ANTIBACTERIAL EFFECTS OF PHOMOPSIDIONE ISOLATED FROM *DIAPORTHE FRAXINI* AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) WITH GENE EXPRESSION AND METABOLOMICS PROFILING

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LIST OF SYMBOLS

˚C	Temperature, Degree Celsius
Da	Weight, Dalton
g	Weight, gram
h	Hour
k	Kilo
L	Volume, Litre
m/z	Mass-to-charge ratio
m	Length, meter
m	Milli (1×10^{-3})
min	Minutes
µ	Micro
M	Molar concentration
n	Nano
%	Percent
±	Plus-minus
psig	Pounds per square gauge
rpm	Revolution per minute
s	Second
V	Voltage

LIST OF ABBREVIATIONS

ANOVA	Analysis Of Variance
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CDC	Centre for Disease Control and Prevention
CLSI	Clinical & Laboratory Standards Institute
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
ESI	Electrospray ionization
HA-MRSA	Hospital-acquired methicillin-resistant *Staphylococcus aureus*
HCA	Hierarchical cluster analysis
IDSA	Infectious Diseases Society of America
INT	*p*-Iodonitrotetrazolium violet salt
LC-HRMS	Liquid chromatography-high resolution mass spectrometry
MBC	Minimum bactericidal concentration
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
MLS_B	Macrolide-lincosamide-streptogramin B
MRSA	Methicillin-resistant *Staphylococcus aureus*
mRNA	Messenger RNA
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MVDA	Multivariate data analysis

PBS	Phosphate buffer saline
PC	Principal component
PCA	Principal component analysis
PIA	Polysaccharides intercellular adhesins
PLS	Partial least squares
PLS-DA	Partial least squares-discriminant analysis
*p*NPP	*p*-Nitrophenyl palmitate
Q-TOF	Quadrupole Time-of-Flight
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
TMP-SMX	Trimethoprim-sulfamethoxazole
tRNA	Transfer RNA
US	United States
VIP	Variable importance in projection
WHO	World Health Organisation

LIST OF APPENDICES

KESAN ANTIBAKTERIA PHOMOPSIDIONE YANG DIPENCILKAN DARIPADA *DIAPORTHE FRAXINI* TERHADAP METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) BERSERTA EKSPRESI GEN DAN PROFIL METABOLOMIK

ABSTRAK

Peningkatan kelaziman patogen *multidrug-resistant* (MDR) telah menyumbang kepada kadar kematian yang tinggi akibat penggunaan berlebihan dan penyalahgunaan antibiotik. Antara patogen MDR, methicillin-resistant *Staphylococcus aureus* (MRSA) adalah yang paling mengancam dan memberi impak yang terbesar kepada kesihatan awam. Setakat kini, banyak derivatif keton yang bioaktif telah dilaporkan sebagai agen anti-MRSA. Phomopsidione ($C_7H_{10}O_4$), suatu derivatif keton bioaktif yang dipencilkan daripada *Diaporthe fraxini*, telah menunjukkan kesan antibakteria. Kajian ini bertujuan untuk mengkaji kesan antibakteria dan anti-biofilem phomopsidione terhadap MRSA dan mengenalpasti kesan modulasi phomopsidione dalam faktor virulensi. Tambahan pula, perubahan dalam ekspresi gen dan profil metabolit MRSA sebagai tindak balas terhadap phomopsidione turut dikaji. Dalam ujian mikropencairan kaldu, phomopsidione menunjukkan aktiviti perencatan yang ketara terhadap MRSA dengan kepekatan perencatan minimum (MIC) dan kepekatan keracunan bakteria minimum (MBC) pada 62.5 dan 500.00 µg/mL. Dalam ujian biofilem kristal ungu, phomopsidione mampu merencat dan memusnahkan pembentukan biofilem. Phomopsidione menunjukkan pengurangan yang ketara dalam faktor virulensi MRSA pada MIC dan MBC apabila dinilai dengan ujian kuantifikasi bahan polimer ekstraselular (EPS) dan ujian aktiviti enzim katalase serta lipase. Pemprofilan transkripsi menunjukkan phomopsidione

merendahaturkan ekspresi gen yang berkaitan dengan virulensi MRSA, terutamanya gen *agrA*, *agrC*, *RNAIII*, *hld*, dan *icaA*. Selain itu, analisis metabolomik tanpa sasaran yang menggunakan kaedah spektrometri jisim resolusi tinggi-kromarografi cecair (LC-HRMS) telah menunjukkan perbezaan yang ketara dalam profil metabolit MRSA yang telah didedahkan dengan phomopsidione berbanding dengan kumpulan kawalan. Berdasarkan dapatan, kajian ini mencadangkan bahawa phomopsidione menunjukkan kesan antibakteria, anti-biofilem dan anti-virulensi yang ketara terhadap MRSA. Kajian lanjut adalah perlu untuk menilai keberkesanan dan keselamatan phomopsidione sebagai agen antibakteria alternatif terhadap jangkitan MRSA dengan menggunakan model haiwan.

ANTIBACTERIAL EFFECTS OF PHOMOPSIDIONE ISOLATED FROM *DIAPORTHE FRAXINI* AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) WITH GENE EXPRESSION AND METABOLOMICS PROFILING

ABSTRACT

The rising prevalence of multidrug-resistant (MDR) pathogens has contributed to a high mortality rate due to overuse and misuse of antibiotics. Among the MDR pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) is the most threatening and poses the greatest impact on public health. Thus far, many bioactive ketone derivatives have been reported as anti-MRSA agents. Phomopsidione ($C_7H_{10}O_4$), a bioactive ketone derivative isolated from *Diaporthe fraxini*, has previously demonstrated antibacterial effects. The present study was aimed to investigate the antibacterial and anti-biofilm effects of phomopsidione against MRSA and determine the phomopsidione-mediated modulation in virulence factors production. Additionally, the changes in gene expression and metabolites profile of MRSA in response to phomopsidione were examined. In broth microdilution assay, phomopsidione exhibited significant inhibitory activity against MRSA with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 62.5 and 500.00 µg/mL, respectively. In crystal violet biofilm assay, phomopsidione inhibited and eradicated biofilm in a concentration-dependent manner. Phomopsidione showed significant reduction in the virulence factors production of MRSA at MIC and MBC when assessed using quantification of extracellular polymeric substances (EPS), catalase and lipase production assays. Transcriptional profiling showed that phomopsidione downregulated the expression of several virulence-associated genes in

MRSA, specifically *agrA, agrC, RNAIII*, *hld*, and *icaA* genes. Moreover, an untargeted metabolomics analysis using liquid chromatography-high resolution mass spectrometry (LC-HRMS) revealed a significant difference in the metabolite profiles of MRSA treated with phomopsidione as compared to the untreated control group. Based on the findings, the present study suggested that phomopsidione exhibited potent antibacterial, anti-biofilm and anti-virulence effects against MRSA. Further studies are necessary to assess the efficacy and safety of phomopsidione using animal models as an alternative antibacterial agent against MRSA infections.

