Review of Literature
2. Review of literature

*Staphylococcus* is a gram positive spherical bacterium that commonly causes surgical and skin infections, respiratory disease and food poisoning. In 1880, Scottish surgeon Sir Alexander Ogston first described staphylococci as “the masses looked like bunches of grapes” in surgical abscess collected from a knee joint.\(^{35}\) In 1884, German physician Friedrich Julius Rosenbach differentiated two different strains of staphylococci based on their colony pigmentation: *S. aureus* (golden yellowish large colonies) and *S. albus* (white colored colonies).\(^{36}\)

2.1 General taxonomical description of *Staphylococcus aureus*


Taxonomically the staphylococci belong to the Family *Micrococcaceae*. Classification of the micrococci and staphylococci based on physiological and biochemical tests has been proposed by Baird-Parker (1963), where the Family *Micrococcaceae* divided into Group I (*Staphylococcus* Rosenbach emend. Evans) and Group II (*Micrococcus* Cohn emend. Evans). *Staphylococcus* can be differentiated from the other three members in the family, *Micrococcus, Stomatococcus*, and *Planococcus*, based on the guanine plus cytosine content of the DNA, cell wall composition, and the ability to grow and ferment glucose anaerobically.\(^{37}\) Only three species of *Staphylococcus* (*S. aureus, S. epidermidis* and *S. saprophyticus*) were included in the genus in 1974. These species were differentiated on the
basis of the ability to produce coagulase, ferment mannitol (both aerobically and anaerobically) and produce heat-stable endonuclease and by the cell wall composition.\textsuperscript{38,39}

Kloos and Schleifer outlined a simplified method for the routine identification of human \textit{Staphylococcus} species in 1975. They divided \textit{Staphylococcus} species into 11 species on the basis of coagulase activity, hemolysis, nitrate reduction, and acid production from several sugars.\textsuperscript{40} Since then the number of species and sub-species had increased to 32 as of 1994.\textsuperscript{41} Currently (2015), according to the List of Prokaryotic Names with Standing in Nomenclature, the genus \textit{Staphylococcus} comprises 52 species and 28 subspecies.\textsuperscript{42}

\textit{S. aureus} is non-motile, non-spore forming, catalase positive, gram positive cocci ranging in diameter from 0.5 to about 1.5 μm. The organism may appear singly, in pairs or in clusters. It is a facultative anaerobe; produces coagulase enzyme and some strains produce capsules. It ferments mannitol. The surface of this bacteria is coated with Protein A and it is not found on the surface of coagulase negative staphylococci (CONS).\textsuperscript{41} The cell wall of \textit{S. aureus} is resistant to lysozyme and sensitive to lysostaphin. The bacterium is able to grow in a wide range of temperatures (7°C to 48°C with an optimum of 30°C to 37°C), Sodium chloride concentrations (up to 15% NaCl) and pH (4.2 to 9.3, with an optimum of 7.0 to 7.5). These characteristics enable the bacteria to survive in a wide variety of foods; especially in processed foods and fermented food products like cheeses.\textsuperscript{43} On blood agar, colonies of \textit{S. aureus} appear golden (due to the presence of a membrane-bound carotenoid staphyloxanthin) surrounded by β-hemolytic zones.\textsuperscript{44}
The *S. aureus* cell wall is a multilayered (20-40 nm), comprising a copolymer of peptidoglycan and teichoic acid. Peptidoglycan represents ~50% of the cell wall by weight and is a polymer consisting of repeating units of sugars of 1, 4 β-linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). The glycan chains are cross-linked by tetrapeptide chains (L-alanine, D-glutamine, L-lysine and D-alanine) bound to NAM and a pentaglycine bridge that links tetrapeptide chains on adjacent glycan strands (Figure 1). This cross-linking is catalyzed by the transpeptidase (TPases) activities of penicillin binding proteins (PBPs). Another major cell wall component is teichoic acid, an anionic polymer that consists of repeating alditol phosphate groups covalently linked to the muramic acid residue of peptidoglycan. Teichoic acid composition is also different among the staphylococcal species. The cell wall contains ribitol teichoic acid (polysaccharide A) in *S. aureus*, glycerol teichoic acid (polysaccharide B) in *S. epidermidis*.

**Figure 1:** Schematic diagram of the peptidoglycan layer of *S. aureus*

In some *S. aureus* strains, the cell wall is coated with an extracellular polysaccharide capsule. More than 90% of clinical strains of *S. aureus* can produce polysaccharide capsules that are usually thin (<0.05 μm) and consist of aminouronic acid sugars and fucosamine. Serotyping has identified 11 serotypes, with serotypes 5 and 8 being responsible for about 75% of human infections. Most MRSA strains are serotype 5.
*S. aureus* is found in the external environment and in the anterior nares of 20 to 40% of healthy people. Other sites of colonization include intertriginous skin folds, the perineum, the axillae and the vagina. Although this organism is frequently a part of the normal human microflora, any defect in the host immune system enables the bacteria to gain entry into the host tissue and it can cause infections ranging from localized abscess to serious, invasive infections such as SSTIs, pneumonia, bacteraemia, endocarditis, osteomyelitis, septic arthritis and toxin-mediated infections such as toxic shock syndrome, food poisoning, scalded skin syndrome.47,51

### 2.1.1 Virulence factors and their role in pathogenesis

*S. aureus* is an opportunistic pathogen able to persist and multiply in various environments and cause a diverse range of diseases in both humans and animals. Disease causing ability has been attributed to two major mechanisms: 1) Invasion and inflammation and 2) Toxin production.52 The ability of the bacterium to produce an array of virulence factors that contributes effectively to establish and maintain the pathogenicity is listed in Table 1.53

*S. aureus* produces two types of exotoxins; pyrogenic toxin superantigens (PTSAgs) and hemolysins. Toxic shock syndrome (TSS), staphylococcal food poisoning (SFP) and staphylococcal scalded skin syndrome (SSSS) are known to be caused by PTSAg toxicity. The PTSAgs toxin presently includes toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SE) (SEA, SEB, SEC,SED, SJE, SEG, SHE and SEI) and the exfoliative toxins (ETA and ETB).54-56
**Review of Literature**

*S. aureus* secrete hemolysins producing β-barrel pores in the plasma membrane and cause leakage of the cell’s content and lysis of the target cells, which includes α-hemolysin, β-hemolysin, γ-hemolysin and Panton-Valentine leukocidin.\(^{57,58}\)

**Table 1:** Virulence factors involved in the pathogenesis of *S. aureus*\(^ {53}\)

<table>
<thead>
<tr>
<th>Type of virulence factors</th>
<th>Selected factors(^a)</th>
<th>Genes</th>
<th>Associated clinical syndromes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Involved in attachment</td>
<td>MSCRAMMs (e.g., clumping factors, fibronectin-binding proteins, collagen, and bone sialoprotein-binding proteins)</td>
<td><em>clfA, clfB, fnbA, fnbB, cna, sdr, bbp</em></td>
<td>Endocarditis, osteomyelitis, septic arthritis, and prosthetic-device and catheter infections</td>
</tr>
<tr>
<td>Involved in persistence</td>
<td>Biofilm accumulation (e.g., polysaccharide intercellular adhesion), small-colony variants, and intracellular persistence</td>
<td><em>Ica locus, hemB mutation</em></td>
<td>Relapsing infections, cystic fibrosis, and syndromes as described above for attachment</td>
</tr>
<tr>
<td>Involved in evading/destroying host defenses</td>
<td>Leukocidins (e.g., PVL and γ-toxin), capsular polysaccharides (e.g., 5 and 8), protein A, CHIPS, Eap, and phenol-soluble modulins</td>
<td><em>lukS-PV, lukF-PV, hlg, cap5and 8 gene clusters, spa, chap, eap, psm-a gene cluster</em></td>
<td>Invasive skin infections and necrotizing pneumonia (CA-MRSA strains that cause these are often associated with PVL) abscesses (associated with capsular polysaccharides)</td>
</tr>
<tr>
<td>Involved in tissue invasion/penetration</td>
<td>Proteases, lipases, nucleases, hyaluronate lyase, phospholipase C, and metalloproteases (elastase)</td>
<td><em>V8, hysA, hla, plc, sepA</em></td>
<td>Tissue destruction and metastatic infections</td>
</tr>
<tr>
<td>Involved in toxin-mediated disease and/or sepsis</td>
<td>Enterotoxins, TSST-1, exfoliative toxins A and B, α-toxin, peptidoglycan, and lipoteichoic acid</td>
<td><em>sea-q (no sef), tsfH, eta, etb, hla</em></td>
<td>SFP, TSS, SSSS, bullous impetigo, and sepsis syndrome</td>
</tr>
<tr>
<td>With poorly defined role in virulence</td>
<td>Coagulase, ACME, and bacteriocin</td>
<td><em>Arc cluster, opp-3 cluster, bsa</em></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** ACME (arginine catabolic mobile element); CA-MRSA (community-acquired methicillin resistant *S. aureus*); CHIPS (chemotaxis inhibitory protein of staphylococci); Eap (extracellular adherence protein); MSCRAMMs (microbial surface components recognizing adhesive matrix molecules); PVL (Panton-Valentine leukocidin).

