EFFICIENCY OF POVIDONE-IODINE AGAINST METHICILIN RESISTANT *Staphylococcus aureus* BIOFILM ON STAINLESS STEEL IMPLANT DEVICES

By

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Biofilm formation on implant devices and antibiotic resistance of bacteria make managing implant-related infection challenging. Irrigation of the infected joint with antiseptic agents is crucial in periprosthetic joint infection (PJI) with debridement, antibiotics, and implant retention. Various antiseptic agents are commonly used for irrigation, but the optimal choice, concentration, and irrigation time for complete biofilm elimination still need to be determined. The current research aims to investigate the effectiveness of povidone- iodine (PVP) irrigation on stainless steel implants coated with Methicillin-resistant Staphylococcus aureus (MRSA) using an in vitro method. The mature biofilms were analyzed using a scanning electron microscope (SEM) to study their morphology and physiology. Later, the mature MRSA biofilm was treated with PVP irrigation at three different time intervals (1, 3, and 5 minutes) with six different doses (0.3%, 0.5%, 0.75%, 1%, 2.5% and 5%) to compare its efficacy using standard treatment methods. The results showed that low concentrations of PVP (0.75%) treated for a duration of 5 mins, effectively eliminated the MRSA biofilm. It suggests that irrigating with low-concentration PVP could be a beneficial treatment for PJI in the presence of biofilm. Additionally, the study provides evidence that irrigation with PVP is critical in removing biofilm during PJI treatment.

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KECEKAPAN POVIDONE-IODINE UNTUK MEMBASMI BIOFILEM METHICILIN RESISTANT *Staphylococcus aureus* DARIPADA IMPLANT KELULI TAHAN KARAT

Oleh

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Pembentukan biofilem pada peranti implan dan ketahanan antibiotik bakteria menjadikan pengurusan jangkitan berkaitan implan mencabar. Irigasi sendi yang dijangkiti dengan agen antisepsik adalah penting dalam jangkitan persendian periprostetik (PJI) dengan debridement, antibiotik, dan retensi implan. Pelbagai agen antisepsik biasanya digunakan untuk irigasi, tetapi pilihan, kepekatan, dan masa irigasi yang optimum untuk penyingkiran biofilem sepenuhnya masih perlu ditentukan. Kajian semasa bertujuan untuk menyelidik keberkesanan irigasi povidone-iodin (PVP) ke atas implan keluli tahan karat yang dilapisi dengan Staphylococcus aureus tahan Methicillin (MRSA) menggunakan kaedah in vitro. Biofilem matang dianalisis menggunakan mikroskop elektron imbasan (SEM) untuk mengkaji morfologi dan fisiologi mereka. Kemudian, biofilem MRSA matang dirawat dengan irigasi PVP pada tiga selang masa yang berbeza (1, 3, dan 5 minit) dengan enam dos yang berbeza (0.3%, 0.5%, 0.75%, 1%, 2.5% dan 5%) untuk membandingkan keberkesanannya menggunakan kaedah rawatan standard. Hasil kajian menunjukkan bahawa kepekatan rendah PVP (0.75%) yang dirawat selama 5 minit, secara berkesan menghilangkan biofilem MRSA. Ini menunjukkan bahawa irigasi dengan PVP kepekatan rendah boleh menjadi rawatan yang bermanfaat untuk PJI dalam kehadiran biofilem. Selain itu, kajian ini memberikan bukti bahawa irigasi dengan PVP adalah penting dalam menyingkirkan biofilem semasa rawatan PJI.

Kata Kunci: Antimikrob, Antiseptik, Biofilms, Chlorhexidine Gluconate, Orthopedik

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LIST OF ABBREVIATIONS

THR	total hip replacement
TKR	total or unicompartmental knee replacement
PJIs	prosthetic or periprosthetic joint infections
MDR	multidrug-resistant
MRSA	methicillin-resistant *Staphylococcus aureus*
S. aureus	*Staphylococcus aureus*
DAIR	implant retention and antibiotic treatment
TSE	two-stage exchange
OSE	one-stage exchange
CHG	chlorhexidine gluconate
PVP	povidone-iodine
PMMA	poly(methyl methacrylate)
UHMWPE	ultrahigh-molecular weight polyethylene
MSSA	methicillin-sensitive *S. aureus*
E. coli	*Escherichia coli*
P. aeruginosa	*Pseudomonas aeruginosa*
C. albicans	*Candida albicans*
SSIs	surgical site infections
MSIS	musculoskeletal infection society
IDSA	Infectious Disease Society
CRP	C-reactive protein
ESR	erythrocyte sedimentation rate
WBC	white blood cell
CT	computed tomography

MRI	magnetic resonance imaging
SCCmec	staphylococcal cassette chromosome mec
HA-MRSA	hospital-associated MRSA
CA-MRSA	community-associated MRSA
LA-MRSA	livestock-associated MRSA
WHO	World Health Organization
CDC	Centers for Disease Control and Prevention
NICE	National Institute for Health and Care Excellence
EPS	extracellular polymeric substances
MIC	minimum inhibitory concentration
MBC	minimum bactericidal concentration
AMR	Antimicrobial resistance
SEM	scanning electron microscope
CFU	colony forming unit
TSB	Tryptic Soya Broth
MHA	Muller Hinton Agar
XTT	2, 3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide
PBS	phosphate buffered saline
N/S	normal saline

CHAPTER 1

INTRODUCTION

1.1 Background

Joint replacements, particularly total hip replacement (THR) and total or unicompartmental knee replacement (TKR), are the most well-known joint replacement surgeries performed worldwide, with more than 1,000,000 surgeries per year in the United States (Seebach & Kubatzky, 2019). Arthroplasty surgeries improve patients' lives by restoring function and relieving pain (Cheng Li et al., 2018). These surgeries have evolved with minimally invasive surgical methods (Galakatos, 2018), protocols to reduce blood transfusions (Turcotte et al., 2020), perioperative pain management (Stevenson, Neuwirth, & Sheth, 2018), robotic navigation systems (Perets et al., 2020), and advanced prosthetic biomaterials (Das & Chakraborti, 2018). However, complications related to implant-associated bone infections are becoming a leading problem in orthopedic surgery, with severe consequences for affected patients.

Prosthetic or periprosthetic joint infections (PJIs) lead to severe complications that affect patients' quality of life by causing rheumatoid arthritis (Gausden et al., 2021) and osteomyelitis (Lamret et al., 2020), and can lead to death in some situations (Lum et al., 2018), as well as posing a socioeconomic burden (Thakrar et al., 2019). The principal mechanism causing infections within a year of surgery is pathogen entry into joints through direct contact or aerosolized contamination during a surgical procedure (Zardi & Franceschi, 2020). Various Gram-positive and Gram-negative bacteria are generally responsible for these infections, while fungal infections are rare (Koutserimpas et al., 2019). Aerobic Gram-positive cocci are responsible for 65% of PJIs, anaerobic bacteria cause about 4% of infections, followed by Gram-negative rods causing 6% of infections, and fungi causing 1% of infections (Bassetti et al., 2019). In particular, multidrug-resistant (MDR) microorganisms are a leading problem in PJIs management.

Among them, Methicillin-resistant *Staphylococcus aureus* (MRSA) has become the prime concern among clinicians, as guidelines for optimal treatment for patients affected by PJIs are unavailable (Xiang, Xuan, & Li, 2018). *Staphylococcus aureus* (*S. aureus*) is a Gram-positive coccus commonly found in healthcare facilities (Miller et al., 2020). It is known for its resistance to methicillin (oxacillin or nafcillin) and most β-lactam antibiotics of the penicillin class (Miller et al., 2020). In addition, MRSA biofilm formation on the prosthetic surface plays a critical role in the pathogenesis of PJI (Benito et al., 2019), where the prosthesis allows bacteria to adhere to it, forming a biofilm and causing infection in adjacent tissues (Lamret et al., 2020). Because biofilm is difficult to eradicate due to antimicrobial resistance, it becomes a challenge in treating PJIs. As a result, PJIs force prosthesis detachment, amputations, or in some cases, lead to patient death and also pose a financial burden to the community.

Currently, multiple methods of antimicrobial treatment with an appropriate surgical approach are used as the gold standard for the treatment of PJIs (Bassetti et al., 2019). The surgical techniques include debridement, implant retention and antibiotic treatment (DAIR), two-stage exchange (TSE) (Vaz, Taylor, Kendrick, & Alvand, 2022), and one-stage exchange (OSE), resection arthroplasty, arthrodesis, and amputation as alternative approaches (Bezel et al., 2019). Although none of the clinical trials identified the best surgical method for PJIs, treatment was based on the specialist's decision, taking into account the type of infection, the severity of infection, microbiological strains, the durability of the prosthesis, condition of the periprosthetic tissue, and clinical condition of the patient (Bassetti et al., 2019). In addition, several practices are also used to prevent postoperative infections, including irrigation before wound closure (Hernandez et al., 2019). The use of numerous irrigation solutions such as saline, antibiotics, antiseptics, and surfactants is increasing regardless of concerns about cytotoxicity (Siddiqi et al., 2021).

Most significantly, antiseptic solutions such as acetic acid (CH_3COOH) (Tsang et al., 2018), chlorhexidine gluconate (CHG) ($C_{22}H_{30}Cl_2N_{10}$) (Frisch et al., 2017), hydrogen peroxide (H_2O_2) (Lu & Hansen, 2017), hypochlorous acid (HClO) (Rembe et al., 2020), povidone-iodine (PVP) (Hernandez et al., 2019), and sodium hypochlorite (NaClO) (Wright, Kahler, & Walsh, 2017) have been applied to alleviate joint infections. As of 2012, dilute PVP irrigation of the wound before closure became widely used in orthopedic surgeries, including spine surgery (Balato et al., 2020). PVP is composed of 99% polyvinylpyrrolidone and 1% iodine, with PVP using hydrophilic properties to release iodine directly at the cell surface. Iodine entering the cell acts as an oxidant on several cytoplasmic membrane components and inactivates numerous cytoplasmic molecules, resulting in bacterial, viral, and fungal cell integrity damages (Balato et al., 2020). Therefore, the toxicity of PVP directly corresponds to the concentration of iodine delivered to the cell wall (Siddiqi et al., 2021). However, the efficacy of diluted PVP in treating PJI by eradicating chronic biofilms is still a challenge due to limited data from *in vitro* and *in vivo* studies.

1.2 Problem statement

Orthopedic implants are used to fix fractures, correct deformed and replace joints, which give mechanical support for the bone to maintain alignment and fully function during physical activity. Various joints prostheses of the elbow, hip, knee, and shoulder with different osteosynthetic materials like plates, pins, screws, and wires are categorized as orthopedic implants (Filipović et al., 2020). They are made up of diverse biomaterials, including alumina (Rahmati & Mozafari, 2019), cobalt (Co) alloys (Garcia-Falcon et al., 2021), hydroxyapatite (Arcos & Vallet-Regí, 2020), magnesium (Mg) and magnesium-based alloys (Mozafari, Bordbar-Khiabani, & Yarmand, 2020), poly(methyl methacrylate) (PMMA) (Hasandoost et al., 2020), stainless steel (Pattanayak, Panda, & Dhupal, 2020), titanium (Ti) and titanium-based alloys (Kaur & Singh, 2019), ultrahigh-molecular weight polyethylene (UHMWPE) (Ren et al., 2019), and zirconia (Magnani et al., 2021). Even though different biomaterials are used for implants manufacture, it is always related to infection possibility that may lead to secondary surgical procedures for implant replacement, amputation or cause death with substantial economic loss.

Bacterial contamination on implants mostly originated from hospital environments like surgical equipment, operation room surfaces and air ventilation system, healthcare personnel clothing, and patients' skin and body (Filipović et al., 2020). Orthopedic implant infections are often caused by *S. aureus* with other pathogens that may also infect with a low infection rate (Arciola, Campoccia, & Montanaro, 2018). Bacterial adhesion is an initial step in implant infection, where it supports bacterial colonization (microcolonies) at the implant by generating protective biofilms. The biofilm enables the bacterial colonies to sustain themselves in the host. So, adhesion and biofilm formation play a significant role in the pathogenesis of implant infection. Besides, biofilms also become resistant to the host's endogenous defenses and need to be treated with a combination of antibacterial therapies and tissue debridement. Consequently, a large amount of antibiotics usage in treating biofilms lead to the development of antibiotic-resistant bacteria strains like MRSA and methicillin-sensitive *S. aureus* (MSSA). Hence, understanding on biofilm formation mechanism is vital in preventing implant-associated infections.

In the clinical setting, surgical treatment with aggressive irrigation and debridement is performed once implant infection is detected. An antiseptic irrigation solution is a significant component of the irrigation and debridement method (Ruder & Springer, 2017). Irrigation is used in orthopedic procedures to keep tissues moist, remove tissue debris and bacterial contaminants from the wound, and, in the case of surgical debridement for infection, remove bacteria and fragments of biofilm from biofilm-based infections (Schmidt et al., 2018). PVP remains a popular irrigation solution for antisepsis and wound healing due to its favorable efficacy and tolerability. PVP's broad spectrum of activity, ability to penetrate biofilms, lack of associated resistance, anti-inflammatory properties, low cytotoxicity, and good tolerability contribute to PVP's popularity (Bigliardi et al., 2017). The microbicide effect of iodine seems to be associated with bacterial cellular inhibition mechanisms and structures. It oxidizes nucleotides, fatty and amino acids in bacterial cell membranes and cytosolic enzymes involved in the respiratory chain, denaturing, and deactivating them (Bigliardi et al., 2017). Past research suggest that PVP can use in lower concentrations and causes minimal damage to healthy tissue. Goswami et al. (2019) reported that 0.3% PVP showed high efficacy in eradicating MSSA and *Escherichia coli* (*E. coli*) and had low cytotoxicity against human osteoblasts, chondrocytes, and fibroblasts in comparison to CHG. Diluted PVP was highly efficient compared to other antimicrobials in removing biofilms of *Pseudomonas aeruginosa*, MRSA and *Candida albicans* (*C. albicans*) (Hoekstra, Westgate, & Mueller, 2017). Besides, a study by Johani et al. (2018) stated that PVP eradicated biofilms of *S. aureus* and *P. aeruginosa* within 15 minutes, whereas CHG eradicate *S. aureus* biofilm only.