CHAPTER 1

INTRODUCTION

1.1 Research background

Staphylococcus aureus is a common Gram-positive bacterium residing in the nasal cavity or on the skin of humans (Chao et al., 2008). *S. aureus* has become resistant to various antibiotics used in clinical practice over time (Rajput et al., 2019). Following the introduction of methicillin during the late 1950s, it took only a few years to discover the methicillin-resistant *S. aureus* (MRSA) isolates (Chao et al., 2008). Since then, MRSA infections have increased yearly and becoming one of the most threatening multidrug-resistant (MDR) pathogens. It is widespread in the human population and approximately 40% of adults are asymptomatic carriers (Okwu et al., 2019). MRSA infections encompass mild skin infections to chronic infections, including bloodstream infections and pneumonia. According to Subarna et al. (2023), the pooled prevalence of MRSA in India, Pakistan, Sri Lanka, and Bangladesh was 5.65%, 17.20%, 22.56%, and 4.93% respectively.

The term virulence refers to the capability of an organism to cause infection (Sharma et al., 2017). MRSA produces virulence factors which are crucial in cellular survival, pathogenesis, and colonisation (Sharma et al., 2017). The formation of multicellular communities known as biofilms and extracellular polymeric substances (EPS) enable MRSA to accomplish host colonisation and resistant to unfavourable environmental conditions. Toxins assist MRSA in evasion and cause a range of symptoms to the infection, depending on the specific toxins involved. Lipase breaks

down host tissue and contribute to the spread of the infection while catalase provides protection to the cells from oxidation (Lakshmi et al., 2020).

MRSA establishes infections due to the presence of distinct virulent genes encoding distinct virulent components (Rivera et al., 2015). The regulation of quorum sensing in MRSA is primarily controlled by the accessory gene regulator (*agr*) system (Lakshmi et al., 2020). The *agr* system comprises of the genes *RNAIII*, *agrB, D, C* and *A* as quorum sensing regulatory genes. It directly aids in biofilm formation and virulence factors production (Lu et al., 2023). Additionally, *icaA* gene is involved in the biosynthesis of polysaccharides intercellular adhesins (PIA) which maintains bacterial surface persistence (Sinsinwar et al., 2022). *Hld* gene that encodes δ-hemolysin has the ability to induce membrane damage and harmful effects in erythrocytes and immune cells (Sinsinwar et al., 2022). These virulence factors and a broad spectrum of antibiotic resistance limits the treatment against MRSA infections. Patients with MRSA infections require prolonged hospitalisation and intensive care. Thus, it has brought significant burden to the healthcare system (Rajput et al., 2019).

An antibiotic is a chemical substance used to disrupt the bacteria or their growth (Kourkouta et al., 2018). Since 1998, there are only 10 new antibiotics that have been introduced in the market (Chao et al., 2008). Antibiotic resistance refers to the state where bacteria are no longer susceptible to an antibiotic (Church & McKillip, 2021). For decades, antibiotic resistance has been a serious problem in public health globally (Dadgostar, 2019). The rising prevalence of MDR pathogens has contributed to a high mortality rate and prevalence of infectious diseases (Okwu et al., 2019). The Centre for Disease Control and Prevention (CDC) estimated that antibiotic resistance pathogens has caused over 2.8 million infections and 35,900 deaths every year in United States (US) in 2019 (Kadri, 2020).

Ineffectiveness of conventional antibiotics and the lack of accessibility of new anti-MRSA agents have accelerated the need for alternatives. In this aspect, new and effective antibacterial agent could be an alternative to treat and/or manage the spread of MRSA infections. Bioactive metabolites from endophytic fungi are promising alternative antibiotics against MRSA (Ustun et al., 2019). Endophytic fungi produce secondary metabolites in defence against harmful microorganisms or insects (Faheem et al., 2022). They are documented as potent anti-biofilm and anti-virulence in MRSA (Faheem et al., 2022). These bioactive metabolites possess diverse chemical classes and play a key role in providing novel chemical skeletons in searching for lead compounds (Nagarajan et al., 2021). The endophytic fungus *Diapothe fraxini* was previously reported to produce a ketone derivative, phomopsidione ($C_7H_{10}O_4$) (Yenn et al., 2017). According to the study, it exhibited significant antibacterial action against a broad range of bacteria, suggesting its potential against MRSA infections (Chin et al., 2020). Therefore, the compound is worthy of further investigations.

Transcriptional and metabolomics studies have allowed the understanding of a biological process at a molecular level (Stuart et al., 2020). Transcriptional profiling refers to the process of quantifying gene expression in the biological samples at the level of ribonucleic acid (RNA) transcription (Atshan et al., 2013). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) is commonly used for transcriptional profiling (Rivera et al., 2015). The virulence gene expression of MRSA will aid in improving the comprehension of virulence mechanisms of anti-MRSA compounds.

Metabolomics is a comprehensive approach involving the analysis of metabolites with molecular weight lower than 1000 Da in a biological system under specific conditions (Sebak et al., 2019). Among the analytical tools, liquid

chromatography-high resolution mass spectrometry (LC-HRMS) is primarily used in metabolomics studies to examine the mass-to-charge ratio (*m/z*) of the metabolites in the biological samples (Nagarajan et al., 2022). Additionally, multivariate data analysis (MVDA) is used to compare and visualise the differences among the metabolites in different sample groups (Sebak et al., 2019). The post-treatment metabolic perturbation is important to understand the inhibitory effects of anti-MRSA compounds (Schelli et al., 2017b).

