# SYNERGISTIC ACTIVITY OF CEFTRIAXONE AND *POLYALTHIA LONGIFOLIA* LEAVES EXTRACT AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

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**by**

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## LIST OF SYMBOLS

| | |
|---|---|
| $\sum$ | Sum of |
| $\beta$ | Beta |
| $<$ | Less than |
| $>$ | Greater than |
| $\leq$ | Less than or equal to |
| $\geq$ | Greater than or equal to |
| C | Concentration |
| OD | Optical Density |
| V | Volume |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 16S rRNA | 16S ribosomal RNA |
| AST | Antimicrobial Sensitivity Test |
| CA-MRSA | Community-Associated Methicillin-Resistant *Staphylococcus aureus* |
| CEPs | Cephalosporins |
| CLSI | Clinical and Laboratory Standards Institute |
| CNS/CoNS | Coagulase-Negative Staphylococci |
| CPS | Coagulase-Positive Staphylococci |
| D-Ala-D-Ala | D-alanyl-D-alanine |
| DMSO | Dimethlysulphoxide |
| DNA | Deoxyribose Nucleic Acid |
| ECDC | European Centre for Disease Prevention & Control |
| EDTA | Ethylene Diamine Tetraacetic |
| FIC | Fractional Inhibitory Concentration |
| FnBP | Fibronectin Binding Protein |
| HA-MRSA | Hospital Associated Methicillin-Resistant *Staphylococcus aureus* |
| HPLC | High Performance Liquid Chromatography |
| MBC | Minimum Bactericidal Concentration |
| PLLME | *P. longifolia* leaf methanol extract |
| MHA | Muller Hinton Agar |
| MHB | Muller Hinton Broth |
| MIC | Minimum Inhibitory Concentration |
| mRNA | Messenger Ribo Nuclei Acid |
| MRSA | Methicillin-Resistance *Staphylococcus aureus* |
| MSSA | Methicillin-Susceptible *Staphylococcus aureus* |
| NA | Nutrient Agar |
| NaCl | Sodium Chloride |
| NB | Nutrient Broth |
| NCBI | National Center for Biotechnology Information |
| NCCLS | National Committee on Clinical Laboratory Standards |
| ORFs | Open reading Frames |
| PBP2a | Penicillin-Binding Protein 2a |

| | |
|---|---|
| PCR | Polymerase Chain Reaction |
| PG | Peptidoglycan |
| PMF | Proton Motive Force |
| PNA-FISH | Fluorescence *In Situ* Hybridization Using Peptide Nucleic Acid Probes |
| PVL | Panton-Valentine Leukocidin |
| RNA | Ribo Nucleic Acid |
| SCC*mec* | Staphylococcal Cassette Chromosome *mec* |
| tRNA | Transfer Ribo Nucleic Acid |
| TSB | Tryptone Soya Broth |
| TSST-1 | Toxic Shock Syndrome Toxin-1 |
| WHO | World Health Organization |

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**AKTIVITI SINERGI CEFTRIAXONE DAN ESTRAK DAUN *POLYALTHIA LONGIFOLIA* TERHADAP *STAPHYLOCOCCUS AUREUS* RINTANGAN METISILIN (MRSA)**

## ABSTRAK

Jangkitan Methicillin-resistant *Staphylococcus aureus* (MRSA) telah menjadi antara salah satu jangkitan bakteria patogenik yang signifikan dalam isu-isu kesihatan dalam negara-negara sedang maju. Seterusnys, gen *mecA* yang paling penting menyumbang kepada penentangan metisilin dalam kacukan MRSA, di mana mengekodkan protein pengikat penisilin PBP2a novel. Oleh yang demikian, strategi alternatif yang baharu diperlukan untuk mengatasi isu-isu ini dengan memajukan ejen antimikrobial yang baharu, mengubahsuai aktiviti antibiotik yang sedia ada dengan kombinasi ekstrak tumbuhan sebagai penentang ejen pengubahsuai atau dengan menggunakan kombinasi ekstrak tumbuhan dengan antibiotik yang sedia ada terhadap penentang bakteria. Oleh itu, kajian baharu yang telah diadakan untuk memeriksa aktiviti pengubahsuaian bakteria dan kesan sinergi oleh ekstrak methanol *Polyalthia longifolia* (Sonn.) Thwaites (Annonaceae famili) terhadap MRSA dalam kombinasi dengan ceftriaxone. Pengubahsuain aktiviti antibiotik dan kesan sinergi oleh ekstrak methanol daun *P. longifolia* (PLLME) diperiksa dalam kajian oleh kaedah peresapan cakera, kaedah rebusan mikro-cairan, kaedah mikro-cairan "*checkerboard"*, dan pengesanan penekanan gen *mecA* dihasilkan oleh kaedah Tindak Balas Rantai Polimerase multiplex "multiplex PCR". PLLME telah menunjukkan aktiviti pengubahsuain antibioktik yang bagus dengan mengurangkan nilai Kepekatan Inhibit Minimum (MIC) PLLME (16000 µg/mL) dan ceftriaxone (8000 µg/mL) kepada 2000 µg/mL untuk PLLME dan 1000 µg/mL untuk ceftriaxone masing-masing dalam

Kepekatan Inhibitori Pecahan (FIC) mencerakinkan terhadap bakteria MRSA. Disamping itu, PLLME yang digabungkan dengan ceftriaxone didapati benar-benar menekan ekspresi gen *mecA* pada bakteria MRSA. Dalam kesimpulan, PLLME mampu menjadi ejen signifikan dalam terapi kombinasi dan sumber prinsip pengubahsuaian ketahanan yang dapat menjadi pilihan sebagai pilihan rawatan untuk rawatan jangkitan MRSA.