\(^a\)Several factors may have >1 role in *S. aureus* pathogenesis.
2.1.1.1 Panton-Valentine Leukocidin

Panton-Valentine Leukocidin (PVL) is a bi-component exotoxin produced by *S. aureus*, was first described by Van de Velde (1894). Clinical studies propose the PVL being a virulence factor in necrotizing diseases.\(^{59,60}\) PVL was significantly associated with community-acquired infections (85% of strains), compared with hospital-acquired (0%). PVL was also found to be associated with strains causing invasive skin infections such as furunculosis (93%) and cutaneous abscess (50%), compared with superficial folliculitis (0%). PVL was not detected in strains causing infective endocarditis, TSS or mediastinitis, although only few strains were tested.\(^{60}\)

PVL forms pores in the membranes of leukocytes, causing their lysis. This pore is an octameric β-barrel molecular complex perpendicular to the plane of the cell membrane; similar to that made by *S. aureus* α-toxin.\(^{61,62}\) Pore formation requires two components of the toxin, LukS-PV and LukF-PV encoded by two genes *lukS-PV* and *lukF-PV*. Sub-lethal concentrations of purified PVL toxin induce pronounced histamine release from human basophils and stimulate human neutrophils to release enzymes (β-glucuronidase and lysozyme), chemotactic components (leukotriene-B4 and IL-8), and oxygen metabolites.\(^{61,63,64}\) PVL was shown to exhibit a potent dermonecrotic effect with an increased local tissue destruction through the release of neutrophil components such as oxygen radicals.\(^{65}\) Rasigade et al., 2010 reported that PVL-bearing MSSA strains may form a reservoir for acquiring PVL gene in MRSA strains.\(^{66}\)
2.1.1.2 α-Toxin (α-Hemolysin)

Alpha (α) toxin was the first bacterial exotoxin identified as a pore former and the major cytotoxic agent released by *S. aureus*.\(^6\) \(α\)-toxin plays an important role in the pathogenesis of staphylococcal diseases, as *S. aureus* mutants lacking *hla* gene (Structural gene encoding for α-Toxin) display reduced virulence in invasive disease models.\(^6\)\(^8\)–\(^7\) \(α\)-toxin is secreted as a water soluble monomer forming a heptameric, β-barrel structure in host membranes. \(α\)-hemolysin diffuses into the eukaryotic membrane and oligomerizes into β-barrel forms a pore which is particularly cytolytic toward human platelets and monocytes.\(^7\) \(α\)-toxin facilitates the secretion of newly synthesized chemokines into the airway and exaggerates neutrophil-mediated inflammatory lung injury.\(^7\)

2.1.1.3 β-Toxin (β-Hemolysin) (Sphingomyelinase C)

Beta (β) toxin is a Mg\(^{2+}\)-dependent neutral sphingomyelinase hydrolyzes sphingomyelin of the host cell plasma membrane to phosphocholine and a bioactive secondary messenger, ceramide. These ceramides may have a number of effects in eukaryotic cells, including stimulation of second messenger systems, activation of mitogen activated protein kinase (MAPKs), changes in cell shape and apoptosis.\(^7\)\(^3\),\(^7\)\(^4\) The cytotoxic effect of β-toxin is cell type-specific and species-specific, suggesting that its primary virulence activity is to modulate host processes that affect pathogenesis, rather than to directly kill host cells. Instead of lysing host cells β-toxin makes them susceptible to other lytic agents, such as α-toxin and PVL.\(^7\)\(^5\) β-toxin has been shown to maximize lung injury not through its cytotoxic activity, but rather through enhancing the neutrophil infiltration in a syndecan-1-dependent manner.\(^7\)\(^5\) Moreover, this toxin can activate different cell signaling pathways involved in the induction of *c-Fos* (proto-oncogene product) expression through
the NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) and p38 MAPK signaling cascades.\textsuperscript{73, 76}

2.1.1.4 \(\gamma\)-Toxin (\(\gamma\)-Hemolysin)

Gamma (\(\gamma\)) toxin and PVL are bicomponent toxins made by \textit{S. aureus}. Each of these toxins is made up of two non-associated secreted proteins, i.e. F and S for PVL and H\(\gamma\)I and H\(\gamma\)II for \(\gamma\)-hemolysin.\textsuperscript{77, 78} \(\gamma\)-hemolysin is secreted by virtually every strain of \textit{S. aureus}, while PVL is produced only by 3 to 5\% of strains. The toxins affect neutrophils and macrophages, and \(\gamma\)-hemolysin is additionally able to lyse many varieties of mammalian erythrocytes. However, \(\gamma\)-hemolysin is not distinguishable on blood agar plates.\textsuperscript{79} Prevost et al., 1995 reported that the components of these proteins arise from two distinct loci within the \textit{S. aureus} genome.\textsuperscript{80} In strains containing both cytotoxins, three S components (HlgA, HlgC and LukS-PV) and two F components (HlgB and LukF-PV) are available. The genes for \(\gamma\)-hemolysin are transcribed from a single locus, located on a 4.5-kb \textit{ScaI}-digested chromosomal fragment. Extracts from a clone containing this fragment were hemolytic and leukotoxic.\textsuperscript{61, 78, 81}

2.1.1.5 Pyrogenic toxins

\textit{S. aureus} produces PTSAgs are associated with TSS, SSSS and SFP. Most PTSAgs cause TSS in deep-tissue infections, whereas only TSST-1 is associated with menstrual, vaginal TSS. In contrast, SFP has been linked only with SE.\textsuperscript{82} Although the pathogenesis of TSS has not been completely explained, systemic toxicity in this disease results in part from the immunomodulatory superantigen activity of the toxins. The massive T lymphocytes cells proliferation of the toxins results in excessive release of cytokines, at abnormally high
levels, affects the cardiovascular system, resulting in shock. In addition, a multitude of other organ systems (i.e., renal, hepatic, gastrointestinal, and nervous) are affected, producing less-specific symptoms such as diarrhea, emesis and reduced kidney and liver function. ETA and ETB are involved in SSSS. The exfoliative toxins possess mitogenic activity toward T lymphocytes, but it remains still controversial, whether they should be implicated as superantigens. Another human illness, SFP is acquired by ingestion of preformed toxin and has been linked only to the SE. The hallmark symptom, emesis, is often accompanied by diarrhea and abdominal cramping, but the absence of a fever suggests that toxemia is minimal.

2.1.1.6 Biofilm production by S. aureus

Biofilm have been defined as surface-attached communities of bacterial cells encased in an extracellular polymeric matrix composed of polysaccharides, proteins, nucleic acids, and host factors. Biofilm formation helps microorganisms to evade host immune responses and shows much greater resistance to antibiotics than free living cells. The biofilm formation is an important virulence factor in S. aureus especially on medical implants such as prosthetic joints, prosthetic heart valves, intravascular catheters and cerebrospinal fluid shunts, which creates increasing health care problems.

S. aureus produce a multilayered biofilm embedded within a glycocalyx or slime layer composed of teichoic acids (80%) and 20% of staphylococcal host proteins. Mack et al., 1996 isolated a specific polysaccharide antigen named polysaccharide intercellular antigen composed of β-1, 6-linked NAM residues (80 to 85%) and an anionic fraction with a lower content of non-N-acetylated D-glucosaminyl residues that contains phosphate and
ester-linked succinate (15 to 20%).\textsuperscript{91} In \textit{S. aureus}, biofilm formation comprises multiple steps, starting with adherence of the bacteria either directly to artificial surfaces or through host factors that act as bridging molecules such as the extracellular matrix and plasma proteins (i.e. fibrinogen or fibronectin or platelets). In the second step, the bacteria proliferate and accumulate into a biofilm requiring intercellular adhesion.\textsuperscript{92, 93} This step is promoted by polysaccharide intercellular adhesion (PIA) and β-1, 6-N-acetylglucosaminoglycan, which is synthesized by gene products encoded by the ica\textit{ADBC} operon.\textsuperscript{92, 94} However, studies found biofilm-associated proteins (Bap) and \textit{S. aureus} surface proteins (SasG) are also involved in regulation of biofilm formation during \textit{ica}-independent biofilm development.\textsuperscript{95} Bacteria can adhere to components of the extracellular matrix of host tissues, leading to colonization. Adherence is promoted by protein of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family.\textsuperscript{96} \textit{S. aureus} biofilms, once established, are recalcitrant to antimicrobial treatment and the host response, results in many recurrent infections. Current therapies for treating and preventing chronic biofilm mediated infections are limited to surgical intervention and prolonged antibiotic administration or addition of antimicrobial compounds to indwelling medical devices.\textsuperscript{97}

### 2.2 Mechanism of antibiotic resistance in \textit{S. aureus}

In 1961, Jevons first reported MRSA in UK.\textsuperscript{98} At the beginning of the 1971, MRSA represented only 5\% of all \textit{S. aureus} isolates at the general hospital in Birmingham.\textsuperscript{99} According to the CDC and National Nosocomial Infection Surveillance System (NNISS), the infection of MRSA in USA hospitals was 2.4\% in 1975 which increased to 29\% in 1991 and then reached 43\% by 2002. Since then, the rate of MRSA strains has increased
significantly every year worldwide. In India, the prevalence of MRSA has increased from 6.9% in 1988, to 54% in 2003, 63% in 2013, varying from 8 to 71% across India.