1.2 Problem statement

The overuse and misuse of antibiotics, as well as poor infection control have accelerated the spread of MRSA infections. MRSA is recognised as a high-priority MDR bacteria by the World Health Organisation (WHO) since 2017 (Cascioferro et al., 2020). People with MRSA infections are vulnerable to higher mortality rate (up to 64%) when compared to people with drug-sensitive infections (Gurung et al., 2020). The Infectious Diseases Society of America (IDSA) has included MRSA in the top six of the most threatening MDR microorganisms (Hussein et al., 2020). Conventional therapeutic options for MRSA infections are progressively becoming limited due to the widespread of multidrug resistance and various virulence factors (Quave et al., 2011). These have urged researchers to search for alternatives and effective anti-MRSA compounds. Endophytic fungi from the genus *Diaporthe* are one of the potential sources for bioactive metabolites with antibacterial, antifungal, anti-malarial and anti-inflammatory activities (Nagarajan et al., 2021; Xu et al., 2021). A ketone derivative, phomopsidione, isolated from *D. fraxini* was chosen in the present study to investigate its antibacterial potential. The potent anti-candidal activity of phomopsidione has been previously reported (Yenn et al., 2017). However, there are

lack of studies on its antibacterial potential against Gram-positive bacteria, in particular, MRSA. Thus, this study aims to examine the antibacterial effects and virulence factor expression of phomopsidione against MRSA. Additionally, comparative analyses involving the gene expression and untargeted metabolomics were conducted on the MRSA culture with the presence and absence of phomopsidione.

1.3 Research objectives

The research objectives of this study are:

1. To investigate the antibacterial and anti-biofilm effects of phomopsidione against MRSA.
2. To determine phomopsidione-mediated modulation in virulence factor production of MRSA.
3. To examine the changes in gene expression and metabolite profile of MRSA in response to phomopsidione.

CHAPTER 2

LITERATURE REVIEW

2.1 Methicillin-resistant *Staphylococcus aureus*

Sir Alexander Ogston identified *S. aureus* in 1880. *S. aureus* can cause a wide variety of diseases (Divyakolu et al., 2019). *S. aureus* is a commensal bacterium commonly found in humans and can cause infections both in healthcare settings and in community. (Divyakolu et al., 2019). *S. aureus* is a Gram-positive coccoid bacterium characterised by a spherical shape. It is non-motile and not spore-forming, yet is versatile to evolve resistance to antibiotics (Algammal et al., 2020; Craft et al., 2019; Okwu et al., 2019). Penicillin-resistance *S. aureus* was the first strain of *S. aureus* to exhibit antibiotics resistance. Penicillin-resistance *S. aureus* was first identified in 1940 and reached a pandemic level by 1960s (Craft et al., 2019). Mechanistically, penicillin resistance is due to the cleavage of β-lactam ring in penicillin and its derivatives by the enzyme penicillinase (Otto, 2012).

Methicillin was introduced in 1959 and authorised into clinical practice in 1961 to combat penicillin resistance (Craft et al., 2019; Vestergaard et al., 2019). Unfortunately, the first clinical isolates of MRSA were documented in the United Kingdom and the US in 1962 and 1968, respectively (Aslam et al., 2018). As reported, MRSA was discovered two years shortly after methicillin introduction. MRSA is now a historical emergent pathogen that causes persistent human and veterinary infections (Algammal et al., 2020; Craft et al., 2019). MRSA was described as the first "superbug" in history. Superbugs are defined as the types of bacteria which have developed resistance to a commonly used antibiotic or medication (Uddin et al., 2021). *S. aureus,*

Acinetobacter baumannii, and *Mycobacterium tuberculosis* are known as superbugs that can develop MDR and extensively drug-resistant (XDR). MDR bacteria possess resistance to multiple drugs, while XDR bacteria are resistant to nearly all clinically approved antibacterial drugs (Leon-Buitimea et al., 2020). Each year, these superbugs are capable of committing to the mortality of about 1 million individuals globally due to incurable antibiotic-resistant infections (Sharma et al., 2021).

Murray et al. (2022) reported that MRSA infections have resulted in more than 100,000 deaths in 2019. Approximately 323,700 cases of hospitalised patients, 16,000 deaths, and 1.7 billion dollar healthcare costs were documented in the report of Antibiotic Resistance Threats in the US (CDC, 2019). Uddin et al. (2021) stated that MRSA accounted for less than 74% of all *S. aureus* infections worldwide. The median incidence of MRSA bloodstream infections was 12.11% in the Global Antimicrobial Resistance and Use Surveillance System report 2019 (Uddin et al., 2021). The death rate among patients with both MRSA bacteraemia and infective endocarditis is significant, ranging from 17 to 50% (Shimizu et al., 2022). In comparison to the non-resistant form of *S. aureus* infection, MRSA patients have a higher rate of mortality at 64% (Sharma et al., 2021). Thus, MRSA is recognised as a high-priority MDR bacteria by the WHO since 2017 (Cascioferro et al., 2020).

