CHAPTER 1: INTRODUCTION

Statement of problem

1.1. Sepsis:

According to 'The Third International Consensus Definitions for Sepsis and Septic Shock' sepsis is defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection" [1]. Sepsis and severe sepsis are one of the most common causes of death among patients in medical intensive care units. Clinical studies of sepsis reports show, higher incidences in aging population and in patients surviving sepsis long term physiological defects have been reported [2].

1.1.1. Epidemiology

Sepsis comprises a significant health burden worldwide [3]. Among the known bacterial causes of sepsis, Gram positive organisms such as *Staphylococcus aureus* and *Streptococcus pneumonia;* and Gram negative organisms such as *Escherichia coli, Klebsiella* species, and *Pseudomonas aeruginosa* are predominant. Sepsis is second leading cause of death worldwide and a health care expense of $23.7 billion was accounted in year 2013 [4, 5].

According to Indian statistics, among Gram-positive bacteremia (sepsis), *Staphylococcus aureus* sepsis is one of the most prevalent and difficult to treat infections and is associated with significant morbidity and mortality. MRSA constituted 54% of all *S. aureus* episodes and with high rates of MRSA infections both in community and hospital settings causing significant morbidity and mortality [6].

1.1.2. Clinical features of sepsis

Sepsis symptoms are generally non-specific and include fever, chills, anxiety, breathing difficulty, fatigue, vomiting and nausea. But these symptoms in general are also presented in many clinical conditions. Sometimes, the main symptoms are absolutely absent in case of severe sepsis especially in aged patients [1, 7].

Local infection symptoms in organ system may add to the etiology of sepsis. Such symptoms include the following:

- Pulmonary infection
- Head and neck infections

- Cardiac infections
- Abdominal and gastrointestinal (GI) infections
- Pelvic and genitourinary (GU) infections
- Bone and soft-tissue infections

The other symptoms associated with sepsis are, inflammatory variables (high Leukocytosis, elevated C-reactive proteins), hemodynamic variables (arterial hypotension), Organ-dysfunction variables (Coagulation abnormalities, Thrombocytopenia), Tissue perfusion variables (Hyperlactemia), Severe sepsis and septic shock) [8].

1.1.3. Pathogenesis of sepsis

During sepsis, host response against the invading pathogen is very critical with anti-inflammatory and pro-inflammatory mechanisms leading to either the clearance of the infection and tissue recovery, or it may lead to organ injury due to exaggerated immune response [9, 10]. Several bacterial factors like, PGN, lipoteichoic acid and lipoproteins etc [collectively called pathogen-associated molecular patterns (PAMP)] are powerful activators of innate immune responses during sepsis. The PAMPs interact with host receptors [collectively called pattern recognition receptors (PRRs) such as toll like receptors, nucleotide-binding oligomerization domain–like receptors and C-type lectin receptors etc] presented on immune cells and activate the immune response to release cytokines which is very important in pathogenesis of sepsis [8, 11]. Enhanced cytokine production and exaggerated inflammatory response causing necrotic cell death and collateral tissue damage [8, 12, 13].

The initial biochemical events, which takes place during sepsis in described in **Figure 1.**

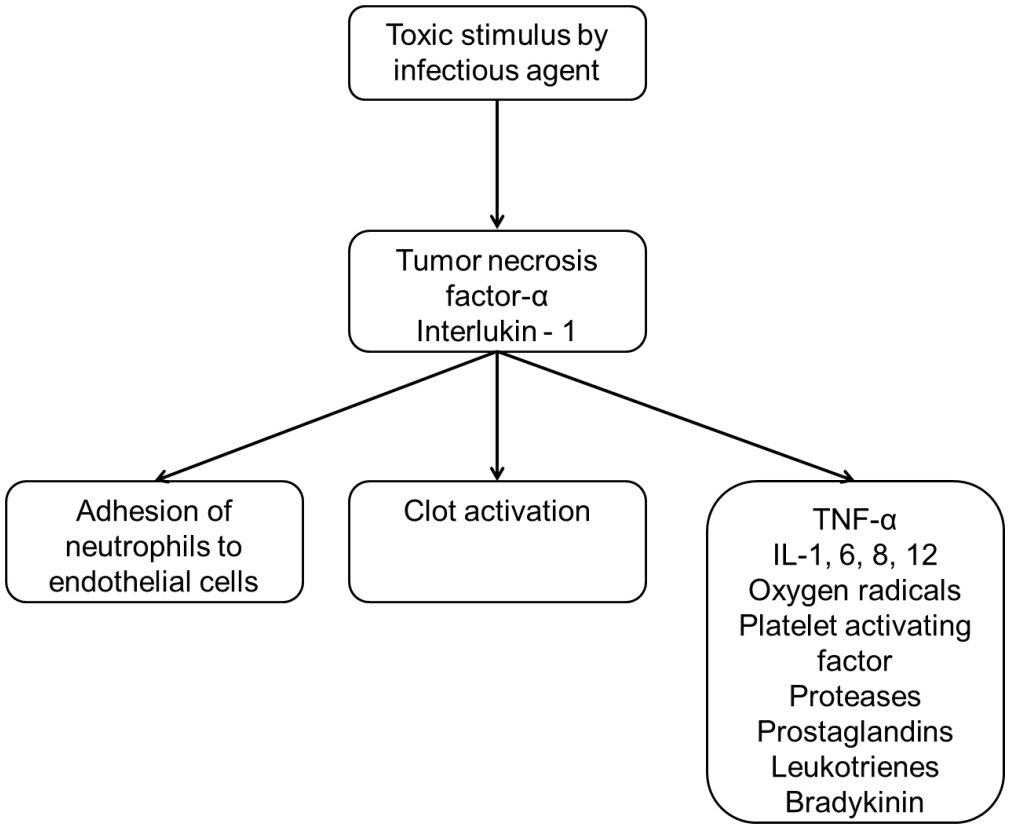

Figure 1. The initial biochemical events of sepsis. In sepsis patients lead to neutrophil-endothelial cell adhesion, coagulation, and the generation of secondary inflammatory mediators. (Adopted from the book chapter 2 of Contemporary Diagnosis and Management of Sepsis by Wesley and Bernard-first edition)

1.1.4. Management of sepsis

Patients with sepsis, severe sepsis, and septic shock require hospital admission. Based on the current literature the management of sepsis includes;

- Early identification of the causative microorganism
- Early and adequate antibiotic therapy after identifing the causative agent and based on the detected sensitivity of the microorganism antibiotic or antimicrobial therapy is given
- Proper management of ventilator
- Early hemodynamic resuscitation and continued support

Once sepsis is diagnosed and the site of infection is identified, drainage or removal of the infected site, interventions to treat and prevent effects of harmful host responses, and treatment of complications are added in treatment modality. One essential component for managing sepsis is to control the source of infection.