2.2.1 Methicillin resistance

The mechanism of resistance to methicillin was described in 1981, with the identification of reduced-affinity PBP2a in MRSA. Methicillin resistance in staphylococci is due to the acquisition of a large mobile DNA element (20 to 100kb in size), namely “Staphylococcal cassette chromosome mec” (SCCmec). As shown in Figure 2, eight SCCmec types (I to VIII) have been identified. The characteristics of SCCmec elements are presented in Table 2. MRSA produces a modified PBP2a that can complete cell wall synthesis when the transpeptidation activities of the native PBPs are inactivated by the β-lactam antibiotics.

<table>
<thead>
<tr>
<th>SCCmec type</th>
<th>mec complex</th>
<th>ccr genes</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Class B-E</td>
<td>ccrA1B1</td>
<td>34kb</td>
</tr>
<tr>
<td>II</td>
<td>Class A</td>
<td>ccrA2B2</td>
<td>52-58kb</td>
</tr>
<tr>
<td>III</td>
<td>Class A</td>
<td>ccrA3B3</td>
<td>67kb</td>
</tr>
<tr>
<td>IV</td>
<td>Class B-E</td>
<td>ccrA2B2 or ccrA4B4</td>
<td>20-25kb</td>
</tr>
<tr>
<td>V</td>
<td>Class B-E</td>
<td>ccrC</td>
<td>28kb</td>
</tr>
<tr>
<td>VI</td>
<td>Class B</td>
<td>ccrB4</td>
<td>20-25kb</td>
</tr>
<tr>
<td>VII</td>
<td>Class C</td>
<td>ccrC2, ccrC8</td>
<td>28-30kb</td>
</tr>
<tr>
<td>VIII</td>
<td>Class A</td>
<td>ccrA4, ccrB4</td>
<td>32kb</td>
</tr>
</tbody>
</table>
The altered protein PBP2a, retains effective TPase activity while having reduced affinity for penicillin and other available β-lactam antibiotics. PBP2a exhibits a reduced rate-constant for acylation by β-lactams and elevated dissociation constants. These factors, acting together, prevent acylation of PBP2a and thus result in β-lactam resistance.\textsuperscript{106} PBP2a is encoded by the \textit{mecA} gene. The mobile \textit{mecA} gene complex is comprised of \textit{mecA} together with its regulator genes, \textit{mecR1-mec1}, and resides within a genomic island, the \textit{SCCmec} that constitutes 1 to 2% of the ~2.9 million bp \textit{S. aureus} chromosome.\textsuperscript{107-109}

The \textit{SCCmec} contains the insertion sequence, \textit{IS431} which is considered to be involved in translocation of resistance genes, and the recombinases necessary for site-specific integration and excision. Some \textit{SCCmec} types also contain various additional genetic elements, such as Tn554 (which encodes resistance to macrolides, clindamycin and streptogramin B), pUB110 (Which encodes resistance to kanamycin and tobramycin) and pT181 (which encodes resistance to tetracycline).\textsuperscript{110} Another regulatory gene is \textit{femA}, functioning with \textit{mecA} gene is required for the expression of high-level methicillin resistance in MRSA was found to be higher than in low-level resistant MRSA and MSSA\textsuperscript{111, 112} and this gene is able to differentiate \textit{S. aureus} from other CONS (lacking \textit{femA}).\textsuperscript{113} Not only \textit{mecA} and \textit{FemA} gene, a number of environmental and genetic factors have also shown to influence the methicillin resistance. Depending on the genetic background of the strain that acquired \textit{mecA}, resistance levels range from phenotypically susceptible to highly resistant. Genes involved in cell-wall precursor formation and turnover, regulation, transport, and signal transduction may determine the level of resistance that is expressed.\textsuperscript{114, 115}
**Figure 2:** Basic structures of SCC*me*c elements. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC)\textsuperscript{103}
2.2.2 Resistance to macrolides, lincosamides and streptogramins

The increasing prevalence of MRSA has led to renewed interest in the usage of Macrolide-Lincosamide-Streptogramin B (MLS\textsubscript{B}) antibiotics to treat MRSA infections. However, widespread use of MLS\textsubscript{B} antibiotics has led to an increase in the number of staphylococcal strains acquiring resistance to MLS\textsubscript{B} antibiotics.\textsuperscript{116-118} The emergence of macrolide resistance in \textit{Staphylococcus} is mediated by an efflux mechanism encoded by \textit{msrA} gene (conferring resistant to macrolides and Streptogramin B only) or may be due to ribosomal target site modification mediated by \textit{erm} gene, affects the binding of macrolide, lincosamide and Streptogramin B (MLS\textsubscript{B} resistance).\textsuperscript{117} Expression of MLS\textsubscript{B} phenotype is either constitutive (cMLS\textsubscript{B} phenotype) or inducible (iMLS\textsubscript{B} phenotype). In constitutive resistance, r-RNA methylase is always produced (cMLS\textsubscript{B}); whereas as in inducible, methylase is produced only in the presence of an inducing agent i.e erythromycin (iMLS\textsubscript{B}).\textsuperscript{119} \textit{S. aureus} isolates with constitutive resistance are resistant to both erythromycin and clindamycin whereas those with inducible resistance are resistant to erythromycin and appear sensitive to clindamycin (iMLS\textsubscript{B}). The treatment of patients harboring iMLS\textsubscript{B} staphylococci with clindamycin leads to the development of constitutive resistance, subsequently leading to therapeutic failure.\textsuperscript{120, 121}

2.2.3 Resistance to mupirocin

Mupirocin (pseudomonic acid A or Bactroban) derived from \textit{Pseudomonas fluorescens} NCBI 10586, is a topical antibiotic used for treating MRSA associated SSTIs, surgical site infections and eliminating nasal colonization of MRSA among patients and medical staff.\textsuperscript{122-124} It was first introduced in the UK in 1985 to treat staphylococcal and streptococcal wound infections and to eradicate nasal carriage of \textit{S. aureus} including
MRSA.\textsuperscript{125} Mupirocin contains the 9-hydroxy-nonanoic acid moiety and acts by inhibition of bacterial protein synthesis with specifically and reversibly binding to bacterial isoleucyl tRNA synthetase (IRS), thereby preventing isoleucine incorporation into growing protein chains.\textsuperscript{126, 127} Within two years after its introduction, mupirocin resistance among MRSA isolates emerged. Mupirocin resistance strains are categorized into two types: low- and high-level resistance (Minimum inhibitory concentrations (MIC) 8 to 256 and >256 μg/ml, respectively). Low-level resistance is related to changes in the native IRS, due to point mutations in the chromosomally encoded \textit{ileS} gene.\textsuperscript{128} The clinical isolates exhibiting high-level resistance to mupirocin is due to a plasmid mediated gene, \textit{mupA} (also referred to as \textit{ileS2}), which encodes an additional modified IRS.\textsuperscript{129, 130} Another novel gene, \textit{MupB} is also responsible for high-level of mupirocin resistance. Insertion sequences have been identified flanking the \textit{MupA} gene in plasmids, which might facilitate movement of the \textit{MupA} gene between bacterial isolates.\textsuperscript{131} High-level mupirocin resistant strains cannot be eradicated with mupirocin and constitute a serious clinical problem.\textsuperscript{132} However, low-level mupirocin resistance appears to be more prevalent in clinical isolates than high-level resistance, and the emergence of low-level mupirocin resistance has been shown to increase failure rates for nasal decolonization of MRSA.\textsuperscript{130, 133, 134}

