2.0 REVIEW OF LITERATURE

Although a voluminous literature on *Staphylococcus aureus* is available but the literature pertinent to the proposed research project has been reviewed in this chapter.

2.1 General background

*S. aureus* was first discovered by Alexander Ogoston in late 1870’s from post operative wound sepsis. In 1883, it was named *Staphylococcus* (the clusters of grape-like organisms), after the Greek word ‘*Staphyle*’ meaning bunch of grapes and ‘*coccus*’ which means granules. The species name ‘*aureus*’ originated from the Latin word ‘*aurum*’ which refers to the golden colour produced by the organism when grown on solid media (Howard and Kloos, 1987). *S. aureus* belongs to family Staphylococcaceae. Being facultative anaerobe this organism grows rapidly on blood agar and some non selective media like nutrient agar under aerobic and anaerobic conditions (Yu and Washington, 1985). The colonies appear as smooth, convex and sharply defined and produces β-hemolysis on blood agar (Lowy, 1998). The cell wall of *S. aureus* is hard, protective covering, about 20-40 nm thick (Shockman and Barrett, 1983). The main component of the cell wall is peptidoglycan which makes up to 50% of the total cell wall mass (Waldvogel, 1990). The peptidoglycan structure varies from one species to another but several structural or functional elements are conserved (Schleifer and Kandler, 1972). Another important component of cell wall is a group of phosphate containing polymers called teichoic acid which makes up about 40% of the weight, whereas rest of the cell wall is composed of surface proteins, exoproteins and autolysin (peptidoglycan hydrolases). Some of these components are involved in attachment of the bacteria to the host cell surface. About 90% clinical strains of *S. aureus* possess capsular polysaccharides. This organism is Gram positive, non-motile and non spore-forming (Thakker et al., 1998). The members of genus *Staphylococcus* are catalase positive and oxidase negative. These biochemical properties distinguish them from *Streptococci* (Wilkinson, 1997). The major habitats of *Staphylococci* bacteria are the skin, sebaceous glands and mucosal surfaces in humans as well as in animals (Kasprowicz et al., 2011).

2.2 Genome organization

The complete genome sequences of ten strains of *S. aureus* have been determined so far while genomes of many others have been partially sequenced (Diep et al., 2006). The genome of *S.
*Staphylococcus aureus* is a single, circular molecule, 2.8 - 2.9 Mbps in size with G+C content of about 33 % (Crossley and Archer, 1997). It is composed of about 2700 protein coding sequences (CDS) as well as structural and regulatory RNAs. The genome consists of core genome, accessory component and foreign genes. The core genome constitutes the backbone of the genome. The organization of core component is highly conserved and identity of individual genes between isolates is 98-100%. These genes are mainly responsible for fundamental functions linked to central metabolism. The accessory components consist of genetic regions which contain transposons, the genetic elements which have the ability of horizontal gene transfer between strains. Transposons include: pathogenicity islands (PIs), genomic islands, prophages, chromosomal cassettes and transposons (Lindsay & Holden, 2004). The diagrammatic representation of *S. aureus* genome is schematically presented in Fig 2.1.

![Fig: 2.1 Schematic circular diagram of the SCC-like MSSA463 chromosome. Where appropriate, categories are shown as concentric circles representing coding strands. From the outside to inside: the colored segments on the gray outer ring represent genomic islands and horizontally acquired DNA. Inside the gray outer circle, the second circle represents the nucleotide position in Mb. The third and fourth circles show open reading frames (ORFs) on the plus and minus strands, respectively. Different colors represent different Clusters of Orthologous Groups (COG) functions. The color coding for coding sequences (CDS) is listed to the right of the circular diagram. The fifth circle indicates the location of predicted tRNAs. The sixth and seventh circles represent rRNA predicted on the plus and minus strands, respectively. The eighth and ninth circles show G+C content and G+C skew, respectively (> 0%, green; <0%, purple). Adapted from: De Zhi et al., 2013](image)
Pathogenicity islands
The family of Staphylococcal pathogenicity islands carries genes for superantigen toxins, 15–20 kb elements located at constant positions in the chromosome. Pathogenicity islands also contain genomic islands from the νSA family, which codes about half of Staphylococcal toxin and virulence factors. These islands are mainly responsible for pathogenicity of the species. The genomic islands include νSA1 (carrying enterotoxin genes seb, tst, ear) and νSA2 (containing genes encoding enterotoxin-sec and toxic shock syndrome toxin- tst) (Gill et al., 2005).

Prophages
Depending upon the size of their genome, prophages of S. aureus can be classified into three groups. The prophages of less than 20 kb fall in class I, about 40kb in class II and more than 125 kb in class III (Kwan et al., 2005). The prophages are responsible for evolution of pathogenicity and horizontal transfer of genetic information. S. aureus strains sequenced so far contain 1-3 prophages and most of them carry virulence determinants like enterotoxins, exofoliative toxins, Staphylokinase and Panton-Valentine Leukocidin (pvl) (Diep et al., 2006).