Timely administration of antibiotics (4-6 h of presentation) is essential. Almost 10% patients fail to get timely treatment which results in 10-15% higher mortality among the patient who does not receive prompt antibiotic therapy. The classes of antibiotics that are most commonly prescribed for sepsis patients include β-lactams, Vancomycin, Oxazolidinones, Aminoglycosides, Quinolones, Azalaides and Macrolides. For some sepsis-related infections, surgical approaches to treatment may be required, including drainage of external and intra-abdominal abscesses and debridement of devitalized tissue (Contemporary Diagnosis and Management of Sepsis by Wesley and Bernard-first edition).

1.1.5. Complication of sepsis

Sepsis is a dynamic and complex disease in which imbalanced inflammatory response can lead to severe complications [10], many of them due to reduced blood flow to vital organs or sufficient infectious agents reaching to tissues through hematogenous route and affecting organs like brain, kidneys, joints and heart and causing complications like septic arthritis, endocarditis and abscess formation in internal organs like kidney, liver [14]. Enhanced apoptosis of lymphoid cells has been observed in both human patients and mice models of sepsis. Further complications associated with sepsis could be sudden drop in blood pressure, which is termed as septic shock.

1.2 Septic arthritis

One of the serious outcomes associated with sepsis or hematogenous route of infections is septic arthritis although, the disease can also be due to local traumatic injury or acquired post medical procedures like prosthetic joints and other surgical interventions [15-17].

1.2.1. Epidemiology

Septic arthritis is a serious inflammatory disease caused due to infection of the joint cavities. It can be acute or chronic and has high mortality and morbidity rates. Although people of all ages can be affected it usually affects elderly people and young children below three years of age. Immunocompromised individuals, patients with preexisting joint pathologies such as rheumatoid arthritis, gout, osteoarthritis, crystal arthropathy or prosthetic joint surgery, intravenous drug users, patients with implanted medical devices, diabetes and cutaneous ulcers are at increased risk of developing septic arthritis [17-20].

Septic arthritis is caused primarily by bacteria although viruses and fungi also account for a small number of septic arthritic cases. The mortality in septic arthritis varies from 10-25% and *S. aureus* alone is responsible for 60% of all joint infections. *S. aureus* produces a wide variety of virulence factors which play an important role in initiating septic arthritis [20]. Other microbial isolates include streptococci, *Neisseria gonorrhoeae, Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens, Haemophilus influenzae*, *Mycobacterium spp*. and fungi. *H. influenzae* is the most common pathogen isolated from young children affected with septic arthritis, but not in children who are vaccinated with *H. influenzae* b vaccine. Infections by *Neisseria gonorrhoeae* are commonly seen in sexually active young adults while infections with Gram-negative bacilli are often seen in neonates, elderly patients, intravenous drug users and patients suffering from immune deficiency disorders. Fungal arthritis is seen in immunocompromised patients [21, 22].

1.1.2. Clinical features of septic arthritis

The typical clinical presentation of septic arthritis includes;

- Severe pain in the affected joint, especially with movement
- Swelling (increased fluid within the joint)
- Inability to move the limb with the infected joint

- Chills
- Fever
- Fatigue and generalized weakness
- Warmth (the joint is red and warm to touch because of increased blood flow)
- Arthrocentesis is a clinical procedure used for surgically drawing samples of joints fluid (synovial fluid) for an accurate diagnosis of septic arthritis.

1.1.3. Pathogenesis of septic arthritis

Joint cavities in general are sterile and contain synovial fluid that is secreted by the synovial cells present in the synovial membrane. Synovial fluid is composed of interstitial fluid filtered from the blood plasma, hyaluronan (secreted by fibroblast-like cells in the synovial membrane), lubricin/ proteoglycan 4 (secreted by surface chondrocytes) and phagocytic cells. It provides nutrients and lubricates the articulating joints and helps in removing the debris that results from normal wear and tear in the joint [23, 24].

Infection of the joints usually results by hematogenous spread during transient or persistent bacteremia or by direct inoculation during trauma, joint surgery, hemodialysis, rarely through local steroid injections or joint aspirations. It has been reported that if the circulating bacterial cells are sufficiently high in number may invariably extravagate into most if not all of the parenchymal tissues of the experimental mouse [22, 25]. However, the innate immunity system of the host helps in clearing the infection from most of the organs within a week. Kidney and joints are two major exceptions, where the bacterium survives for longer period of time. Kidney failure is less likely to be observed in case of *S. aureus* infected kidneys, upon entry into the joint space bacteria gets deposited into the synovial fluid or synovial membrane and trigger an inflammatory reaction that initiates synovial membrane hyperplasia along with the release of cytokines and proteases [26] This results in cartilage degradation and eventually irreversible subchondral bone loss. The most commonly affected joint is the knee which accounts for approximately 50% of bacterial arthritis cases as infection usually involves a single large joint, such as the knee, but many joints may be involved.

1.1.4. Management of septic arthritis

The main points of managing septic arthritis are:

- Timely and adequate drainage of the infected synovial fluid
- Administrating appropriate antimicrobial therapy
- Immobilization of the joint to control pain

Medical cure of septic arthritis is possible with antibiotic therapy if detected early [27, 28].