\textbf{2.2.4 Resistance to glycopeptides}

After the emergence of methicillin resistance in \textit{S. aureus} in the 1960s, the glycopeptides, particularly vancomycin discovered by Eli Lilly in the 1950s.\textsuperscript{135} It became the first line of antimicrobial therapy for serious MRSA infections. Vancomycin inhibits cell wall synthesis in \textit{S. aureus} and other gram-positive organisms. It binds to the C-terminal of acyl-D-alanyl-D-alanine (D-Ala-D-Ala) residue of the peptidoglycan precursor to forms a
stable, noncovalent complex and inhibits the transglycosylation reaction in peptidoglycan biosynthesis. This process results in the intracellular accumulation of Undecaprenyl diphospho-N-acetylmuramyl-(L-Ala-D-Glu-L-Lys-D-Ala-D-Ala)-β-1,4-N-acetylglucosamine. Any process that interferes with vancomycin binding to D-Ala-D-Ala residues in the cell wall will decrease the potency of the drug. Vancomycin resistant strains avoid cell death by the addition of “false” binding sites (e.g., a D-Alanyl-D-Lactate-containing ligand) leads to a reduction of vancomycin activity. Emergence of vancomycin resistant strains of S. aureus (VRSA) is due to the acquisition of the vanA gene from vancomycin resistant enterococci were first reported from USA in 2002. Despite being the drug of choice for the treatment of MRSA infections, the susceptibility of MRSA to this antibiotic is decreasing, with the increased reports of clinical failure. Initial reports of reduced vancomycin susceptibility in clinical isolates of S. aureus was observed from Japan in 1997, followed by nine cases from USA. Additionally, two cases were also reported, one from India and one from Iran generating significance concern in the medical community. Increasing MIC seems to be related to vancomycin use. As the MIC increases, the frequency of heteroresistance also has been observed to increase. Studies evaluating the outcome of severe S. aureus infections, increased MIC of vancomycin were associated with an increased nephrotoxicity. These findings suggest that there is a need to change from vancomycin therapy in light of clinical response, not MIC alone.

2.2.5 Resistance to linezolid

Linezolid is an oxazolidinone, with antimicrobial activity against multidrug-resistant gram positive bacteria, including MRSA, vancomycin-resistant enterococci and Streptococcus species. Linezolid acts by inhibiting bacterial protein synthesis through
binding to the Peptidyl Transferase Center (PTC) of the 50S ribosomal subunit, preventing it from complexing with the 30S subunit, mRNA, initiation factors and formylmethionyl-tRNA. The net result is to block assembly of a functional initiation complex for protein synthesis, thereby preventing translation of the mRNA. Linezolid may also inhibit the synthesis of staphylococcal and streptococcal virulence factors (e.g. coagulase, hemolysins and protein A). Data from the global surveillance reported linezolid resistance in 1% of \textit{S. aureus} and 2% of CONS. The most common mechanisms responsible for linezolid resistance in clinical isolates of \textit{S. aureus} were: (i) mutations in the domain V region of one or more of the five or six copies of the 23S rRNA gene, (ii) acquisition of the plasmid-mediated ribosomal methyltransferase \textit{cfr} gene and (iii) deletions or mutations in the ribosomal protein L3 of the PTC. Additional mutations in domain V of the 23S rRNA genes and substitutions in ribosomal protein L4 of the PTC are also reported in laboratory-derived linezolid resistant \textit{S. aureus} strains. The first report of linezolid resistant bacteria showed the presence of point mutations at the drug target site. The most frequent mutation is G2576T, although other mutations have been found in clinical isolates and \textit{in vitro}, indicating that resistance was apparently generated \textit{de novo} through spontaneous mutations rather than genetic exchange. Resistance develops slowly, and it is not transmissible between species.

A new mechanism (non-mutational) of linezolid resistance has been reported in veterinary staphylococcal isolates which involves acquisition of a natural resistance gene, \textit{cfr} (chloramphenicol-florfenicol resistance). The \textit{cfr} gene was initially described in a bovine \textit{Staphylococcus sciuri} isolate. The product of the \textit{cfr} gene is a methyltransferase that catalyzes methylation of A2503 in the 23S rRNA gene of the large ribosomal subunit,
conferring resistance to chloramphenicol, florfenicol, clindamycin, lincosamides, oxazolidinones, pleuromutilins and streptograminA, but not to macrolides.\textsuperscript{157} Toh et al., 2007 reported the first cfr-mediated, linezolid-resistant clinical isolate of MRSA.\textsuperscript{158} Two new cases of cfr-mediated resistance in clinical isolates of \textit{Staphylococcus epidermidis} and \textit{S. aureus} were reported from the USA, in 2008.\textsuperscript{159} Rajan et al., 2014 from India reported cfr-mediated resistance in clinical isolates of \textit{S. aureus} from a patient undergoing dialysis treatment.\textsuperscript{160} In human isolates, the gene was located in the chromosome, unlike the animal isolates, but it was probably part of an integrated plasmid that was potentially capable of excision and mobilization. Therefore, the gene could be transmitted to other pathogenic strains and spread quickly.\textsuperscript{158}

\subsection*{2.2.6 Resistance to daptomycin}

Daptomycin a cyclic lipopeptide derived from the fermentation of \textit{Streptomyces roseosporus}, is a cyclic lipopeptide antibiotic with potent bactericidal activity against gram positive organisms including \textit{S. aureus} (both MSSA and MRSA), \textit{Enterococcus faecalis} (both vancomycin susceptible and resistant).\textsuperscript{161} It was discovered by Eli Lilly and Co. Indianapolis, Ind., in 1980.\textsuperscript{162} Daptomycin confers a novel mode of action involving the disruption of amino acid transport by the cell membrane and alterations of the cytoplasmic membrane potential. This leads to the alteration in membrane function resulting in impairment of potassium dependent macromolecular synthesis following efflux of potassium from the cell and the bactericidal activity is concentration dependent and is influenced by pH and ionized calcium concentrations.\textsuperscript{163-165} An interesting property of daptomycin is that it is effective at all growth phases, including the stationary phase. Due to this property daptomycin is useful in the treatment of indolent, deep seated infections, such as endocarditis and osteomyelitis.\textsuperscript{166}
Clinical trials explained that daptomycin was efficacious in patients with skin and skin structure infections and bacteraemia (investigator’s brochure, Eli Lilly & Co.). However, treatment failures were noted in patients with *S. aureus* endocarditis (Eli Lilly & Co., personal communication). Possible reasons for failure included the high degree of protein binding and the degree of penetration into cardiac vegetation. Clinical studies have reported that daptomycin was active against *S. aureus* isolates resistant to vancomycin, linezolid, quinupristin-dalfopristin and teicoplanin. The synergy of daptomycin with oxacillin and other β-lactams against MRSA has been proposed for the treatment of MRSA infection, however further studies are needed to determine the *in vivo* efficacy of the combination.

The emergence of daptomycin resistance in *S. aureus* has been described. The mechanisms of daptomycin resistance in *S. aureus* appear to be quite diverse and complex. Daptomycin resistant strains often accumulate single nucleotide polymorphisms in several trademark gene loci, especially involving *mprF* (which encodes lysylphosphatidylglycerol synthetase), *yyG* (which encodes sensor histidine kinase), and *rpoB* and *rpoC* (which encode the β and β′ subunits, respectively, of RNA polymerase) results in higher phosphatidylglycerol (PG) and lysyl-PG (LPG) ratios in the membrane outer leaflet and bind less daptomycin than the wild-type strain. In addition, phenotypic changes in the cell membrane and cell wall have been identified, such as; resistance to cell membrane depolarization and permeabilization, reduced cell wall surface binding of daptomycin, enhanced expression of the *dlt* operon (involved in D-alanylation of cell wall teichoic acids) and progressive cell wall thickening.
2.3 Epidemiology of MRSA

Epidemiology is the study of the spread of a bacterial pathogen including their distribution pattern and risk factors for control of contagious diseases in populations. In recent years, MRSA has emerged as an important nosocomial pathogen with an increased rate of morbidity and mortality worldwide. An understanding of the epidemiological characteristics of *S. aureus* is essential for the management of infections in both the hospital and community. The prevalence of MRSA is no longer confined to hospital patients since MRSA infections have been increasingly reported in the community. MRSA is differentiated in two types; Community-acquired MRSA (CA-MRSA) and Hospital-acquired MRSA (HA-MRSA). Characteristics of hospital associated MRSA compared with community associated MRSA is listed in Table 3. Though CA-MRSA and HA-MRSA are microbiologically and epidemiologically different, characterizing these two types remains problematic as no consistent case definitions exist. Currently, CA-MRSA strains have become more prevalent and their infections are no longer confined to the community but have started to replace HA-MRSA in health care settings.