Insertion sequences and transposons
At least one gene carried by insertion sequence which codes for transposon and participates in recombination required for transposition. Most of the insertion sequence elements contain inverted terminal repeats of short size which act as transposase binding sites (Baba et al., 2004). In the genome of S. aureus, insertion elements are distributed both in coding and non coding regions. In the MRSA strains such as N315 and Mu50, eight copies of IS-1181 have been found (Kuroda et al., 2001). The strains of S. aureus carrying large mobile genetic elements, staphylococcus chromosome cassette mec (SCCmec) are regarded as MRSA. The mecA gene is carried by this element which codes for alternative penicillin binding protein (PBP2a) which has low binding affinity to all β-lactam antibiotics (Ito et al., 1999).

Staphylococcal chromosome cassette (SCCmec)
SCCmec is the genetic material of 21-67 kb in size which is present in the chromosome of MRSA strains on the unique site known as attBsc. This site is located near origin of replication. attBsc occurs as open reading frame of unknown function which is identified as orfX. This frame is well conserved among S. aureus strains. SCCmec are variable genetic materials which
have certain conserved features. Among the conserved elements, SCCmec contains the mec operon which is composed of mecA and its regulatory gene as well as the cassette chromosome recombinase complex ccr (Holden et al., 2004). The ccr locus consists of cassette chromosome recombinase genes, ccrA, ccrB or ccrC, which are involved in the integration into chromosomes and precise excision of the SCCmec element from the chromosome (Ito et al., 2001). The variable regions of SCCmec are known as J-regions which contain integrated genetic elements like plasmids (pT181, pUB110 and p1258), transposons (Tn554) and insertion sequences (IS431, IS1272 and IS256). Based on the combination of different mec and ccr complexes, 8 types of SCCmec elements have been defined on the basis of combination of different mec and ccr complexes till date. The characteristics of SCCmec elements are presented in the table 2.1 (Zhang et al., 2009).

Table 2.1 Characteristics of different types of SCCmec elements

<table>
<thead>
<tr>
<th>SCCmec type</th>
<th>mec complex</th>
<th>ccr genes</th>
<th>Size</th>
<th>Other resistance determinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Class B-E</td>
<td>ccrA1B1</td>
<td>34kb</td>
<td>None</td>
</tr>
<tr>
<td>II</td>
<td>Class A</td>
<td>ccrA2B2</td>
<td>52-58kb</td>
<td>Erythromycin, spectinomycin, bleomycin, tetracycline</td>
</tr>
<tr>
<td>III</td>
<td>Class A</td>
<td>ccrA3B3</td>
<td>67kb</td>
<td>Erythromycin, spectinomycin, bleomycin, tetracycline, mercury cadmium</td>
</tr>
<tr>
<td>IV</td>
<td>Class B-E</td>
<td>ccrA2B2 or ccrA4B4</td>
<td>20-25kb</td>
<td>None</td>
</tr>
<tr>
<td>V</td>
<td>Class B-E</td>
<td>ccrC</td>
<td>28kb</td>
<td>None</td>
</tr>
<tr>
<td>VI</td>
<td>Class B</td>
<td>ccrB4</td>
<td>20-25kb</td>
<td>None</td>
</tr>
<tr>
<td>VII</td>
<td>Class C</td>
<td>ccrC2, ccrC8</td>
<td>28-30kb</td>
<td>None</td>
</tr>
<tr>
<td>VIII</td>
<td>Class A</td>
<td>ccrA4, ccrB4</td>
<td>32kb</td>
<td>Erythromycin, spectinomycin</td>
</tr>
</tbody>
</table>

2.3 Clinical manifestations of S. aureus

S. aureus has the ability to cause wide variety of disease conditions ranging from minor infections of skin to serious post-operative wound infections, bacteremia, and necrotizing pneumonia (Lowy, 2003). This organism colonises and infects both hospitalized patients as well as healthy individuals in the community. Although, S. aureus is present as commensal on the skin and nasopharynx of the human body, this organism also causes local infections of the skin,
nose, urethra, vagina and gastrointestinal tract (Shulman and Nahmias, 1972). Due to breach in
the skin and mucous membrane by trauma or surgery, the basic skin lesion caused by *S. aureus* is
pyogenic abscesses. On reaching the lymphatic channels or blood, it causes septicemia. *S. aureus*
also produces wide range of extracellular toxins and exfoliative toxins (Projan and Novick, 1997).
Toxic shock syndrome toxin-1 is responsible for toxic shock syndrome (TSS) which is caused by
the strains having the *tst* gene (Waldvogel, 1990). This syndrome is characterized by fever, rash,
diarrhoea and inability to maintain proper haemostasis. In severe cases, the disease may progress
further involving multiple organs, desquamation of skin over the entire body and death in some
instances (Todd *et al*., 1978). Another syndrome known as Staphylococcus scalded skin
syndrome (SSSS) is produced by exfoliative toxin. This syndrome is characterized by toxic
epidermal necrosis, scarlatiniform erythema which damages epidermal layer of the skin. Patients
infected with exfoliative toxin producing strains can result in endocarditis leading to reduced
cardiac output and septic embolization (Murray *et al*., 2002). *S. aureus* frequently colonizes in
many animal species and can cause conditions such as dermatitis, pneumonia, and septicemia. In
swine, it causes osteomyelitis and meningitis. In bovines, it is an important pathogen causing
mastitis (Quinn *et al*., 2000). Mastitis is inflammation of mammary gland tissue which is caused
by number of bacterial pathogens but *S. aureus* is the most important and common etiological
agent. *S. aureus* cause chronic and deep infection in mammary glands which is very difficult to
treat (Hassan *et al*., 2010). Bovine mastitis is responsible for reduced milk production, decline in
milk quality, and increased labour cost (Beck *et al*., 1992). Clinical and subclinical mastitis due
to *S. aureus* are recognised most important disease conditions which cause huge economic losses
to the dairy industry (Taverna *et al*., 2007). Economic losses due to mastitis in dairy industry
occurs worldwide (Kavitha *et al*., 2009).