Based on the above findings, we conclude that further research is needed to understand the suitable concentration of PVP and the time taken for the biofilm eradication for better PVP utilization as an antiseptic. Thus, the current study tested MRSA as an experimental bacteria due to its tendency to form biofilm and cause a higher infection rate. Our prosthetic model was stainless steel implant, the first metallic biomaterial implant on the market and PVP served as an antiseptic solution.

1.3 Research questions

i. How long does it take to povidone-iodine to eradicate the mature MRSA biofilm on the stainless steel implants?

ii. What concentration is required for povidone-iodine irrigation to eradicate the mature MRSA biofilm on the stainless steel implants?

1.4 Objectives

1.4.1 General objective

To determine the efficacy of irrigation using povidone-iodine on MRSA-coated implants.

1.4.2 Specific objectives

i. To determine time taken for the formation of mature MRSA biofilm on the stainless steel implants.

ii. To determine the irrigation time of povidone-iodine needed to eradicate mature MRSA biofilm on the stainless steel implants.

iii. To determine the percentage of povidone-iodine required to eradicate the mature MRSA biofilm on the stainless steel implants.

1.5 Hypothesis

Povidone-iodine is an effective antiseptic in eradicating MRSA biofilm on stainless steel implants.

1.6 Definition of terms

It refers to an infection that occurs in the tissues surrounding a joint replacement implant. This infection can involve various microorganisms such as bacteria, fungi, or viruses and may result from contamination during surgery, spread from other infections in the body, or through the bloodstream. PJI can lead to pain, inflammation, swelling, and potentially compromise the function and longevity of the joint replacement. Treatment typically involves a combination of antibiotics, surgical debridement, and in some cases, revision surgery to remove and replace the infected implant (Lamret et al., 2020).

Biofilm: Microbe-derived sessile group of cells irreversibly attached to a substrate or interface, or to each other, embedded in a matrix of extracellular polymeric substances produced by themselves, and exhibiting an altered phenotype in terms of growth rate and gene transcription (Lamret et al., 2020).

1.7 Significance of the study

The study aims to determine the maturation time of MRSA biofilm on a stainless steel implant and to determine the timing and concentration of PVP treatment to eradicate the matured MRSA biofilm. It's important to identify the timing and concentration of PVP treatment for the safe and effective use of PVP by the healthcare providers.

CHAPTER 2

LITERATURE REVIEW

2.1 Prosthetic joint infection

The most widely used clinical classification for PJIs depends on time since primary onset of the symptoms (Izakovicova, Borens, & Trampuz, 2019). An early infection (less than three months after surgery) occurs during the insertion of the implant device before closing the wound, where the main symptoms start as erythema (Kaufman, Meaike, & Izaddoost, 2016) and oedema at the implant insertion site (Richold, 2018), followed by wound drainage, joint pain, effusion, and fever. Delayed onset infections happen after early onset and before two years (from 3 months to less than two years). In this stage, the patient suffers from continuous joint pain with or without implant loosening (Gallo & Nieslanikova, 2020). Besides, the late-onset infection occurs after more than two years of surgery, caused by the haematogenous spreading of bacteria from the infection source inside the body (Rakow et al., 2019). Yet, this classification is not clinically useful. Therefore, researchers Zimmerli & Sendi (2017) proposed a more meaningful clinical classification to guide surgical therapy, as shown in Table 2.1. Based on that, symptoms appear within one month of the operation, referred to as early post- operative PJIs, and are named chronic PJIs when symptoms arise more than one month after surgery. Hematogenous PJIs are classified as acute when symptoms appear within three weeks of an uneventful post-operative period and chronic when symptoms persist longer than three weeks.

Table 2.1 : Prosthetic joint infection classification

	Early	**Delayed**	**Late**
Time of infection	Within 1–3 months of surgery	After early onset, but before 24 months	After the delayed period / 24 months
Type of organism	Virulent pathogens; i.e., *S. aureus* or gram-negative *bacilli*	Less virulent pathogens; i.e., coagulase negative *staphylococci* or *Propionibacterium acnes*	
Rote of infection	During implantation	During implantation	Hematogenous spread from distant source of infection
Treatment	Surgical debridement	Two-stage arthroplasty exchange	Two-stage arthroplasty exchange

2.2 Treatment of prosthetic joint infection

The treatment of PJI involves a combination of surgical and antimicrobial therapy. Surgical management may include the removal of the implant, debridement of the

infected tissue, and replantation of a new implant. The decision to remove the implant depends on several factors such as the duration of infection, type of organism, and the extent of bone and tissue damage. Antimicrobial therapy is an essential component of the treatment of PJI. The choice of antibiotic depends on the causative organism and the sensitivity of bacterial cultures. In most cases, empiric antimicrobial therapy is initiated before the culture results are available. A prolonged course of antimicrobial therapy is necessary to eradicate the infection, and treatment duration varies depending on the type of prosthetic joint and the type of organism causing the infection. In cases where surgical intervention is not possible, an antibiotic therapy alone may be considered. This may be the case in elderly patients or patients with significant medical comorbidities who may not tolerate surgery.

DAIR is a standard surgical procedure used to treat implant-associated infections. The primary objective of DAIR is to save the implant by removing the infected tissue and providing ample antibiotics support. The advantages of DAIR over other treatment options are numerous, as discussed below:

a Preservation of implant:

One of the primary advantages of DAIR is that the implant is preserved. The procedure eliminates the need for a new implant, which would significantly increase the cost of treatment. Additionally, the preservation of the implant requires less invasive surgery, reducing the risk of complications and shortening the patient's recovery period.

b Reduced morbidity:

DAIR reduces the rate of morbidity caused by implant-related infections. For instance, deep surgical site infections (SSIs) can lead to implant loosening, poor joint function, chronic pain, and even amputation in severe cases. DAIR curtails the spread of infections and minimizes their impact.

c Cost-effectiveness:

DAIR is a cost-effective treatment method compared to other options such as removing the implant and inserting a new one. DAIR utilizes antibiotics and debridement to manage implant infections, which saves a considerable amount of money for both the patient and the hospital.

d Limited hospital stay:

DAIR is an outpatient procedure, meaning that the patient can go back home on the same day. The short hospital stay reduces the patient's exposure to hospital-acquired infections, thereby improving their recovery rate.

e Reduced risk of complications:

DAIR has a lower risk of complications compared to other treatments for implant-associated infections. The procedure is less invasive and limits the risk of exposing the patient to the adverse effects of anesthesia.

f Increased success rate:

DAIR has a high success rate in treating implant-related infections. The procedure eliminates the source of the infection, reduces bacterial load, and provides sufficient antibiotics support.

When appropriately carried out, DAIR can swiftly manage implant infections and help patients recover without the need for a new implant. The treatment of PJI can be challenging and requires a multidisciplinary team approach. The treatment may involve surgical intervention, antimicrobial therapy, or a combination of both, depending on the severity and type of infection. Early recognition and management are essential to prevent further complications and optimal outcomes for patients with PJI.

2.3 Diagnosis of prosthetic joint infection

2.3.1 The musculoskeletal infection society (MSIS)

The definition of PJI has been standardized by the MSIS and the Infectious Disease Society (IDSA), who proposed earlier guidelines (Baker et al., 2015). However, the MSIS Modified Musculoskeletal Infection Society criteria are now considered to be the gold standard for diagnosing PJI (Berns et al., 2020). In Table 2.2, all the criteria, their associated point values, and the corresponding conclusion on infection are outlined (Parvizi et al., 2018). The criteria include CRP (C-reactive protein), ESR (erythrocyte sedimentation rate), WBC (white blood cell), and PMN.

2.3.2 Isolation of organism

While culture is considered the gold standard for diagnosing PJI, it has several disadvantages. For instance, it can take a long time to incubate and sometimes fails to achieve bacterial growth due to prolonged antibiotic treatment or formation of biofilm on the implant device (Larsen et al., 2012).

2.3.3 Serum biomarkers

Serum biomarkers play a crucial role in the assessment and diagnosis of periprosthetic joint infection (PJI) in the hip and knee. This article delves into the evidence surrounding novel serum markers and examines factors influencing traditional serum

testing, with the aim of aiding clinicians in accurately interpreting these assessments. Presently, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) maintain their position as primary screening tests for PJI. However, their lack of specificity necessitates supplementation with more specific synovial tests and microbiology findings. Therefore, they should not be relied upon in isolation for diagnosing PJI. Despite efforts to identify alternative serum biomarkers, none have exhibited superior sensitivity to replace CRP and ESR as the initial screening tests for PJI (Saleh et al., 2018).

In addition to culture, there are other synovial indicators that can be used to diagnose PJI. These include inflammatory proteins, cytokines, and antimicrobial peptides. One such antimicrobial peptide is alpha-defensin, which is commonly used as an immunodiagnostic test for diagnosing PJI (Ahn et al., 2011).

2.3.4 Imaging techniques

Standard X-ray is primarily used to recognize bone loosening and joint osteolysis, while computed tomography (CT) provides a clear evaluation of bone structure. Additionally, magnetic resonance imaging (MRI) is very helpful for surgeons because it provides valuable information about the soft tissues surrounding the implant device (Andersson et al., 2016).

Table 2.2 : All criteria and their associated point values and corresponding conclusion on infection

MSIS criteria		Score	Conclusion
Major criteria	Two positive periprosthetic cultures with phenotypically identical organisms or a sinus tract communicating with the joint.		Infected
Minor criteria			
Serum biomarkers	Elevated CRP (>1 mg/dL) or D-dimer (>860 ng/mL)	2	More than 6 infected
	Elevated ESR (30 mm/h)	1	
Synovial biomarkers	Elevated WBC count (>3000 cells/mcg/L) or LE (++)	3	2–5 possible infected
	Positive alpha- defensin (signal-to- cut off ratio >1)	3	
	Elevated synovial PMN percent(>80%)	2	Less than 1 not infected
	Elevated synovial CRP (>6.9 mg/L	1	

2.4 MRSA and Stainless steel implant

S. aureus is a Gram-positive coccoid bacteria of Firmicutes phylum (Lee et al., 2018). These bacteria commonly found in human commensal microbiota of the nasal mucosa among 20-80% of population (Sakr et al., 2018). *S. aureus* can gain access to the underlying tissues or the bloodstream when the cutaneous and mucosal barriers are disrupted, leading to the infection. Right after the introduction of methicillin (celbenin) in clinical practice as a treatment option against penicillin-resistant *S. aureus* clones, the *S. aureus* clones start evolved into MRSA via horizontal gene transfer of staphylococcal cassette chromosome mec (SCCmec). SCCmec is a mobile genetic material that encodes mecA or mecC genes, which provide resistance to methicillin and most of β-lactam antibiotics. poses a significant challenge in healthcare settings and communities alike due to its formidable resistance mechanisms and adaptability. Its resistance to multiple antibiotics, including beta-lactams, renders traditional antibiotic therapies ineffective against MRSA infections. Additionally, MRSA's ability to form biofilms provides a protective environment that shields the bacteria from the host immune response and antimicrobial agents. This biofilm formation contributes to the persistence of MRSA in the environment, particularly in healthcare facilities where transmission between patients and surfaces is common. Furthermore, individuals can carry MRSA asyptomatically, unknowingly serving as reservoirs for transmission. The adaptability of *S. aureus*, including MRSA strains, allows them to evolve and persist despite efforts to control their spread through infection control measures and antimicrobial stewardship programs. As a result, combating MRSA requires a multifaceted approach that addresses antibiotic resistance, environmental persistence, and asymptomatic carriage, emphasizing the importance of comprehensive infection control strategies and antibiotic stewardship programs in mitigating its impact. MRSA also showed resistant to other antibiotic classes, which hampered current treatment options of this bacteria. The term MRSA still carry over although methicillin now no longer applied for human use due to its toxicity.

At present, there are seven commonly used antibiotics to combat MRSA, namely: vancomycin, daptomycin, linezolid, Sulfamethoxazole and trimethoprim (TMP-SMZ), quinupristin-dalfopristin, clindamycin, and tigecycline. However, the effectiveness of these antibiotics is gradually diminishing as MRSA strains evolve resistance to them. Therapeutic options for infections caused by MRSA and S. aureus with reduced vancomycin susceptibility are currently limited. Consequently, there is an urgent global need for the development of novel drugs capable of effectively treating S. aureus exhibiting multidrug resistance, in order to address the significant public health challenge posed by this microorganism worldwide (Okwu et al., 2019).

Since its introduction, an epidemiology change was observed in MRSA as it evolved from hospital-associated MRSA (HA-MRSA), followed by community- associated MRSA (CA-MRSA), and livestock-associated MRSA (LA-MRSA). The MRSA spreading via two mechanisms. The first mechanism involved spreading of existing resistant clones and second mechanism involved acquisition of SCCmec by a MSSA strain. The mechanism of horizontal transfer of SCCmec are not well understood, yet the epidemiological studies recorded that this resistance mechanism has covered most of *S. aureus* clones infecting human and animals. Currently, MRSA

is one of the most common antibiotic-resistant bacteria resulting 171,000 invasive infections each year in Europe alone. Individuals with invasive medical devices or compromised immune systems are more susceptible to *S. aureus* infection.

Stainless steel implants are widely utilized in orthopedic and dental surgeries due to their exceptional combination of mechanical properties and biocompatibility. Renowned for their strength and durability, stainless steel implants can withstand the mechanical stresses and load-bearing requirements typical of orthopedic implants. Additionally, they exhibit excellent corrosion resistance, ensuring longevity and stability within the body's physiological environment. Biocompatibility is another key advantage, as stainless steel implants generally provoke minimal adverse reactions or immune responses when implanted. Moreover, the ease of fabrication allows for the customization of implants to suit the specific anatomical needs of patients and surgical requirements. Cost-effectiveness further enhances the appeal of stainless steel implants, particularly in cases where budget considerations play a significant role. With a long-standing clinical track record and proven reliability, stainless steel implants continue to be a preferred choice for orthopedic and dental surgeons worldwide, providing patients with durable and effective solutions for various medical conditions and procedures.