Livestock-associated MRSA infects domestic animals and the first incidence was reported from dairy cows in 1970. Algammal et al. (2020) hypothesised that the infected animals served as permanent reservoirs for MRSA, resulting in human infections. MRSA causes significant morbidity and mortality globally as it has emerged as one of the most common pathogens of hospital-acquired (HA-MRSA) and CA-MRSA infections (Algammal et al., 2020; Cascioferro et al., 2020; Kourtis et al., 2019). According to Gupta et al. (2018), MRSA isolates were found in pus (51.6%), invasive

devices (17.7%), bodily fluids (12.9%), urine (8.0%), blood (4.8%), and sputum (4.8%). A majority of HA-MRSA was detected in the respiratory tract, bloodstream, urinary tract, and intravenous site infections. They affect elderly patients with associated comorbidities or prior antibiotic use (Khan et al., 2018; Nichol et al., 2019). According to Algammal et al. (2020), HA-MRSA strains are mainly colonised in front nares of healthy people. Occasionally, new resistant variants of HA-MRSA are often emerged, and they are mostly MDR. Therefore, therapeutic options are progressively becoming limited (Quave et al., 2011). It was once postulated that MRSA was restricted to people who had been exposed to healthcare facilities (HA-MRSA). Until the mid-1990s, CA-MRSA has been identified when the number of cases has increased among individuals who have never been exposed to healthcare settings (Khan et al., 2018). CA-MRSA typically infects young, healthy patients and manifests as respiratory, circulatory, or skin and soft tissue infections (Algammal et al., 2020; Khan et al., 2018; Nichol et al., 2019). Unlike HA-MRSA, CA-MRSA tends to colonise at multiple body areas other than front nares, with a majority of 23% at the inguinal regions. The primary colonisation sites of CA-MRSA for children and young patients are the rectum and throat, respectively (Algammal et al., 2020). According to previous studies, MRSA cause invasive infections, including superficial skin, soft tissue and bloodstream infections, sepsis arthritis, bacteremia, pyogenic endocarditis, osteomyelitis, necrotising pneumonia, otitis media and death (Algammal et al., 2020; Cascioferro et al., 2020; Kourtis et al., 2019).

Biofilm development is a key pathogenic mechanism of MRSA that confers resistance to antibiotics. It increases bacterial persistence and modulates the host's immune response (Hosseini et al., 2020). It enhances adherence to biotic and abiotic surfaces and accumulates more bacterial cells to form multi-layered cell clusters

(Meroni et al., 2019; Resch et al., 2005). Cascioferro et al. (2020) stated that biofilm-associated infections account for over 80% of bacterial infections. In regard to this, MRSA is one of the excellent biofilm formers. The environmental and physiological diversities such as growing conditions and nutrient intake of biofilm cells are significant different among various layers (Resch et al., 2005). The biofilm cells embedded in a slimy matrix are generally more tolerant as compared to planktonic cells. Hence, they provide significant protection against antibiotics and host defense (Meroni et al., 2019). The biofilm development also contributes to colonisation and the spread of MRSA infections (Otto, 2012).

With the rising of MDR virulent in MRSA strains, emphasis should be placed on minimising the risk factors for MRSA infections (Algammal et al., 2020; Gupta et al., 2018). In developed countries, MRSA infections mostly affect vulnerable groups, including elderly individuals, recipients of transplants, patients with cancer or receiving immunosuppressive drugs (Adedeji, 2016). Abidin et al. (2020) stated that prolonged duration of hospitalisation and major surgery procedure will increase the risk for MRSA infection. Therefore, appropriate steps in preventing MRSA infections in both healthcare and community settings are needed. These include good infection control practices and rational antibiotic policy (Gupta et al., 2018; Kourtis et al., 2019). Significant improvement has been achieved in preventing MRSA bloodstream infections in the US hospitals after enhancing infection control measures. Despite the declining numbers of HA-MRSA bloodstream infections, the decrease trend have slowed down over the last few years (Kourtis et al., 2019).

2.2 Virulence factors of MRSA

Virulence is defined as the capability of an organism to initiate infections. Virulence factors are defined as the molecules, regulatory systems and cellular structures that assist in the host colonisation at the cellular level (Sharma et al., 2017). They enable microorganisms to accomplish: (I) colonisation, (II) evasion, (III) obtaining nutrition from the host, and (IV) resistance to unfavourable environmental conditions (Algammal et al., 2020). Several virulence factors including toxins, surface protein, adhesins, and enzymes are crucial in MRSA survival, pathogenesis, host's cells and tissues invasion, surface adherence, immunological response evasion, and colonisation (Divyakolu et al., 2019; Otto, 2012). MRSA generates infections due to the presence of distinct virulent genes encoding distinct virulent components (Rasheed & Hussein, 2021).

2.2.1 Accessory gene regulator group

Most of the virulence-associated factor expression genes of MRSA such as toxin secretion and biofilm formation are governed by *agr* (Lakshmi et al., 2020; Rasheed & Hussein, 2021; Selvaraj et al., 2019). Through quorum sensing, *agr* regulates the significant changes in virulence gene expressions that are important for MRSA pathogenesis at a specific cell density (Cheung et al., 2011; Rasheed & Hussein, 2021). *Agr* positively impacts the expression of toxins and exoenzymes, but has a negative effect on surface proteins, such as fibronectin-binding proteins, fibrinogen-binding proteins, and protein A (Cheung et al., 2011; Otto, 2012; Rasheed & Hussein, 2021). This is to ensure the generation of essential virulence factors when MRSA is most needed. Surface proteins are essential for initial colonisation and adherence. After

initiating an infection, toxins and exoenzymes are produced for immune evasion (Cheung et al., 2011). However, a study conducted by Otto (2012) has presented conflicting findings that *agr* may upregulate the surface proteins, presumably in a strain-dependent manner.

When the population density of MRSA rises, the quorum sensing system is activated. This has led to an elevation in the pathogenicity of the infection by boosting the expression of virulence factors. *AgrB* and *agrD* of MRSA cell combine and produce an auto-inducing peptide to bind with a signal receptor *agrC* (Rasheed & Hussein, 2021). The binding results in the phosphorylation of the response regulator *agrA* and further activates the P2 and P3 promoters while the communication in MRSA is maintained by positive feedback (Divyakolu et al., 2019; Lakshmi et al., 2020). Operons P2 and P3 promote the transcription of *RNAII* and *RNAIII*, correspondingly (Lakshmi et al., 2020). *RNAIII* transcribes the set of pathogenesis-related genes such as *hld*. Hence, their expression serve as a proxy marker to evaluate *agr* function (Divyakolu et al., 2019; Lakshmi et al., 2020). Moreover, *agr* has a significant role in the formation of biofilm (Lakshmi et al., 2020). Cheung et al. (2011) stated the potential relationship between the *agr* group types and the resistance development of MRSA to glycopeptides.