### 2.4 Virulence factors and pathogenesis of *S. aureus* infections

*S. aureus* possesses a variety of virulence factors. These factors are structural as well as products
that contribute to the pathogenesis of Staphylococal infections. There are two main mechanisms
in the pathogenesis. The first one involves invasion of the host by this organism and resulting
inflammatory response. A number of events are involved in this process such as colonization of
the organism, synthesis of extracellular molecules that facilitate adherence and stimulation of
host immune response. The second mechanism involves the production of various toxins.
Exotoxins produced by *S. aureus* are responsible for host tissue damage and promotion of further
spread in the body. The biofilm production is also one of the contributing factors. Biofilm are complex aggregation of bacteria encapsulated by an extracellular matrix and usually attached to the mucosal surface of the host. The biofilm formation is of great clinical significance because bacteria in biofilm have increased resistance to environmental stress, host immunological defence and antimicrobial agents (Otto, 2008). A wide range of other virulence factors which contribute to pathogenesis include: α-hemolysin, pvl (pore forming toxins), superantigen (enterotoxins, toxic shock syndrome toxin-1), polysaccharide capsule, protein A (phagocytosis inhibitors) and immune evasion molecules, chemotaxis inhibitory proteins, staphylokinase, aureolysin etc. (Barlett and Hulten, 2010). The extracellular environment of S. aureus overcomes opsonisation by complement and antibodies. These molecules are directly or indirectly responsible for killing this organism. Opsonophagocytosis is inhibited by expression of capsule on its surface, clumping factors, protein A and number of complement inhibitors all that inactivate or inhibit host opsonins from binding or targeting the bacterium for destruction (Foster, 2005; Rooijakkers et al., 2005). Various virulence factors of MRSA are depicted through Fig. 2.2.

![Fig 2.2 Virulence factors of MRSA (Adapted from: Gordon and Lowy, 2008)](image)

S. aureus can infect and remain intact within epithelial cells, endothelial cells and even in macrophages but not in neutrophils. S. aureus secretes two molecules i.e. chemotaxis inhibitory proteins (CHIP) and extracellular adherence protein (Eap) which block neutrophil recognition of
chemotactic factors (de Haas et al., 2004) and neutrophil binding to endothelial adhesion molecule respectively (Chavakis et al., 2002). When neutrophils reach the site of infection, they release antimicrobial substances, including antimicrobial peptides, reactive oxygen species (ROS), reactive nitrogen species (RNS), protease and lysozymes. Defense against ROS in \textit{S. aureus} is mediated by deploying large number of antioxidant enzymes such as catalase, pigment superoxide dismutase which neutralize ROS and RNS (Foster, 2005). Stevens et al., 2007 investigated the effect of cell wall active antibiotics and inhibitors of protein synthesis on transcription and translation of genes for \textit{pvl}, \textit{α}-hemolysin, \textit{tst}-1 in both MSSA and MRSA strains. Subinhibitory concentration of nafcillin induced mRNA for \textit{pvl}, \textit{α}-toxin and \textit{tst}-1 which resulted in increased toxin production. Clindamycin and linezolid suppressed translation but not transcription of toxin genes. The inhibition of protein synthesis is an important criterion in the selection of antimicrobial agents to treat serious infections caused by toxin producing gram positive pathogens and by inducing and enhancing toxin production by deliberate use of \textit{β}-lactam antibiotics to treat MRSA infections would lead to poor clinical outcome (Stevens et al., 2007). Although, infection with \textit{S. aureus} was considered a major problem in hospital earlier but the incidence of community acquired infection has been on the increase during past few decades. A new mode of transmission of CA-MRSA has recently been identified. It is through currency note which is frequently transferred from one person to another. Kumar and co-workers (2009), isolated \textit{S. aureus} strains from paper currency and screened them for virulence associated genes. These workers successfully demonstrated that the paper currency can serve as carrier for the spread of resistant pathogens. Since \textit{S. aureus} has the ability to colonize healthy asymptomatic individuals, the carriers of \textit{S. aureus} are at higher risk of infection because they are presumed to be an important source for the spread of \textit{S. aureus} strains among individuals (Chambers and Deleo, 2009).

