive therapy with bacteriophage combined with antibiotic eradicated chronic relapsing periprosthetic joint infection and femur osteomyelitis (276). These reports suggest how close an adjuvant therapy such as that using bacteriophage is from human clinical trials.

In addition to ticagrelor or savirin-antibiotic study, experiments to assess the *in-vitro* antibacterial and antibiofilm activity of synovial fluid from arthroplasty patients against *S. aureus,* including MRSA, were also conducted (see chapter 7). Building on earlier study that demonstrated the antibacterial property of synovial fluid (25), the findings in this thesis demonstrated the efficacy of synovial fluid alone and that containing cefazolin in reducing biofilm and planktonic growth of *S. aureus,* including MRSA. While earlier study only reported the antibacterial activity of synovial fluid, this study has advanced knowledge that showed inhibition of biofilm formation and anti-MRSA activity. In an era of growing concern over antibiotic resistance, the demonstrated efficacy of synovial fluid from arthroplasty patients in these *in-vitro* studies may explain the effect of cefazolin observed in the prevention of MRSA PJI (264, 277). In addition to the novel findings outlined here, the results have also provided valuable insights for future research in this field and these are outlined in the next section.

8.2. Recommendations for future research

The experimental data presented in this thesis provide more evidence on the effectiveness of antiplatelet drug ticagrelor and antivirulence molecule savirin for the treatment of biofilm-related *S. aureus* prosthetic joint infection (PJI) and the molecular mechanisms for their antibiofilm activities. Additionally, the data also showed the *in-vitro* efficacy of synovial fluid, alone and when containing cefazolin, from patients undergoing elective arthroplasty surgery to prevent the planktonic and biofilm growth of MRSA. This study provides a platform for future studies to enhance efficacy of the current treatment and prophylaxis for *S. aureus* PJI. Recommendations for future research aimed at the treatment and prophylaxis of PJI include:

8.2.1. Assessment of an extended dose of savirin and ticagrelor in the treatment of *S. aureus* PJI

To assess the doses required to eradicate *S. aureus* infections completely, extended therapy using higher concentrations of the drugs for a longer period of time than used in this study are worth investigating. There are limited data on the toxicity of savirin in animals, therefore studies investigating different doses (of the ticagrelor or savirin molecule) and its' associated toxicity on animals are needed. Additionally, the pharmacokinetics and pharmacodynamics of savirin are not known and require further studies on the optimal dose and route for drug administration in animal models.

Similarly, although ticagrelor is a Food and Drug Administration (FDA) approved drug, its pharmacokinetics and pharmacodynamics especially in relation to its antimicrobial and antibiofilm property, and as an adjunct to cefazolin in mice are unknown. Measuring savirin and ticagrelor concentration in the blood and knee joint tissue of mice at different time points may yield a better understanding on the concentration of the drugs to which *S. aureus* was exposed *in-vivo,* and may help to further optimise the doses for pre-clinical studies. For this blood and joint tissues including synovial fluid could be collected from experimental animals at different time points after administering of the drugs and the concentrations can be measured by liquid chromatography-tandem mass spectrometry (257). The overall hypothesis of this project was that the infection would be sterilised with the combined therapies. Therefore due to time and resource constraints, determination of drug concentration was out of the scope of the current aims of this project.

8.2.2. Studies in larger animal models, using the materials and techniques used in modern arthroplasty surgery

In this study, a K-wire mouse model of *S. aureus* PJI was investigated. Orthopaedic grade stainless steel K-wire protruding into the knee joint space was used as a proxy, representative of a prosthetic joint (203). Although clinically relevant, this mouse model is very simple and may not represent the pathogenesis in human PJI. No baseline data was available for the use of savirin and ticagrelor for the treatment of PJI in an animal model. The use of mice is less demanding and relatively less expensive, yet effective for the generation of preliminary animal

data before starting an expensive large animal model study. Modern arthroplasty uses titanium implants and other biomaterials. Therefore based on the findings of this project, studies that use large animal PJI models with the same materials and techniques used in modern arthroplasty are recommended to yield results more aligned to the actual treatments used in contemporary clinical practice.

8.2.3. Efficacy of combination treatments of savirin and ticagrelor, or in combination with other antivirulence molecules, or other antibiotics

Savirin and ticagrelor in combination may be tested for their efficacy to treat *S. aureus* PJI. Additionally, testing savirin or ticagrelor with other antivirulence molecules or antimicrobial peptides or other antibiotics is recommended. This might give more understanding on the combined effect of these molecules with other drugs and might help to identify the best combination for development of an adjuvant therapy. Additionally, savirin or lower ticagrelor doses that do not cause bleeding are recommended to be tested for their effectiveness as adjuvant prophylactic agents for *S. aureus* PJI.

8.2.4. The efficacy of savirin or ticagrelor on MRSA PJI mouse models

MRSA PJI is on the rise and there are limited treatment options (4). This project showed the *in-vitro* antibacterial and antibiofilm activity of savirin or ticagrelor against MRSA. Since this bacterium is among the most important bacterial pathogens in PJI, adjuvant therapies with

savirin or ticagrelor may be tested for their effectiveness in the treatment of MRSA PJI in an animal model.