1.2.5. Complication of septic arthritis

Failure in early phase recognition can lead to many complications of septic arthritis such as osteomyelitis, bony erosions, fibrous ankylosis, sepsis, and even death. Even though surgical as well as medicinal approaches are used to treat the disease but still successful management is limited due to and inadequate drainage of joints [29-31].

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1.3. *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive golden yellow color opportunistic pathogen and belongs to Micrococcaceae family. This bacterium was first identified by the surgeon Sir Alexander Ogston in 1980 from patients wound samples. Around 30-40% human population are always colonized with *S. aureus.* This bacterium colonizes our skin, anterior nares, axilla and groins. Although this bacterium is in general regarded as harmless to healthy people, it causes life threatening infections in immune-compromised individuals [32-35].

1.3.1. Morphology:

S. aureus cell size ranges between 0.5 to 1.5 μm, and appeared as single, pairs or as grape-like clusters under microscope **(Figure 2)**. It is non-spore-forming, non-motile, and as facultative anaerobe. The bacterial colonies appear yellow on nutrient rich media [29].

1.3.2. Biochemical characteristics:

Biochemically, it is characterized as coagulase and catalase positive. The catalase protects intraphagocytic *S. aureus* by destroying hydrogen peroxide produced by the phagocytes. Catalase converts hydrogen peroxide to water and nascent oxygen (forming the characteristic bubbles), which is one of the major strategies employed by these organisms in immune evasion

[36-41]. *S. aureus* is positive for DNase production and mannitol fermentation [42]. When cultured on sheep blood agar plates, hemolysis can be observed. *S. aureus* is β-hemolytic [43, 44]. *S. aureus* produces exotoxins and enzymes that act on distant sites from their infection foci. These toxins are the cause of pathologic conditions like the scalded skin syndrome, toxic shock syndrome etc. [45, 46].

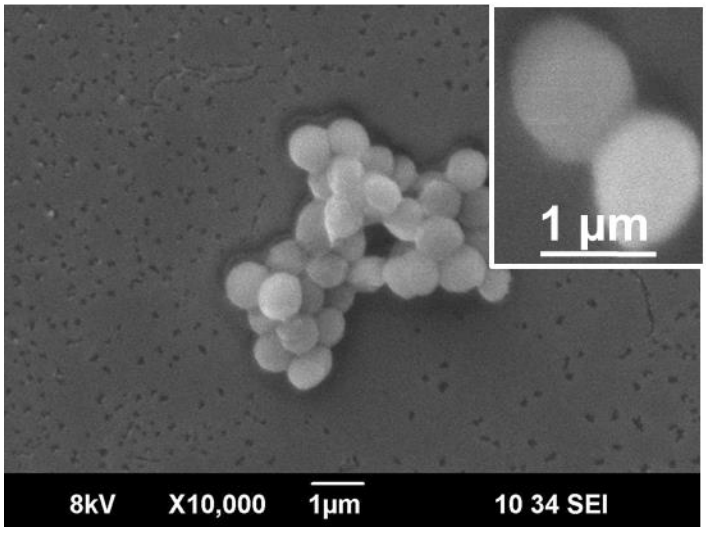


Figure 2. *S. aureus* SEM image. *S. aureus* cell size ranges between 0.5 to 1.5 µm.

1.3.3. *S. aureus* infection:

S. aureus frequently colonize mucosal surfaces and nares of healthy individuals; a breach in immune system triggers them to turn into opportunistic pathogens [47, 48]. Around 20-40% of human populations harbor *S. aureus* in their anterior nares without actually contracting an infection [49-51]. *S. aureus* is the major cause of morbidity and mortality in hospital settings and also causes wide spread community associated infections [52-56]. *S. aureus* causes a plethora of infections ranging from minor skin infections like boils, acne and impetigo to major invasive diseases like endocarditis, soft tissue abscesses, pneumonia, sepsis and septic arthritis (**Figure 3**) [57-60]. It is known to cause infection in human of all ages, specifically in immune-compromised patients [61].

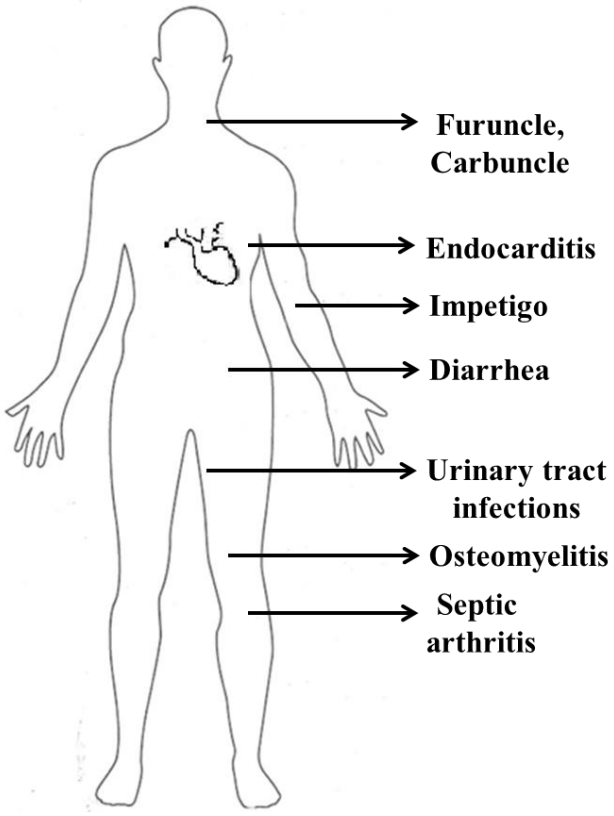


Figure 3. Staphylococcal infections. Once *S. aureus* breaches the immune barriers of host it can lead to multiple infectious diseases.