\textbf{Adherence factors (Adhesins)}

Following attachment of \textit{S. aureus} to the surface of host cell, this organism colonizes on the surfaces. The adherence is mediated by several adhesins. One of the major classes of adhesins of \textit{S. aureus} is a protein that is covalently anchored to the cell wall peptidoglycans and specifically attached to the plasma or extracellular matrix (ECM) components. This is collectively known as microbial surface component recognising adhesive matrix molecules (MSCRAMMs). The \textit{Staphylococcal} protein A (\textit{spa}), fibronectin binding protein A and B (\textit{fnbpA} and \textit{fnbpB}), collagen
binding protein and clumping factors (clf) A and B are other typical members of MSCRAMM family (Speziale et al., 2009).

**The capsule and cell wall**

Eleven types of capsular polysaccharides have been identified in *S. aureus*. The capsule inhibits phagocytosis (Lowy, 1998).

**Cell lysis and tissue invasion**

In the host cell, some toxins of *S. aureus* enhance inflammation by the formation of pores and lead to lysis of the infected cell. The pore forming hemolysins are α, β & δ (Cheung et al., 1994). α- hemolysin is cytotoxic for endothelial cells, thrombocytes and monocytes. It binds to specific receptor and stimulates several host cell signalling cascades, resulting in the release of pro-inflammatory cytokines and induction of apoptosis (Dinges and Orwin, 2000).

**Small-colony variants (SCVs)**

*S. aureus* produces small colony variants which have different phenotypic and pathogenic characters. These variants cause persistent as well as recurrent infections several years later after the initial infection (Proctor et al., 2006). The SCVs are defective in their electron transport pathways. These variants generally form non-hemolytic, non-pigmented small colonies on solid media (Kaneko and Kamio, 2004). Also, they are less virulent, but due to slow growth and reduced cell wall synthesis they can tolerate more β-lactam antibiotics than their wild type parents. The low membrane potential of SCVs makes them resistant to antibiotics of aminoglycoside group (Proctor et al., 2006).

**Exoproteins**

A group of exoprotein such as nucleases, lipases, protease, hyluronidase and collagenase are secreted by all *S. aureus* strains. These proteins convert the local host tissue into nutrients required for their growth. The cytolytic toxin forms pores in the plasma membrane which lead to lysis of the target cell due to leakage of cellular contents (Foster, 2005). Several types of cytolytic toxins are secreted by *S. aureus* e.g. hemolysin (Kaneko and Kamio, 2004). α- hemolysin become inserted into eukaryotic membrane and oligomerizes into a β-barrel which forms a pore and cause osmotic cytolysis. This toxin is responsible particularly cytolysis of human platelets and monocytes (Menestrina et al., 2001). Panton-Valentine Leukocidin (PVL) is another important cytolysin which has two components, LukF-PV and LukS-PV. This toxin inserts into plasma membrane of the host and ultimately leads to pore formation. PVL possesses
high affinity for leukocytes, while other bi-component toxins γ-hemolysin and lukocidin are cytotoxic to erythrocytes and leukocytes, respectively (Kaneko and Kamio, 2004). Toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins and exfoliative toxin constitutes another group of S. aureus toxins and are referred to as pyrogenic toxin super antigens (PTSAgs) (Lina et al., 2004). As the name suggests the superantigenicity is the important characteristic of this group. These toxins stimulate proliferation of T-lymphocytes and are responsible for toxic shock syndrome and food poisoning, while exfoliate toxins A and B are involved in staphylococcal scalded skin syndrome (SSSS) (Melish and Glasgow, 1970). Other protein such as staphylococcal component inhibitors (SCIN), chemotaxis inhibitory protein of S. aureus (CHIPS), staphylokinase (SAK), extracellular fibrinogen binding protein (Efb), extracellular adherence proteins (Eap) and formyl peptide receptor like-I-inhibitory protein (FLIPr) are also involved in modulation of the immune response. SCIN is C3 convertase inhibitor, which blocks the formation of C3b on the surface of S. aureus, therefore the ability of human neutrophils to phagocytose the bacteria (Rooijakkers et al., 2005). CHIPS and FLIPr block neutrophil receptor for chemoattractant (Prat et al., 2006). Epa blocks migration of neutrophils from blood vessels to the tissue (Chavakis et al., 2006). SAK binds to α-defensins which abolishes their bactericidal activities. Efb inhibits activity of both classical and alternative pathways of the complement system (Bokarewa et al., 2006). The enterotoxin A is produced by MRSA but not by MSSA strains. Hemolysin α, β & δ are produced by almost all strains at similar level but MRSA produced significantly more coagulase than MSSA.

**Superantigenic exotoxins**

Nineteen S. aureus superantigenic exotoxins have been described which include: one TSST-1 and 18 staphylococcal enterotoxins (SE) A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, R & U.