2.3.1 Epidemiology of HA-MRSA

MRSA infections usually result in an increase in morbidity and mortality rates among hospital patients. The first report of nosocomial *S. aureus* appeared in 1961 and since that time the prevalence of MRSA infections is increasing in intensive care units (ICU) and health care settings all over the world. The NNIS reported increase in the rate of MRSA (40%) in 1999 compared to 1994 to 1998. MRSA infections are associated with increased morbidity, mortality and length of hospital stay which leads to a major financial burden on healthcare services. From 1998 to 2002, about 51.3% of *S. aureus* isolates
from the ICU patients were methicillin-resistant according to NNISS.\(^{181}\) According to European antimicrobial surveillance system (EARSS) the proportion of MRSA isolated from blood between the years 1999 and 2002 was 41 to 45% in the UK (Greece, Ireland, Malta and Italy) but less than 1% in Iceland, Denmark, Netherlands and Sweden. This inequality was not only observed between countries but also sometimes between hospitals within same country.\(^{182, 183}\) The Indian Network for Surveillance of Antimicrobial Resistance (INSAR) in 2013 reported, that the MRSA rate among ICU patients was 47%.\(^{184}\) A high prevalence of MRSA (35% in ward and 43% in ICU) was observed from blood culture specimens in Delhi.\(^{185}\)

The most common MRSA nosocomial infections are SSTIs, bloodstream infections, bone and joint infections, endovascular infections, osteomyelitis and meningitis.\(^{186, 187}\) The HA-MRSA strains carry the SCC\textit{mec} I (approximately 34 kb in size), II (53 kb) and III (67 kb) types. HA-MRSA strains are multidrug-resistant MRSA showed resistance to various antibiotics (\(\beta\)-lactam, chloramphenicol, erythromycin, streptomycin and tetracycline antibiotics).\(^{188, 189}\) As per epidemiological studies, MRSA infections are difficult and expensive to manage (screening, treatment and isolation) than other types of infection.\(^{190}\) Earlier studies have revealed that the mortality rate associated with MRSA bacteremia is higher than that associated with MSSA bacteremia.\(^{8, 179}\) In summary, MRSA are now endemic in many hospitals, causing nosocomial pneumonia and surgical site infections and the second leading cause of nosocomial bloodstream infections.
2.3.2 Epidemiology of CA-MRSA

CA-MRSA infections among young people without healthcare-associated risk factors have emerged during the past decade. In February 2005, the CDC defined CA-MRSA infection as identification of MRSA in a patient with signs and symptoms of infection, either in the outpatient setting or within 48 hours after admission to a hospital, with no history of MRSA infection or colonization, no history of admission to a hospital or a nursing home during the previous year, and no history of dialysis, surgery, permanent indwelling catheters, or medical devices that pass through the skin to the body. The majority of CA-MRSA strains carry the SCCmec IV (21 to 24 kb) and V (28 kb). SCCmec types IV and V are smaller than types I, II and III and lack antibiotic resistance genes other than mecA, which explains why resistance in CA-MRSA is mostly limited to the β-lactam class of antibiotics. However, the size of SCCmec IV and V can allow for increased mobility of the cassettes and relatively higher fitness of the CA-MRSA strains, which facilitates the efficient spread and persistence of this pathogen in the community.

CA-MRSA is associated with the PVL production which leads to severe community acquired pneumonia and SSTIs. Most common CA-MRSA infections are bacteremia, SSTIs, septic arthritis, TSS, necrotizing fasciitis and necrotizing pneumonia. In 1993, CA-MRSA was first reported in Western Australia and between 1997 to 1999, four deaths were reported among children due to CA-MRSA infections in North Dakota and Minnesota, USA. SSTIs associated with CA-MRSA have been reported in prisons (Los Angeles, San Francisco, Mississippi, Georgia and Texas), homosexuals, competitive athletes military, and certain native populations, day-care...
Maree et al., 2007 reported increase in the prevalence rate of CA-MRSA from 17% in 1999 to 56% in 2003. Previous studies have shown a difference in the rate of CA-MRSA infections varying from 10 to 67%. The surveillance study conducted by Asian Network for Surveillance of Resistant Pathogens (ANSORP), from September 2004 to August 2006 reported that the prevalence of CA-MRSA in Asian countries was 25.5% of which India accounted for 4.3% and the most common circulating CA-MRSA clones were ST59-MRSA-SCCmec type IV-spa type t437, ST30-MRSA-SCCmec type IV-spa type t019 and ST72-MRSA-SCCmec type IV-spa type t324.

Risk factors associated with spread of CA-MRSA include overcrowding, sharing of contaminated items and direct contact with skin surfaces or abrasions and improper maintenance of hygiene. MRSA carriage is a significant risk factor for subsequent development of SSTIs. Crum, 2005 reported a fatality rate of 64% due to severe CA-MRSA infections, while 40% of the surviving individuals suffered from significant disabilities afterwards. Clinicians need improved guidance on diagnosis and treatment of CA-MRSA disease, including wound management, differential diagnosis of specific clinical presentations, culture and susceptibility testing, selection of empirical therapy for adults and children with possible CA-MRSA infections.
### Table 3: Characteristics of HA-MRSA compared with CA-MRSA

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HA-MRSA</th>
<th>CA-MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients characteristics</strong></td>
<td>Older age; underlying diseases common</td>
<td>Younger age; underlying diseases rare</td>
</tr>
<tr>
<td><strong>Specific groups at risk</strong></td>
<td>Patients in hospital or other healthcare facilities</td>
<td>Athletes, military recruits, children attending day care centers, prisoners</td>
</tr>
<tr>
<td><strong>Spectrum of disease</strong></td>
<td>Bacteremia, surgical site infection, ventilator associated pneumonia, and urinary tract infection</td>
<td>SSTIs (such as abscesses, cellulitis, folliculitis, and impetigo), necrotizing pneumonia</td>
</tr>
<tr>
<td><strong>SCCmec types</strong></td>
<td>I, II and III</td>
<td>IV and V</td>
</tr>
<tr>
<td><strong>Antibiotic susceptibility</strong></td>
<td>Multidrug-resistant: including β-lactams, macrolides, TMP-SMX, lincosamide, tetracycline, quinolones and growing resistance to glycopeptides also</td>
<td>Resistant to β-lactams. Variable susceptibility to macrolides, TMP-SMX, tetracycline, lincosamide</td>
</tr>
<tr>
<td><strong>Presence of PVL</strong></td>
<td>Rare</td>
<td>Common</td>
</tr>
<tr>
<td><strong>PFGE types</strong></td>
<td>USA 100 or USA 200</td>
<td>USA 300 or USA 400</td>
</tr>
<tr>
<td><strong>Multilocus sequence typing (MLST) Clones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Europe</strong></td>
<td>ST 239</td>
<td>ST 8</td>
</tr>
<tr>
<td><strong>USA</strong></td>
<td>ST 5</td>
<td>ST 1, ST 8</td>
</tr>
<tr>
<td><strong>Australia</strong></td>
<td>ST 22, ST 239</td>
<td>ST 93, ST 30</td>
</tr>
<tr>
<td><strong>Asia</strong></td>
<td>ST 5, ST 239</td>
<td>ST 59, ST 30, ST 72</td>
</tr>
</tbody>
</table>

TMP-SMX: trimethoprim-sulfamethoxazole; PFGE: pulsed-field gel electrophoresis
2.4 **Importance of development of new antistaphylococcal agents**

The emergence of *S. aureus* strains with resistance to penicillin and methicillin in 1948 and 1961, respectively and the recent reports of vancomycin resistant strains indicate that the battle against this versatile pathogen is not yet over and emphasizes the need to develop newer antibacterial agents. The treatment of *S. aureus* infections are still a challenge for clinicians. Currently, Linezolid is a drug of choice for the treatment of pneumonia and SSTIs caused by MRSA. The major advantage of linezolid is its availability in intravenous and oral form. Generally, oral therapy is less expensive than its intravenous counterpart, and provides the clinician and patient with greater flexibility in an effective therapeutic regimen. However, recurrent infection with MRSA was observed in a patient with endocarditis after treating linezolid.\textsuperscript{212} The meta-analysis of randomized controlled trials found the success rate of 57.6\% for linezolid and 46.6\% for vancomycin for the treatment of hospital-acquired pneumonia due to proven MRSA and suggested that linezolid was not superior to glycopeptide antibiotics for clinical success, microbiologic success, adverse event or mortality.\textsuperscript{213} The effectiveness of daptomycin in all growth phases has been noted to be important for the treatment of deep-seated infections, such as endocarditis or osteomyelitis caused by MRSA. However, the main drawbacks of daptomycin include its high cost, lack of an oral formulation, poor penetration to pulmonary tissue, and possible rhabdomylosis.\textsuperscript{214}

As *S. aureus* continues to exhibit resistance to a wide range of antimicrobial agents, further research is in needed to understand how best to preserve the existing drugs, including recycling some older drugs for which there are currently only limited clinical data for the treatment of staphylococcal infections.\textsuperscript{215} There is urgent need to develop antistaphylococcal agent. Scientists have renewed interest to investigate lysostaphin efficacy in the treatment of *S. aureus* infections.
2.5 Antistaphylococcal activity of lysostaphin