**SYNERGISTIC ACTIVITY OF CEFTRIAXONE AND *POLYALTHIA LONGIFOLIA* LEAVES EXTRACT AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)**

## ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) infection has become one of the most significant pathogenic bacterial infection health issues in developing countries. Furthermore, the most important *mecA* gene that is encodes a novel penicillin-binding protein PBP2a contributes to the methicillin resistance in MRSA strains. Therefore, new alternative strategies are needed to address this issue by developing a new antimicrobial agent, modifying the existing antibiotic activity with a combination of plant extracts as resistance modifying agents, or using the plant extract combined with existing antibiotics against resistant bacteria. Consequently, the current study was conducted to evaluate the antibiotic modifying activity and synergistic effects of methanol extract *Polyalthia longifolia* (Sonn.) Thwaites (Annonaceae family) against MRSA in combination with ceftriaxone. The antibiotic modifying activity and synergistic effects of *P. longifolia* leaf methanol extract (PLLME) were evaluated in this study by disc diffusion method, broth microdilution method, *checkerboard* microdilution method, and detection of the *mecA* gene suppression by multiplex Polymerase chain reaction (PCR). The PLLME showed a good antibiotic modifying activity by decreasing the Minimum Inhibitory Concentration (MIC) values of PLLME(16000 µg/mL) and ceftriaxone (8000 µg/mL) to 2000 µg/mL for PLLME and 1000 µg/mL for ceftriaxone respectively in the Fractional Inhibitory Concentration (FIC) assay against the MRSA bacteria. Furthermore, PLLME combined with ceftriaxone was found to completely suppress

the *mecA* gene expression in the MRSA bacteria. In conclusion, PLLME could be a significant agent in combination therapy and a source of resistance modifying principles that could be valuable as treatment options for MRSA infection treatment.

# CHAPTER 1

# INTRODUCTION

The third most important reason for death worldwide is microbial infectious diseases as projected by the World Health Organization (WHO). The infection caused by Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a worldwide health problem primarily in health care centers such as hospitals, clinics and care centers that start from simple non-life-threatening skin infections to severe deadly infections related diseases (Garoy et al., 2019). *S. aureus* is frequently an important source for public acquired post-operative infections, endocarditis, toxic shock syndrome, food poisoning, and osteomyelitis (Monecke et al., 2011). MRSA infection has become a severe problem in human health worldwide since it already highly exists in the community and not just as primary nosocomial infectious microbes as previously viewed (David et al., 2010). Growing information on the existence of vancomycin-resistant MRSA and other antibiotic-resistant pathogenic microbes indicates the nastiest state that the human population has to face in upcoming decades in the fight against antibiotic-resistant pathogenic microbial infections since Fleming's great finding on antibiotic.

As a result, concentrated struggles again have been made to discover the novel antimicrobial agents from medicinal plants by the scientist. Moreover, the increasing incidence of drug-resistant pathogens has drawn the pharmaceutical company and scientific communities' attention to study the medicinal plant-derived phytochemicals, which are commonly used in traditional medicine. Consequently, the search for drugs derived from medicinal plants by scientists has accelerated in recent years worldwide. Medicinal plant, a popular health care agent, is used daily by billions of people globally as their primary healthcare. In traditional medicine, the medicinal

plants were considered a panacea with various curative values, including anti-infectious activity. One of such important medicinal plants with multiple curative values is *Polyalthia longifolia* var. angustifolia Thw. (Annonaceae). The leaves of *P. longifolia* are testified to have great medicinal value (Rajeshkumar et al., 2010), therefore *P. longifolia* is considered as an ancient remedy to be discovered. Furthermore, *P. longifolia* is a medicinal plant with linear-lanceolate leaves found in Sri Lanka, India's tropical parts, and Malaysia. This tree is normally planted along roadsides and in gardens for its beautiful appearance. *P. longifolia* is one of the most important traditional indigenous medicinal plants commonly used in traditional medicine as febrifuge and tonic (CSIR, 1969).

Until 2019, there was rather limited experimental evidence of the synergistic activity between *P. longifolia* leaves extract and synthetic antibiotics against MRSA. The previous experiments conducted in our laboratory have demonstrated the *in vitro* interaction of ampicillin and *P. longifolia* Leaf Ethyl Acetate Fraction (PLEAF) by Checkerboard Method and Microscopic Technique against MRSA (Kirubakari et al., 2020a). We found that the PLEAF fraction worked well synergistically in combination with ampicillin to kill MRSA's local resistance strain. Moreover, the PLEAF fraction also exhibited a good antioxidant activity. The fraction also enhanced the Vero cell viability in the presence of ampicillin, which is an important finding that showed the nontoxic nature of ampicillin in the presence of PLEAF in combinational therapy. Further study was also conducted to observe the *in situ* antimicrobial synergistic effects between PLEAF and ampicillin against MRSA local isolate using modern Scanning Electron Microscopy (SEM) observation (Kirubakari et al., 2020b). PLEAF and ampicillin combination exhibited significant antibacterial activity against MRSA to destroy the resistance bacteria observed via SEM.

As the problem statement, genetic mutation was involved in the development of resistance to the antibiotic. The attainment of the *mecA* gene by horizontal transmission by conjugation was the main cause of antibiotic resistance in *S. aureus* (Wielders et al., 2002). This important *mecA* gene is attributed to methicillin resistance in MRSA strains, which encodes a novel penicillin-binding protein 2A (PBP2A) (Stapleton et al., 2002). Therefore, new alternative strategies are needed to address this issue by developing a new antimicrobial agent, modifying the existing antibiotic activity with a combination of plant extracts as resistance modifying agents, or using the plant extract combined with existing antibiotics against resistant bacteria to suppress the expression of *mecA* gene in MRSA bacteria. Thus, MRSA bacterial infection increasing incidence has drawn the pharmaceutical and scientific communities' attention towards studies on the potential antimicrobial activity of plant-derived substances, which are used in traditional medicine in different countries. Scientists from divergent fields are investigating medicinal plants with an eye on their antimicrobial usefulness. However, as a further study, in understanding multidrug-resistant bacteria's challenges, *P. longifolia* leaves' antibacterial activity, antibiotic-modifying activity, and synergistic effects combined with different first-line antibiotics commonly used against infectious agents need to be investigated. The synergistic antimicrobial effects of *P. longifolia* leaves' methanolic extract combined with β-lactam antibiotics such as ceftriaxone will advance new knowledge because this study has not been done yet. In addition, ceftriaxone has a broad spectrum of potency against Gram positive bacteria. Since, ceftriaxone was used in combination with different antibiotics to treat MSSA infections (Patel et al., 2014, Lother and Press, 2017, Kamfose et al., 2020). Therefore, in this study ceftriaxone has been combined with *P. longifolia* leaf methanol extract (PLLME) for the examination against MRSA.

Moreover, the combined effects of ceftriaxone and PLLME against MRSA bacteria in terms of antibiotic modifying activity and synergistic effects are still uncertain and fewer studies were conducted in this area of study.