1.3.4. Incidences of *S. aureus* infection:

According to the Centers for Disease Control and Prevention (CDC) report all bacterial infections in the world are gradually becoming resistant to antibiotics (https://www.cdc.gov/drugresistance/index.html) and the category of bacteria gaining resistance towards most of the antibiotics are called superbugs. According to a recent report, Centers for disease control and prevention (CDC), USA has listed *S. aureus* among one of the most critical pathogen in hospital and community settings. According to the study conducted by Indian Network for Surveillance of Antimicrobial Resistance (INSAR) group, India, the overall prevalence of MRSA from hospital outpatients was an appalling 41%. Isolation rates of MRSA from ICU were 47%, ward patients 49% and outpatients were 27%, in 2009. This study clearly shows the high risk of hospital acquired *S. aureus* infections in nosocomial settings [62, 63]. *S.aureus* is the most common pathogen associated with septic arthritis, which is the most dangerous joint disease [22, 64]. Patients with rheumatoid arthritis, orthopedic prosthesis and compromised immune status are at higher risk for *S. aureus* arthritis. The mortality in septic arthritis varies from 10-25% and *S. aureus* alone is responsible for 60% of joint infections in rheumatoid arthritis patients [64, 65].

Data from Amrita Institute of Medical Science, Kochi, India for year 2015-16 showed that out of all blood culture samples tested for Gram positive organism, 44% were positive for *S. aureus* while in the same year 14 patient's synovial fluid samples were found to be positive for *S. aureus*.

1.3.5. Current line of management and challenges in treating *S. aureus* infections:

Current available treatment for staphylococcal infection is antibiotics. Treatment is determined based on the type of infection and antibiotic resistance of the infecting strain [66]. According to CDC, common antibiotics used to treat *S. aureus* are: Vancomycin, clindamycin, tetracyclines like doxycycline, minocycline, trimethoprim-sulfamethoxazole, linezolid.

At the time of discovery of antibiotics, penicillin was the drug of choice in treating most cases of bacterial infections including *S. aureus*. Emerging antimicrobial resistance and continuous change in spectrum of clinical disease remains the challenges to treat the bacterium [60]. Horizontal gene transfer leading to the acquisition of various genes and pathogenicity islands in the staphylococcal genome is the key feature responsible for its antibiotic resistance

(Figure 4) [67-69]. Currently most *S. aureus* clinical strains are resistant to one or more antibiotics, which includes penicillin and other β-lactam class of antibiotics. The most significant among these drug resistant strains are the Methicillin Resistant *S. aureus* (MRSA) and Vancomycin Resistant *S. aureus* (VRSA). Apart from Methicillin and Vancomycin, resistance to Linezolid, Gentamicin, Tetracycline, Chloramphenicol and Clindamycin has also been observed among different *S. aureus* clinical isolates [70, 71].

Staphylococcal sepsis and septic arthritis are clinical challenges difficult to treat because of the above mentioned problems.

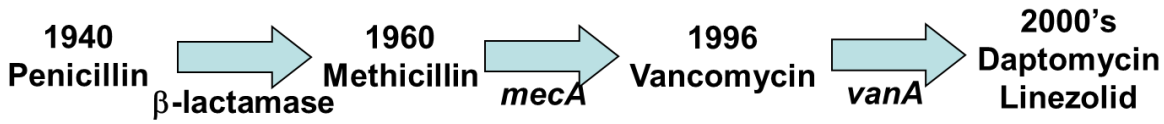


Figure 4. Development of antibiotic resistant strain of *S. aureus.* Due to horizontal gene transfer leading to the acquisition of various genes and pathogenicity islands in the staphylococcal genome is the key feature responsible for its antibiotic resistance. MRSA remains to be a main challenge to treat clinically. More advance strains like vancomycin resistant and linezolid resistant strain has also been reported recently.

1.3.6. Role of *S. aureus* in sepsis:

A dramatic increase in the incidence of *S. aureus* (particularly caused by methicillin-resistant *S. aureus* (MRSA)) induced sepsis has been reported in past few years [72]. To establish sepsis (infection in blood) or septic arthritis, *S. aureus* evades host innate immune response. Frequent recurrence is a key feature of *S. aureus* diseases, which is observed around 8-33% in case of skin and soft tissue infections and bloodstream infections [73]. Prior exposure does not triggers protection against subsequent *S. aureus* infection [74]. Diseases from *S. aureus* can range from superficial (e.g. boils) to deeply invasive (e.g. meningitis) and can be life threatening. *S. aureus* up-regulates many of its virulent factors during stressful conditions like increased cytokine response during a blood borne infection and this trait of the bacterium is the key factor for its survival in the blood stream. *S. aureus* possesses a plethora of virulence factors that enables it to cause the infection while a subset of these factors produced by *S. aureus* perform various functions in evasion of the immune system, also has certain structural features adds on to thwart the host immune response. For examples, *S. aureus* secretes protein A and anti-opsonizing proteins preventing its phagocytosis by neutrophils [75], as well as superatingens and toxins like leukotoxins also subverts the natural immune response. Moreover, formation of biofilms may lead to resist immune responses and inhibits penetration of antibiotics [72].

It is critical to understand the pathogenesis of this organism to develop effective and sustainable treatments for *S. aureus* infections. But, it is equally important to elucidate the main evasive mechanisms by which the organism causes disease, which may be possible to identify new drug targets either for preventative or curative treatments.

1.3.7. Role of *S. aureus* in septic arthritis:

During septic arthritis, *S. aureus* enters into the joint via direct introduction or through extension from an adjacent site of infection or by hematogenous spread during transient or persistent bacteremia. The low fluid shear conditions in the joint cavity enable bacterial adherence and cause infection.

Several *S. aureus* adhesins such as fibrinogen binding proteins, fibronectin binding proteins, collagen binding protein, laminin binding protein, protein A, elastin binding protein and bone sialoproteins have been reported to play a role in its joint colonization and adherence to the

joint extracellular matrix. This results in high influx of inflammatory cells like neutrophils and macrophages. Bacterial internalization takes place through receptor mediated cytosis [18, 76].

1.4. *S. aureus* cell wall: important virulence factor in sepsis and septic arthritis:

The cell wall of staphylococcus is composed of murein, teichoic acids and cell surface associated proteins. Cell wall, along with providing structure to the bacterial cell, also provides protection from external stimuli. It is a macromolecular structure consisting of glycan strands that are cross-linked by short peptide chains interconnected by penta-glycine cross-bridge [46, 77, 78]. *S. aureus* has a thick coating of peptidoglycan layer that is lysozyme resistant helping them to evade the primary line of immune defense. Teichoic acid present on the staphylococcal surface also contributes to the lysozyme resistance [79, 80]. *S. aureus* are known to express capsule, made up of polysaccharide units. [81-83].