2.2.2 Hemolysin

Hemolysins can damage the erythrocytes of the host during infection (Wang et al., 2020). Different MRSA strains produce different levels of toxins for red blood cell lysis (Algammal et al., 2020; Senon et al., 2021). MRSA has been reported to express neutral sphingomyelinase and several pore-forming toxins including α-, δ-, and γ-hemolysins (Algammal et al., 2020; Wang et al., 2020). These hemolysins are crucial

for pathogenesis and involve in the destruction of host cells and tissues, thereby, suppressing the host immune system (Fu et al., 2021).

2.2.2(a) α-Hemolysin

Among all the exotoxins, α-hemolysin is the primary virulence factor and has been shown to linked with severe disease in MRSA (Algammal et al., 2020; Wang et al., 2020). The *agr* contains *RNAIII* to stimulate the translation of α-hemolysin encoded by *hla* genes (Fu et al., 2021). α-Hemolysin is a 33 kDa pore-forming toxin that stimulated the release of cytokines and chemokines for host damage (Burnside et al., 2010; Divyakolu et al., 2019; Rasheed & Hussein, 2021). α-Hemolysin is cytotoxic against human and mammalian cells such as erythrocytes, epithelial cells, endothelial cells, macrophages, monocytes, and T-cells except for human neutrophils (Otto, 2012; Rasheed & Hussein, 2021). A high concentration of α-hemolysin induces high susceptibility against rabbit erythrocytes and causes lysis of human lung epithelial cells and mammary gland necrosis (Algammal et al., 2020; Burnside et al., 2010; Divyakolu et al., 2019). MRSA harbouring the *hla* gene can cause pneumonia, sepsis, skin and soft tissue infections, brain abscess, septic arthritis, and corneal infections (Burnside et al., 2010; Rasheed & Hussein, 2021). Otto (2012) has reported that *hla* gene considerably affects the virulence of CA-MRSA in the models of skin and lung infections.

The upregulation of the *agr* system increases the production of *hla*, leading to a multi-step pore formation process (Fu et al., 2021). Firstly, the secretion and bonding capability of water-soluble α-hemolysin monomers cause oligomerisation into the heptameric structure in the host membrane (Rasheed & Hussein, 2021). Exposure of different type cells and concentrations of α-hemolysin induce different intracellular signalling pathways. In general, when α-hemolysin binds to the host membrane, it is

oligomerised into a heptamer. Subsequently, a 1 to 3 nm β-hairpin lined amphipathic pore is formed (Fu et al., 2021; Oliveira et al., 2018). The cell membrane is perforated, leading to a rapid release of small cytoplasmic contents such as adenosine triphosphate and potassium ions (K^+). This has caused changes in ion gradients and facilitates epithelial barrier breakdown by interaction with the ADAM10 receptor (Otto, 2012). *Hla* gene activates the enzyme and degrades E-cadherin. E-cadherin is the main substrate for ADAM10 and compromises the epithelial tissue (Oliveira et al., 2018). Eventually, MRSA invasion occurs via pore and triggers necrotic which leads to cell death (Fu et al., 2021; Rasheed & Hussein, 2021).

2.2.2(b) β-Hemolysin

β-Hemolysin was initially identified by Glenny and Stevens in 1935 (Oliveira et al., 2018). It is called sphingomyelinase or hot-cold hemolysin which hydrolyses host sphingomyelin. It is the predominant sphingolipid in eukaryotic membranes and increases hemolytic activity after incubation below 10°C (Burnside et al., 2010). In 1989, Projan and his team discovered β-hemolysin encoded by gene sequence *hlb* (Oliveira et al., 2018). β-Hemolysin is a 35 kDa non-pore-forming hemolysin with high specificity. It predominantly expresses in most strains of MRSA (Burnside et al., 2010; Rasheed & Hussein, 2021). The host specificity is highly depending on the sphingomyelin contents of erythrocytes in the host membrane (Oliveira et al., 2018). For example, sheep erythrocytes are more susceptible to sphingomyelinase than humans (Rasheed & Hussein, 2021). Unlike α-hemolysin, β-hemolysin exhibits high hemolytic activity against red blood cells of sheep, but not against rabbit (Burnside et al., 2010; Oliveira et al., 2018). Human cells are vulnerable to β-hemolysin, including skin dermal cells, neutrophils, T lymphocytes, polymorphonuclear leukocytes, keratinocytes and

monocytes (Divyakolu et al., 2019; Rasheed & Hussein, 2021). Epidemiological studies have addressed the role of β-hemolysin in the reappearance of pneumonia, furunculosis, chronic osteomyelitis and endocarditis, respiratory and corneal infections in humans (Divyakolu et al., 2019; Rasheed & Hussein, 2021). The ability of β-hemolysin to prevent the movement of cilia on the cells lining the nasal cavity is reported in MRSA infections (Burnside et al., 2010). A large number of β-hemolysin is reported in the MRSA strains obtained from chronic skin infections in human and bovine mastitis (Oliveira et al., 2018). In cases of pneumonia and murine ear skin infection models, the virulence of diseases is decreased in MRSA strains lacking the gene *hlb* (Oliveira et al., 2018).

As a phosphoric diester hydrolase, sphingomyelinase enters erythrocytes and hydrolyses sphingomyelin of the host membrane into phosphorylcholine and ceramide during an infection (Rasheed & Hussein, 2021). Ceramide activates mitogen-activated protein kinases by stimulating the second messenger system and inhibits the interleukin-8 (IL-8) expression by endothelial cells. This has resulted in biofilm development and phagosomal escape of MRSA cells (Oliveira et al., 2018). Additionally, it increases host cell vulnerability to α-hemolysin and Panton-Valentine Leukotoxin, leading to alterations in cell morphology and cell death (Divyakolu et al., 2019; Rasheed & Hussein, 2021). In a rabbit endocarditis model, a mutant strain that expressed altered form of β-hemolysin showed less pathogenesis significantly (Oliveira et al., 2018).