## 1.1 Objectives

The study embarks according to the following objectives:

a. To study the antibiotic modifying activity of PLLME against *mecA* gene-positive human strains of *Staphylococcus aureus*.

b. To investigate the possible synergistic effect of PLLME against *mecA* gene presence in MRSA DNA in combination with ceftriaxone.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Gram Positive Staphylococcus

Staphylococcus genus is composed of 41 rationally described species, which are categorized and distinguished by the genus associates based on a variety of phenotypic characteristics, including their morphology and biochemical reactions. Staphylococcus is usually grouped into positive coagulase (CPS) and negative coagulase (CNS) (Resch et al., 2008). Besides that, Staphylococcus coagulase-positive *S. aureus*, which is pathogenic to humans and animals, is classified as the largest species of CPS that instigates pyogenic infections in humans. Apart from *S. aureus*, there are six more CPS species classified as *S. lutrae*, *S. pseudintermedius*, *S. intermedius*, *S. schleiferi subs. coagulans*, *S. delphini*, and the coagulase variable *S. hyicus* (Bannerman and Peacock, 2007). Although CNS might be an opportunistic pathogen, anyway it consistently exists by medical devices or colonizes exposed wounds (Faria et al. 2009). Human beings are a natural repository for *S. aureus* as the mucous membranes is the primary habitat for them and this is followed by human and animal skin and skin glands (Kasprowicz et al., 2011).

### 2.1.1 *Staphylococcus aureus*

*Staphylococcus aureus* is associated with the genus Staphylococcus and is a Gram-positive bacterium. *S. aureus* as a major human pathogen induces a wide range of hospital and community acquired diseases worldwide. Also, this bacterium is a causative agent for multiple human infections as they can produce skin infections and food poisoning through medical devices over the well-known clinical complications of colonization and often are associated with wound-related infections. Manifold diseases along with infective endocarditis, bacteraemia, infections of skin and soft

tissue, for instance scalded skin syndrome, impetigo, cellulite and folliculitis (CDC, 2003). Other than that, relating to life-threatening diseases for examples deep postsurgical infections, septicaemia, toxic shock syndrome (Yee-Guardino et al., 2008), septic arthritis, prosthetic device infections, pulmonary infections such as pneumonia and empyema (Kohli et al., 2011), meningitis osteomyelitis, and urinary tract infections (Moreillon et al., 2005).

Coagulase-negative staphylococci (CoNS) were tested to be inoffensive skin commensals before the 1970s, despite that, CoNS is now growing at the root of hospital-acquired infection, specifically nosocomial bacteraemia. The National Nosocomial Infection Survey (NNIS) is a constant collaborative surveillance system sponsored by the Centers for Disease Control (CDC) to attain national data on nosocomial infections. NNIS has found that the prevalence of CoNS caused by nosocomial bacteraemia has risen from 9 to 27%, while the NNIS survey also showed a 20 to 60% increase in the resistance proportion of nosocomial CoNS to methicillin, oxacillin, and nafcillin and most of these methicillin-resistant CoNS were also resistant to many other antimicrobial agents (Schaberg et al., 1991). Such multi-resistant CoNS typically colonize the skin of hospitalized patients that assists as a potential reservoir of multi-resistant isolates which can cause infections (Archer, 1991). Thus, CoNS anticipated a significant cause of infections among humans (Sharma et al., 2011). Furthermore, these colonizing isolates facilitate reserving for the transmission of antibiotic-resistance genes between CoNS and subsequently be acquired by *S. aureus* (Forbes et al., 1983). As a consequence of *S. aureus* has been tolerated genetic modification over the past 50 years, hence this organism developed antibiotic-resistant strains (McCallum et al., 2010). Penicillin and its derivatives have been used in treating infections contributed by *S. aureus*. Penicillin is the foremost β-lactam antibiotic

presented in treatment (Davies et al., 2010). Penicillin is medicine. Through the development of β-lactamase in *S. aureus*, this organism becomes resistant to penicillin since β-lactamase hydrolysis penicillin and induces alteration of the target PBPs, thereby inactivates the antibiotic (Chambers, 1999). In addition, β-lactamases as a clinically vital resistance mechanism are almost utterly found in staphylococci. On the other hand, Staphylococcal β-lactamases are narrow-spectrum penicillinases with comparatively declined activity against semi-Synthetic anti-Staphylococcal penicillin, in that event, penicillin derivative of new semisynthetic penicillinase-resistant antimicrobial drugs including methicillin, oxacillin, cephalosporins, and carbapenems progressed in the treatment of staphylococcal infections caused by β-lactamase-producing strains.

β-lactamase is an enzyme that binds β-lactams and expeditiously hydrolyses them into biologically inactive metabolites. So, these kinds of inactive metabolite compounds cannot be bound to PBPs. Besides, development of reinforcement of β-lactamase resistant antibiotics including methicillin, oxacillin, cephalosporins as well as carbapenems (Wright, 1999) treatments in *S. aureus* infections (Rayner and Munckhof, 2005). However, nosocomial infections through of *S. aureus* in nursing homes and hospitals become progressively resistant to numerous antibiotics including methicillin the first penicillin derivative as a substitution in the treatment of penicillin -resistant strains, and thus these antibiotic resistant strains termed as MRSA (Casey and Chasens, 2009). *S. aureus* is a significant pathogen linked to severe hospital and community-acquired diseases. MRSA is responsible for the growth of nosocomial infections which have a tremendous impact on morbidity, mortality, and hospitalization costs (Feizabadi et al., 2011).

### 2.1.2 Classification

Gram-positive *S. aureus* cells are spherical. They frequently resemble bunches and clusters of grapes when observed *S. aureus* Gram staining under a light microscope and named it accordingly as 'Staphylococcus'. In Greek words, 'staphyle' resembles the clusters and a bunch of grapes and 'coccus' means granule (Figure 2.1) (Licitra et al., 2013). As the colonies grew on solid media golden in colour, and hence it developed its specific sobriquet of aureus.