**2.5 Resistance of S. aureus to antimicrobial agents**

The development of resistance to antimicrobial agents in S. aureus presents difficulty in treating infections due to resistant strains. A number of factors are associated with this development e.g. suboptimal use of antimicrobials for prophylaxis and treatment of infection, non compliance with infection-control practices, prolonged hospitalization, increased number and duration of stays in intensive care-units, increased use of invasive devices and catheters, ineffective infection-control practices, transfer of colonized patients from hospital to hospital, grouping of colonized patients in long-term-care facilities, use of antibiotics in agriculture and household tasks, and increase in
national and international travels. The level of antibiotic resistance is dependent on the following: the population of organism that spontaneously acquires resistance mechanism as a result of selective pressure either from use of the antibiotic or otherwise, the rate of introduction from the community of resistant organism into healthcare settings. Several biochemical mechanisms may be involved for the development of resistance: the presence of an enzyme that inactivates the antimicrobial agent; the presence of an alternative enzyme that is inhibited by the antimicrobial agent; mutation in the antimicrobial agent’s target site which reduces the binding of the antimicrobial agent; post-transcriptional or post-translational modification(s) of the antimicrobial agent’s target which reduces binding of the antimicrobial agent, reduced uptake of the antimicrobial agent i.e. active efflux of the antimicrobial agent and; overproduction of the target of the antimicrobial agent, expression or suppression of a gene in vivo (Aarestrup et al., 2001).

2.5.1 Mechanism of action of β-lactam antibiotics

β- lactam antibiotics include penicillins, cephalosporins, methicillin and oxacillin. These antibiotics are bactericidal and act on the cell wall and target the transpeptidation step of the peptidoglycan synthesis by inactivating and binding the transpeptidase domain of penicillin binding proteins (PBPs) in the cell wall (Chambers, 2004). β- lactam binds with the PBP non-covalently. This association may either separate or undergo irreversible reaction of acylation when PBP covalently binds the antibiotic at its active site and cleave the cyclic amide bond in the β-lactam ring. The natural substrate for PBP undergoes sudden deacylation by hydrolysis that liberates the PBP for a next round of transpeptidation. On the other hand, when substrate is β-lactams antibiotic, the deacetylation process is extremely slow and the PBP is effectively inactivated. Functional PBPs, cell wall synthesis is inhibited and cell death occurs (Chambers, 2003). Being located outside the cytoplasmic membrane, targets of β- lactams are easily accessible. Also, the drug targets are specific to bacteria (Wilke et al., 2005).

β- lactamases also contribute to the resistance to β-lactam by inactivating several antibiotics in a reaction similar to the β-lactams which inhibit PBPs. Four different types of β-lactamases in S. aureus have been described until now depending upon the sequence similarity. These enzymes differ in their substrate specificity (Zygumnmt et al., 1992). β- lactamase is encoded by β-lactamase gene, blaZ which is carried on plasmid located on transposone. The binding of β-lactamase to β-lactam, results in the formation of an acylated intermediate, but in contrast, the
reaction catalyzed by PBPs during peptidoglycan biosynthesis, resolution of the acylated intermediate results in the cleavage of the amide bond of the β-lactam ring. The inactivated β-lactam antibiotic and active β-lactamase are released. The expression of the \textit{blaZ} gene is induced by the presence of β-lactam antibiotics through a regulatory system composed of a repressor, \textit{Bla-I}, and a signal transducer, \textit{Bla-RI}. These are located in a two-gene operon that is divergently transcribed from \textit{blaZ}. A membrane protein \textit{BlaRI} which is composed of an extracellular sensor domain is acylated by β-lactam antibiotics, both side membrane domain that transduces the signal across the membrane and the intracellular zinc metalloprotease domain which is proteolytically activated upon acylation of the sensor domain. \textit{BlaI} repressor forms a homodimer which binds to the operator region of the operon encoding \textit{BlaI} and \textit{BlaRI} and containing the β-lactamase structural gene (\textit{blaZ}). Homodimer formation by \textit{BlaI} repressor that binds to operator region of the operon encoding \textit{BlaI} and \textit{BlaRI} and contains the β-lactamase structural gene (\textit{blaZ}). After binding of β-lactam antibiotic to the sensor domain of \textit{BlaRI}, its metalloprotease domain undergoes autocleavage, following cleavage of \textit{BlaI}, which prevents its dimerization and leads to derepression of \textit{blaZ} transcription (Zhang \textit{et al.}, 2001).

Beside, β-lactam antibiotics, some semi-synthetic β-lactamase-insensitive β-lactams such as methicillin, oxacillin and nafcillin are also used for treating \textit{S. aureus} infections. As discussed earlier, the development of resistance in \textit{S. aureus} to this class of β-lactam has been attributed to the acquisition of the \textit{meca} gene located on the SCCmec element. \textit{S. aureus} strains which contain this gene are known as methicillin resistant \textit{S. aureus} (MRSA). The \textit{meca} gene encodes an alternative penicillin binding protein PBP2a, which has low affinity for β-lactams. The PBP2a belongs to a group of high molecular mass (78 kDa) family of PBPs and consists of a transpeptidase domain and a non-penicillin binding domain of unknown function. The protein PBP2a possesses low affinity for β-lactams which allows MRSA strains to grow in antibiotic concentrations and to inactivate all native PBPs (Gaisford and Reynolds, 1989). To a certain extent PBP2a appears a proactive enzyme as compared to other native PBPs which synthesized highly cross-linked peptidoglycan (de Jonge and Tomasz, 1993). Even when the transpeptidase activity of all native PBPs is inhibited in the presence of methicillin, PBP2a has shown its effect on the transglycosylase, β-lactam-insensitive domain of the native PBP2 to confer resistance (Pinho \textit{et al.}, 2001).