2.2.2(c) δ-Hemolysin

δ-Hemolysin was identified by Willian and Harper in 1947 (Divyakolu et al., 2019). Approximately 97% of *S. aureus* isolates generate δ-hemolysin with molecular weight of around 3 kDa (Burnside et al., 2010; Divyakolu et al., 2019). It is a 26-amino

acid peptide categorised under the phenol-soluble modulin family. It is encoded by *hld* and under the control of the *agr* system (Burnside et al., 2010; Su et al., 2020). *Hld* gene encodes a 514-nucleotide transcript inside the *RNAIII* locus of *agr* to regulate the production of virulence factor (Divyakolu et al., 2019). It is active against numerous mammalian cells and organelle including erythrocytes, spheroplasts and protoplasts (Burnside et al., 2010). Unlike α and β-hemolysins, δ-hemolysin lacks a cleavable signal sequence (Burnside et al., 2010). Furthermore, δ-hemolysin is the only phenol-soluble modulin which promotes mast cell degranulation. It increases the severity of atopic dermatitis which affects 15 to 30% of children and 5% of adults in industrialised nations (Su et al., 2020).

There are three steps involved in the cell lysis by δ-hemolysin (Divyakolu et al., 2019). Firstly, it aggregates and binds to the cell surface to form transmembrane pores on the host membrane (Alkhafaji & Alsaimary, 2020). Then, the curvature of the membrane elicits a pro-inflammatory response or cytolysis and destabilises the membrane at high concentrations (Alkhafaji & Alsaimary, 2020; Su et al., 2020). This has resulted a prompt entry of calcium ions (Ca^{2+}) and stimulated the free radicals generation in granulocytes (Divyakolu et al., 2019). δ-Hemolysin was found to be essential for disease severity, demonstrating a significant virulence factor in a CA-MRSA bacteremia mouse model (Su et al., 2020).

2.2.2(d) γ-Hemolysin

Smith and Price first reported the pore-forming γ-hemolysin in 1938. However, its biological and biochemical roles were assigned in the late 1970s as a result of improved purification techniques (Oliveira et al., 2018). γ-Hemolysin consists of three proteins, hlgA, hlgB and hlgC, which are categorised under two transcription units

(Staali & Colin, 2021). Class S component encodes γ-hemolysin A (hlgA) while class F and S components encode hlgC and hlgB. HlgA and hlgC have a molecular weight of 32 kDa while hlgB has a molecular weight of 36 kDa. HlgA and hlgC display lytic activity when combined with hlgB (Oliveira et al., 2018). Within the γ-hemolysin group, they can interact with different subunits such as in combinations of hlgAB, hlgCB or hlgACB. They function as synergy and exhibit analogous actions (Oliveira et al., 2018; Staali & Colin, 2021). According to Divyakolu et al. (2019), *S. aureus* expresses up to five distinct bicomponent leukocidins involved in the MRSA pathogenesis, including γ-hemolysins (hlgAB and hlgCB), Panton-Valentine Leukocidin (PVL or lukSF), lukED and lukGH. For example, γ-hemolysins form hlgAB and hlgCB share the same F subunit component (hlgB) but have distinct S subunit components (hlgA or hlgC) (Jing et al., 2018; Oliveira et al., 2018). According to Staali & Colin (2021), over 99% of clinical *S. aureus* strains were found to produce γ-hemolysin. It effectively damages the host defense cells and erythrocytes of mammalian. Therefore, it plays a crucial role in the evasion of innate immune response (Jing et al., 2018). It is reported that rabbit erythrocytes were more responsive to γ-hemolysins than fowl (Divyakolu et al., 2019). HlgAB possesses cytolytic action towards human and rabbit leukocytes and is particularly efficient at lysing human erythrocytes. On the other hand, hlgCB has a limited effect on erythrocytes (Oliveira et al., 2018).

It is known that hlgAC is strongly related to strains which are capable of causing human and bovine colonisation (Oliveira et al., 2018). Jing et al. (2018) stated that γ-hemolysin plays a supplementary role in the virulent infections in mice and rabbits. Similar to α-hemolysin, γ-hemolysins are membrane-bound hetero-oligomers which form barrel-like pores on the host cell membrane. The binding of both S and F

components is essential. The conformational change is induced by the binding of S component to cellular receptors. This allows the dimerisation and recruitment of F component to phagocytic cells (Divyakolu et al., 2019). These dimers create a pre-pore that precedes the membrane insertion of the β-barrel transmembrane channel and oligomerisation (Jing et al., 2018). The cell lysis occurs after the complex multi-step process. It is also reported that γ-hemolysins stimulate the generation of toxic shock syndrome (Divyakolu et al., 2019). Through macrophage evasion and the release of iron ions (Fe^{2+}) from erythrocytes, hlgAB has been shown to be an essential component for the growth of MRSA in bloodstream (Oliveira et al., 2018).

2.2.3 Biofilm formation

MRSA is a renowned biofilm producer and the pathogenicity was attributed to distinct cell surfaces virulence factors, including biofilm-associated protein, capsular polysaccharides, collagen-binding protein, clumping factors, fibronectin-binding proteins, microbial surface components recognising adhesive matrix molecules, and PIA. Staphylococcal protein A and staphyloxanthin were expressed to promote adherence and persistence of MRSA cells during host colonisation (Resch et al., 2005; Selvaraj et al., 2019). Biofilm formation in MRSA starts from attachment onto a surface, followed by an expansion in biomass and accumulation of adhesive molecules. In the final step, microcolonies form, and biofilm matures and disperses on the surface (Vijayakumar et al., 2020). Biofilm forming strategy is relatively less aggressive compared to the active stage of toxin stimulation during MRSA infection. Biofilms are agglomeration of cells that attach to bacterial surfaces in the extracellular tissues. They give significant protection against antibiotics and host defenses. Biofilms allow MRSA

cells to adhere to biotic or abiotic surfaces, prolong infection and colonisation, and disseminate infection in hospital and community settings (Otto, 2012).