2.5.1 Discovery of lysostaphin

In the early 1960’s Charles Schindler from the laboratory of Vernon Schuhardt at the University of Texas discovered the lysostaphin as the serendipitous scientific discovery similar to the discovery of penicillin. While doing transduction experiments with staphylococci, He found a small white colony surrounded by an area of growth inhibition on a *S. aureus* lawn and the colony was designated as K-6-W1 and identified to be of *Staphylococcus simulans* biovar *staphylolyticus*.\(^{28}\) The bacteriolytic factor was named as lysostaphin and was found to lyse all staphylococcal strains including *S. aureus* as well as *S. epidermidis*, although lysed at markedly slower rate than the *S. aureus* strains. Lysostaphin was not active against any other genus of bacteria, but was effective against heat-killed *S. aureus* suggesting that lysostaphin itself was sufficient for the lytic activity and that the activities of endogenous bacterial factors were not required.\(^{28}\)

2.5.2 General properties of lysostaphin

Among the bacteriocins produced by staphylococci, lysostaphin is the prototype class III bacteriocin, described with a high degree of antistaphylococcal bacteriolytic activity.\(^{216}\) Lysostaphin (EC 3.4.24.75) is a zinc dependent glycylglycine endopeptidase metallo-enzyme with molecular mass of ~27 kDa. It contains one molecule of zinc per mole of protein with a pI of 9.5 and a pH optimum of 7.5. The enzymatic activity is destroyed by pepsin or trypsin and inhibited by Hg\(^{2+}\), Cu\(^{2+}\) ions.\(^{22,217}\) The gene encoding for lysostaphin of *Staphylococcus simulans* biovar *staphylolyticus* has been cloned and expressed in *Bacillus sphaericus* and *Escherichia coli* (*E. coli*). The complete coding region comprises 1440 base pairs corresponding to a precursor of 480 amino acids (molecular weight 51,669 Da).\(^{218}\)
Lysostaphin is synthesized as a pre-proenzyme of 493 amino acids, which entered the secretory pathway by an N-terminal leader peptide of 36 amino acids. The proenzyme is released into the culture medium and contains 13 N-terminal tandem repeat. Pro-lysostaphin is 4.5-fold less active than mature lysostaphin and the N-terminal repeats are removed in a growth phase dependent manner by a cysteine protease to yield the fully activated lysostaphin molecule consists of 246 amino acid residues with molecular weight 26926 Da (Figure 3). The lysostaphin molecule consists of two distinct domains: (i) an N-terminal domain with glycyglycine endopeptidase responsible for the catalytic activity of the protein which cleaves pentaglycine cross bridges and (ii) a C-terminal cell-wall-targeting (CWT) domain promotes bacteriocin binding to the staphylococcal peptidoglycan substrate. The C-terminal 92 amino acid residues of lysostaphin are nonessential for enzymatic activity but necessary for directing lysostaphin to bind the cell wall of S. aureus. A study using a reporter system of CWT-GFP (green fluorescent protein) demonstrated that CWT binds to the pentaglycine bridge and carrying lysostaphin into proximity with the cell wall.

2.5.3 Lysostaphin mode of action

The peptidoglycan of gram positive bacteria is an important component of the cell wall, conferring strength and rigidity to the cell, maintaining cell shape, and protecting against osmotic lysis. The extreme mechanical strength of S. aureus cell walls is probably dependent on the high degree of cross-linking of the pentaglycine bridges between adjacent tetrapeptides in peptidoglycan layer of the cell wall. Lysostaphin has glycyglycine endopeptidase enzymatic activity, which lyses staphylococcal cells by hydrolyzing glycyglycine bonds in the pentaglycine bridges in the cell wall. Lysostaphin seems to
Cleave specifically between the third and the fourth glycine residues of the pentaglycine cross bridge (Figure 4). The peptidase activity of lysostaphin liberates N-terminal glycine and alanine from cell wall of S. aureus.\textsuperscript{22,217,223}

![Figure 3: Lysostaphin synthesis and maturation; lysostaphin is synthesized as a preproenzyme, and released into the extracellular medium. Proteolytic cleavage of the propeptide of 211 amino acids, from which 195 residues are organized in 15 tandem repeats of 13-amino acid length, generates the biologically active lysostaphin. The lysostaphin molecule consists of two distinct domains: an N-terminal peptidase domain (PD) responsible for its catalytic activity and the C-terminal wall targeting domain (CWT), which directs lysostaphin to its receptor on the staphylococcal surface. Adapted from Bastos et al., 2010.\textsuperscript{222}](image)

The peptidoglycan of staphylococcal species relatively resistant to lysostaphin contains higher amount of serine than glycine residues. Lysostaphin is unable to hydrolyze glycyllserine and serylglycine peptide bonds. \textit{S. simulans} biovar \textit{staphylolyticus} peptidoglycan is resistant to enzymatic activity of lysostaphin, since the cells produce a
resistance factor (Lif) causes the incorporation of serine residues into the third and fifth positions of the cell wall pentaglycine cross bridges.\textsuperscript{32,224}

![Diagram of lysostaphin target site on staphylococcal peptidoglycan](image)

**Figure 4:** Target site of lysostaphin on the staphylococcal peptidoglycan layer; *S. aureus* peptidoglycan structure and site of primary hydrolysis of lysostaphin on the staphylococcal peptidoglycan. NacGlu, \(N\)-acetylglucosamine; NacM, \(N\)-acetylmuramic acid; A, L-alanine; D-Q, D-glutamine; K, L-lysine; D-A, D-alanine; G, L-glycine. *Replacement of these Glycine residues by Serine residues results in resistance to lysostaphin.* Adapted from Bastos et al., 2010.\textsuperscript{222}

Francius et al., 2008 used atomic force microscopy (AFM) to study the structural and physical dynamic changes of *S. aureus* cells exposed to lysostaphin. They observed that, following exposure of *S. aureus* to lysostaphin revealed major structural changes in the form of substantial swelling of the cells and alterations of their surface structure (septum splitting, Nano-scale perforations, and increased roughness). Progressive disintegration of the cell wall and separation from the plasma membrane after exposure to lysostaphin were also observed. These structural changes correlate with major differences in mechanical properties, i.e., with a decrease of bacterial turgor pressure and of cell wall stiffness,
eventually leading to the formation of osmotically fragile cells and favoring cell lysis. Lysostaphin rapidly lysed actively growing and non-dividing cells including *Staphylococci in biofilms, whereas most antibiotics require actively dividing cells for their action.*

Hence, it has once again aroused the interest of researchers to investigate the therapeutic values of lysostaphin.

### 2.5.4 Development of lysostaphin as antistaphylococcal agent

After its discovery, lysostaphin was licensed to the Mead Johnson & Co. of Evansville Indiana. In the early 1970s, scientists at Mead Johnson began a clinical development program using lysostaphin extracted from *Staphylococcus staphylolyticus*. It was shown that lysostaphin was lytic for 252 strains of *S. aureus* from various clinical sources and lysostaphin was able to inhibit metabolically inactive cells and capsule producing strains. However, lysostaphin failed to enter polymorph nuclear leukocytes to kill engulfed *S. aureus*. Preclinical animal studies showed that lysostaphin was significantly better than oxacillin for protecting intravenously challenged mice against *S. aureus* and clearing kidney infection in these mice. While it was shown that lysostaphin could induce anti-lysostaphin antibodies in mice and rabbits, these antibodies did not appear to be associated with any toxicity or neutralization of the drug. Goldberg et al., 1967 tested lysostaphin in an experimental endocarditis model in dogs and found that from 5-50 mg/kg, at intervals of 1 to 24 hours, administered for up to 6.5 days after initiation of infection significantly improved the clinical condition of the infected dogs. These treatments also decreased the bacterial count in lung, liver, spleen, kidney and aortic and mitral valves.
The topical application of lysostaphin is more effective than other available antimicrobial agents in transiently eliminating the staphylococcal nasal carrier state. Quickel et al., 1971 tested the efficacy of lysostaphin on nasal carriage of \textit{S. aureus}. 152 subjects were treated intranasally with 0.5\% lysostaphin spray, Neosporin, or no treatment. Five days of three treatments a day with lysostaphin appeared to be more effective than Neosporin for eradication and prevention of recolonization by \textit{S. aureus}. No lysostaphin resistant \textit{S. aureus} variants were isolated following treatments and there were no local or systemic reactions to lysostaphin. Antibody formation to lysostaphin was reported in most of the treated subjects.\textsuperscript{229}

The first ten years of lysostaphin research were supportive of this unique enzyme as a potential therapy for staphylococcal infections. However, study of lysostaphin as an antistaphylococcal agent was discontinued due to the lack of homogeneous preparations of lysostaphin and the availability of other effective treatments.