\textbf{2.5.2 Quinolone resistance}
Quinolones were introduced in the year 1980 for treating infections due to some gram negative bacteria and later due to pneumococci and staphylococci. The emergence of quinolone resistance among MRSA strains occurs quickly and more prominently. For this reason, the significance of fluoroquinolones as anti-staphylococcal agents has been reduced. The resistance to quinolones occurs due to spontaneous chromosomal mutations in the target of the antibiotic, topoisomerase IV or DNA gyrase, or by the induction of a multidrug efflux pump. Hoibyet et al., (1997) demonstrated that therapy with ciprofloxacin quickly increased the percentage of CoNS strains colonizing the nares and skin which were resistant to both ciprofloxacin and methicillin. As S. aureus is a part of normal flora, similar selection process may apply for S. aureus also. A mechanism of resistance to quinolones is a stepwise acquisition of chromosomal mutations (Hooper, 2002). The quinolones act on DNA gyrase, which relieves DNA supercoiling, and topoisomerase IV which separates concatenated DNA strands. Amino acid changes in the critical regions of the enzyme DNA complex (quinolone resistance determining region [QRDR]) reduce quinolone affinity for both of its targets. The accumulation of the resistance mutation in QRDR sets might be responsible for increased resistance. The increased expression of NorA multidrug resistance efflux pump in S. aureus can also result in low-level quinolone resistance (Ng et al., 1994).

2.5.3 Vancomycin resistance
Resistance to vancomycin was first observed in a clinical isolate of S. hemolyticus (Schwalbe et al., 1987). Due to increased use of vancomycin for the treatment of MRSA infections, enterococcal infections preceded the emergence of vancomycin-resistant staphylococci (Kirst, 1998). Vancomycin intermediate resistant S. aureus (VISA) were first reported from Japan in 1997 and later on from other parts of the world (Smith et al., 1999). The VISA isolates were all MRSA. The VRSA and VISA infections differ in their degree of resistance as well as mechanism of discrimination. VRSA strains acquire resistance by conjugal transfer of vanA from Enterococcus faecalis, which is more efficient means for dissemination of resistance gene among strains of Staphylococcus but in VISA resistance is chromosomally mediated. Also, the resistance is complete in VRSA whereas, its level is low and variable in VISA. Hiramastu et al. (1997) reported a minimum inhibitory concentration (MIC) of 8-16 µg/ml of vancomycin against some VISA strains. The reduced susceptibility to vancomycin results from changes in peptidoglycan biosynthesis. In VISA strains, it is noted that additional quantities of
peptidoglycan have been synthesized which result in thickening of cell wall. Also, there is
decreased cross-linking of peptidoglycan strands. This leads to the exposure of more D-Ala-D-
Ala residues (Hanaki et al., 1998). As a result, there are more D-Ala-D-Ala residues available to
bind and trap vancomycin. The bound vancomycin then acts as a further barrier to drug
molecules reaching their target on the cytoplasmic membrane. The VRSA isolates express
complete vancomycin resistance, with MICs of ≥128 µg/ml. Resistance in these isolates is
caused by alteration of the terminal peptide to D-Ala-D-Lac instead of D-Ala-D-Ala. Synthesis of
D-Ala-D-Lac occurs only with exposure to low concentrations of vancomycin (Hiramastu et al.,
2004).

2.6 Epidemiology

*S. aureus* is ubiquitous in nature. Coagulase negative *Staphylococci* (CoNS) colonize on the
skin, especially moist skin folds and perineal area of neonates. *S. aureus* and CoNS are also
found in the nasopharynx, oropharynax, gastrointestinal tract and urogenital tract. Approximately 15% of healthy adults carry *S. aureus* in nasal cavity (Murray et al., 2002). Most
common hospital acquired infections include; bacteremia, surgical wound infections, urinary
tract infections and pneumonia (Fischetti et al., 2000). Due to high risk areas in hospitals like
newborn nursery, intensive care unit, cancer chemotherapy wards and operation theatre, introduction of epidemic pathogenic *S. aureus* may lead to serious clinical illness (Brooks et al.,
2001). Several outbreaks associated with hospital settings have been reported from Taiwan
(Wang et al., 2001). More than 50% outbreaks have been reported in Asian countries (Joshi et al.,
2013), while 25% prevalence has been recorded from other countries including South Africa,
Brazil, Australia, US, Norway and Sweden (Borg et al., 2007). The global prevalence of hospital
acquired MRSA is presented in Fig. 2.3a. However, the higher prevalence (> 50%) in Asian
countries is given in the Fig. 2.3b.