In past two decades it has been shown that cell wall of *S. aureus* plays an important role in virulence mechanism caused by the bacterium. Cell wall composed of a thick layer of peptidoglycan (PGN). PGN of staphylococci is a β-1-4 linked polymer of alternating *N*-acetyl-muramic acid (NAM) and *N*-acetylglucosamine (NAG) residues. The D-lactyl moiety of NAM is linked to the short stem peptides (L-Ala-D-iGln-L-Lys-D-Ala-D-Ala) that are cross-linked to each other *via* penta glycine bridges [84, 85]. The structure and component of staphylococci cell wall has been described in **(Figure 5)**. It has been reported previously that, even the dead *S. aureus* bacterium [20] or its purified PGN alone can induce arthritic symptoms in mice [86].

1.4.1 Teichoic acid:

In order to maintain the membrane integrity the peptidoglycan layer of Gram positive bacterial cell wall is generally modified with carbohydrate-based anionic polymers. These anionic polymers mediate extracellular interactions, influence membrane permeability, as well as add on to the stability to the plasma membrane, and PGN layer. Sometimes it may also act as scaffolds for extra cytoplasmic enzymes required for cell-wall degradation and growth. The major class under the category of anionic glycopolymer are Teichoic acids (TA), found in a wide range of pathogenic and non-pathogenic Gram-positive bacteria alike [87].

There are two types of TAs: (i) lipo-TA (LTA) and (ii) wall TA (WTA). LTAs are anchored to the inner membrane and WTAs are covalently linked to PGN **(Figure 5)**. LTA and

WTA are polymers of repeating units of glycerol or ribitol phosphates that are modified by D-alanylation [88, 89]. In *S. aureus* induced septic arthritis model it was found that Δ*dlt* mutant which lacks D-alanine in their teichoic acids developed less severe clinical symptoms of septic arthritis [90].

1.4.2. *O*-acetylation of bacterial peptidoglycan:

The normal, unmodified glycan strands of bacterial PGN consist of alternating residues of β-1,4-linked *N*-acetylmuramic acid and *N*-acetylglucosamine. *O*-acetylation is one of the various modifications of PGN layer effecting its pathobiological features. *O*-acetylation of C-6 hydroxyl group of NAM residues in PGN caused by *O*-acetyl transferase (OatA) protein, expressed in the cell membrane of *S. aureus* **(Figure 5)** [84, 91].

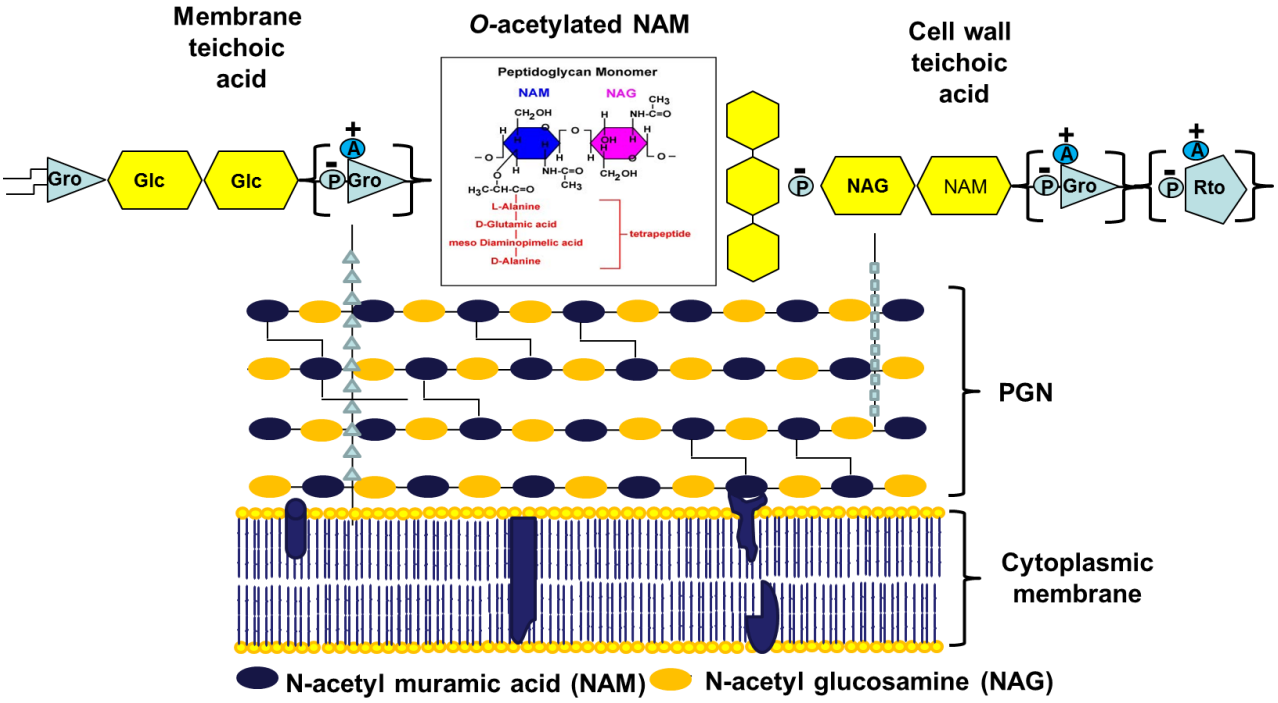


Figure 5. Structure of *S. aureus* cell wall. The major structure present in cell wall which gets modified and responsible for pathogenesis of *S. aureus* are Teichoic acids, *O*-acetylation of NAM subunit (Adopted from http://www.arabslab.com/vb/showthread.php?t=577 and further modified [80, 89])

1.5. Evasion mechanisms of *S. aureus* against host immune response:

Innate immunity is the major barrier for any invading pathogen. The host immune response against *S. aureus* mainly depends on the bactericidal effect of professional phagocytes specially macrophages and neutrophils. The mechanism of phagocytosis and bacterial killing is highly influenced by opsonization of the bacterial cells either by complement system and/or antibodies. Post phagocytosis, the neutrophils or macrophages mediates the bacterial killing by several mechanisms like production of reactive oxygen species (ROS), and secretion of cationic antimicrobial peptides (cAMPs).