2.5 Povidone-Iodine (PVP) as antiseptic in wound healing

Povidone-Iodine (PVP) is a commonly used antiseptic agent in wound healing that is known for its broad-spectrum antimicrobial properties. PVP is derived from iodine, which has been known to possess antiseptic properties for many years. However, iodine's use as an antiseptic agent was limited primarily due to its toxicity and staining properties. Povidone, a water-soluble polymer, was added to iodine to create PVP, which is highly effective in inhibiting the growth of microorganisms in wounds. The appropriate timing for treatment with PVP varies depending on the medical context.

In preoperative settings, it is commonly utilized for skin preparation just before surgery to mitigate the risk of surgical site infections. During surgical procedures or wound care, PVP may be employed for wound irrigation to cleanse the site and reduce bacterial contamination. Additionally, it serves as an antiseptic for disinfecting the skin prior to invasive procedures like injections or catheterizations. In minor wound care, such as cuts and abrasions, PVP can be applied topically to prevent infection. The timing and method of PVP treatment should always align with the specific medical condition and the guidance of healthcare professionals, ensuring its safe and effective use (Monstrey et al., 2023).

2.5.1 Mechanism action of PVP

PVP is a mixture of povidone, hydrogen iodide, and elemental iodine that targets critical bacterial structures necessary for survival and replication (Eggers, 2019). PVP contains polyvinylpyrrolidone and 1% free iodine, which slowly releases free iodine. The free iodine is chemically harmful to the cell walls of bacteria and proteins, causing oxidation of amino acids and nucleic acids in biological structures. This damage is

difficult, if not impossible, to repair or counteract (Kanagalingam et al., 2015). PVP is commonly used as a skin disinfectant before or after surgery (Eggers, 2019). It can be applied topically to the skin as a liquid or powder, and its broad antimicrobial spectrum helps limit the spread of microorganisms.

There are various mechanisms by which PVP works as an antiseptic agent in wound healing. PVP is highly effective in penetrating bacterial cell walls and disrupting their metabolic processes, leading to cell death. Additionally, PVP has been shown to inactivate bacterial enzymes and proteins, preventing the microbial colonization of wounds. Its mechanism of action involves disrupting bacterial metabolic processes, inhibiting microbial colonization, reducing wound exudate, promoting granulation tissue formation, and protecting wounds from oxidative stress. Its prolonged antimicrobial activity also makes it a valuable agent in preventing bacterial infection recurrence, ultimately leading to optimal wound healing. Fig 2.1 showed action mechanism of PVP.

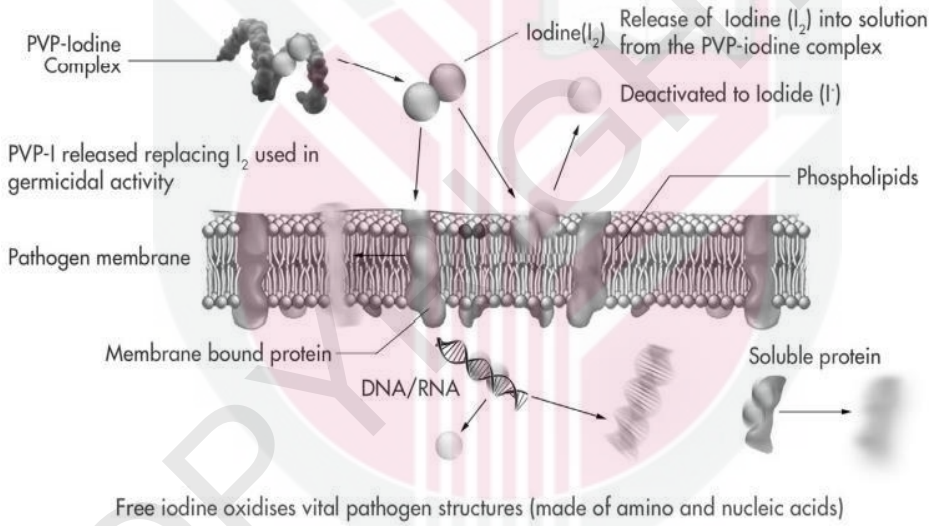


Figure 2.1 : Mechanism of action of PVP in equilibrium with free iodine, where the active moiety is iodine, oxidising pathogen nucleotides and fatty/amino acids and thus deactivates proteins as well as DNA/RNA
(Adapted from Bigliardi et al., 2017)

In orthopedic surgery, it is recommended to irrigate the wound with PVP before closure and use it as a preoperative disinfectant. The World Health Organization (WHO), the Centers for Disease Control and Prevention (CDC), the Department of Health and Human Services in the USA, and the National Institute for Health and Care Excellence (NICE) have all recommended the use of PVP in these ways, as they have found that it reduces the rate of postoperative infections. During the second international consensus meeting on musculoskeletal infection, the topic of using PVP for intraoperative wound irrigation was discussed. It was suggested that rather than

using infected or non-infected solutions, dilute PVP should be used for irrigation (Goswami & Austin, 2019).

In order to address the difficulty of getting iodine to the cell surface, Hydrophilic Polyvinylpyrrolidone can be used to deliver the iodine (Gershenfeld et al., 1954; Oduwole et al., 2010). Once the iodine enters the organism, it causes damage to the cell membrane and molecules in the cytoplasm through oxidation (Rodeheaver et al., 1982). Additionally, iodine binds with cytosol enzymes in the respiratory chain and causes denaturation and inactivation (Kanagalingam et al., 2015). Studies have shown that iodine possesses not only antimicrobial properties but also anti-inflammatory properties (Bigliardi et al., 2017; Schreier et al., 1997).

2.5.2 Antimicrobial activity of PVP

PVP is an antimicrobial product that has broad-spectrum activity against bacteria, fungi, protozoa, viruses, and bacterial spores (Gatti et al., 1998; J. Lachapelle et al., 2013). Unlike some other antiseptics and antibiotics, cross-resistance to iodine has not been observed, perhaps due to the many ways in which iodine affects organisms (Eggers, 2019; Sibbald et al., 2011; Leaper, 2008; Sibbald et al., 2007).

2.5.3 Anti-biofilm activity

The World Union of World Healing Societies' 2016 position document identifies iodine as an effective antibacterial agent for biofilm management (Alves et al., 2021). In vitro studies have shown that PVP and CHG have antibacterial and antibiofilm activity. Studies have demonstrated that PVP at a concentration of 0.25% completely removed biofilms of MSSA, *Klebsiella pneumoniae*, and *P. aeruginosa* (K et al., 2018). In a study by Wang et al. (2022) different concentrations of PVP (0.5%–10%). were tested on *S. aureus and P. aeruginosa* biofilm development. The study investigated the toxicity and antibacterial effects of PVP irrigation in fracture surgery. Researchers examined the impact of using Povidone-Iodine solution during surgical procedures to clean and disinfect fracture sites. They analyzed its effectiveness in eliminating bacteria and preventing infections, as well as any potential adverse effects or toxicity associated with its use. The study likely involved experiments or clinical observations to assess the outcomes of Povidone-Iodine irrigation in fracture surgery, aiming to provide valuable insights for orthopedic surgeons and healthcare professionals regarding the safety and efficacy of this commonly used technique. Similarly, in a study by Schmidt et al. (2018), complete eradication of *Staphylococcus epidermidis* biofilm required only 1 minute of irrigation with 10% PVP, while 3.5% PVP needed 10 minutes of irrigation to remove the biofilm. A recent study using an *in vitro* model of PJI grew MSSA biofilm for 72 hours and exposed it to 10% and 0.35% PVP for 3 minutes. The study found that only 10% PVP effectively removed the biofilm (Premkumar et al., 2021). In contrast, Presterl et al. (2007) showed that in the eradication of *S. epidermidis* biofilms, hydrogen peroxide and alcohol were superior to PVP.

2.5.4 Adverse effects

Some investigations have raised concerns about the potential cytotoxic effects of PVP on *in vitro* human cells, such as osteoblasts, myoblasts, chondrocytes, and fibroblasts. However, these concerns have not yet been verified *in vivo* studies (Goswami & Austin, 2019). Despite these concerns, it is noteworthy that the FDA has authorized the use of Surgiphor Wound Irrigation System, a commercially available sterile dilute PVP solution for clinical use (Siddiqi et al., 2021). It is essential to keep in mind that PVP should still be used with caution and under the guidance of a healthcare professional.

2.6 Biofilm on medical devices

Biofilms are complex communities of microorganisms that are encased in a matrix of extracellular polymeric substances (EPS), which can be made up of polysaccharides, proteins, nucleic acids, and lipids. These biofilms adhere to both biological and non-biological surfaces, and can pose challenges in terms of infection prevention and treatment (Van Giau et al., 2019).

Although surface-associated bacteria were first observed by Anthony van Leeuwenhoek in 1684, it was not until the 1978 study by Costerton et al. that the term 'biofilm' was used and described. Biofilm described as a structured community of bacteria enclosed in a self-produced polymeric matrix that adheres to a surface (Percival et al., 2011). Biofilms are found in various environments and are formed by both homogeneous and heterogeneous microbial communities. One of the biggest challenges associated with biofilms is that the microorganisms present within them are often less susceptible to antibiotics than their individual forms. In addition, biofilms are highly resistant to the host immune system, as they develop immune resistance through repeated changes in surface antigens resulting from gene expression changes. These factors make biofilms difficult to treat and remove, and can lead to chronic infections (Høiby et al., 2010; Vasudevan, 2014).

Previously, antibiotic-resistant bacteria were mainly found in intensive care units, but now they are being isolated in various healthcare settings, including hospitals and other healthcare centers (Patil et al., 2019; Römling & Balsalobre, 2012). While biomedical implants such as prosthetics, catheters, and other devices have improved modern medicine, they can also increase the risk of infection. The presence of these implants can provide a surface for bacteria to adhere to and form a biofilm, which can lead to infections that are difficult to treat (Costerton et al., 1999; Darouiche, 2004). As a result, there is a growing need for effective strategies to prevent and manage biofilm-related infections associated with the use of biomedical devices.

Biofilm-associated infections have affected millions of people worldwide and can lead to severe health complications, including death (Arciola, Campoccia, Ehrlich, et al., 2015; Montanaro, Speziale, et al., 2011). According to the CDC, approximately 65% of all chronic infectious diseases are attributed to microbial biofilms (Joo & Otto, 2012). These statistics underscore the importance of developing effective strategies for

preventing and managing biofilm-related infections in patients with medical implants. Antimicrobial resistance in biofilms has significant consequences, including an estimated 30 million deaths and a substantial financial burden on healthcare systems (Balouiri et al., 2016). In the United States, infections associated with medical devices account for 25.6% of all healthcare-related infections (Magill et al., 2014). These infections can cause prolonged hospital stays, increased healthcare costs, and even mortality, highlighting the urgent need for effective strategies to prevent and manage biofilm infections associated with medical devices.

Infections associated with medical implants involve complex interactions between the pathogen, the biomaterial, and the host immune response. Host immune defenses are usually effective at clearing tissue infections caused by opportunistic pathogens in the absence of a foreign body (Arciola et al., 2018). However, the presence of a medical implant can disrupt the normal immune response and provide a surface for bacteria to adhere to and form a biofilm, leading to a chronic infection that is difficult to eradicate. Understanding the interactions between the pathogen, biomaterial, and host immune response is crucial for developing effective strategies to prevent and manage biofilm-related infections associated with medical implants.

In the case of implant-associated infections, the biomaterial can cause a local tissue response that includes acute and chronic inflammation, a foreign body reaction, the development of granulation tissue, and eventually, fibrous encapsulation (Anderson, 2016). The presence of an implant can also provide an ideal surface for bacterial cells to adhere to and form a biofilm, which can exacerbate the local tissue response and lead to chronic infections. Effective strategies to prevent and manage biofilm-related infections associated with medical implants should take into account the local tissue response and the potential for bacterial adherence and biofilm formation. The presence of medical implants can promote the development of biofilms, which can be challenging to treat.

While antimicrobial drugs such as imipenem, colistin, and others may be effective at reducing biofilm growth, they are often unable to completely remove the entire biofilm. Additionally, the biofilm bacterial cells have higher minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) levels, which makes antibiotic treatment less effective (Hengzhuang et al., 2011; Hoiby et al., 2011; Wu et al., 2015). As a result, there is a need for alternative approaches to prevent and manage biofilm-related infections associated with medical implants.

2.6.1 Formation of biofilms

Biofilms are complex, dynamic communities of microorganisms that adhere to surfaces and secrete a matrix of EPS. Medical devices, such as catheters, implants, and prostheses, are particularly susceptible to biofilm formation, as they provide a surface for bacteria to attach to and grow on. The formation of biofilms occurs in several stages: attachment, colonization, proliferation, and maturation.

Attachment: This phase involves the initial adhesion of planktonic bacteria to a surface. This is a critical stage, as it sets the foundation for biofilm formation. Bacteria attach to the surface using various mechanisms, such as fimbriae, flagella, and pili.

Colonization: In this phase, bacteria begin to multiply and form microcolonies. The first cells to colonize the surface are called primary colonizers, and they pave the way for secondary colonizers by modifying the surface and producing EPS.

Proliferation: During this stage, microcolonies merge to form a mature biofilm. Bacteria within the biofilm experience changes in gene expression, metabolism, and growth rate. This allows them to adapt to their environment and survive under adverse conditions.

Maturation: In this final stage, the biofilm is fully developed, and cells within the biofilm assume different roles, forming structured communities. The EPS matrix provides protection and nutrients to the cells, which promote their growth and survival. Overall growth cycle of biofilm explained in Fig 2.2.