\textbf{2.5.5 Production of recombinant lysostaphin (r-lysostaphin)}

Early work with lysostaphin was conducted with lysostaphin protein extracted from it natural host \textit{S. simulans} biovar \textit{staphylolyticus} which yielded fairly low amounts of lysostaphin of inconsistent purity.\textsuperscript{230} The cloning and sequencing of the lysostaphin gene allowed lysostaphin to be produced recombinantly in higher amount that could be purified to lots of consistent purity from various expression systems. In 2000, Biosynexus Incorporated of Gaithersburg, MD licensed the rights to the lysostaphin patents from AMBI and began producing lysostaphin in a \textit{Lactococcus lactis}. Recombinant production of lysostaphin was moved into a commercial grade pPOP expression system in \textit{E. coli} at Avecia in Stansted,
UK for production of lysostaphin for clinical use. Expression of lysostaphin in \textit{E. coli} allowed for production of more than 5 g of lysostaphin per liter of culture. \textit{E. coli} was capable of producing highly active lysostaphin with activity similar to that produced by \textit{Lactococcus lactis}.\textsuperscript{231} A recombinant lysostaphin expressed in \textit{E. coli} is sold commercially for staphylococcal genetic studies, being used for DNA isolation, formation of protoplasts and differentiation of staphylococcal strains. It was used for further therapeutic developments.

\textbf{2.5.6 \textit{In vitro} bactericidal activity of r-lysostaphin}

Antibiotic resistant strains of \textit{S. aureus} including MRSA and vancomycin intermediately susceptible (VISA) strains of \textit{S. aureus} were found to be as sensitive to lysostaphin. MIC of lysostaphin against various strains of \textit{S. aureus} was ranged from 0.001 to 2.0 µg/ml.\textsuperscript{31, 232-234} Bacteria growing in biofilms are less susceptible to most antibiotics; it is very difficult to treat a biofilm infection in-place with conventional antibiotics. Lysostaphin has the capacity to degrade staphylococcal biofilms \textit{in vitro}, not only killing the staphylococci, but also stripping the extra-cellular biofilm matrix from the artificial surface at concentrations as low as 1 µg/ml. Scanning electron microscopy confirmed that lysostaphin eradicated both the sessile cells and the extracellular matrix of the biofilm. For \textit{S. epidermidis}, higher concentrations of lysostaphin were needed to achieve the same effect.\textsuperscript{226} Shah et al., 2004 demonstrated that lysostaphin-coated catheters were completely cleared of bacteria as compared to control catheters.\textsuperscript{235} The inhibitory effect of lysostaphin-coated catheters was maintained for at least four days after coating. This lysostaphin activity presents a new treatment option for patients with difficult to treat staphylococcal infections of indwelling devices like artificial heart valves and other
prosthetic devices. The use of combinations of antimicrobials is common in the clinical setting since it expands the spectrum of organisms that can be targeted, prevents the emergence of resistant organisms, decreases toxicity by allowing lower doses of both agents, and can result in synergistic inhibition. Synergy has been observed in vitro between lysostaphin and β-lactams, bacitracin, polymixin B and daptomycin. 236, 237

2.5.7 In vivo bactericidal activity of r-lysostaphin

In vivo efficacy of lysostaphin was tested in various animal models. Lysostaphin proved highly efficacious for clearance of S. aureus infections associated with biofilm production. Jugular vein catheterized mice were infected with S. aureus and allowed to form biofilm on catheter. Higher dose of lysostaphin (20 mg/kg), three times a day for four days was able to eradicate biofilm from the catheter. These findings were consistent with higher concentration of lysostaphin required to inhibit the biofilm formation in in vitro experiments. 226 In a rabbit model of MRSA endocarditis, animals were treated thrice daily for three days with 5 mg/kg of lysostaphin, produced sterile vegetation in 10 out of 11 treated rabbits with a mean reduction in vegetation bacterial counts of 8.5 log10 CFU/g (Colony Forming Units) compared to the counts in the untreated controls. In this study, vancomycin administered at 30 mg/kg, twice daily did not sterilize the vegetation and reduced vegetation bacterial counts by only 4.8 log10 CFU/g. The evaluation of the immunological effects of lysostaphin administration was carried out; and there was no evidence of immunological reactions following up to nine weeks of intravenous administration. 239 Lysostaphin is also effective in reducing the vegetation produced by VISA strain in endocarditis model. 240 It was observed that, the rates of sterilization of aortic valve vegetations were significantly better for animals treated with either a single dose of
lysostaphin (43%) or lysostaphin given twice daily for 3 days (83%) than for animals treated with vancomycin. Vancomycin was ineffective on sterilization of aortic valve vegetation. Kiri et al., 2002 demonstrated the synergistic effect of lysostaphin with β-lactam against oxacillin resistant S. epidermidis isolates in the central venous catheter infections. The rabbits treated with the combination of nafcillin (200 mg/kg intramuscularly) and lysostaphin (1 mg/kg intravenously) had a significant reduction in mean log_{10} vegetation counts (5.3 log_{10} CFU/g) compared to rabbits treated with r-lysostaphin or nafcillin alone.\textsuperscript{241}

Lysostaphin has been found effective in the treatment of S. aureus systemic infection in mouse model. Lysostaphin (5 mg/kg) was administered once over three days, which consistently cleared bacteremia and solid organ infections in the S. aureus (MSSA and MRSA) challenged mice. In vivo synergistic activity of lysostaphin with oxacillin reduced the dose of lysostaphin to 1 mg/kg and vancomycin showed additive effect with lysostaphin.\textsuperscript{242}

In developing countries, neonatal bacteremia is more prevalent, S. aureus is the most common pathogen for late-onset sepsis among very low birth weight infants, and nearly 20% die as a result of the infection. In a neonatal mouse model, pups were infected subcutaneously and littermates randomized to receive either vancomycin 15 mg/kg, lysostaphin 10 mg/kg or 15 mg/kg, intraperitoneally at time points 0.5, 6, 24 and 30 hours after the infection. Pups were observed for survival rate during seven days, and quantitative blood cultures were obtained 24 hours after infection. Lysostaphin was more effective than vancomycin for treatment of MRSA infection and also significantly improved the survival of the lysostaphin-treated pups (52%) versus vancomycin-treated pups (34%). The serum
concentrations of lysostaphin and vancomycin were found to be 2.34 μg/ml and 1.72 μg/ml, respectively. Notably, both serum concentrations were greater than the MICs and MCBs and lysostaphin serum concentrations were higher than vancomycin, suggesting a higher half-life for this bacteriocin.\textsuperscript{243} This pharmacokinetic data is in agreement with similar results seen in Oluola et al., 2007\textsuperscript{244} and Kokai-Kun et al., 2007.\textsuperscript{242}

Clara Sei et al., 2011 observed that treatment of systemic \textit{S. aureus} with lysostaphin actually blunts the inflammatory cytokine mediated response leading to reduced expression of TNF and IL-6 in response to \textit{S. aureus} challenge. Lysostaphin treatment was able to reverse systemic shock caused by \textit{S. aureus} as indicated by changes in core body temperature.\textsuperscript{245} These findings may be explained by the recent publication (Ip et al., 2010) which demonstrated that prior to TLR-dependent cytokine production; whole staphylococci must be engulfed and delivered into acidic phagosomes. Lysostaphin rapidly lyses all free bacteria thus preventing their uptake in phagosomes.\textsuperscript{246}

Dajcs et al., 2000 reported, 0.28\% lysostaphin was found to be very effective in comparison to 5\% vancomycin for treating keratitis mediated by MSSA or MRSA in a rabbit model. The lysostaphin was administered every 30 minutes up to 15 hours post infection and was found to be capable of penetrating the cornea to kill the staphylococci. Lysostaphin treated eyes were found to recover from infection. The residual bacteria in lysostaphin treated eyes were significantly lesser than in vancomycin treated eyes (0.58 ± 0.34 vs. 5.83 ± 0.16 log CFU/cornea, respectively; P ≤0.0001).\textsuperscript{247} Lysostaphin was also found to persist in the aqueous humor for days while retaining its antistaphylococcal activity and did not show any adverse reactions.\textsuperscript{248}
Walsh et al., 2003 in adult mice model, compared the pharmacokinetics of lysostaphin with a Polyethylene glycol-lysostaphin conjugate (PEGylated). It was found that 24 h after a single dose of 40 mg/kg of lysostaphin, the serum drug concentration dropped 500-fold, whereas for the PEGylated derivative the drop was only 10-fold. This reflects an increase in lysostaphin stability due to PEG conjugation. This improved retention of lysostaphin would reduce the dosage and frequency needed to maintain plasma drug concentrations above therapeutically effective drug concentrations. Maintaining these high levels of lysostaphin for longer periods of time may also result in more rapid clearance of bacterial infections and decrease the probability of emergence of lysostaphin resistance strains.  