2.2.3(a) Polysaccharide intercellular adhesin

MRSA requires PIA, a glycan composed of β-1,6-linked 2-acetamido-2-deoxy-D-glucopyranosyl residues to facilitate intercellular aggregation and promote cell adhesion (McCarthy et al., 2015; Otto, 2012). PIA is a slimy substance embedded in cells. They are protected from the host immune defense system and antibiotics (Resch et al., 2005). Generally, MRSA produces *ica* operon-encoded PIA-dependent biofilm. The *ica* operon consists of *icaA, icaD, icaB,* and *icaC,* and a repressor called icaR (McCarthy et al., 2015; Meroni et al., 2019). *Ica* expression is modulated by *sigB, icaR* and unknown elements. Its activation and deactivation can be accomplished by insertion elements and based on various environmental factors (Resch et al., 2005). The *icaA* and *icaD* are the most essential genes for PIA production and intercellular adhesion (Hosseini et al., 2020; Vijayakumar et al., 2020). MRSA isolates which produce a strong slime layer are more virulent and typically more challenging to treat (Hosseini et al., 2020). A majority of clinical MRSA strains carry the *ica* locus (McCarthy et al., 2015). Other than PIA, the synthesis of *ica* operon-encoded poly-*N*-acetylglucosamine (PNAG) was also reported in the biofilm formation (McCarthy et al., 2015). Moreover, biofilm formation by *ica*-independent operon has been reported in Staphylococci such as *S. epidermidis* and *S. aureus* (McCarthy et al., 2015; Meroni et al., 2019). One of the earliest findings of *ica*-independent biofilm formation was found in bovine mastitis *S. aureus* isolates, which produced biofilm through the biofilm-associated protein (McCarthy et al., 2015). Another study with *ica*-independent biofilm formation was found in the *S. aureus* isolate UAMS-1 (McCarthy et al., 2015).

2.2.3(b) Surface-associated proteins

In Gram-positive bacteria, surface-associated proteins include biofilm-associated protein, protein A, clumping factors, fibronectin-binding proteins, and *S. aureus* surface protein G. They are produced by an *ica*-independent pathway to promote biofilm production. They are belonging to the family of microbial surface components recognising adhesive matrix molecules. They are attached to the peptidoglycan through covalent bonds and are mostly encoded in the core genome (Otto, 2012; Wertheim et al., 2008). They play a critical role in bacterial cell wall synthesis, biotic surface binding, immune evasion, bacterial aggregation and biofilm formation (Otto, 2012). On the other hand, the effect of surface proteins on virulence may be attributed to intercellular bacterial aggregation. They increase biofilm and the immune evasion capacity by reducing neutrophil phagocytosis (Algammal et al., 2020; Otto, 2012). For example, Staphylococcal protein A is a cell wall component encoded by *spa*. It inhibits complement system opsonisation and shields the MRSA phagocytosis process (Algammal et al., 2020; Heilmann et al., 2004).

Clumping factors such as ClfA and ClfB are present on the MRSA cell surface to initiate fibrinogen adherence. Fibrinogen is a basic constituent of the host's extracellular matrix protein (Algammal et al., 2020). According to a study, ClfB attaches to human protein cytokeratin 10 (CK10). It expresses and manifests on squamous epithelial cells (Wertheim et al., 2008). Corrigan et al. (2009) described that both ClfB and iron-regulated surface determinant protein A (IsdA) are able to promote *in vitro* adherence to desquamated epithelial cells. It has been reported that clfB was a key determinant in mouse nasal colonisation *in vivo*, thus, suggesting anti-ClfB antibodies might provide protection against infection in mice (Wertheim et al., 2008).

Fibronectin-binding proteins, such as FnBPA and FnBPB, are highly prevalent in MRSA isolates. They are predominantly present on the cell surface during the logarithmic phase (Foster & Höök, 1998; Speziale & Pietrocola, 2020). The *N*-terminal A domains of FnBPA share 24% of similar identities with FnBPB. In general, the A domains of fibronectin-binding proteins share about 25% similarity with ClfA but they bind to elastin (O'Neill et al., 2008). The transcription of these genes is governed by *agr* and *sar*. *Sar* stimulates the production of fibronectin-binding proteins in the exponential growth phase while *agr* inhibits the transcription (Speziale & Pietrocola, 2020). During the late logarithmic phase, only a limited quantity of fibronectin-binding proteins remains on the bacterial cell surface to retain binding onto fibronectin. However, the majority of fibronectin-binding proteins may be broken down by proteases and subsequently released from the cell (Speziale & Pietrocola, 2020).

2.2.3(c) Extracellular polymeric substances

EPS are composed of oligosaccharides, proteins, humic substances, and nucleic acid. These biochemicals are secreted by the organic material in the medium and released from cell lysis (Craft et al., 2019; More et al., 2014). The main function of EPS is aiding in cell aggregation and adherence to the biotic or abiotic substratum (Lakshmi et al., 2020). Moreover, EPS are crucial in the flocs (a specialised type of microbial aggregate) and biofilm formation, cell protection, adsorption of metabolites and ions, as well as enzymatic reactions (More et al., 2014). EPS constitutes more than 50% of the total organic material in the viscous biofilm matrix (More et al., 2014). MRSA cells encased in this EPS matrix are generally sessile. They can thrive in harsh conditions such as an anoxic environment through lowered metabolic rate and cessation of cell division (Lakshmi et al., 2020). EPS protects MRSA cells from antibiotics, host immune

cells, and environmental desiccation (Vickery et al., 2011). In a mature biofilm layer, the EPS matrix is hydrolysed to disperse MRSA cells into the environment (Lakshmi et al., 2020). Generally, MRSA cells in biofilms are at least 100 times more virulent and resistant than planktonic cells (Vickery et al., 2011). Such behavioural characteristic worsens the prevalence of HA-MRSA infections as they can contaminate implants and indwelling devices (Lakshmi et al., 2020). Based on the nature, EPS are categorised as capsular (C-EPS), loosely bound (LB-EPS), slime (S-EPS), and tightly bound (TB-EPS) (More et al., 2014). It is a challenge to remove EPS through the use of detergent, leading to a lower effectiveness of disinfecting processes. Eventually, persistence of bacteria is elevated in the environment (Vickery et al., 2011).