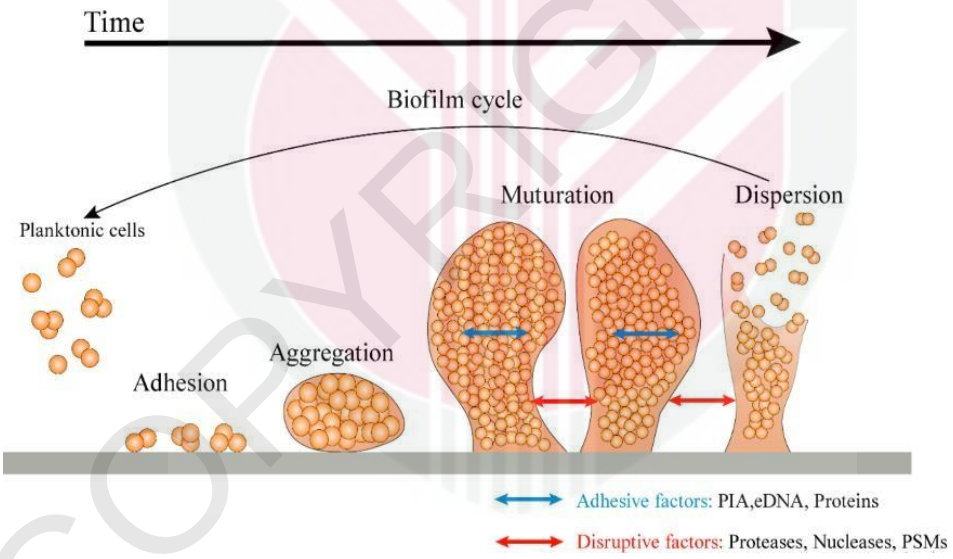


Figure 2.2 : A model of the growth cycle of biofilm
(Adapted from Penget al., 2023)

2.6.2 Biofilm evasion on immune system

One of the hallmarks of biofilms is their ability to evade the immune system, leading to persistent infections that are difficult to treat. Biofilms have several mechanisms to evade the immune system. One such mechanism is the production of a slimy

extracellular matrix that provides a physical barrier against immune cells' infiltration. The extracellular matrix is composed of exopolysaccharides, proteins, and DNA, which are essential for biofilm formation and resistance to antibiotics. This matrix not only physically hinders immune cells' ability to reach the biofilm but also sequesters immune molecules such as antibodies, complement, and cytokines, making them ineffective in clearing the infection.

Another mechanism biofilms use to evade the immune system is quorum sensing. Quorum sensing is a communication system used by bacteria to coordinate their behavior and regulate gene expression. In biofilms, bacteria use quorum sensing to control the conditions within the biofilm, such as nutrient availability and waste disposal. However, quorum sensing also allows biofilms to evade the immune system by altering their gene expression to down regulate virulence factors that would attract immune cells. Additionally, quorum sensing can lead to the production of biofilm-specific molecules that prevent immune cells from recognizing and responding to the biofilm.

Biofilms can also manipulate the host immune response by inducing a low-grade chronic inflammation that is ineffective at clearing the infection. This sustained inflammatory response is thought to promote the formation of new blood vessels in the biofilm, which enhances the bacteria's nutrient supply and promotes biofilm stability. Furthermore, biofilms can alter the host's immune response by recruiting immune cells that have an anti-inflammatory phenotype, further exacerbating the chronic inflammation and promoting biofilm survival.

Biofilms are highly adaptive communities of microorganisms that have evolved to evade the host immune system through multiple mechanisms. Understanding these mechanisms is crucial for developing effective therapies to combat chronic biofilm infections.

2.7 Summary

Antimicrobial resistance (AMR) is a growing concern due to the emergence of biofilms in medical devices. Biofilms are inherently resistant to antimicrobial agents, which makes them difficult to eradicate. The EPS matrix acts as a physical barrier that prevents antimicrobial agents from penetrating the biofilm. Additionally, bacteria within the biofilm adopt a slow-growing, dormant state, which further protects them from the effects of antimicrobial agents.

To combat AMR in biofilms, various strategies have been proposed, such as using quorum sensing inhibitors, enzymes that degrade the EPS matrix, and optimizing device design to prevent biofilm formation. In summary, biofilm formation in medical devices is a complex process that involves multiple stages. AMR in biofilms is a growing concern and requires a multi-pronged approach to address.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study design

Bacterial adhesion to orthopedic implants and its adverse effects are vital flaws in PJIs. Although several pieces of research demonstrated the underlying mechanisms of bacterial adhesion to implants, we still do not have a sufficient understanding of the topic to date. Thus, investigation of biofilm formation in different biomaterials of implants could help to hinder implant-related infections. Besides, an irrigation approach to mediate biofilm is ideal for implant infections. Although the irrigation concept is popular in the medical field, there are limited comparative data that show irrigation solution efficiency on sessile bacteria in both *in vitro* and *in vivo* case series. The antiseptic agent types, concentration, and exposure time of antiseptics are significant factors in determining irrigation outcomes of biofilms on medical implant infections (Schmidt et al., 2018).

Therefore, the current study was designed to determine the efficacy of PVP irrigation on MRSA coated stainless steel implants. MRSA biofilm was grown on stainless steel plates via *in vitro* method, and the matured biofilms were viewed using a scanning electron microscope (SEM) to understand the biofilm morphology and physiology. Later, the mature MRSA biofilm was examined using PVP irrigation with three different time responses and six dose responses to compare standard treatment cares. The overall study design is shown in Figure 3.1. The materials used are listed in Table 3.1, and details methods are discussed in subsection 3.3.

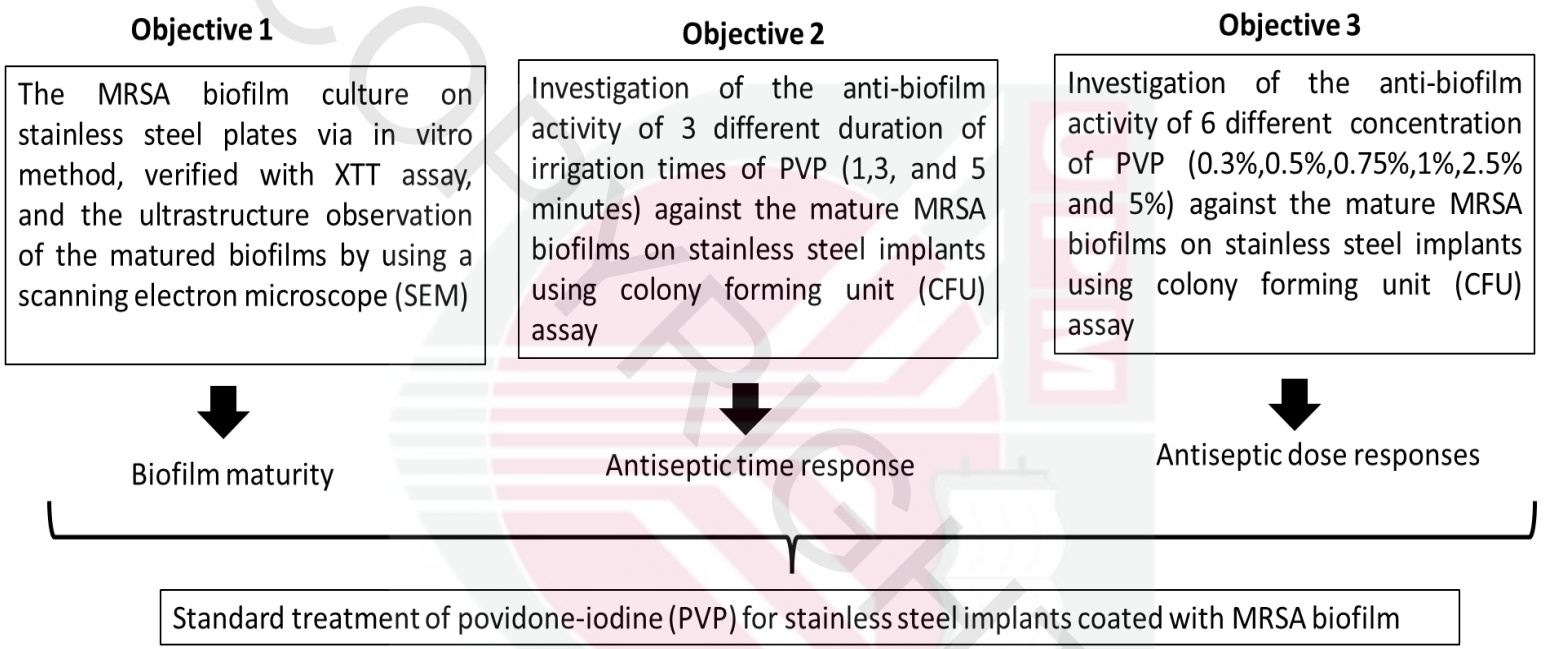


Figure 3.1 : The flowchart of the overall study design

3.2 Materials

Listed below are all the materials, reagents, and chemicals used in this study.

Table 3.1 : List of reagents and chemicals based on alphabetical order

Materials and chemicals	Brand & manufacturing country
Betadine® Antiseptic Solution (povidone- iodine, 10%)	Betadine, US
Cell culture dishes	TrueLine
Cell scraper	BIOLOGIX
Glutaraldehyde 4% in 0.1m Phosphate Buffer, pH 7.3	
Menadione	Sigma-Aldrich
Minisart® Syringe Filter, Polytetrafluorethylene (PTFE), Pore Size 0.2 µm	Sartorius, Germany
Muller Hinton Agar (MHA)	Oxoid
Normal saline	RinsCap
Osmium Tetroxide Solution	
Phosphate buffered saline (PBS)	Sigma-Aldrich
Sodium cacodylate, 0.1M buffer soln., pH 7.0	
Sterile PS L-Shaped cell spreaders	BIOLOGIX
Tryptic Soya Broth (TSB)	Oxoid
XTT sodium salt	Sigma, Steinheim, Germary
3.5 mm stainless steel implant 316L	Siora Surgicals Pvt. Ltd., India

3.3 Methods

3.3.1 Assessing the maturity of MRSA biofilm

3.3.1.1 Media preparation for biofilm production

Tryptic Soya Broth (TSB) and Muller Hinton Agar (MHA) medium were produced based on the manufacturer's protocol. Precisely, 40 g of TSB powder was dissolved in 1 L distilled water, and the mixture was autoclaved at 121°C for 15 minutes (Ishii, Matsumoto, & Sekimizu, 2015). For MHA media, 38 g of MHA powder was suspended in 1 L distilled water, and the mixture was autoclaved at 121°C for 15 minutes. After autoclaving, the agar medium was poured into sterile petri dishes, and let it cooled and solidified. The plates stored at 2-8°C for later use.

3.3.1.2 Preparation of stainless steel plates

Totally 20 stainless steel alloy (316L) implants with 1.5 cm length and 3.5 mm thickness were used for this experiment. All implants were washed with sterilized distilled water, and autoclaved at 121°C for 15 minutes (Govindaraj & Muthuraman, 2015). Later, the implants dried in the oven for 30 minutes and stored at room

temperature for next experimental steps.

3.3.1.3 Bacterial strains and culture preparation

The MRSA SCCmec type I strain obtained from the Microbiology laboratory, Department of Medical Microbiology, Faculty of Medicine and Health Sciences, UPM. The clinical isolate was characterized using PCR and sanger sequencing. A single colony of MRSA cultured on MHA plates, incubated at 37°C for 24 hours and later stored at 4°C (Wu et al., 2019). Bacteria were sub-cultured weekly and maintained in colony form on the agar plate. A single colony of MRSA was used to prepare the inoculum for all biofilm production. MRSA colony from a freshly streaked MHA plate was inoculated into 20 mL TSB and incubated for 18 hours at 37°C with a shaking incubator of 120 rpm maximum speed (Chen et al., 2019).

3.3.1.4 MRSA biofilm formation on stainless steel plates via *in vitro*

After the growth of bacterial cells to the logarithmic growth stage, the optical density of the bacterial solution was measured and adjusted with fresh TSB broth to standardize the inoculum a McFarland standard of 0.5 as detected by a cuvette spectrophotometer at 600nm (Eppendorf BioPhotometer Plus), which was equivalent to $1.0x10^8$ cells/mL. A fresh inoculum used for each trial. Seven sterile 12-well culture plates were taken, the prepared sterilized stainless-steel plates were put into 12 well, and three wells were selected in each plate each. 12 well plate contained 3 implants. Stainless-steel implants were immersed into 2 mL of bacterial inoculum. The 12 well plates were incubated in incubator shaker at 50 rpm at 37°C for 7 days. After cultured for correspond time, stainless steel implants were taken out for bacterial biofilm determination and analysis. TSB broth media was aspirated and replenished daily basis.

3.3.1.5 Evaluating the dynamic of bacterial biofilm formation using XTT method

To determine the degree of biofilm formation, an XTT [2, 3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay was used. XTT is a tetrazolium salt and mitochondrial dehydrogenase substrate that can be deoxidized by cytoplasmic respiratory chain enzymes into a water-soluble formazan (Stockert et al, 2018), which results in an absorbance measurement that directly indicates the level of biofilm formation. Pilot experiments were conducted to establish the optimal timeline for biofilm maturity. The XTT-menadione solution was prepared by dissolving 55 mg of menadione in 100 mL of acetone and adding it to a freshly saturated solution of 4 mg XTT in 10 mL of normal saline (Sabaeifard et al., 2014). The XTT solution was filtered through a 0.22 μm membrane before being prepared instantly before each assay. Stainless steel implants were examined daily for 1 week and transferred to new 12 well plates, washed with sterile PBS to remove planktonic bacteria, immersed in sterile PBS, and scraped with a cell scraper for 2 minutes on both sides of the implants. A 100 μL PBS suspension was then pipetted into a flat bottom polystyrene 96 well plate, and 100 μL of XTT-menadione solution was added to the washed suspension.

A blank control well with sterile PBS and a well with bacteria suspension were also included. The 96 well plate was covered with aluminum foil, sealed with parafilm, and incubated for 3 hours at 37°C in the dark without shaking. The colorimetric change of the XTT solution was measured at 490 nm using a microtiter plate reader, and the resulting data indicated the metabolic activity of the biofilm (Lopes, Azevedo, & Pereira, 2014). Triplicate biofilm assays were performed and repeated for three individual weeks, with a total of 80 biofilms analyzed for maturity.