8.2.5. Testing cefazolin antibiotic prophylaxis for the prevention of MRSA PJI in animal model

Synovial fluid including when containing cefazolin showed the *in-vitro* antibacterial and antibiofilm activity against MRSA. Therefore, it is recommended to investigate the efficacy of cefazolin to prevent biofilm-related MRSA PJI in an animal model.

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CHAPTER 9: APPENDICES

9.1. Preparation of Luria-Bertani (LB) broth

Ten grams tryptone, 10gm sodium chloride, 5gm yeast extract, (5gm d-glucose for 0.5% glucose LB broth) were dissolved in distilled water and final volume was adjusted to 1000ml. The solution thus formed was autoclaved for 15min at 15psi pressure and 121^{O}C temperature. The LB broth was then stored at 4^{O}C.

9.2. Preparation of Luria-Bertani (LB) agar

Ten grams tryptone, 10gm sodium chloride, 5gm yeast extract, 15gm agar were dissolved in distilled water and final volume was adjusted to 1000ml. The solution thus formed was autoclaved for 15min at 15psi pressure and 121^{O}C temperature. The agar was left for some time to cool to 56^{O}C and poured into disposable plastic petriplates. The plates were left at room temperature for solidification and the solidified plates were stored at 4^{O}C.

9.3. Preparation of mannitol salt agar (MSA)

One hundred and eleven grams of mannitol salt agar powder was dissolved in 1000ml of distilled water, boiled to dissolve, and autoclaved at 121^{O}C for 15min at 15psi pressure. The MSA plates were prepared and stored following the methods used for LB agar plates.

9.4. Preparation of phosphate buffer solution (PBS)

Five phosphate buffer tablets from Sigma-Aldrich were dissolved in 1000ml distilled water and autoclaved at 121^{O}C and 15psi for 15min. The PBS solution was then stored at 4^{O}C.

9.5. Preparation of ticagrelor solution

For the *in-vitro* and molecular studies, 5mg ticagrelor (stored at -20^{O}C) was dissolved in 1ml of absolute dimethylformamide (DMF). Ticagrelor 200µg/ml solution was prepared by using 1 part absolute DMF and 5 parts 0.5% glucose containing LB (GLB) broth. The solution was prepared fresh and diluted as required. For animal studies, a 90mg ticagrelor tablet was dissolved in sterile PBS, diluted as required, and used within 2hrs of its preparation.

9.6. Preparation of savirin solution

For the *in-vitro* and molecular experiments, 50mg savirin powder (stored at room temperature) was dissolved in 1ml absolute dimethylsulphoxide (DMSO). The final volume was adjusted to 40ml by adding GLB. The pH was adjusted to 9.5 by the addition of 1.15ml of 1M NaOH. The solution was diluted to final savirin concentration 80µg/ml, pH 7, and DMSO concentration 0.16%. The solution prepared thus was aliquoted in 15ml volume and stored at -80^{o}C. For the animal experiments, 50mg savirin powder was dissolved in 2ml absolute DMSO. Then the solution was diluted with sterile PBS to final savirin concentration 0.4mg/ml and the pH was adjusted to 11 using 1M NaOH. For positive control 1.6% DMSO in sterile PBS at pH 11 was used.

9.7. Preparation of vancomycin and cefazolin solution

Lyophilized vancomycin, and cefazolin powders (Alphapharm pharmaceutical company, Australia) were reconstituted using sterile water. The solutions were then diluted as required.

9.8. Preparation of rifampicin solution

Rifampicin powder (32mg) from Thermo-fisher scientific was dissolved in 2ml DMSO and then diluted using GLB broth as required.

9.9. Preparation of ketamine/xylazine for anaesthesia

Ketamine (100µl) and xylazine (10µl) were added to sterile distilled water to make a final 1ml solution. Each mouse was injected with 10µl per gram of it's body weight.

9.10. Preparation of buprenorphine for analgesia

Analgesic injection was prepared as follows: 2ml buprenorphine (stock solution of 0.3mg/ml) was added to 38ml PBS to make a final 40ml solution. Seven µl of this solution was injected per gram of mouse body weight. Oral analgesic was compiled by adding 5ml buprenorphine stock solution to 160ml of drinking water.

9.11. Preparation of crystal violet and sodium acetate

Ten grams crystal violet powder was first dissolved in 200ml methanol followed by the addition of 800ml distilled water. The solution formed thus was 1% crystal violet. Two percent sodium acetate was prepared by dissolving 10gm sodium acetate in 500ml distilled water.

9.12. Electrophoresis, nanodrop, and qubit

Two grams agarose powder was dissolved in 200ml of Tris-acetate-EDTA (TAE) buffer using a microwave oven. The gel formed was cooled in running tap water and 10µl gel-red was added. The solution was poured into a gel holder and left until set. A comb was used to make wells in gel. The gel was placed in a gel tank and the tank was filled with TAE buffer until the liquid ran over gel. DNA loading dye was added to DNA samples (2µl in 10µl DNA sample) and the DNA samples were added to wells in gel. Gel tank was adjusted at 90V for 1.5hrs and allowed to run until the samples moved two-third down the gel sheet (from black to red). Power for gel tank was turned off and a final image was captured using a UV imager and imaging software. Nanodrop, and qubit were used as per the instruments' instruction manuals.