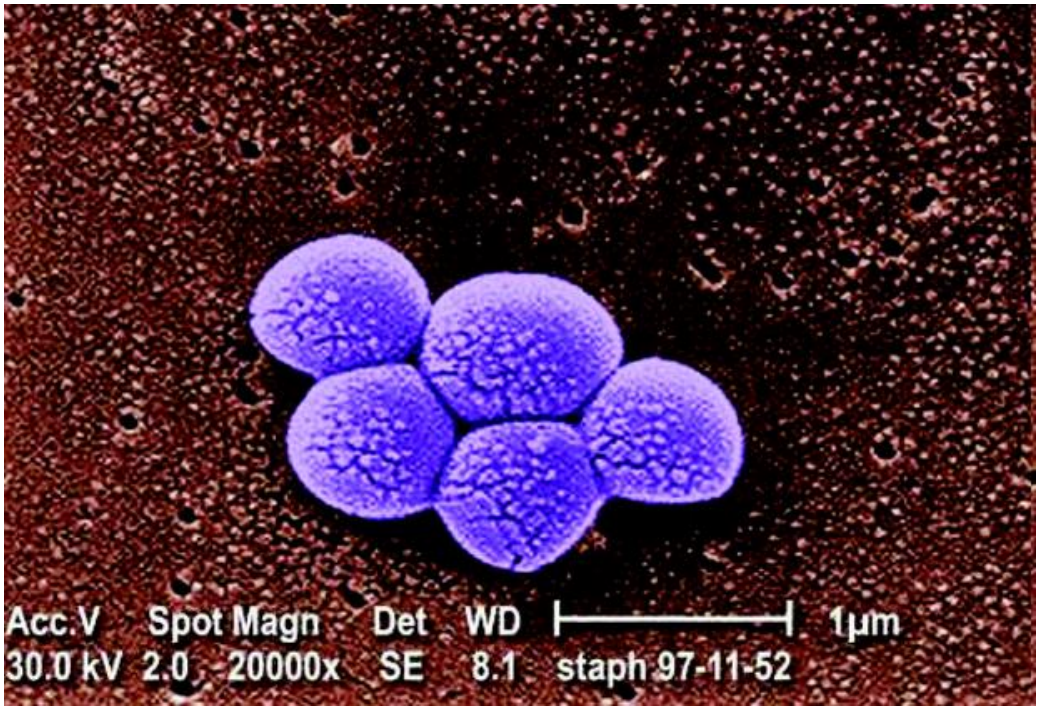


Figure 2.1 An electron micrograph of MRSA bacteria
(Source: Klevens et al., 2007)

- **Scientific Classification**
  - Domain: Prokaryotes
  - Kingdom: Eubacteria
  - Phylum: Firmicutes
  - Class: Bacilli
  - Order: Bacillales
  - Family: Staphylococcaceae
  - Genus: Staphylococcus
  - Species: aureus

- **Binomial name**
  - Staphylococcus aureus
    (Source: Rosenbach, 1884)

### 2.1.3 Structure of *Staphylococcus aureus*

Gram-positive bacteria display a wide range of cell wall structure from simple to very complex (Figure 2.2). Referring to figure 2.2, section A demonstrates the superficial as well as secreted proteins, and hence synthesis of all these secreted proteins is reliant on the phase of growth as presented in the graph, and is prearranged by regulatory genes for example *agr*. Whereas, in section B and C illustrates the cell envelope cross-sections. Especially in viewing section C which contains various surface proteins with the structural assembly that are identical to the clumping factor as well as repetitive segments of amino acid. Furthermore, TSST-1 indicates toxic shock syndrome toxin 1. The Staphylococcal cell wall is 50% of peptidoglycan by weight, while this peptidoglycan inheld of alternate polysaccharide subunits with 1,4 β-linkages of N-acetylglucosamine and N-acetylmuramic acid. Normally, extraordinarily crosslinked types with relatively unusual structural design Staphylococcal cell walls are in the right place. Further to this, the peptidoglycan chains in *S. aureus* are crosslinked by the tetrapeptide chains bound through a pentaglycine linkage to the N-acetylmuramic acid. Correspondingly, differences in peptidoglycan structure of staphylococcus strains result in differences in its capacities, which are why intravascular coagulation was disseminated (Kessler et al., 1991). Consequently, macrophages inducing the release of cytokines complement activation and platelets aggregation through peptidoglycan may have endotoxin-like activity. The main components of Ribitol teichoic acids of the cell wall covalently linked to peptidoglycan. The cell wall envelopes are an integral part of the exoskeleton that prevents bacterial cell breakdown under low osmolar pressures, while the bacteria are protected from their environment by the host tissue instead of the physical barrier. Also, the cell wall of the microorganisms presents a significant role in the infection

and susceptibility of pathogenicity (Van Heijenoort and Gutmann, 2000). Besides, *S. aureus* structure is made up of murein, teichoic acids and, wall-associated surface proteins (Mazmanian et al., 2001). As a matter of fact, murein contains peptide bridge glycan strands that provide the structural integrity of the sacculus. It is typically a staphylococcal characteristic function that is detected by murein degrees of a cross and these are estimated as a linked peptides ratio to the overall amount of all peptide ends and is extremely high from 80 to 90% (Gally and Archibald, 1993). In addition, carbohydrate antigen, teichoic acid is a compound in *S. aureus* containing N-acetylglucosamine and polyribitol phosphate. Patients with ingrained staphylococcal infections were examined in normal human serum through the elevated antibody titres of teichoic antibodies.

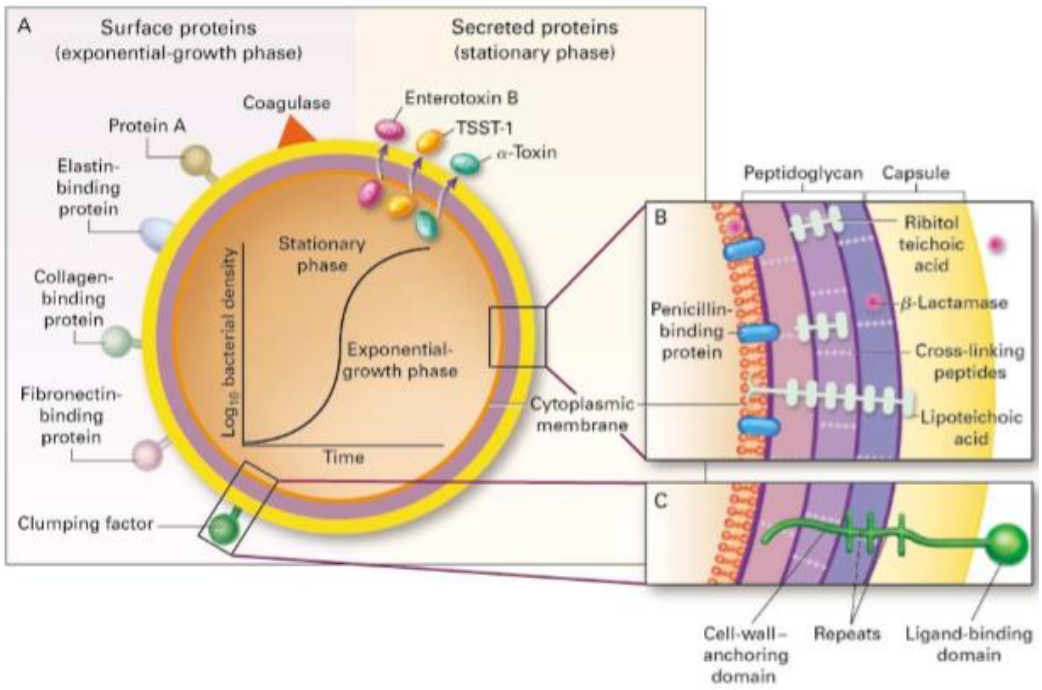


Figure 2.2 Structure of *S. aureus*
(Source: Lowy, 1998)