3.3.1.6 Observation of bacterial biofilm ultrastructure using scanning electron microscope (SEM)

To determine the structure of the biofilm on the surface of the implant, a scanning electron microscope was utilized (Azzola et al., 2020). Stainless steel implants were removed daily for 10 days and the coated biofilm implants were transferred to a new 12- well plate each day. The implants were then washed with PBS three times to eliminate any planktonic bacteria from the surface. After the PBS wash, the implants were fixed with 4% buffered glutaraldehyde at 4°C for 6 hours and then washed with 0.1 sodium cacodylate buffer for three cycles of 10 minutes each. Osmium tetroxide (OsO_4) was used to further fix the implants (1% in distilled water) for 2 hours at 4°C. The implants were then washed again with 0.1 sodium cacodylate buffer for three cycles of 10 minutes each and placed in 0.1 sodium cacodylate buffer at 4°C. Dehydration of the implants with acetone was performed at the Institute of Bioscience laboratory. Subsequently, the morphology of the biofilm was analyzed and images were obtained with the assistance of the Microscopy Unit at the Institute of Bioscience, Universiti Putra Malaysia. A stainless-steel implant without bacterial suspension was used as a positive control. A total of 21 samples of coated biofilm implants were used in the study.

3.3.2 Investigation of the PVP against MRSA biofilm maturity

3.3.2.1 Preparation of media for biofilm production

TSB powder (40 g) was dissolved in 1 L distilled water, and the mixture was autoclaved at 121°C for 15 minutes (Ishii, Matsumoto, & Sekimizu, 2015). For MHA media, 38 g of MHA powder was suspended in 1 L distilled water, and the mixture was autoclaved at 121°C for 15 minutes. After autoclaving, the agar medium was poured into sterile petri dishes, and let it cooled and solidified. The plates stored at 2-8°C for later use.

3.3.2.2 Preparation of implants

Totally 30 stainless steel implants with 1.5 cm length and 3.5 mm thickness were used for this experiment. All implants were washed with sterilized distilled water, and autoclaved at 121°C for 15 minutes (Govindaraj & Muthuraman, 2015). Later, the implants dried in the oven for 30 minutes and stored at room temperature for next

experimental steps.

3.3.2.3 Preparation of PVP

PVP 5% solution (BETADINE®) was purchased directly from pharmacy and diluted to 2.5%, 1.0%, 0.75% and 0.3% by using normal saline (Rinscap).

3.3.2.4 Bacterial strain and preparation of bacterial suspension

MRSA strain obtained from the Microbiology laboratory, Department of Medical Microbiology, Faculty of Medicine and Health Sciences, UPM. A single colony of MRSA cultured on MHA plates, incubated at 37°C for 24 hours and later stored at 4°C (Wu et al., 2019). Bacteria were sub-cultured weekly and maintained in colony form on the agar plate. A single colony of MRSA was used to prepare the inoculum for all biofilm production. MRSA colony from a freshly streaked MHA plate was inoculated into 20 mL TSB and incubated for 18 hours at 37°C with a shaking incubator of 120 rpm maximum speed (Chen et al., 2019).

3.3.2.5 *In vitro* biofilm production on implants

After the growth of bacterial cells to the logarithmic growth stage, the optical density of the bacterial solution was measured and adjusted with fresh TSB broth to standardize the inoculum a McFarland standard of 0.5 as detected by a cuvette spectrophotometer (Eppendorf biophotometer plus, Germany) at 600 nm. A fresh inoculum was created for each trial. The prepared sterilized stainless-steel plates were put into 12 well, and three wells were selected in each plate. Stainless-steel implants were immersed into 2 mL of bacterial inoculum. The 12 well plates were incubated in incubator shaker with rotation 50 at 37°C for 72 hours. After cultured for correspond time, stainless steel implants were taken out for testing the efficiency of PVP. TSB broth media was aspirated and replenished daily. The implant with bacterial inoculum was used as positive control while implant with normal saline (N/S) 0.9% was used as negative control.

3.3.2.6 Efficiency of PVP to irradicate mature biofilm on stainless steel implant

The activity of PVP to eradicate the mature biofilm was assessed by using CFU assay method. The concentration of PVP used was 5.0 %, 2.5 %, 1.0 %, 0.75 % and 0.3 %, and the time duration of exposure was 1, 3 and 5 minutes. After 72 hours incubation, the coated biofilm implants were transferred to new 12 well plate, and then gently washed 3 times with sterile PBS to remove planktonic bacteria from the surface of implants. After washing with sterile PBS, the implants were exposed to 5.0 %, 2.5 %, 1.0 %, 0.75 % and 0.3 % of PVP for 1, 3 and 5 minutes. The implants were then rinsed with PBS to remove the PVP and submersed with PBS and scraped with cell scraper

for 2 minutes in both side of implants.

The resulting suspension of any residual biofilm bacteria in PBS was then homogenized, serially diluted, and plated onto Muller-Hinton agar (MHA) plates. Count the colonies on the plate and use the serial dilution factor to estimate the number of cells in the original culture for each replicate. Serial dilutions of 10-fold from $1x10^{-1}$ to $1x10^{-5}$ were made in test tubes containing 0.9 mL of PBS. Aliquots of 100 µL of each dilution were seeded onto MHA plate, using the spread plate technique. Afterwards, the petri dishes were incubated at 37ºC for 24 hours.

All biofilm experiments were done in triplicates with both positive (Presence of MRSA biofilm) + no PVP wash but with PBS wash) and negative controls (Absence of MRSA biofilm + PVP wash) to ensure reliability. The logarithm numbers of the colony forming unit per mL (log_{10} CFU/mL) of the samples were measured by observing and counting the total colonies formed after incubation at 37°C for 24 hours and 48 hours using colony counter (Isolab Laborgeräte GmbH, Germany).

The number of viable bacterial cells was expressed in following formulae:

= number of colonies X (dilution factor / the volume of culture plated)

The formula used to calculate the total plate count as stated below:

CFU/mL = (average number of colonies X total dilution factor) / volume plated.

(Brown, 2022)

3.4 Statistical Analysis

The results were presented using the mean and standard deviation. GraphPad Prism (version 9.0) was used to conduct statistical analysis. One-way and two-way analysis of variance (ANOVA) were selected to determine statistical significance with P value <0.05 followed by Tukey's post hoc test.

CHAPTER 4

RESULTS

4.1 Biofilm quantification using XTT method

Stainless steel implants coated with MRSA biofilm were cultured for seven days. To study the kinetics of MRSA biofilm formation on stainless steel implants, cultured samples were observed daily. The XTT-reduction assay was used to quantify the biofilm produced by MRSA, with optical density (OD) representing each strain tested. The quantification assays were done in triplicate and repeated three times independently; the absorbance value of tested MRSA biofilm is presented in Figure 4.1. Results showed that MRSA biofilm production increased with the density of sessile cells, as reflected in the increased production of soluble coloured formazan salt. Planktonic living cells also increased in number during the first two days of incubation but decreased thereafter. On the fourth day of culture, MRSA biofilms displayed high metabolic activity, and living cells started to decrease after the fourth day (starvation/death phase). Therefore, biofilm cultures between day 3 and day 4 were considered to be in their mature form.

4.2 Surface characteristics of MRSA biofilm

Using SEM, we examined bacterial biofilms formed on stainless steel implants over various time points. Our observations revealed three distinct stages of MRSA biofilm formation: after only one day, MRSA attachment to the implant surface was loose and unorganized, as shown in Figure 4.2. By the second day of growth, cocci bacteria began to attach in micro colonies, marking the start of the cell adhesion stage Figure 4.2. On day three, a mature biofilm with a 3-dimensional structure containing densely packed cell clusters had formed. Our findings suggest that a mature biofilm can be achieved after 3 days of bacterial growth on the implant surface.

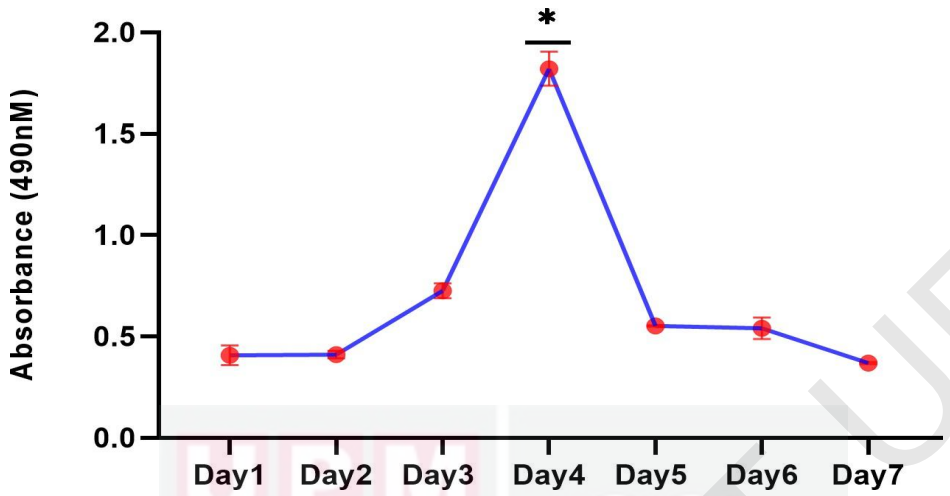
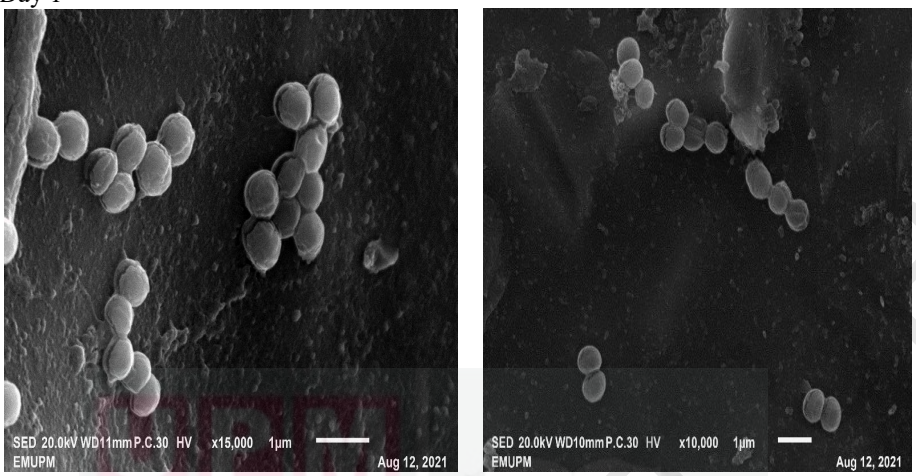


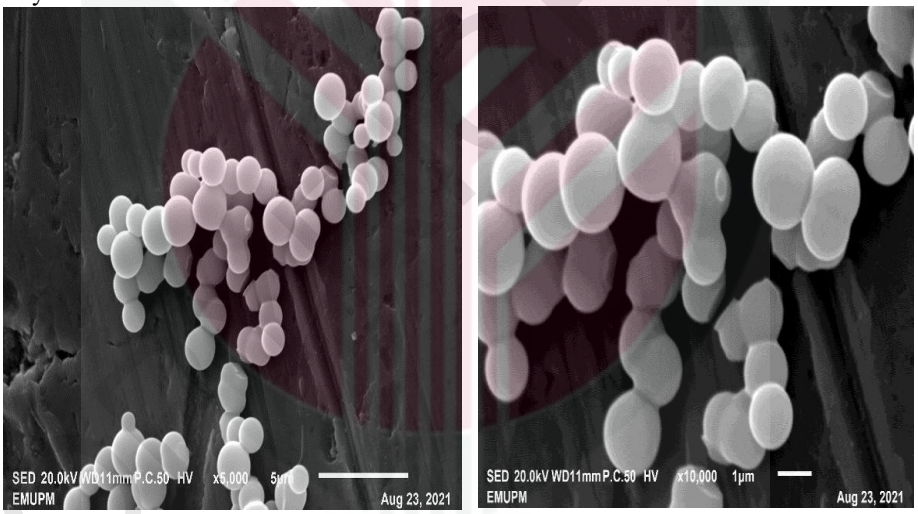
Figure 4.1 : The dynamic formation of MRSA biofilm was monitored for 7 days by measuring absorbance at 490 nm. Bacterial activity increased from day 3 onwards, reaching maximum XTT absorbance on day 4, indicating the presence of mature biofilm between day 3 and day 4. The results are presented as mean ± standard deviation. The experiment was conducted in triplicate. Statistical analysis was performed using one- way ANOVA followed by Tukey's test. The p-value was set to be nonsignificant(ns) P> 0.05, and *P≤ 0.05

Day 1



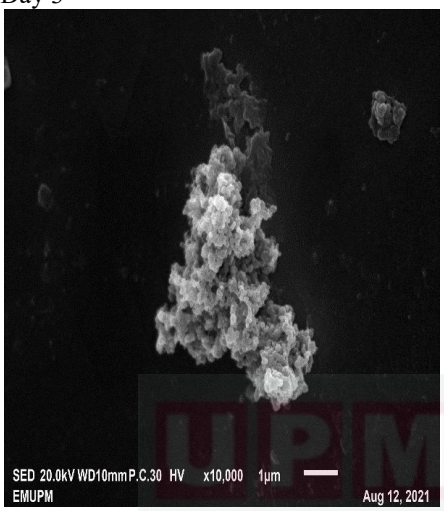
MRSA biofilm formation on stainless steel implant after 1 day of incubation under magnification of ×15000 and ×20000. SEM observation showed that attachment of bacterial cells to an implant surface.

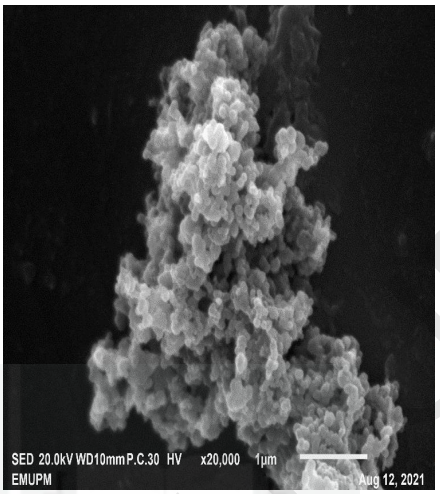
Day 2



MRSA biofilm formation on stainless steel orthopedic implant after 2 days of incubation under magnification of ×2500 and ×5000. SEM observation showed that formation of micro colonies and the beginning of biofilm.