2.6.1 Methicillin-resistant *Staphylococcus aureus* (MRSA)

The first penicillin-resistant *S. aureus* strain was isolated in a hospital in 1942. During early
1950s and 1960s, the resistant strains became pandemic in the community (Chambers and De
Leo, 2009). Most *S. aureus* strains have now become resistant to this drug. *S. aureus* strains also
developed resistance towards methicillin and vancomycin by the acquisition of *meca* and *vanA*
genesis respectively (Lowy, 2003). These strains are not only resistant to methicillin but also to
other β- lactam antibiotics as well other antibiotic classes (Blanc et al., 2007). Out break of
MRSA infections were reported from European hospitals during early 1960, subsequently MRSA clones were spread in health-care institutions world over. MRSA is responsible for 25% of nosocomial infections in the US. The community acquired MRSA infections are also on the increase (Boyce et al., 1994). MRSA is responsible for 29% of nosocomial infections in some medical institutions in New York with 50% of associated deaths (Rubin et al., 1999). Not only MRSA has been associated with mortality but MSSA also resulted in high mortality. The mortality rate of 36% has been observed due to MRSA whereas 24% mortality was due to septicemia caused by MSSA. The overall mortality rate with bacteremia caused by MRSA continues to rise in entire population as coagulase positive Staphylococci became resistant to methicillin across the world. The MRSA infections result in serious sequel as compared to MSSA despite the use of appropriate chemotherapy (Gonzalez et al., 1999; Blot et al., 2002). Transmission takes place by direct contact with a colonized carrier, through injection by drug users, insulin dependent diabetic patients, those with dermatological conditions and patients with long term intravascular catheters constitute high risk group (Wadlvogel, 2000). Young children are more prone to higher colonization rate because of their frequent contact with respiratory secretions (Adcock et al., 1998). MRSA strains confined to hospital earlier are now emerging in the community. The indiscriminate use of antibiotics is the main reason for this emergence as these agents not only enhance overgrowth but their pathogenicity is also increased (Dancer, 2008). Due to evolutionary changes and epidemiological expression of the MRSA strains, new MRSA strains have now emerged which differ from the initial isolates. The frequency of MRSA has been on the rise continuously in hospital setting and now incidences continue to grow in community setting in United States and globally. The reason for such a trend can be explained on the basis that S. aureus has the ability to adapt to the environment and also due to advances in patient care in the hospitals. As compared to nosocomial infection, the community associated MRSA strains can cause higher morbidity and mortality since person to person transmission has been also reported in CA-MRSA infection (Boucher and Corey, 2008). The knowledge of circulating MRSA clones under the prevailing scenario can be helpful in controlling transmission and optimizing treatment (Dauwalder et al., 2008). MRSA as nosocomial human pathogen first reported in hospitals has become a matter of great concern after 1990s for those individuals who had not been hospitalized but undergone invasive procedure. The infection with such MRSA strains were referred to as community-acquired MRSA (Tenover and Goering, 2009; Otter and
French, 2010). CA-MRSA first appeared in high risk populations such as intravenous drug users, patients in nursing homes, chronically ill person, but has also been reported in healthy children (Tenover and Goering, 2009). These strains were susceptible to many antibiotics except beta-lactams until recently but now the resistance to multiple antibiotics is on increase (CDC, 2005). Some epidemic strains which are more prevalent tend to spread within or between hospital and countries, other sporadic strains do not spread so widely. The clonal lineages of \textit{S. aureus} have tendency to colonize specific species and may adapt either human or animals. Extended host spectrum genotypes have appeared which have different lineages, host specificity and tendency to infect wide range of species (Cuny et al., 2010). For example MRSA ST22-IV, EMRSA15 have been reported in pigs (rarely), turtles, bats, cats, dogs, and humans (Van de et al., 2009). The presence of \textit{mecA} genes defines MRSA. Some researchers define MRSA depending upon antibiotic susceptibility testing (Van Duijkeren et al., 2004). In US hospitals, most commonly identified antibiotic resistant pathogen is MRSA (Diekema et al., 2004) and 25.9% of \textit{S. aureus} strains isolated from out patients were methicillin resistant (National infectious surveillance system report, 2003). Most of these strains were isolated from persons who acquired them from healthcare environment (Tacconelli et al., 2008). A genetic evolutionary analysis demonstrated that the \textit{mecA} gene has been transferred into methicillin-susceptible \textit{S. aureus} (MSSA) more than 20 times and emerged in more than 5 phylogenetically different lineages as well as re-emerged within individual lineages (Hiramastu, 2004 ). The introduction of the \textit{mec A} gene from putative donor species into MSSA strains which are already adapted successfully to the hospital environment and to the community, created successful epidemic HA-MRSA and CA-MRSA clones (Baba et al., 2002). It is believed that ancestral MRSA genotypes, ST250- MRSA originated in Denmark which possesses SCCmec type I, were recovered in the 1960s. This genotype arose as it acquired the \textit{mec} gene by ST250-MSSA, which itself had arisen from ST8-MSSA by chromosomal point mutation ((Enright et al., 2002). ST250-MRSA evolved by a single point mutation, an important hospital pathogen in Europe and has been reported to cause outbreaks in New York hospital (Roberts et al., 1998). The emergence of CA-MRSA strains has repeatedly occurred as a result of the introduction of SCCmec type IV into a different genetic MSSA background (Okuma et al., 2002). MDR usually seen in HA-MRSA strain and antibiotic resistance is limited to beta-lactams in CA-MRSA. The size of SCCmec type IV is small which prevents its carriage of additional genetic material, in contrast to the characteristic presence of
additional genetic material in SCC\textit{mec} type II and SCC\textit{mec} type III (Robinson and Enright, 2004).