Protein A is one of the cell wall protein components and is the prototype for the proteins with anti-phagocytic properties, and these properties are dependent on the ability of the protein's cell wall to bind the Fc portion-immunoglobulins. Most superficial staphylococcal proteins typically have certain structural features. So, these types of features comprise a secretory signal sequence at the N terminal, extended positively charged amino acids in the cytoplasm, a spanning domain hydrophobic membrane, and a cell wall attaching area, while altogether at the carboxyl-terminal. Moreover, an exposed N terminal ligand-binding domain on the surface of the bacterial cell, enables some of these proteins to function as adhesins (Foster and McDevitt, 1994). Regardless, bacterial protein A can be unconfined from the bacterial surface utilizing staphylococcus lysostaphin treatment since this protein can respond with normal human serum IgG (Lowy, 1998).

Lysostaphin is a glycyl-glycine endopeptidase that breaks and splits the cell wall's pentaglycyl cross passage. In addition, lysozyme is an N-acetyl-muramidase, splits the glycan strands and releases the protein with the variable in their masses because of the existence of interconnected peptidoglycan fragments in diverse sizes while those proteins are the molecule as a spectrum of fragments (Navarre et al., 1998). Not to mention, some functional and structural elements are preserved even though there are divergences in the peptidoglycan structure from one species to another species of the bacteria. Short cell wall peptides cross-linked the glycan chains which encompasses of repeating disaccharide units, N-acetyl-glucosamine-(β1–4)-N-acetyl-muramic acid (GlcNAc–MurNAc) and these linkages formed a three-dimensional molecular network in order to preserves the bacterium integrity. Finally, it produces constitutes for the main component of the bacterial cell wall, cross-linked peptidoglycan by way of the process of PBPs catalyzes the polymerization of lipid II

subunits by means of trans-glycosylation and trans-peptidation reactions (Perry et al., 2002).

### 2.1.4 Chronology of *Staphylococcus aureus*

*Staphylococcus aureus* was first identified in the late 1870s, as a cause of postoperative wound sepsis by Professor Sir Alexander Ogston. The discoverer then later named this population of grape-like bacteria (Figure 2.1) as staphylococcus (Ogston et al., 1984). On the other hand, Gram-positive Staphylococcus is encircled in a thick cell wall, without a surface membrane. Also, it was first discovered at the end of the 19th century, in the pus of human abscesses (Moreillon et al., 2005). For the period of these years, *S. aureus* infection usually generated conditions of irritate skin and soft tissue for examples burns, scalded-skin disease and impetigo. For even more serious *S. aureus* infection type can evolve into both lethal bacterial pneumonia and bloodstream bacteria. *S. aureus* may lead to food poisoning, in case of this organism acquired from poorly cooked or processed food.

Beforehand, invasive infections caused by *S. aureus* were often lethal before antibiotics became available. With the production and breakthrough of antibiotic drugs including penicillin, medical treatment for *S. aureus* infections was regular and effective, throughout the 1940s. However, over the next two decades, this pathogen subsequently developed tolerance against penicillin. Besides, the misuse and overuse of antibiotics have facilitated the production of natural bacteria through those species that have become resistant to drugs designed to treat such infections. From that point on, almost every *S. aureus* strain has become immune to those specific penicillins, aminopenicillins, and antipseudomonal penicillins (Chambers, 2009). Subsequently, this drug resistance evolves because of the genes acquired by *S. aureus* which encodes drug inactivating enzymes originally referred to as penicillinases but are now referred

to as β-lactamases (Rice, 2006). During the 1960s the incidence of penicillin-resistant strains was augmented. A new class of antibiotics targeting specifically on *S. aureus* pathogens that was found during this time. To meet the snowballing of penicillin-resistant *S. aureus* issues, other formulae similar to penicillin were developed as methicillin. Afterward, this methicillin eventually becomes one of the most common antibiotic types used in treating *S. aureus* infections. Nevertheless, a British scientist has identified the first strains of *S. aureus* bacteria that are resistant to methicillin after a year of this class of antibiotics discovered in 1960. The strain found is then known as MRSA (Zaoutis et al., 2006). On top of this, the earliest case was reported in humans who were infected with MRSA in the United States in 1968. Successively, another distinct strain of bacteria has been developed which can withstand previously effective methicillin drugs and other interrelated antibiotics as well. While methicillin was known to resist the degradation of β-lactamases almost immediately after methicillin was introduced into clinical practice, the MRSA strains were identified which are totally resistant to all β-lactam antibiotics (Fischbach and Walsh, 2009). In fact, MRSA is resistant to the entire class of penicillin-like antibiotics called β-lactams which contain penicillin, amoxicillin, oxacillin, and methicillin. By the year 1980 they had spread all over the world. Commonly, MRSA was a pathogen developed in the hospital from the late 1970s to the early 1990s, causing the whole antibiotic β-lactam class to become inactive. Then, another form of Community-Acquired MRSA (CA-MRSA) was identified for the first time in the late 1990s, and of the kind that caused serious and lethal infections in children deprived of disclosure of preventive health care (Herold et al., 1998).

MRSA has spread throughout the world in healthcare settings over the last 20 years. As a result, *S. aureus* grows even more until resistance to supplemental

antibiotics begins to show. Vancomycin, as the lastest choice, is used against *S. aureus* and was one of a handful of antibiotics when physicians in the United States documented the first *S. aureus* strains resistant to the antibiotic in 2002. The first clinical vancomycin (Mu50) resistant *S. aureus* strain was identified in Japan in 1997 (Hood et al., 2000). While vancomycin-resistant strains are still rare to be identified (Srinivasan et al., 2002), this could rapidly become a major concern in antibiotic resistance. On the other hand, in the research on new and different compounds with a wide range of activities as well as their current therapeutic approaches, some degree of medication choices for infections instigated by type of multidrug-resistant microorganisms is recommended (Entenza and Moreillon, 2009).