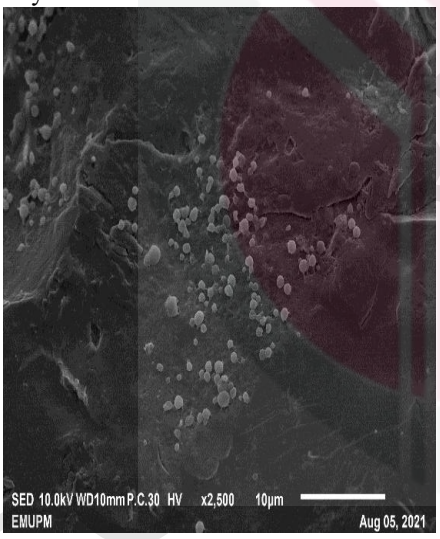
Day 3

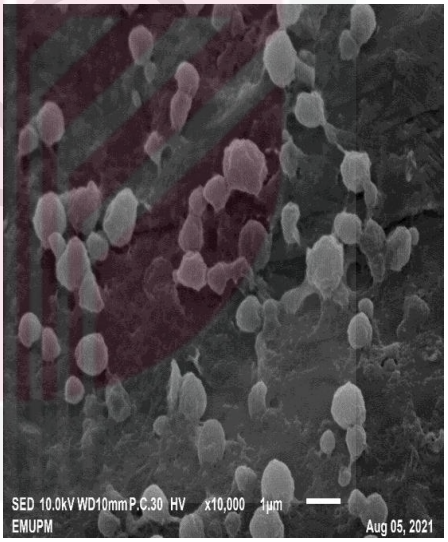




MRSA biofilm formation on stainless steel implant after 3 days of incubation under magnification of ×10000 and ×20000. SEM observation showed that formation of 3-dimensional structure of mature biofilm.

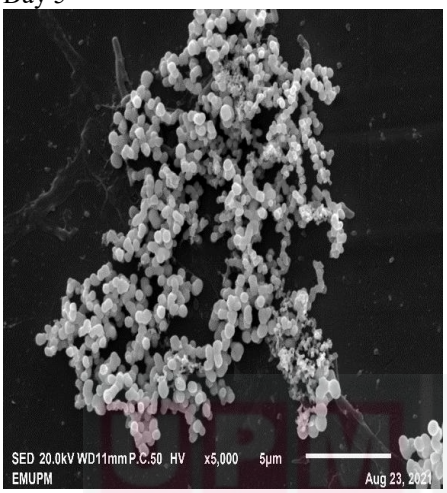
Day 4

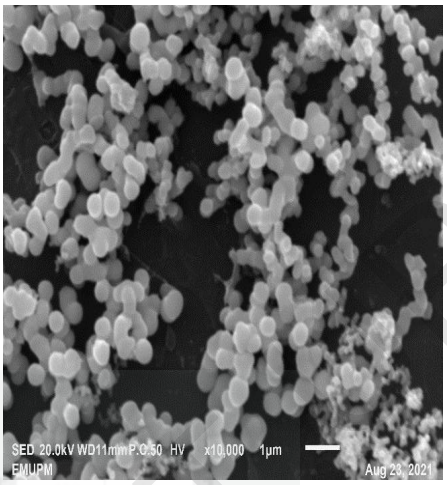




MRSA biofilm formation on stainless steel implant after 4 days of incubation under magnification of ×2500 and ×10000. SEM observation showed that reattachment of
bacteria on the surface of implant.

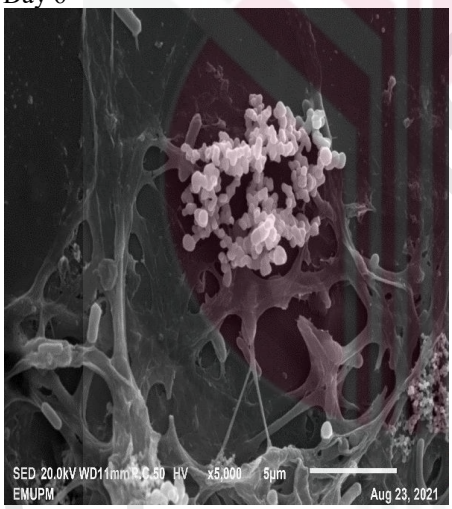
Day 5

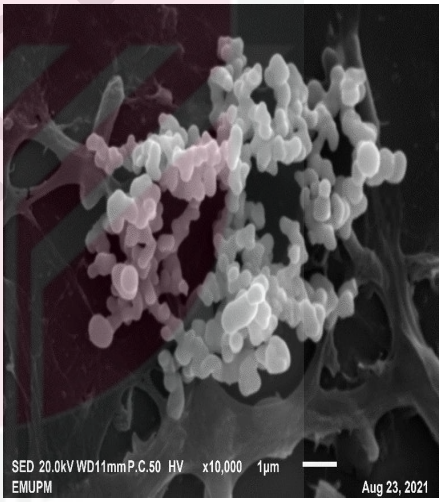




MRSA biofilm formation on stainless steel implant after 5 days of incubation under magnification of ×5000 and ×10000. SEM observation showed that formation of micro colonies and the beginning of biofilm.

Day 6





MRSA biofilm formation on stainless steel implant after 6 days of incubation under magnification of ×5000 and ×10000. SEM observation showed that formation of 3-dimensional structure of mature biofilm.

Figure 4.2 : SEM images for biofilm formation on stainless steel implant from day 1 to 6

4.3 Anti-biofilm activity of different concentration PVP

PVP significantly reduced bacterial cell density compared with the negative control for all microbes at most time points.

4.3.1 Anti-biofilm activity of 5% PVP against different eradication time

Figure 4.3 provides the results obtained from log CFU/mL counts after irrigation with 5% PVP for 1 min, 3 min, 5min, and the results from log CFU/mL counts after 24 hours reincubation. CFU/mL significantly reduced for more than 3 $\log_{10}$ CFU/mL, which was from 6.40 ± 0.022 $\log_{10}$ CFU/mL to zero upon exposure to 5% for 1, 3 and 5 min. After reincubation, the value significantly reduced from 6.20 ± 0.089 $\log_{10}$ CFU/mL for all irrigation time. Log_{10} CFU/mL of positive control before reincubation was 6.41 while after reincubation was 6.20. After treatment with 5% PVP, the count found to be zero at all treatment times. The CFU assays were done in triplicate and repeated two times independently. 5% PVP significantly reduced bacterial cell density in MRSA biofilms of all maturities after just (time) compared with the negative control (all $P < 0.05$) but did not result in complete biofilm eradication at any time point.

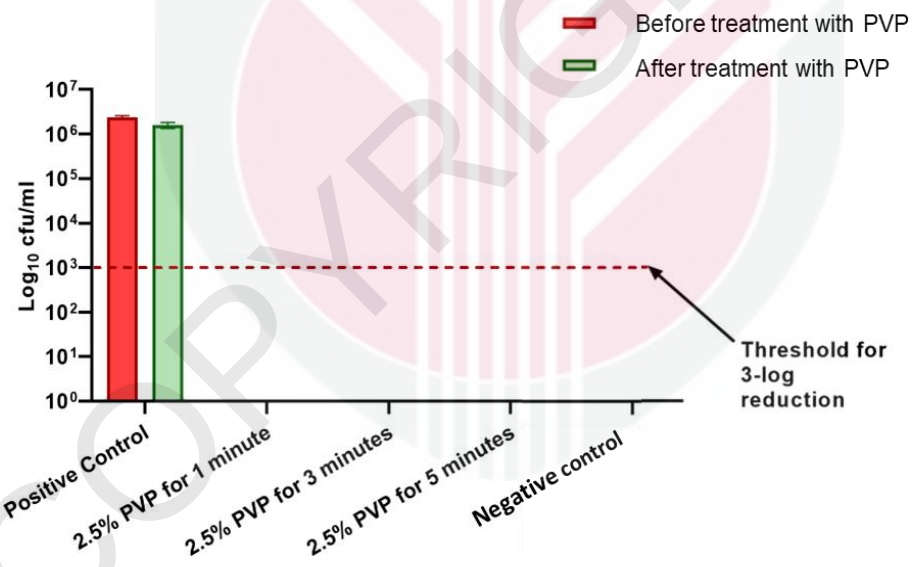


Figure 4.3 : Anti-biofilm activity of 5% PVP against 1, 3 and 5 minutes. The results are presented as mean ± standard deviation. The exposure to 5% PVP reduced the log10 cfu/ml of biofilm to zero in 1, 3, and 5 minutes, both before reincubation and after incubation of stainless steel implants. The experiment was conducted in triplicate. Statistical analysis was performed using two-way ANOVA followed by Tukey's test. The p-value was set to be nonsignificant(ns) P> 0.05, and *P≤ 0.05

4.3.2 Anti-biofilm activity of 2.5% PVP against different eradication time

Figure 4.4 provides the results obtained from log CFU/mL counts after irrigation with 2.5% PVP for 1, 3 and 5 minutes. The results from log CFU/mL counts after 24 h reincubation. CFU/mL significantly reduced for more than 3 log_{10} CFU/mL, which was from 6.37 ± 0.039 to zero upon exposure to 2.5% for 1, 3 and 5 minutes. After reincubation, the count further significantly reduced from 6.19 ± 0.061 to zero CFU/mL for all irrigation time. Log_{10} CFU/mL of positive control before reincubation was 6.41 while after reincubation was 6.19. After treatment with 5% PVP, the count found to be zero at all treatment times. The CFU assays were done in triplicate and repeated two times independently.

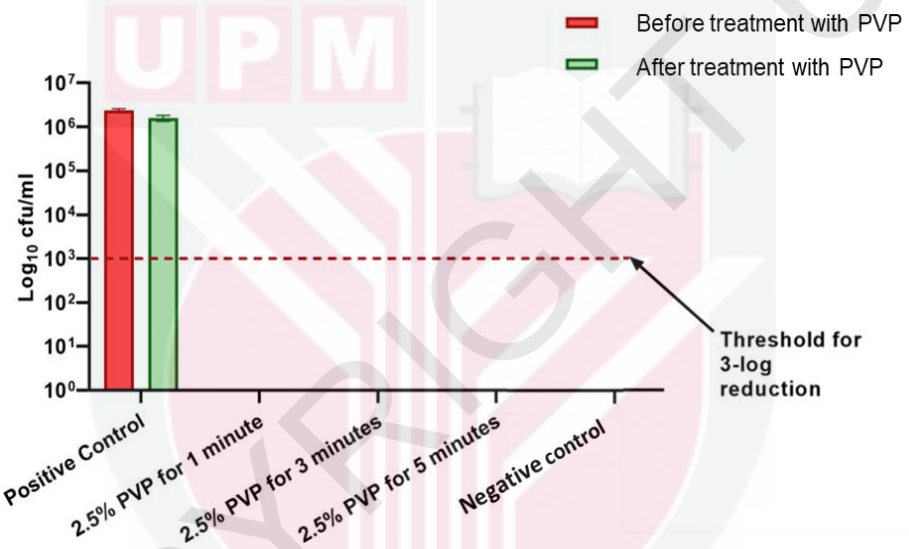


Figure 4.4 : Anti-biofilm activity of 2.5% PVP against 1, 3 and 5 minutes. The results are presented as mean ± standard deviation. The exposure to 2.5% PVP reduced the log10 cfu/ml of biofilm to zero in 1, 3, and 5 minutes, both before reincubation and after incubation of stainless steel implants. The experiment was conducted in triplicate. Statistical analysis was performed using two-way ANOVA followed by Tukey's test. The p-value was set to be nonsignificant(ns) P> 0.05, and *P≤ 0.05

4.3.3 Anti-biofilm activity of 1% PVP against different eradication time

Figure 4.5 provides the results obtained from log CFU/mL counts after irrigation with 1% PVP for 1, 3, 5 min, and the results from log_{10} CFU/mL counts after 24 h reincubation. CFU/mL significantly reduced for more than 3 log_{10} CFU/mL, which was from 6.323 ± 0.053 to zero upon exposure to 1% for 1, 3 and 5 min. After reincubation, the count further significantly reduced from 6.21 ± 0.058 to zero CFU/mL for all irrigation time. Log_{10} CFU/mL of positive control before reincubation was 6.32

while after reincubation was 6.21. After treatment with 1% PVP, the count found to be zero at all treatment times. The CFU assays were done in triplicate and repeated two times independently.

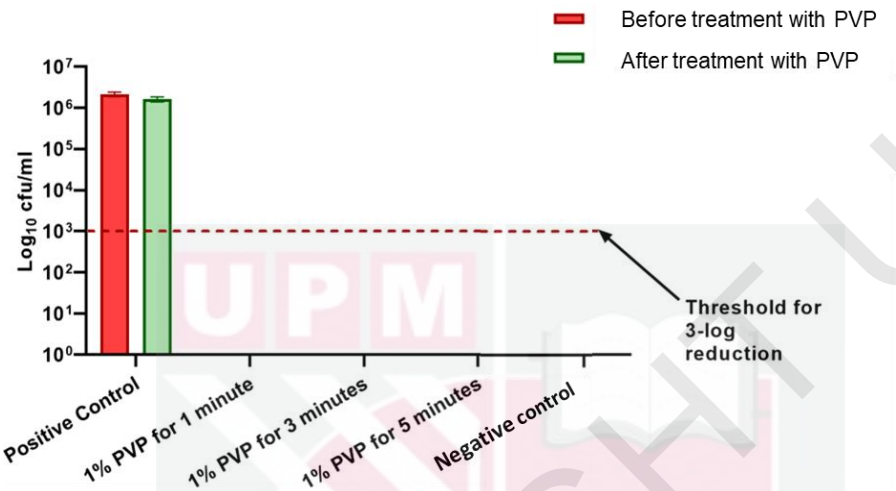


Figure 4.5 : Anti-biofilm activity of 1% PVP against 1, 3 and 5 minutes. The exposure to 1% PVP reduced the log10 cfu/ml of biofilm to zero in 1, 3, and 5 minutes, both before reincubation and after incubation of stainless steel implants. The results are presented as mean ± standard deviation. The experiment was conducted in triplicate. Statistical analysis was performed using two-way ANOVA followed by Tukey's test. The p-value was set to be nonsignificant(ns) P> 0.05, and *P≤ 0.05

4.3.4 Anti-biofilm activity of 0.75% PVP against different eradication time

Figure 4.6 provides the results obtained from log CFU/mL counts after irrigation with 0.75% PVP for 1, 3, 5min, and the results from log_{10} CFU/mL counts after 24 h reincubation. Irrigation with 0.75% PVP for all time of exposure significantly reduced log_{10} CFU/mL. Log_{10} CFU/mL value for 1 minute and 3 minutes irrigation decreased from 6.42 ± 0.012 to 3.11 ± 0.04 and to 2.69 ± 0.029, respectively. Five minutes irrigation reduced log_{10} CFU/mL to zero. After 24 h reincubation, 0.75% PVP that produced significant reductions of log_{10} CFU/mL counted on plates before reincubation compared with control; produced statistically significantly decrease after 24 h reincubation. Log_{10} CFU/mL of positive control before reincubation was 6.42 while after reincubation was 6.21. After treatment with 0.75% PVP, the count was found to be zero at 5 minutes treatment time, while irrigation with 1 and 3 min reduced log_{10} CFU/mL compared to control. The CFU assays were done in triplicate and repeated two times independently.