2.5.8 Topical application of r-lysostaphin

Nasal carriage of S. aureus has been frequently shown to have a significant epidemiological link with subsequent development of endogenous staphylococcal infections. Nasal decolonization can reduce the risk of development of infections by MRSA in addition to reducing the community and hospital spread of these microorganisms. Mupirocin ointment (Bactroban Nasal; 2% mupirocin calcium ointment; SmithKline Beecham) is the current standard of care for clearance of S. aureus nasal colonization, but resistance to this antibiotic has emerged. Currently, limited interventions are available for the clearance of S. aureus nasal colonization including Neosporin ointment and Polysporin ointment.

Kokain-Kun et al., 2003 investigated the efficacy of lysostaphin to clear S. aureus from nasal colonization. A single dose of lysostaphin (formulated at 0.5% in a petrolatum-based cream) eradicated MRSA and mupirocin resistant S. aureus from the cotton rat nares,
within four hours of application, while three doses of mupirocin ointment over three days were required for eradication of MRSA in the same model. Nisin (~1.5 mg) failed in reducing staphylococcal nasal carriage in this model. The potency of the lysostaphin formulation was also persistent in the nares and maintained its antistaphylococcal activity for at least 24 hours after instillation, suggesting that lysostaphin cream could also prevent subsequent *S. aureus* nasal colonization. No lysostaphin-resistant variants of *S. aureus* were isolated following treatment with lysostaphin cream.\(^{34}\)

Wound infections lead to economic loss, increase in patient morbidity and mortality and many wound infections are caused by Staphylococci that are capable of developing antibiotic resistance. Kumar et al., 2014 suggests the use of lysostaphin as a topical antimicrobial to treat MRSA infected wound in mice model. Lysostaphin cream (~120 and 150 µg) has shown >3 log reduction in bacterial load in skin abrasion infected with MRSA in mouse model.\(^{31}\) Lysostaphin immobilized in biomaterial cellulose fiber has shown bactericidal activity against *S. aureus* with low toxicity towards keratinocytes.\(^{253}\) Chitosan-collagen hydrogel incorporated with r-lysostaphin (CCHL) gauze used for the treatment of MRSA infected third-degree burn wounds, is found to be effective in decolonizing bacteria at the site of infection after two weeks of treatment.\(^{254}\) The synergistic combinations of recombinant lysostaphin and antibiotics have been suggested in controlling cutaneous staphylococcal infections. Desbois et al., 2010 reported that combination of lysostaphin and the cationic peptide ranalexin could represent a novel therapeutic agent for the treatment of wounds infected with MRSA. In a rabbit model of wound infection, ranalexin with lysostaphin reduced MRSA in the wound by 3.5 \(\log_{10}\) CFU compared with the untreated control. The combination of ranalexin and lysostaphin found to be significantly more
effective than treatment with ranalexin or lysostaphin alone.\textsuperscript{255} Since lysostaphin is active against all strains of \textit{S. aureus} tested; lysostaphin has potential biotechnological applications in the treatment of staphylococcal infections.

\subsection*{2.5.9 Mechanism of resistance to lysostaphin}

Staphylococci have the capacity to develop resistance to lysostaphin when selected by lysostaphin pressure \textit{in vitro} or \textit{in vivo}. The mechanism involved in resistance to lysostaphin is usually a mutation of the \textit{femA} or \textit{femB} genes, which result in monoglycine or triglycine cross bridges in peptidoglycan layer of the bacterial cell wall.\textsuperscript{256, 257} The \textit{femAB} operon plays a crucial role in the formation of pentaglycine cross bridges of peptidoglycan layer of staphylococcal cell wall. The \textit{femA} gene is involved in insertion of second and third glycine to the bridge; \textit{femB} is encoded for second and third glycine to the bridge. Any defect in the \textit{femAB} operon, the pentaglycine bridges are replaced with monoglycine or triglycines.\textsuperscript{257} The \textit{femB} gene is not capable of substituting for \textit{femA} and visa-versa.\textsuperscript{258}

Lysostaphin resistant variants isolated \textit{in vitro} by incubation with increasing doses of lysostaphin have been mapped to mutations in \textit{femA} and has monoglycine cross bridges. The frequency of resistance development ranged between $5.3 \times 10^{-1}$ and $1.0 \times 10^{-7}$. All resistant mutants showed $>15$ fold increase in MIC of lysostaphin. Laboratory selected, lysostaphin resistant variants of two MRSA strains were mapped to an insertion/frame shift and a 66 bp deletion mutations in the \textit{femA} gene.\textsuperscript{256, 259} Climo et al., 2001 explained the resistance to lysostaphin was related with three characteristics: increased susceptibility to $\beta$-lactam drugs, mutations in \textit{femA}, and an altered peptidoglycan structure in which the normal pentaglycine cross bridges were replaced with a monoglycine.\textsuperscript{256} Kusuma et al., 2007 reported, the
diminished fitness of the lysostaphin-resistant variants of *S. aureus* was reflected by (i) a reduced logarithmic growth rate; (ii) increased susceptibility to elevated temperatures; and (iii) at least fivefold less virulence of the lysostaphin-resistant variants than their wild-type strains in a mouse kidney infection model.\(^{259}\)

MRSA strains express the PBP2a to perform cell wall transpeptidation activity when the other PBPs are inactivated by the presence of β-lactams.\(^{105}\) PBP2a, however, appears to have a requirement for pure pentaglycine muropeptide monomers for efficient cross linking activity, but in lysostaphin resistant variants, these muropeptides are either mono- or triglycines or of mixed amino acids and thus cannot be used as a substrate for transpeptidation by PBP2a. Because of this, resistance to both lysostaphin and β-lactams is unlikely to coexist. Lysostaphin rapidly degrades the cell walls of all of the staphylococci with pentaglycine bridges, while any lysostaphin resistant variants that are selected become susceptible to β-lactams. Since lysostaphin and β-lactams are synergistic and resistance between these two antibiotics appears to be mutually exclusive, this supports that their combination would be the best choice clinically for treatment of staphylococcal infections.\(^{256}\)

Morikawa et al., 2001 observed that cell wall thickness also plays important roles in lysostaphin sensitivity using *S. aureus* strain N315 and its derivative mutants affected in *sigB* gene (encodes for sigma factor B). Cells depleted in *sigB* developed thinner cell wall and demonstrated increased sensitivity to lysostaphin. These cells were approximately 97% more sensitive than the wild-type N315 cells. On the other hand, over expressing *sigB* cells revealed an increased resistance to lysostaphin and cell wall affecting antibiotics, being 300% more resistant than normal N315 cells due to the increase in cell-wall thickness.\(^{260}\)
Grundling et al., 2006 have identified another gene, lyrA (for lysostaphin resistance protein A) in *S. aureus*, whose inactivation caused a high degree of lysostaphin resistance. *lyrA* encodes a 419-amino acid polypeptide with unknown function. In contrast to the case for *femAB* mutants, transposon insertion in *lyrA* did not cause gross alterations of cell wall cross bridges and did not result in a decrease in β-lactam resistance. The resistance phenotype of *lyrA* mutants has proven difficult to explain. Many factors, including minor alterations in peptidoglycan, pentaglycine bridges and other envelope components were proposed as possible explanations for this phenotype.²⁶¹

### 2.5.10 Lysostaphin toxicity reports

Lysostaphin has been shown to be extremely effective for treating serious *S. aureus* infections in various animal models; however, concerns regarding lysostaphin toxicity are also reported. Chan et al., 1998 reported the cell toxicity test and inflammatory effects of recombinant lysostaphin on normal human epidermal keratinocyte (NHEK). Lysostaphin was did not show toxic effect on NHEK cells, but stimulated the cellular IL-8 production which may cause skin diseases. However, further determining the source, kinetics of production, and the regulation of inflammatory mediators in the skin need to be of validated in predicting various toxicities arising from exposure to lysostaphin.²⁶²

The toxicology studies conducted by Biosynexus with lysostaphin, the pathology were consistent with large vessel vasculitis associated with repeat lysostaphin dosing in rabbits and non-human primates. While no deleterious clinical observations were made during the in-life portion of the study, upon autopsy, there was significant pathology observed including microscopic observations in the kidneys and heart and
glomerulonephritis in all animals receiving the higher two doses of lysostaphin. Arteritis was also observed in these animals. This toxicity could be the formation of antibody: antigen complexes in the presence of excess antigen leading to deposition of the complexes on vessel walls and vasculitis.²³⁹,²⁶³