2.2.4 Lipase

Lipolytic activity in Staphylococci was identified in 1901. Lipase in *S. aureus* is the most abundant enzyme in MRSA (Orjiakor et al., 2020; Vijayakumaran, 2013). SAL1 and SAL2 are the lipases documented in MRSA. They have a broad substrate specificity to hydrolyse glycerol ester and degrade triglycerides into fatty acids (Orjiakor et al., 2020; Tam & Torres, 2019). SAL1 and SAL2 encode *gehA* and *gehB,* respectively. Both of them are encoded in different genome regions but share a similar protein sequence (Tam & Torres, 2019). They are reported to inhibit host granulocyte function and inactivate bactericidal lipids (Vijayakumaran, 2013). The lipolytic activity of MRSA is carried out by lipases to release linoleic acid in human plasma. This reaction is highly sensitive to ethylenediaminetetraacetic acid (EDTA) (Vijayakumaran, 2013). Lipases disintegrate the host tissues by catalysing the breakdown of ester linkages of fatty acids and glycerol. This provide the nutrients to the cells and promote colonisation and the growth of MRSA (Orjiakor et al., 2020; Vijayakumaran, 2013). Vijayakumaran

(2013) described that the lipase-encoding genes were upregulated during biofilm formation in *S. aureus*. The lipase inhibitors protected the host by destructing the biofilm development. Orjiakor et al. (2020) also suggested lipase activity is crucial for the nutrition or dissemination of the *S. aureus* bacteria (Orjiakor et al., 2020).

2.2.5 Catalase

Catalase is a ubiquitous enzyme present in plants, animals and aerobic bacterial cells to protect the cells from oxidative stress (Gruner et al., 2007; Lakshmi et al., 2020). The first bacterial catalase was identified by Gottstein in 1893 (Mustafa, 2014). Catalase production enables MRSA to withstand intracellular and extracellular destruction by hydrogen peroxide (H_2O_2). It is encoded by *katA*, which has a 1,518 bp with 505 amino acids (Gruner et al., 2007). MRSA is generally catalase-positive and facultatively anaerobic. Therefore, catalase is sometimes used to differentiate Staphylococci and Streptococci (Gruner et al., 2007; Mustafa, 2014). During cellular metabolism, catalases, which are also known as hydroperoxidases, catalyse the breakdown of H_2O_2 into water and molecular oxygen (Gruner et al., 2007). It safeguards MRSA cells and acts as an oxidising agent to prevent the destructive activity caused by the accumulation of reactive oxygen by-products (Lakshmi et al., 2020; Mustafa, 2014). Throughout this process, it neutralises the bactericidal impact of H_2O_2 and facilitates cellular detoxification (Mustafa, 2014; Zhang et al., 2022). Moreover, catalase is important for preserving cell viability during long-term deprivation, which is a vital ability for MRSA nosocomial infection transmission. In an aerobic environment, MRSA can coexist with microbes which produce H_2O_2 via the synthesis of catalase (Gruner et al., 2007).

2.3 Antibiotics

Antibiotics can be produced naturally from a wide range of microorganisms including yeasts, bacteria, and fungi to suppress the growth of other microbes or destroy them (Condon & Wittmann, 1991; Kourkouta et al., 2018; Yim et al., 2006). Bactericidal antibiotics destroy bacteria cells, while bacteriostatic antibiotics suppress the growth of bacteria. Nowadays, antibiotics are not only employed to treat infectious diseases but also provide important therapeutics and facilitate medical procedures (Hutchings et al., 2019). The process of manufacturing antibiotics is simple and straightforward. Fermentation is one of the widely used method to produce antibiotics industrially (Condon & Wittmann, 1991; Yim et al., 2006). However, there are also antibiotics manufactured artificially through direct chemical synthesis (Cai et al., 2021).

Antibiotics consumption grew by 65% from 21.1 to 34.8 billion defined daily doses globally in between 2000 and 2015 (Klein et al., 2018). The existence and emergence of antibiotics provide tons of benefits to the medical field. It significantly increases the survival rate of patients with infectious diseases during major epidemics (Kourkouta et al., 2018). Many pathogenic bacteria including Bacillus tuberculosis and diseases like pneumonia and meningitis were successfully combated by the available antibiotics (Kourkouta et al., 2018).

The antibiotics era has successfully transformed and revolutionised the treatment of infectious diseases throughout the world, especially in many developed countries (Adedeji, 2016; Kourkouta et al., 2018). Statistically, antibiotics have increased average human lifespan by 23 years in just over a century (Hutchings et al., 2019). Since the common use of antibiotics, cardiovascular diseases, cancer, and stroke have supplanted communicable diseases as the main causes of death in the US. The nation’s elder population in the US has increased from 4 to 13% and the average life

expectancy at birth has extended to 78.8 years (Adedeji, 2016). On the other hand, the annual consumption of antibiotics in Asian countries, such as India, Pakistan and Sri Lanka was 6.5, 1.3, and 1.4 billion, respectively (Jassal et al., 2023). Even though the revolution brought dramatic improvements in healthcare system in developed countries, the infectious diseases continue to affect all age groups disproportionately in impoverished nations and developing countries due to insufficient public health awareness and vaccination coverage (Adedeji, 2016).

2.3.1 History of antibiotics

There is a historical evidence that ancient cultures such as ancient China, Egypt, Rome, Serbia, and Greece used naturally occurring remedies such as herbs and honey to treat diseases before the introduction of antibiotics (Gould, 2016). Kourkouta et al. (2018) claimed that the ancient Chinese were the first to utilise therapeutic mold and plants to treat certain infections back to 2,500 years. The history of antibiotics started with arsphenamine or known as salvarsan. It was the first modern arsenic-based antimicrobial agent deployed in 1910 (Hutchings et al., 2019). Salvarsan was discovered by Ehrlich and his team in 1909. It was effective against syphilis, which is a type of bacterial infection spread by sexual contact, relapsing fever, and African trypanosomiasis (Gould, 2016).

The first antibiotic, penicillin, was found by Sir Alexander Fleming in 1928. It was isolated from the fungus Penicillium rubens. It is grouped under beta-lactam antibiotics and became a legal prescription drug in 1946 (Adedeji, 2016; Hutchings et al., 2019; Kourkouta et al., 2018). Penicillin G and penicillin V are the only two clinically used natural antibiotics obtained from the genus Penicillium despite a number of them being discovered. The introduction of penicillin was recognised as a modern