S. aureus has evolved with mechanisms to evade macrophages and neutrophils mediated killing. *S. aureus* produces capsule and extracellular fibrinogen binding protein to resist phagocytosis by macrophages and neutrophils [92]. This bacterium secretes catalase, super oxide dismutase and produces Staphyloxanthine to resist ROS mediated killing [93, 94]; and D-alanylate its cell wall to resist cAMPs [95]. This pathogen can also survive in the intracellular compartments of a number of other cell types including osteoblasts, endothelial and epithelial cells, keratinocytes [96-99].

Lysozyme is one of the cAMPs, known to have potential defense action against any invading bacterial pathogens. It has been recently reported that *S. aureus* is completely resistant to lysozyme action by *O*-acetylation of NAM subunit in the peptidoglycan backbone of bacterial cell wall.

1.6. Role of lysozyme in host immune response:

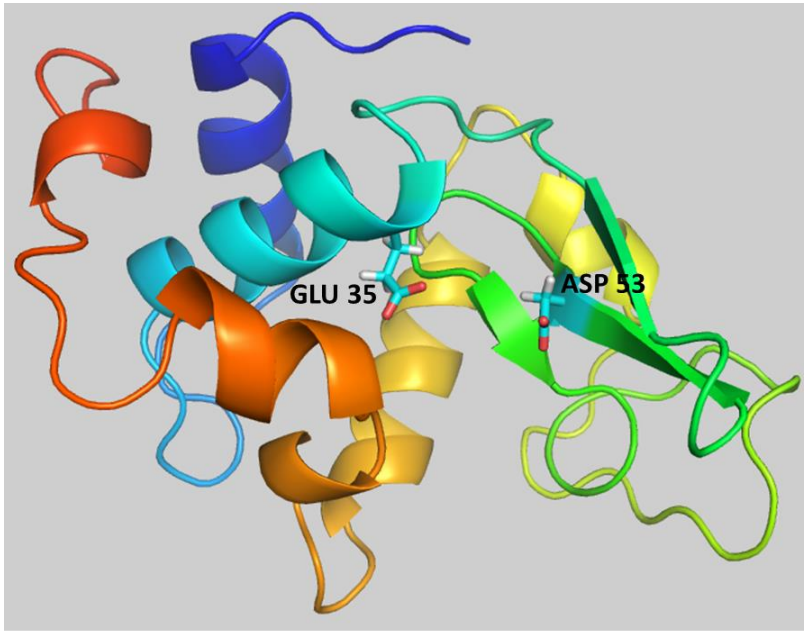
Lysozyme, a principle component of the host innate defense system, is a 14.3 kDa cationic bacteriolytic enzyme **(Figure 6).** Lysozyme has two different antibacterial modes of action: (i) Lysozyme degrades peptidoglycan (PG) by catalyzing the hydrolysis of the β-1,4 glycosidic linkages between N-acetylmuramic acid (NAM) and N-acetyl glucos- amine (NAG) residues in the bacterial cell walls.1,2 (ii) Lysozyme damages bacterial membrane due to its cationic antimicrobial peptide activity [100].

Alexander Fleming had identified lysozyme in 1922, after he splashed some drops of nasal mucus onto a plate of bacteria and noticed the organisms stop growing. Later it was established that Lysozyme, exists inside the body as a natural defender against bacterial invaders.

But it wasn't understood how the enzyme accomplished this task until Phillips and his team determined their 2-Å-resolution X-ray structure of lysozyme from chicken egg white [101].

Lysozyme is present varying concentrations (1 μg/mL−13 mg/mL) in all human biological fluids including serum, tears, saliva, human milk and mucus (**Table 1**). The NCBI-gene data of transcriptome profile of different human tissues also indicates the presence of lysozyme in different tissues in different concentration **(Figure 7).**

Even, the NCBI data of transcription profiling by high throughput sequencing of individual and mixture of 16 human tissues RNA samples also showed presence of lysozyme in different tissues (https://www.ncbi.nlm.nih.gov/gene/4069).



Human Lysozyme (PDB ID: 2ZIL)

Figure 6. Lysozyme structure and its active binding sites. Lysozyme primary structure is comprised of a polypeptide chain made up of 129 amino acids. It has as a globular and compact structure where a long cleft is present on its surface. The cleft acts as the active site where bacterial PGN-sugar moieties binds and cleavage of the bond between NAM and NAG takes place. [100] **.**

Table 1: Lysozyme content in different human biological fluids

Biological fluid	Lysozyme concentration	Reference
Serum	0.8-1.0 μg/ml	[102]
Synovial fluid	100-400 μg/ml	[103]
Saliva	20-200 μg/ml	[104]
Tears	1,700 μg/ml	[105]