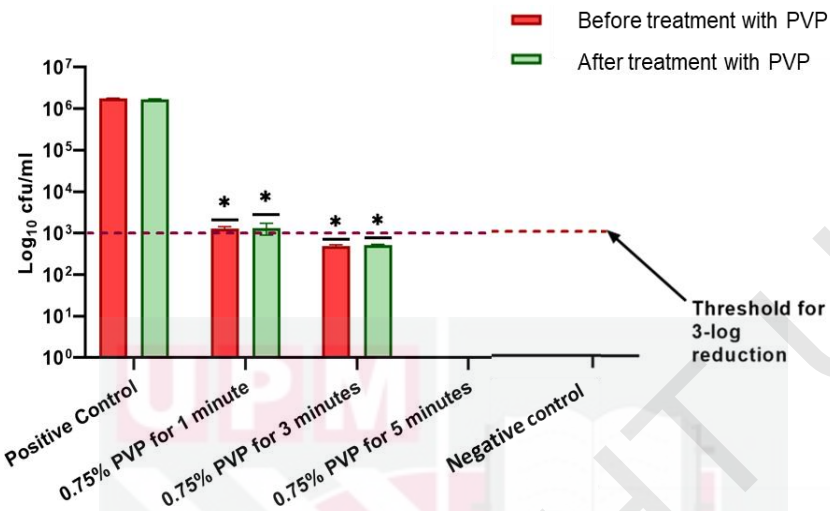


Figure 4.6 : Anti-biofilm activity of 0.75% PVP against 1, 3 and 5 minutes. The exposure to 0.75% PVP reduced the log10 cfu/ml of biofilm to around 10^3 in 1, and 3 minutes and to zero in 5 minutes, both before reincubation and after incubation of stainless steel implants. The results are presented as mean ± standard deviation. The experiment was conducted in triplicate. Statistical analysis was performed using two- way ANOVA followed by Tukey's test. The p-value was set to be nonsignificant(ns) P> 0.05, and *P≤ 0.05

4.3.5 Anti-biofilm activity of 0.5% PVP against different eradication time

Figure 4.7 provides the results obtained from log CFU/mL counts after irrigation with 0.5% PVP for 1, 3 and 5 minutes, and the results from log10 CFU/mL counts after 24 h reincubation. Irrigation with 0.5% PVP for 3 and 5 minutes produced reduction log10 CFU/mL. Three minutes irrigation reduced log10 CFU/mL from 6.24 ± 0.016 to 2.78 ± 0.058, while irrigation for 5 min reduced log10 CFU/mL to 2.49 ± 0.022. Irrigation for 1 min did not yield significant reductions of log10 CFU/mL. After 24 h reincubation, 3 and 5 minutes irrigation decreased for log10 CFU/mL. Log10 CFU/mL of positive control before reincubation was 6.24 while after reincubation was 6.21. After treatment with 0.5% PVP, 1 and 5 minutes significantly reduced for log10 CFU/mL. Irrigation for 1 minute did not yield significant reductions in log10 CFU/mL. The CFU assays were done in triplicate and repeated two times independently.

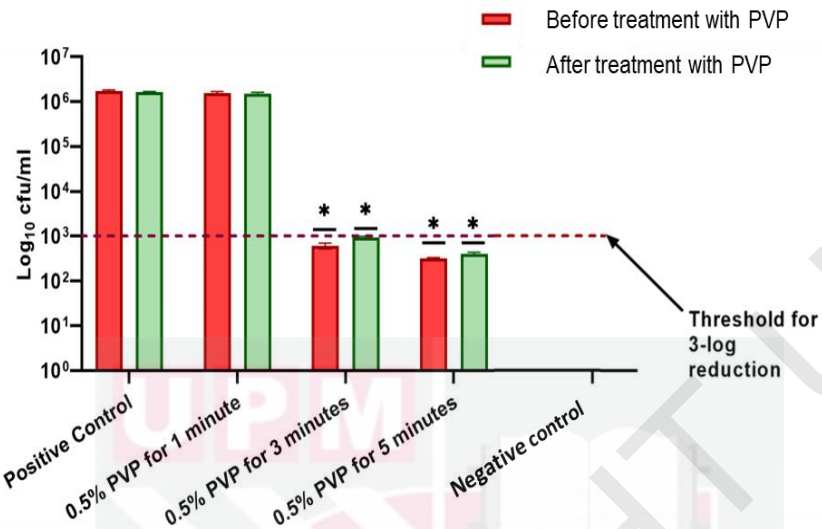


Figure 4.7 : Anti-biofilm activity of 0.5% PVP against 1, 3 and 5 minutes. The exposure to 0.5% PVP reduced the log10 cfu/ml of biofilm to around 10^3 in 3, and 5 minutes, both before reincubation and after incubation of stainless steel implants. The results are presented as mean ± standard deviation. The experiment was conducted in triplicate. Statistical analysis was performed using two-way ANOVA followed by Tukey's test. The p-value was set to be nonsignificant(ns) P> 0.05, and *P≤ 0.05

4.3.6 Anti-biofilm activity of 0.3% PVP against different eradication time

Figure 4.8 provides the results obtained from log CFU/mL counts after irrigation with 0.3% PVP for 1, 3 and 5 minutes, and the results from log10 CFU/mL counts after 24 h reincubation. Irrigation with 0.3% PVP for 3 and 5 minutes produced reduction log10 CFU/mL. Three minutes irrigation reduced log10 CFU/mL from 6.26 ± 0.042 to 2.95 ± 0.03, while irrigation for 5 minutes reduced log10 CFU/mL to 2.78 ± 0.014. Irrigation for 1 minute did not yield significant reductions in log10 CFU/mL. After 24 h reincubation, 3 and 5 minutes irrigation decreased for log10 CFU/mL. Log10 CFU/mL of positive control before reincubation was 6.263 while after reincubation was 6.266. After treatment with 0.3% PVP, 1 and 5 min significantly reduced for log10 CFU/mL. Irrigation for 1 minute did not yield significant reductions in log10 CFU/mL. The CFU assays were done in triplicate and repeated two times independently.

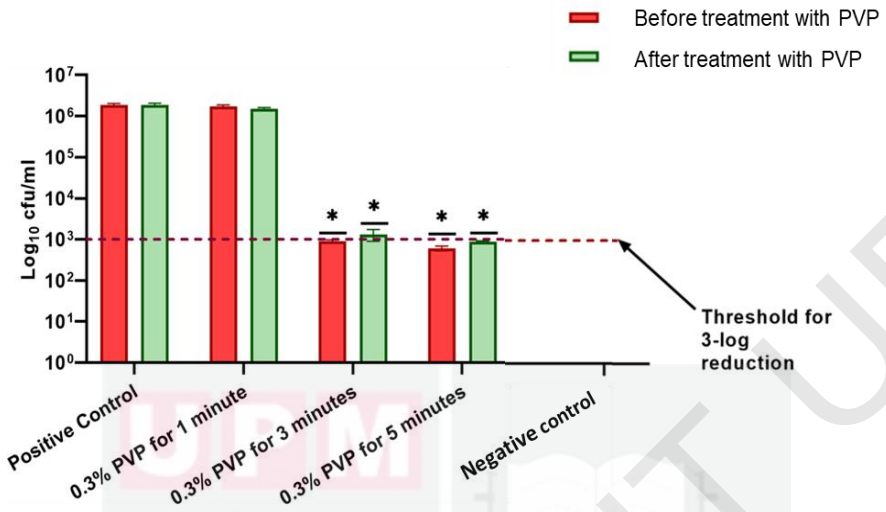


Figure 4.8 : Anti-biofilm activity of 0.3% PVP against 1, 3 and 5 minutes. The exposure to 0.3% PVP reduced the log10 cfu/ml of biofilm to around 10^3 in 3, and 5 minutes, both before reincubation and after incubation of stainless steel implants. The results are presented as mean ± standard deviation. The experiment was conducted in triplicate. Statistical analysis was performed using two-way ANOVA followed by Tukey's test. The p-value was set to be nonsignificant(ns) P> 0.05, and *P≤ 0.05

CHAPTER 5

DISCUSSION

PJI following arthroplasty has significant deleterious consequences for patients' health, despite the fact that PJI occurs in approximately 2.0% total hip and 2.4% of knee arthroplasties, respectively (Fröschen et al., 2022). The frequency of PJIs is steadily rising as TJA becomes more common. Surgical removal and replacement of the infected implant, as well as long-term antibiotic treatment, may be necessary to treat these infections, which can be painful for patients, time-consuming and costly but may be ineffective (Bonanzinga et al., 2017). As evidence has accumulated over the last few decades, bacterial biofilms produced on implant surfaces appear to be a significant factor in PJI pathogenesis, which has been taken into account for the next generation of PJI guidelines (Davidson, Spratt, & Liddle, 2019).

Microorganisms encased in biofilms showed enhanced resistance to typical antimicrobial agents and decreased susceptibility to host immune defense mechanisms (Gebreyohannes et al., 2019). Biofilms are particularly difficult to get rid of because of their intrinsic resistance to therapy (Mishra et al., 2023). Key to biofilm-associated infection's resistance is the complex and varied structure of the biofilm matrix, which is mostly created by the organisms themselves, leading to decreased antibiotic penetration and reduced metabolic activity of biofilm-encased bacteria (Khan et al., 2021). For this, there has been a rise in the number of studies focusing on prospective candidate procedures for destroying the biofilm structure, especially using antiseptic agents during treatment of PJI. The *S. aureus* is widely recognized as one of the most common pathogens responsible for PJI (Kherabi et al., 2022). *S. aureus* is well-known for forming biofilms, which are composed of complex bacterial communities encased in a matrix of polymeric components (Idrees et al., 2021). However, there has been few studies relating to effect on biofilms established on orthopedic implants, include *S. aureus*. In consequence, the aim of this study was to assess the maturity of MRSA biofilm by using XTT method and SEM.

Biofilm quantification was first established in 1985 by using crystal violet (CV) or gentian violet staining, which was enhanced and modified to make the biofilm quantified on a well plate (Haney et al., 2018). CV is an alkaline dye that is attracted to negatively charge surface molecules and polysaccharides present in the extracellular matrix (Asensio et al., 2022). Due to the fact that bacteria, either active or dead, and the substrate are dyed with CV, this method is ineffective for determining the activity of biofilms (Roy et al., 2018). To differentiate living from dead bacteria, to detect and measure metabolic activity in living bacteria, the use of several reactive dyes has become a feasible technique and method including XTT assay (Kamiloglu et al., 2020). The XTT method works by reducing the XTT dye to water-soluble methionine; bacterial supernatant absorbance is proportional to the number of metabolically active microbial cells (Dong et al., 2020). This colorimetric test is non-invasive and non-destructive, requiring less post-processing of samples than other methods (Aslantürk, 2018). Besides, SEM used for the current study as it based on electron absorption and surface scattering. Although weak in vertical resolution, SEM micrographs have a

broad depth of field and produce a 3D appearance, which is important for studying the surface structure of the sample (Donelli, 2014). It is an extremely useful technique for comparative evaluation in biofilm research, particularly when assessing a compound's anti-biofilm activities. SEM imaging has been used to qualitatively verify findings from other quantification methods that show a high degree of correlation (Azeredo et al., 2017). In this study quantitative and qualitative methods were used to observe the formation of MRSA biofilm on stainless steel implant.

The XTT colorimetric assay was used to determine the percentage of viable cells in biofilms established on stainless steel implant. The biofilm biomass represents the total bacteria number in biofilm. The change curve of MRSA biofilm biomass during cultivation actually exhibited the time course of biofilm formation. MRSA biofilm biomass increased after 3 days of inoculation, showing that more cells adhered and accumulated in stainless steel implant surfaces (Figure 4.2). It reached the maximum at 4 days, and then it basically declined, showing that the development of biofilm got into the mature stage. Our study demonstrated that the number of bacterial adhesions was increased with the extension of time. Moreover, the architecture of biofilm at different growth stages was illustrated by SEM, which showed that biofilm consisted of multi-layered cell clusters and displayed the heterogeneity. The stainless-steel implants coated MRSA bacteria were processed and viewed by SEM for 1 week (according to the results of biofilm biomass measurements), to distinguish planktonic bacteria from biofilm bacteria. SEM imaging showed the distinctive shapes of the spherical and coccus shaped MRSA bacteria (Figure 4.2). The SEM analysis showed small cell clusters formation on implants after 24 hours. The SEM analysis revealed no biofilm formation was observed after 48 hours of incubation. After 72 hours of incubation, SEM showed a very complex biofilm coating a stainless-steel implant, with the observation of a large number of bacteria (Figure 4.2). The biofilm was well-structured and firm, with cells attached to each other. Surprisingly, we could not find any EPS matrix around biofilm. A possible explanation for this might be that SEM proceeding includes fixation, dehydration and coating with a conductive material, which can destroy the structure of samples or cause artifacts. Drying causes shrinkage of the biofilm due to the collapse of EPS (Relucenti et al., 2021).