### 2.1.5 Sustained Existence Strategy of *Staphylococcus aureus*

*Staphylococcus aureus* owns significant genetic plasticity as a survival strategy that assigns them to react towards virulence factors with an extensive array and therefore it leads to the resistance against different classes of antimicrobial agents while instigating with ever-increasing menace of their survival. As aforementioned, allocation of the bacteria with antimicrobial producing organisms in the same ecological place has advanced the earliest mechanisms and hence it endures damaging antibiotic molecule effect. Therefore, intrinsic resistance of the bacteria gets furnished to thrive in their existence. Generally, antimicrobial drugs act at three interfaces in microbes scilicet, nucleic acids synthesis, cell wall synthesis and protein synthesis. The survival of *S. aureus* is by producing inhibitors towards the antimicrobial agent's mechanisms.

#### 2.1.5(a) Cell Wall Inhibitors

Bacterial cells are enclosed by peptidoglycan cell wall that goes through glycan strands crosslinking as a result of trans-glycosidase action. As the cell wall is

composed of long sugar polymers and hence this is a foundation for the peptide chains to accelerate from polymers sugar and produces crosslinks from one to another peptide (Kahne et al., 2005). Subsequently, in the presence of PBPs, the D-alanyl-alanine portion of peptide chains is cross-linked by glycine residues and formed cross-linking that makes the bacteria cell wall stronger (Reynolds, 1989).

Subsequently, β-lactam agents are the foremost targets of PBPs. Accordingly, the β-lactams antibiotics in particular penicillin and methicillin drugs which fundamentally inhibit the bacteria cell wall by way of β-lactams antibiotics offering them as pseudo-substrates of the transpeptidase and acylate the active residue. So, when the PBP acts together with the β-lactams ring, immediately that particular PBP is no longer presented for new peptidoglycan synthesis, thus instigating for weak peptide bond formation. Moreover, the weak peptide bond is susceptible to osmotic changes and is sophisticated for the bacterium lysis. Furthermore, five different forms of PBPs have been defined in the category of susceptible strains *S. aureus,* for instance, PBP 1, 2, 3, 3' and 4. Conversely, there is a variation of PBP2a that is accountable for β-lactams resistance (Fuda et al., 2004). Talented PBP2a is capable of taking the place of ordinary PBPs biosynthetic functions even though in the existence of β-lactams ever since it has a low affinity towards β-lactam antibiotics resultantly it prevents cell lysis (Georgopapadakou et al., 1986). In addition, the *mecA* gene is carried in a unique mobile genetic element, integrated into the staphylococcal chromosome, SCC*mec*. A definite methicillin resistance occurs in the presence of the *mecA* gene as it is also constrained to PBP2a synthesis (Grundmann et al., 2006).

The glycopeptides bind to the peptide side chain portion (D-alanyl D-alanine) of the precursor peptidoglycan subunit. Besides, vancomycin, the large drug molecule, induced susceptibility over and done with the process of inhibition of trans-

glycosylation and transpeptidation phases. This is attained through antibiotic action in blocking the D-Ala-D-Ala precursor through hydrogen bonding and preventing the construction of UDP-MurNAc pentapeptide essential for the synthesis of the cell wall. However, vancomycin-resistanta microbes transform the D-alanyl D-alanine precursor to D-Ala-D-Lac utilizing VanH dehydrogenase and VanA ligase enzymes, these two enzymes have low affinity for glycopeptides and by this means it leads to the common hydrogen binding site inaccessible (Chopra, 2003).

### 2.1.5(b) Inhibitor of Protein Synthesis

The most common class of drugs that work by inhibiting protein synthesis are aminoglycosides, tetracyclines, chloramphenicol, oxazolidinones and macrolides. Aminoglycosides are bactericidal, whereas the other class products, such as tetracyclines and macrolides, have a powerful bacterial activity (Bryan et al., 1985). Furthermore, a mechanism is known as transcription, when the bacterial DNA information is used to synthesize a molecule of RNA referred to as messenger RNA (mRNA) (Figure 2.3). And the process is called translation when there is an existing macromolecular form of ribosome synthesis proteins in mRNA. Synthesis of proteins is catalysed through the ribosomes, as well as cytoplasmic factors. Besides that, bacterial 70S ribosome consists of two ribonucleoprotein 30S and 50S subunits (Yoneyama and Katsumata, 2006) and protein synthesis inhibition targeting at this bacterial 30S and 50S ribosome subunit (Johnston et al., 2002).

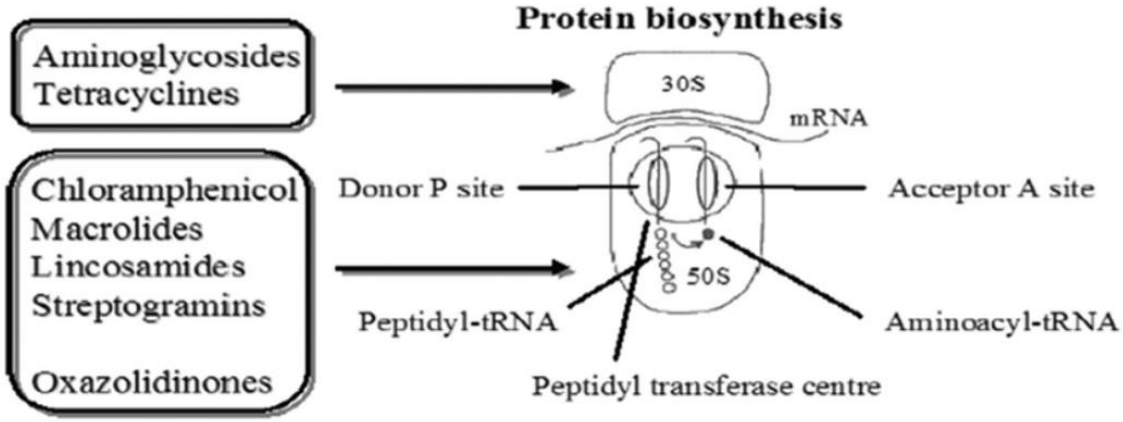


Figure 2.3 Protein biosynthesis inhibitors site of action
(Source: Yoneyama and Katsumata 2006)

Positively charged aminoglycosides molecules attach to the negatively charged outer membrane and the attachment conditioning to the expansion of large pores, thereby tolerating the penetration of antibiotics inside the bacterium. Aminoglycoside activity has the strongest effect on the bacterial ribosome. Other than that, aminoglycosides function together with 16S rRNA of the 30S subunit near 'site A' using hydrogen bonds and this leads to mRNA misreading and premature translation termination (Garima et al., 2017). Sadly, aminoglycosides no longer resist being an essential anti-staphylococcal drug class with the appearance of their structural inactivation in definite amino groups, thus it diminished their ability to block protein synthesis while lacking attraction and binding to the 30S ribosomal subunits and misrepresenting their susceptibility to MRSA (Magnet and Blanchard, 2005). And so, the most commonly observed mechanism of resistance in aminoglycosides is enzymatic inactivation.