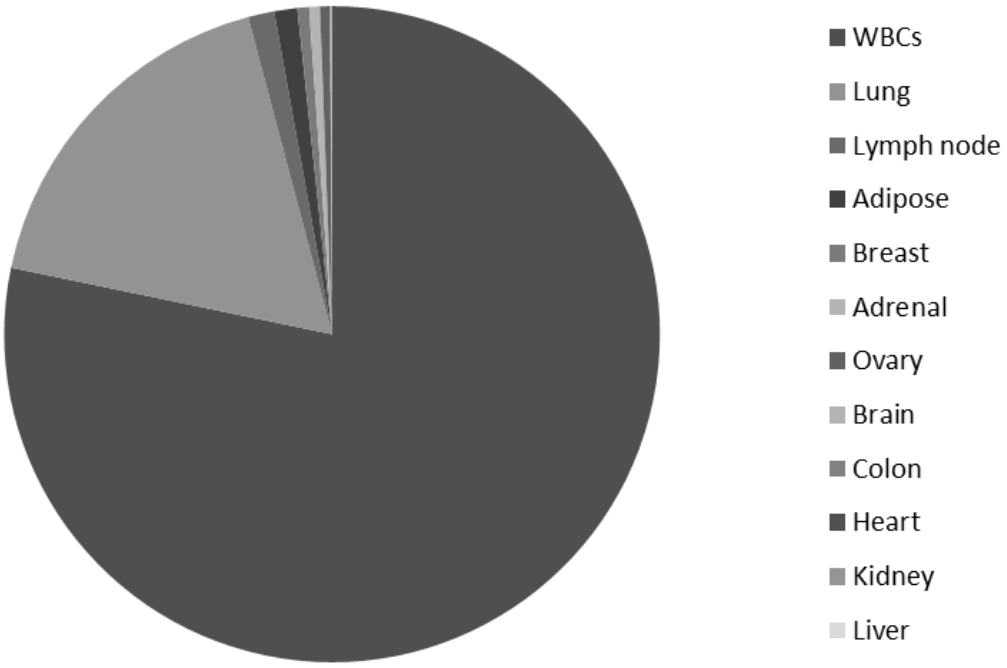


Figure 7. Lysozyme transcriptome profile. Pie diagram indicating the transcriptome profile of 16 different human tissue RNA samples indicating presence of lysozyme in various tissues (https://www.ncbi.nlm.nih.gov/gene/4069).

1.6.1. *S. aureus* sensitivity to lysozyme:

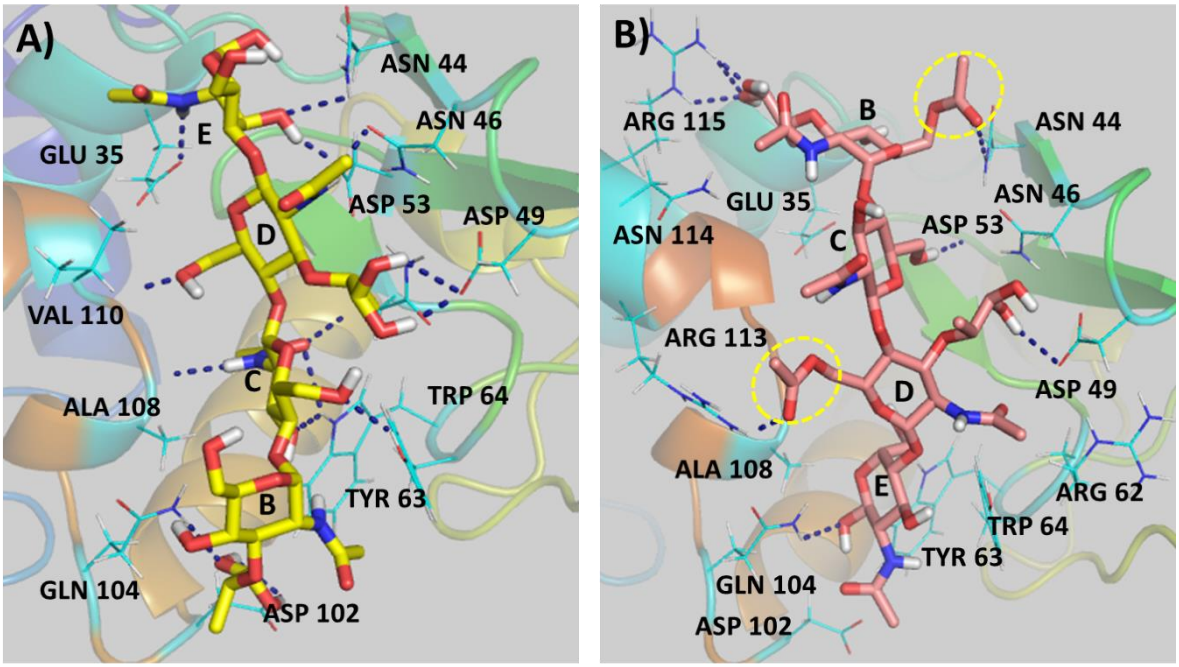
Staphylococcus aureus (*S. aureus*) is completely resistant to lysozyme. Bera *et al.*, in 2005 demonstrated lysozyme resistance in *S. aureus* is due to its PGN modification by *O*-acetylation [79]. *O*-acetyl transferase, a transmembrane protein, is responsible for this PGN *O*-acetylation. Further Shimada *et al.*, showed subcutaneous injection of *oatA* mutant induces enhanced IL-1β production, a key cytokine responsible for host defense against *S. aureus*, compared to wild type *S. aureus* strain in male CD-1 mice [106]. In *S. pneumoniae*, the gene responsible for *O*-acetyl transferase biosynthesis was termed as *adr* (attenuator of drug resistance). The *adr* gene was identified to cause lysozyme and penicillin resistance. Interestingly, *adr* gene showed a significant homology to *oatA* [107].

1.6.1. *O*-acetyl transferase- major determinant of lysozyme resistance:

To date, several pathogenic bacteria like *Listeria monocytogens* [108, 109], Gonococci [110, 111], *Proteus mirabilis* [112], *Micrococus luteus* [113] are known to possess *O*-acetylated peptidoglycan. *O*-acetylation is caused by an enzyme present in cell membrane *O*-acetyl transferase (OatA) expressed by *O*-acetyl transferase gene (*oatA*) (SA2354). Bera *et al.*, studies revealed that the NAM subunit of PGN was *O*-acetylated only in pathogenic strains of staphylococci which were lysozyme-resistant (e.g., *S. lugdunensis, S. aureus, S. epidermidis*) while nonpathogenic species in general were found sensitive towards lysozyme (e.g., *S. xylosus, S. carnosus and S. gallinarum*) and were composed of de-*O*-acetylated PGN.