9.12.1. Preparation of loading dye

DNA loading dye was prepared by mixing 3.9ml glycerol, 500µl 10% sodium dodecyl sulfate (SDS), 200µl 0.5M EDTA, and 0.025g brilliant blue followed by adjusting the volume to 10ml using sterile distilled water.

9.12.2. Preparation of Tris-Acetate-EDTA

First EDTA was dissolved in distilled water by adding NaOH pellets. Then, to prepare 50X TAE buffer 242gm Tris-base was dissolved in 700ml distilled water. The final volume was adjusted to 1000ml (pH 8.5) by adding 57.1ml of 100% glacial acetic acid, 100ml of 0.5M EDTA, and distilled water. The solution was stored at room temperature. The working solution was prepared by adding 1ml TAE buffer to 49ml distilled water.

9.12.3. Reconstitution of primers

Ten µl PCR-grade water per nano-mole of a primer was added and the primer was reconstituted by briefly centrifuging and then vortexing.

9.13. Histological studies

For histology study, the mice samples were fixed in 10% formalin for 24hrs followed by decalcification for 5 days. The decalcification solution was prepared by adding 160ml formic acid and 100ml concentrated formalin to 1740ml distilled water. Tissues were processed using an automated Leica Histocare pearl processor and were embedded in paraffin wax. Then, tissue sections of 5µm thickness were prepared using a microtome. The rodent tissue processing cycle used is presented in Table 9.1.

Reagent	Duration (minutes)	Temperature (^{O}C)
Ethanol 70%	10	45
Ethanol 80%	20	45
Ethanol 95%	20	45
Ethanol 100%	20	45
Ethanol 100%	20	45
Ethanol 100%	20	45
Xylene	25	45
Xylene	25	45
Xylene	25	45
Paraffin	20	58
Paraffin	20	58
Paraffin	20	58

Table 9. 1: Rodent tissue processing cycle

9.14. Haematoxylin and eosin (HE) staining

9.14.1. Preparation of HE staining reagent

Mayer's haematoxylin was prepared by dissolving 0.01gm thymol, 2gm citric acid, 100gm aluminium ammonium sulphate, 0.4gm sodium iodate, and 2gm haematoxylin in 2000ml

distilled water. Young's Eosin was prepared by dissolving 5gm calcium chloride, 5gm erythrosine, and 15gm Eosin in 2000ml distilled water. Similarly, Scott's tap water substitute was prepared by dissolving 8.75gm sodium bicarbonate and 50gm magnesium sulfate in 2500ml distilled water. In HE staining, nuclei stain dark blue while cytoplasm, connective tissue, and red blood cells stain various shades of pink. The HE staining procedure followed is presented in Table 9.2.

Xylene	2min
Xylene	2min
Ethanol	2min
Ethanol	1min
Ethanol	1min
Water wash	1min
Stain	
Mayer's haematoxylin	8min
Water wash	30sec
Scott's tap water substitute	30sec
Water wash	2min
Young's eosin	4min
Water wash until sections appear red-purple	~ 20sec (or 20 dips)
Dehydrate, Clear & Mount	

Ethanol	10dips
Ethanol	1min
Xylene	2min
Xylene	1min
Xylene	Until coverslip was added
Mount coverslip with DPX	

Table 9. 2: HE staining procedure

9.15. Gram's staining

9.15.1. Preparation of Gram's stain reagents

Two percent crystal violet was prepared by dissolving 2gm crystal violet powder in 20ml of 95% ethanol, 0.8gm ammonium oxalate in 80ml distilled water, and then finally mixing them together. Lugol's iodine was prepared by dissolving 2gm potassium iodide in 100ml distilled water and then adding 1gm iodine crystals. Similarly, Twort's stain was prepared by mixing 100ml 0.2% alcoholic neutral red with 11.3ml 0.2% alcoholic fast green. In Gram's staining, Gram positive bacteria stain dark blue/black, Gram negative bacteria stain pink, nuclei stain red, cytoplasm stain light green, and erythrocytes stain green. The staining procedure used is presented in Table 9.3.

Xylene	2min
Xylene	2min
Ethanol	2min
Ethanol	1min
Ethanol	1min
Water wash	1min
Stain	
Stain in 2% crystal violet	2min
Wash off with Lugol's iodine	
Treat with Lugol's iodine	3min
Rinse in water	
Decolourise in acetone	
Rinse immediately in water	
Counterstain in Twort's (dilute in tap water 1:3)	10min
Rinse quickly in water	
Dehydrate, clear, and mount	
Ethanol	10dips
Ethanol	10dips
Xylene	2min
Xylene	1min

Xylene	Until coverslip was added
Mount coverslip with DPX	

Table 9. 3: Gram's staining procedure