By routing 50S ribosomal subunit binding to 23S ribosomal RNA in bacteria (Yoneyama and Katsumata, 2006), macrolide antibiotics disrupt protein synthesis at their primary stage. Bacteria use three methods of fighting against macrolides as described: antibiotic inactivation, antibiotic efflux, and mutation which indicate

methylation to change the target location. In this case, it averts antibiotic binding at the ribosomal target (Matsuoka, 2000). In addition, broad-spectrum antibiotics tetracyclines including chlortetracycline, doxycycline, and minocycline have discovered their uses in the treatment of bacterial infections following penicillin as the second line in that treatment. So, such drugs respond to the 30S ribosomal subunit 16S rRNA sequences as discussed, which prohibit tRNA from binding to 'site A' (Yoneyama and Katsumata, 2006). Finally, minocycline and tetracycline resistance are due to the ribosomal defense mechanisms mediated by genes such as tet(M) (Martin et al., 1986), tet(O) (Sougakoff et al., 1987), tet(Q) (Nikolich et al., 1992) and tet(S) (Charpentier et al., 1993).

Linezolid is a recently approved antibiotic that is fully a synthetic agent affiliated with a newer class of antibiotics. For the effective antibiotic oxazolidinones against Gram-positive multidrug-resistant bacteria, including methicillin-resistant staphylococci, penicillin-resistant pneumococci, and enterococci resistant to vancomycin (Orsi et al., 2011), thus, by interfering at quite a several stages in protein synthesis. Other than that, this mechanism of action involves a selective binding to the 50S subunit 23S rRNA, prevents the production of a complex functional activation as well as suppressing 70S inhibition and thus networking with peptideyl-tRNA (Lambert, 2005; Bozdogan and Appelbaum, 2004). Plus, linezolid was effective in treating *S. aureus* strains of methicillin-susceptible and methicillin-resistant, (Jones et al., 2006) which brought about complex and complicated skin and soft tissue associated infections (Weigelt et al., 2005), as well as nosocomial pneumonia. Besides, susceptibility of abbreviated Linezolid in *S. aureus* occurs over and complete with a specific point of mutations at the central loop of the domain 'V' of 50S subunit’s 23S ribosomal RNA (Nannini et al., 2010). Moreover, domain 'V' here represents catalyses

for the peptide bonds forming and this process is known as a peptidyl transferase. So, there is an alternative function of drug efflux proteins or transporters often known as antibiotic-fighting efflux pumps (Borges-Walmsley et al., 2003).

### 2.1.5(c) Nucleic Acids Breakdown

Resistance due to the mutational alterations is still decisive to particular antibiotic classes under certain settings. And it is factual mainly for rifampicin and fluoroquinolone antibiotics (Alekshun and Levy, 1999). In general, *S. aureus* fluoroquinolone resistance works by either inducing multidrug resistance efflux pump or by transporting an alteration in chromosomes which are accountable for topoisomerase IV or DNA gyrase expression (Wolfson and Hooper, 1989). Plus, the fluoroquinolones with C8 surrogates, for example moxifloxacin and gatifloxacin give the impression to be more effective than the older drugs of this class against *S. aureus*, by the same token it might as well be less possible in the selection of resistant mutants. Along with this, the addition of rifampin can strenghten the reaction (Shopsin et al., 2004). Modification of the gene *gyrA* that encodes for subunit A of gyrase DNA, viz. topoisomerase (Sreedharan et al., 1990) as the initial resistance mechanism. Besides that, a further mechanism that is associated with chromosomal DNA mutation in the SmaI A locus is to achieve fluoroquinolone resistance (Trucksis et al., 1991). Amino acid substitutions in the vibrant regions of DNA complex enzymes are usually discussed as the quinolone resistance determining region (QRDR) decreases quinolones' affinity for both its targets (Ng et al., 1996). However, the distinct condition seems whenever acquired resistance genes are united in chromosomes while obstructing their identification. It is appropriately established by the methicillin resistance factor in *S. aureus mecA* gene (Spratt, 1992).

## 2.2 Background of Methicillin Resistant *Staphylococcus aureus* (MRSA)

MRSA is the most significant source in hospital-acquired MRSA (HA-MRSA) infection that is becoming progressively difficult to overcome since their resistance is emergent to all recent antibiotic classes. In addition, penicillinase-resistant penicillin called methicillin was presented in 1959 as a substitution of penicillin to fight against penicillin-resistant *S.* aureus. However, within a year and over again another first human *S. aureus* strain adopted methicillin-resistant was reported by the late Professor Patricia Jevons in UK hospital (Kim, 2009) then MRSA isolates be present almost immediately discovered in other European countries, thereafter from Japan, Australia and the United States. Subsequently, MRSA occurrence presented a different rate between these countries but in remained increasing rate, potential 70% in Japan (Noskin, 2001), 45% in the United Kingdom, 40% in Italy and Greece, and 30% to 50% in the United States (Jones et al., 2003). Consequently, it has been reported worldwide for outbreaks of the organisms endemically and epidemically (McDougal et al., 2003) while leading the economies of the world to develop destructively. In a short time, MRSA has become a problem in all size hospitals worldwide, healthcare workers, as well as growing in the number of diverse population clusters encountering the difficult of MRSA infections globally and at various communities (Tacconelli, 2009) while they are progressively retrieved from nursing homes and the community (Hussain et al., 2000, Mertz et al., 2010). The reports on increased morbidity and mortality are supported by the augmented frequency of community allied MRSA infection especially the prolonged fever, extended hospitalization, and an advanced pulmonary problem altogether with bone and joint-related infections, and recurrence of severe staphylococcal sepsis syndrome (Gonzalez et al., 2005).