Lysozyme has a large active binding site cleft which can accommodate tetra-saccharide to hexa-saccharide subunits of the glycan chains and denoted as A, B, C, D, E, and F subsites by Imoto *et al.* It was shown that lysozyme cleaves the β-1,4 glycosidic linkage between subsites D and E **(Figure 8)**. Detail molecular mechanism of lysozyme resistance by *S. aureus* was recently reported by Anju *et al.*, using computational analysis. The authors reported that, *S. aureus* cell wall of a mutant lacking the *O*-acetylated PGN when interacts with lysozyme, occupied proper position in the active binding site cleft of lysozyme in order to undergo its normal catalytic mechanism, and the complex was also highly stable during molecular dynamics (MD) simulations. But, the *O*-acetylated PGN as found naturally in wild type strain was highly distorted and the saccharides occupied different positions due to the steric hindrance with higher instability during MD simulations. Further, *O*-acetylated PGN had undergone an escape

mechanism, due to its high strain energy or steric interference leading to lysozyme resistance in *S. aureus* [100].



Docked Structures of Human lysozyme wth A) MGMG B) M(A)GM(A)

Figure 8. Lysozyme mechanism of action. Glu -35 and Asp – 53 are the key catalytic cleavage site residues present in lysozyme, binds at β- 1,4 glycosidic bond between NAM and NAG subunits present in bacterial peptidoglycan and cleaves. (A) In Δ*oat*A, the cleavage site D and E binds perfectly with lysozyme active sites Glu35 and Asp53 of human lysozyme, thus binding leads to cleavage. (B) On the other hand, *O*-acetylated wild type causes steric hindrance as the peptidoglycan binds in the opposite direction at the active site of lysozyme. *O*-acetylation is highlighted with the yellow dotted circle **(Adopted from [100]).**

1.7. *In vivo* models to study *S. aureus* infection:

In the present thesis three *in vivo* models have been used for the study are: *Drosophila melanogaster* as *S. aureus* infection model, Murine model of sepsis and murine model of septic arthritis. The rationale for selecting these models is described in the following section.

1.7.1. *Drosophila melanogaster* model of *S. aureus* infection:

Fruit fly *Drosophila melanogaster* [114], the nematode *Caenorhabditis elegans* [115], and silkworm larvae *Bombyx mori* [116] are novel non-mammalian models of to assess the *in vivo* virulence of *S. aureus* strains. These models are advantageous from the perspectives of low cost, ease of infection, the ability to study large numbers of infected hosts and ethical considerations.

These model organisms do lack a definitive acquired immune system but they reproduce many human immune responses. The fruit fly, for example, possesses elements of the human innate immune system, such as antimicrobial peptides and toll-like receptor expression. Moreover, *Drosophila* and mammalian innate immune recognition and response show high degree of conservation. Even though the definite adaptive immunity as well as vasculature is absent in *Drosophila* with anatomical differences from mammalian system several reports have suggested that *Drosophila* serves as a suitable model for host-pathogen interaction as well as muscle and epithelial homeostasis. Another advantage of using this simple model system is that a large scale screening of systemic identification of microbial factors and the pathogenesis associated with their interaction with host is possible. Reports also suggest that, *Drosophila* precisely activates the genes of specific AMPs against particular pathogens and discriminate between different classes of microorganisms [117, 118]. In addition, staphylococcal infections in these hosts are amenable to antibiotic treatment [119, 120], which supports their use as model systems for screening and assessing the efficacy of antibiotic regimens *in vivo*.

1.7.2. Murine model of sepsis:

Sepsis caused by *S. aureus* is usually the result of hematogenous infection from surgical intervention or from a wound site. Animal models of sepsis in which the bacteria are injected intravenously (i.v.), e.g., in chickens [121] or mice [122-124], closely mimic the hematogenous spread of disease seen in humans and have proved useful in defining the components of the host immune system involved in resistance to colonization and septic shock.

Among all the animal model mice have several advantages over other models:

1. Knockouts/ transgenic strains availability
2. Genetically characterized/inbred strains availability
3. Immunological system similarities with human counterpart
4. Easily established staphylococci infection models - skin colonization
5. Readily compliant to gene therapies and/or drug

Murine models of *Staphylococcus aureus* mediated sepsis and arthritis have been used extensively to gain a better understanding of the host–bacterium relationship as well to develop better methods of prevention and treatment [17].

1.7.3. Murine model of septic arthritis:

Tarkowski *et al*., and Tao *et al.,* greatly contributed in establishing the mice model of septic arthritis. Their model has been extensively used by various research groups. Two routes of *S. aureus* administration can induce the septic arthritis disease (1) through tail vein (hematogenous) mimicking systemic infection and (2) intra-articular injection of bacterial cell suspension in the knee joint to mimic local infection model [17, 20, 26].

There are several similarities between human and mice model:

(1) Staphylococcus can colonize both human and mice

(2) Joint infection of staphylococcus in both species cause severe joint damage

(3) The immune response of human and mice are highly similarity

1.8. Current deficit:

The role of *S. aureus* peptidoglycan *O*-acetylation has never been studied *in vivo* and impact of *O*-acetylated PGN is necessary to evaluate the potential of OatA, as a new drug target for the treatment of *S. aureus* infections. In the present thesis different infection models have been chosen for *in vivo* comparison of *S. aureus* strains with de-*O*-acetylated and *O*-acetylated PGN induced infections. Firstly, *Drosophila melanogaster* as a pre-model where survival and functionality of the infected flies were analyzed, later murine model of sepsis was used to understand the effect of *S. aureus* infection when spread hematogenously in systemic circulation and finally in order to understand the fate of the bacterium when delivered locally, mice model of septic arthritis was used.

Hypothesis

In this doctoral dissertation, we proposed to test the following hypothesis:

"Deletion of O-acetyl transferase gene (oatA) in S. aureus enhances host lysozyme induced bacterial death & attenuates disease severity in mice models of sepsis and septic arthritis"

The proposed hypothesis was tested using the following two aims:

Specific Aim 1

To determine the effect of cell wall peptidoglycan de-*O*-acetylation on lysozyme sensitivity in *S. aureus*.

Specific Aim 2

To determine the effect of cell wall peptidoglycan de-*O*-acetylation on *S. aureus* induced disease sequelae in the drosophila melanogaster and murine models of *S. aureus* infection.