Our finding are in agreement with Dong et al., (2020) findings, which assessed the biofilm maturity of *S. aureus* and *E. coli* on titanium implant using XTT and SEM. At 72 hours after incubation, mature biofilm was formed, and at 24 hours weak biofilm of *S. aureus* was formed, while 6 hours after incubation there were no biofilm formation (Dong et al., 2020). However, the findings of the current study do not support the previous research by Sun et al., (2012), where biofilm of *S. aureus* formed on polystyrene plate was examined using biofilm biomass assay and SEM image; it was recorded that mature biofilm was formed as early after 12 h of incubation (Sun et al., 2012).

Orthopedic surgeons face significant issues with biofilms in periprosthetic infections. No matter how much effort is expended to battle biofilm infections, whether through conservative chemotherapy or surgical management, the required outcomes cannot be guaranteed. It is still difficult to choose the best irrigation agent. An irrigating solution can contain antiseptics, antibiotics, or surfactants (Ruder & Springer, 2017). The

efficacy of using antibiotics in irrigation solutions has not been demonstrated and may lead to the development of antibiotic-resistant bacteria (Edmiston et al., 2017). Antibiotic resistance is not a problem with using antiseptic solutions, however due to their cytotoxicity, they can cause tissue damage and wound problems (Kaiser, Wächter, & Windbergs, 2021). In TJA, the optimal irrigation solution has a short time to effect, has bactericidal activity across a broad range of bacteria, and does not cause tissue toxicity in both the local and systemic tissues (Christopher et al., 2022). The bactericidal requirements for irrigation solutions are modest when attempting to avoid PJI since harmful bacteria are in a planktonic condition and are also in low numbers as a result of a plethora of other successful perioperative preventative strategies (Iannotti et al., 2020). Using the antiseptic solution during treatment of PJI is different from prevention of infection due to formation of biofilm on implant devices and adjective tissues.

Studies have focused on determining the effectiveness of various antiseptic solutions against biofilms. Nonetheless, the majority of antimicrobial treatments aimed at these pathogens rely on the susceptibility of planktonic bacteria *in vitro*, potentially underestimating the resilience of more resistant bacterial forms. For example, biofilms have been documented to exhibit a 100-1,000-fold increase in tolerance compared to their planktonic cell counterparts (Ceri et al., 1999; Yan and Bassler, 2019), attributed to a variety of distinct mechanisms. There are a significant difference planktonic free bacteria, there are limited data on effective of antiseptic solutions against mature biofilm on orthopedic implant surfaces.

In vitro biofilm formation can be affected by various factors such as the choice of growth media, temperature, CO_2 levels, and incubation duration for both induction and maturation phases. While in vitro biofilm formation may not precisely mirror in vivo conditions, investigating biofilm resistance remains crucial for elucidating potential links to clinical outcomes in diseases associated with biofilms (Thieme et al., 2019). A study by Bowler et al. (2012) compared phenotypic antibiotic tolerances between mature and young *P. aeruginosa* biofilms, as well as released planktonic bacteria, for four antibiotics. The findings shown that the resistance levels were comparable for gentamicin and ciprofloxacin but differed for ceftazidime and meropenem. β-Lactamase mapping revealed that mature biofilms produced more β-lactamase than young biofilms after 5 hours of ceftazidime exposure, thereby promoting the growth of released planktonic bacteria. This underscores the significance of early intervention and antibiotic selection in managing *P. aeruginosa* biofilm infections.

The efficacy of povidone iodine against matured biofilm versus planktonic biofilm can vary due to several factors, including the concentration of povidone iodine, contact time, biofilm composition, and microbial species involved. Generally, the effectiveness of povidone iodine may be reduced against matured biofilms compared to planktonic microorganisms due to the protective nature of the biofilm matrix. Planktonic microorganisms are free-floating and not embedded within a protective matrix, making them more susceptible to the antimicrobial effects of povidone iodine. When exposed to povidone iodine, these microorganisms can be killed or inhibited more readily.

In contrast, matured biofilms are highly structured communities of microorganisms embedded within a self-produced extracellular polymeric substance (EPS) matrix. This matrix serves as a protective barrier, reducing the penetration of antimicrobial agents and making the microorganisms within the biofilm more resistant to disinfection. While povidone iodine can penetrate the EPS to some extent and exert antimicrobial activity against the microorganisms within the biofilm, its efficacy may be reduced compared to planktonic microorganisms. The presence of the biofilm matrix can hinder the diffusion of povidone iodine, limiting its ability to reach and kill all microorganisms within the biofilm. Additionally, the concentration of povidone iodine and the duration of exposure play crucial roles in its effectiveness against both planktonic and biofilm-associated microorganisms. Higher concentrations and longer contact times may enhance the antimicrobial activity of povidone iodine against biofilms.

Povidone iodine can penetrate the EPS to some extent and kill the microorganisms within the biofilm. However, its effectiveness can be reduced compared to planktonic (free-floating) microorganisms due to the protective nature of the biofilm matrix. Additionally, the concentration of povidone iodine, contact time, and the nature of the biofilm can influence its efficacy.

Our findings indicate that there was no significant reduction in log CFU/mL with 0.3% and 0.5% PVP treatment during 1-minute, 3-minute, and 5-minute irrigation periods when compared to the positive control. Based on the results, matured biofilm on stainless steel implant is difficult to be eradicated using low concentration of PVP. PVP solutions typically range from 0.5% to 10% concentration. In wound care and mucous membrane disinfection, lower concentrations such as 0.3% or 0.5% may be used. In contrast, for surgical site preparation or preoperative skin disinfection, concentrations of 5% to 10% are more common. Low concentrations of povidone iodine often fail for biofilm treatment due to several factors. These include limited penetration into the biofilm matrix, reduced antimicrobial activity against biofilm-embedded microorganisms, insufficient contact time for effective disruption, the inherent resilience of biofilms to antimicrobial agents, and the potential development of antimicrobial resistance over time. Biofilms' complex structure and protective matrix render them more resistant to low concentrations of PVP, which may not adequately penetrate or disrupt the biofilm to eradicate the microorganisms within. Consequently, higher concentrations or alternative treatment approaches may be necessary to effectively target and eliminate biofilms in various clinical settings.

Our findings comparable to those of the previous studies that have examined the effect of PVP on bacterial biofilm; in which 5% and 1% concentration of PVP needed 10 and 30 secs to kill *S.aureus* (Gryson et al., 2023). Besides, current findings are consistent with other study, where all bacterial strain including *Pseudomonas*, *Klebsiella*, *Proteus*, *Staphylococcus*, and *E. coli* exposed for 15 seconds to 2.5% concentration of PVP, were killed (Scherr & Dodd, 1976). These findings are in agreement with the results of Ernest et al., (2018), tested the ability of Betadine 10%, Dakin's solution, H_2O_2, and

1% chlorine dioxide (ClO_2) to remove *S. aureus* biofilm on different orthopaedic implant (cobalt chrome, stainless steel and titanium alloy discs). PVP had statistically significant reductions in CFU/cm^2 with an average 98% CFU/cm^2 reduction. This was superior to Dakin's solution and H_2O_2 (Ernest et al., 2018). *S. aureus* and *S. epidermidis* bacteria were reduced by PVP (0.17%, 0.35%, and 0.7% w/v), according to Oduwole, and they discovered that PVP also prevented *staphylococcal* production of intercellular adhesion, a polysaccharide bacterial product that is crucial for biofilm development. Oduwole used spectrophotometry to perform a semi-quantitative biofilm assay, which was followed by a quantitative real- time polymerase chain reaction (qPCR), which revealed decreased expression of the icaADBC biofilm gene cluster (Oduwole et al., 2010). Premkumar et al., tested the efficiency of antiseptic solutions against MSSA biofilm established on different orthopedic implant surface. The implant devices were irrigated for 3 min. 10% PVP mixed 1:1 with 4% H_2O_2 were effective against biofilm form of MSSA. However, 0.3% PVP was not effective against the biofilm (Premkumar et al., 2021). PVP is toxic to human fibroblast cells at higher doses (20%), thus dilution is required to decrease the toxicity of PVP to host tissue (Hoekstra, Westgate, & Mueller, 2017). The ability of low concentration of PVP to eradicate bacterial biofilm has been proved in our study. Similar findings has been reported where, PVP at low concentrations (0.25 percent w/w) totally destroyed multi-drug resistant *S. aureus*, *Klebsiella pneumoniae*, *P. aeruginosa*, and *Candida albicans* biofilms (Capriotti K, Pelletier J, 2018).

Several *in vitro* studies have tested the ability of PVP to remove planktonic form of bacteria. Previous research reported that bacterial growth was completely eradicated between 0 and 3 minutes after application of 10% of PVP (Cichos et al., 2019). *S. aureus*, *S. epidermidis*, and *Cutibacterium acnes* tested with 0.35% PVP, 0.05% CHG, 0.5% sodium hypochlorite, polyhexamethylene biguanide, and an acetic acid–based solution for 15, 30, 60, 90, and 120 seconds; 0.35% PVP was able to remove bacteria during 15 seconds (Christopher et al., 2021). According to a recent *in vitro* study, use of 0.3% PVP was found to be the most effective in eradicating MSSA and *E. coli* with the least cytotoxicity against human osteoblasts, chondrocytes, and fibroblasts (Goswami et al., 2019).

Several clinical studies have demonstrated that 5% of PVP did not lead to a greater reduction in bacterial presence in corneal ulcers compared to merely scraping and rinsing (Gregori et al., 2016). Similarly, a study by Shindo et al. (2202) showed 10% PVP solution is safe and effective for skin antisepsis in healthy young adults. Following the development of a periprosthetic joint infection (PJI), surgical intervention is typically necessary, involving thorough irrigation and debridement as part of an aggressive treatment approach (Ruder & Springer, 2017). To improve the successful rate of PJI treatment, vancomycin powder was used in conjunction with intraoperative 0.3% PVP irrigation/debridement, with modular component and liner exchange in the management of PJI. Povidone-iodine solution at a concentration of 10% is mixed with 500-1000cc of normal saline. The resulting diluted PVP solution is used to irrigate the wound for a duration of 3 minutes. Subsequently, the wound is meticulously rinsed with normal saline to remove any residual povidone-iodine solution before proceeding with wound closure (Riesgo et al., 2018). Schmidt et al. (2018) treated *S. epidermidis* biofilm with chlorhexidine (0.025%, 0.05%, and 0.1%),

PI (0.35%, 1.0%, 3.5%, and 10%), sodium hypochlorite (0.125%, 0.25%, and 0.5%), and triple antibacterial solution (bacitracin 50,000 U/L, gentamicin 80 mg/L, and polymyxin 500,000 U/L) for 1, 5, and 10 minutes. The finding reveals 10% at all treatment time and 3.5% PVP at 10 minutes were able to eradicate the biofilm while low concentration of PVP and triple antibacterial solution (bacitracin 50,000 U/L, gentamicin 80 mg/L, and polymyxin 500,000 U/L) solution were not effective.

However, current study findings do not support the previous research, low concentration of PVP (0.75 %) was proven to eradicate the mature biofilm after 5 minutes of treatment. Increasing the concentration to 2.5% reduces the treatment time to 1 min. Similarly, PVP (0.35%), and chlorhexidine gluconate (0.05%) were tested against *S. aureus*, *S. epidermidis*, and *C. acnes* inoculated on cobalt-chrome, titanium and stainless-steel and was proven to be effective than other antiseptic agents (Kia et al., 2021). Utilizing a single agent like PVP at every stage of surgical intervention may offer potential advantages, such as cost-effectiveness, compared to agents limited to specific stages of the surgical procedure.

CHAPTER 6

CONCLUSION

6.1 Conclusion

The incidence rate of implant-related infection after arthroplasty and fracture fixation remains high despite advancements in surgical operations and techniques. The formation of biofilm on implant devices leads to connective tissue destruction, impaired vascularization, implant loosening, and joint dislocation (Campoccia et al., 2006). This antibiotic resistance makes it challenging to manage implant infections (Gbejuade et al., 2015). In the treatment of PJI with DAIR, irrigation of the infected joint with antiseptic agents is a critical step to eliminate bacteria and biofilm from the implant device (Schmidt et al., 2018). Various antiseptic agents, such as PVP, chlorohexidine, hydrogen peroxide, and sodium hypochlorite (Dakin's solution), are commonly used for irrigation in the orthopedic field (Frisch et al., 2017; Riesgo et al., 2018; Ruder & Springer, 2017). However, the optimal choice of antiseptic agents, their optimal concentrations, and the ideal irrigation time for complete elimination of fully mature biofilm from orthopedic implants remains controversial. Therefore, documenting the most effective antiseptic agents that are less likely to lead to the development of resistant infections is necessary (König et al., 1997; Lachapelle et al., 2013; Organization & others, 2016).

Therefore, the aim of current study was to assess the effectiveness of PVP in eradicating MRSA biofilm on stainless steel orthopedic implants. The results showed that a mature biofilm could be formed on the implant after 72 hours of incubation. However, low concentrations of PVP were able to effectively eliminate the MRSA biofilm when given for extended periods of time. These findings suggest that irrigating with PVP at a low concentration could be a beneficial treatment for PJI in the presence of biofilm. Furthermore, the study provides evidence that irrigation with PVP is a critical step in removing biofilm from orthopedic surfaces during PJI treatment.

6.2 Limitations

It is important to note that this study only tested one type of antiseptic agent against one type of bacteria, so further research is needed to determine the effectiveness of other agents and against different bacterial strains. More rigorous study designs are necessary in clinical trials to facilitate meaningful comparisons among antiseptic agents, especially concerning the confounding factors in antiseptic treatment utilizing povidone iodine. These factors include patient characteristics such as age, sex, and underlying health conditions, which may impact treatment response and tolerability. Additionally, wound-specific factors such as size, depth, and microbial composition can influence the effectiveness of povidone iodine in preventing or treating infections. The method of application, concomitant medications, environmental factors, and study design elements such as randomization and blinding further contribute to the complexity of interpreting study results. Addressing these confounding factors is

crucial to accurately assess the true efficacy and safety of povidone iodine treatment and to draw meaningful conclusions regarding its clinical utility. preoperative skin preparation using other bacterial species including. Additionally, this study was conducted *in vitro* and may not fully reflect conditions *in vivo* where bacterial biofilms are more resistant to antibiotics. Moreover, the potential cytotoxicity of PVP was not assessed in this study.

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