## 2.3 Epidemiology of MRSA

*Staphylococcus aureus* infections are progressively reported everywhere globally (Grundmann et al., 2006; Lescure et al., 2006). Humans are major *S. aureus* reservoirs and together with this organism including MRSA which are spotted on the skin as well as mucous membranes of living organisms (Boucher and Corey, 2008). Besides, it is estimated that about a part of every adults' communities are colonized with *S. aureus* and almost 15% of the total population carries *S. aureus* in the anterior nares. Some of the populations have a tendency that ensures a greater colonization rate of *S. aureus* that is up to 80%, for example health care workers, hospitalized patients, immunocompromised individuals and people who consistently using needles especially intravenous drug users (Tong et al., 2015). Hence, the medical problems of these infected patients have turned out to be more complex as well as the consequence of our sophisticated medical system (Fowler et al., 2005; Wisplinghoff et al., 2004). Because of this, the cure for these infections has become further challenging. Furthermore, the organism *S. aureus* can be passed on from one individual to another through direct exposure or via fomites (Rasigade and Vandenesch, 2014). In the latest study, presenting high healthcare outlays intended for altogether *S. aureus* bacteraemia infected sufferers and with the existence of indwelling devices, they remained twofold as in elevation among patients with hospital-acquired *S. aureus* bacteraemia (Chu et al., 2005). So, the incidence of infective endocarditis has been increasing and is the most severe *S. aureus* bacteraemia complication (Fowler et al., 2005).

### 2.3.1 Hospital Associated MRSA (HA-MRSA)

Current studies describe a recurrent spike in hospital MRSA infections (Kuehnert et al., 2005; Lescure et al., 2006; Klevens et al., 2006). An additional of 125,969 hospitalizations in United States with *S. aureus* infections were reported by

CDC inspectors each year in the period 1999-2000, and this counted in pneumonia and also in infections associated with bloodstream (Kuehnert et al., 2005). Also, HA-MRSA occurs when there are hospitalized patients with bloodstream infection, pneumonia, serious infectious skin and soft tissue-associated infections (Klevens et al., 2007) and in addition to that, HA-MRSA generally shows not only susceptibility to β-lactams but also resistance to other types of antibiotics. Furthermore, antibiotic use as well as likely overuse also led to resistance development. Many hospitalized patients will then be weak and their immune-compromised, thus HA-MRSA is a common and very serious infection (Klevens et al., 2007). Thus, the risk factors of HA-MRSA infection, including antibiotic utilization, burn cases, post-operative prolonged hospitalization, intensive care, haemodialysis, MRSA colonization, and exposure to people with MRSA colonization or contamination. This leads to the increase of HA-MRSA infections in both patients and healthcare institutions (Elliott et al., 2010). HA-MRSA strains tend to carry SCC*mec*. Moreover, three varieties of SCC*mec* (Varieties I, II and III) are found in HA-MRSA, which are exposed to have multidrug resistance (Mongkolrattanothai et al., 2003). Type I HA-MRSA does not produce any specific resistance determinants; however, Type II and Type III HA-MRSA are additive resistance determinants with *mecA* and these additional genetic factors are also responsible for tolerance towards many antibiotics and β-lactam agents (Kluytmans-Vandenbergh and Kluytmans, 2006). Both forms of Type II and Type III SCC*mec* have an identical location of chromosomal integration and cassette chromosome recombinase genes which are responsible for SCC*mec* horizontal transition (Daum et al., 2002). Therefore, this HA-MRSA assistant leads resistance to numerous antibiotics while being sophisticated for the selective benefit of spreading between patients by workers and also from polluted surfaces.

### 2.3.2 Community Associated MRSA (CA-MRSA)

CA-MRSA started to arise in the 1990s and is an infection that takes place without healthcare exposure therefore it occurs in patients with no presence of threat factors commonly related by means of HA-MRSA (Zetola et al., 2005). Correspondingly, CA-MRSA is frequently related to skin and soft tissue infections that are present in young and healthy persons who have clear healthcare exposure records (Fridkin et al., 2005). Plus, skin and soft tissue infections are the most common infections generated by CA-MRSA, although more respiratory tract involving invasive infections keep on increasing no matter with or without bacteraemia and septic shock. Typically, CA-MRSA strains show resistance towards β-lactams but anyway it is sensitive to non- β-lactams, for example, trimethoprim-sulfamethoxazole, tetracyclines and clindamycin. Most of the CA-MRSA strains carry type IV or V SCC*mec* (Naimi et al., 2003). Genetically CA-MRSA strains appear to be dissimilar from HA-MRSA but it is further probable to own virulence factors with exclusive combinations (Fey et al., 2003). CA-MRSA strains frequently carry genes for the cytotoxin Panton-Valentine leukocidin (PVL) that confers improved virulence (Diep et al., 2006). Generally, *S. aureus* produces <5% of cytotoxin PVL and shows lytic activity against specific human cells including polymorphonuclear cells, monocytes, and macrophages. Lastly, the lytic cycle is involved with extremely specific activity. Subsequently, these *S. aureus* exoproteins have been correlated to furuncles, cutaneous abscesses, severe necrotic skin infections and severe necrotizing pneumonia (Schwartzman et al., 2007).

### 2.3.3 Virulence Factor

*Staphylococcus aureus* expressing various secreted virulence factors and this promotes through host colonization, including a variety of membranes damaging

toxins that are capable of developing pores in the cytoplasmic membrane of the host cells while promoting cell lysis, as the result it induces propagation over the body, host defence evasion mechanisms and targets both damaged skin and mucosa (Ferry et al., 2005). Along with, *S. aureus* infections virulence and pathogenicity are linked with various bacterial surface components such as capsular protein A and polysaccharide that are able to identify adhesive matrix molecules such as clumping factor (clf) and fibronectin-binding protein (fnBP) in addition to extracellular proteins, for example; coagulase, hemolysins, enterotoxins, toxic shock syndrome (TSS) exfoliative toxin and PVL (Fraser and Proft, 2008). Other than that, virulence factors are generally divided into three categories depending on the adhesive, toxin, and immunomodulatory roles. Also, surface-attached proteins Adhesins withstand the bacteria just before attaching to distinctly different human tissues. Whereas, secreted protein toxins are the cause for tissue damage and it builds bacterial discharge (pus) in abscesses, and it is meant to be an assistant communication between hosts. Lastly, immunomodulatory proteins contribute to drops in host immunity and thus this leads to the inhibition of infection resistance (Collins et al., 2010). Proteins present many of the microbial surface proteins that promote *S. aureus* adhesion for example fibrinogen and fibronectin. The importance of those plasma proteins capable of coating indwelling medical devices in addition to deposited proteins bound to bacteria which are alleged to be a vital element in infections of foreign bodies and pathogenesis of wound (Foster and Hook, 1998). Adhesin genes including *clf* (Ni Eidhin et al., 1998) and *fnb* (Jonsson et al., 1991) in *S. aureus*, encodes correspondingly for the fibrinogen and fibronectin-binding proteins (FnBP). Both FnBP A and FnBP B are encoded with *fnbA* and *fnbB* genes accordingly and therefore this carries distinguished characteristic, host tissues or implanted biomaterials, attachment and colonization of *S. aureus*