2.6.1.1 Hospital acquired methicillin-resistant \textit{S. aureus} (HA-MRSA)

CDC has laid down some criteria for defining HA-MRSA; when a patient is diagnosed with MRSA infection within 48 hours of hospitalization, it is regarded as a case of HA-MRSA infection. If the patient lacks HA-MRSA risk factors like surgery, hemodialysis, resides in long term health care facility or hospitalized within the preceding years or the presence of catheter at the time of culture, it is regarded as CA-MRSA (Morrison \textit{et al}., 2006). The other criteria to differentiate HA-MRSA and CA-MRSA strains include: differences in their antibiotic sensitivity pattern (David & Daum, 2010). Soft tissue infections are the most frequent clinical manifestations of CA-MRSA. The mechanism of methicillin-resistance is same in both HA-MRSA and CA-MRSA but susceptibilities to non-\(\beta\)-lactam antibiotics often differ as CA-MRSA exhibits broader antibiotic susceptibility than HA-MRSA (Louis and Rice, 2006). French \textit{et al}., 1990 reported that strains of methicillin-resistant and methicillin-sensitive \textit{S. aureus} in Hong Kong had similar virulence and equally pathogenic in animal model but some researchers believe that MRSA are relatively a virulent opportunist and their importance has been overstated.

The HA-MRSA infections are continuously on the rise. In a span of one year (1999-2000) 1.25 million patients were hospitalized for \textit{S. aureus} infections such as blood stream infection, pneumonia and 43.2% of the recovered isolates were found resistant to methicillin (Kuehnert \textit{et al}., 2005). In a surveillance program in the US for nosocomial blood stream infections, MRSA isolates increased from 22% in 1995 to 57% in 2001 (Wisplinghoff \textit{et al}., 2004). Similarly, hospital associated \textit{S. aureus} infections were 64.4% in 2003 in ICUs which were caused by MRSA as compared with 35.9% in 1992, (Kleven\textit{ts et al}., 2006).

2.6.1.2 Community associated methicillin-resistant \textit{S. aureus} (CA-MRSA)

CA-MRSA outbreaks have occurred in wide range of groups which include, professional football players (Kazakova \textit{et al}., 2005) and soldiers (Ellis \textit{et al}., 2009). Poor personal hygiene and close body contact have been considered as pre disposing factors (Turabelidze \textit{et al}., 2006). It has been hypothesized that asymptomatic pet dogs might have been the source of humans acquired MRSA infections but there is limited data to support this. One possibility is that, these are descendants of HA- MRSA. In penicillinase–mediated resistance disseminated strains from hospital with a horizontal transfer of the penicillinase gene into sensitive recipient strain
contributed to the emergence of a penicillin resistant strain in the community. Penicillinase is plasmid encoded and transferred by transduction and conjugation. The etiological bacteria was MW2 strain of community

**Fig 2.3 a.** Global prevalence of MRSA (Modified from [http://www.targetmap.com](http://www.targetmap.com))

**Fig 2.3 b.** Prevalence of hospital acquired MRSA (HA-MRSA) in Asian countries. (Showing higher prevalence have been labeled) ([www.landinscan.com](http://www.landinscan.com))
associated MRSA (CA-MRSA), which acquired SCCmec type IV, the \textit{S. aureus} pathogenicity island SaPI3 and bacteriophage Sa2 in its evolution from MSSA476 (Baba \textit{et al.}, 2002). The emergence of CA-MRSA has posed a serious threat to individuals in both community and hospital environment since CA-MRSA are more virulent as compare to HA-MRSA (Etienne, 2005). CA-MRSA strains have started to replace HA-MRSA In healthcare system (Seybold \textit{et al.}, 2006). The association of MRSA infections also has been linked to longer hospital stay which result in increased cost of healthcare system than MSSA infections (Cosgrove \textit{et al.}, 2005).

\textbf{2.7 Bacteriophage Typing}

Phage typing is a conventional epidemiological tool which was first employed for typing \textit{S. aureus} strains in England as early as 1940. This method is widely used even nowadays since it is considered as an ideal method for typing \textit{S. aureus} strains. In combination with other molecular methods, phage typing is used to determine variations among the strains which can be correlated to epidemic strains. In this manner, the significance of spread of different strains in the community and the pathological conditions caused by them can be fully evaluated. Horizontal gene transfer plays a very important role in evolution of bacteria. About 20\% of the extra genetic content of any given bacterial species has been acquired from other organisms by means of mobile genetic elements (MGE) such as bacteriophages, insertion sequences (IS), plasmids, and transposons (Novick \textit{et al.}, 2010). Bacteriophages through horizontal gene transfer and lysogenic phage conversion can convert a non-virulent strain of \textit{S. aureus} to a virulent strain (Boyd \textit{et al.}, 2002). Prophage incorporation into \textit{S. aureus} results in the enhanced ability of the bacteria to colonize the host tissue by ecological adaptation, evasion from the immune system and acquisition of virulence factors (Vojtov \textit{et al.}, 2002).

\textbf{2.8. Molecular diagnosis and typing of \textit{S. aureus} strains}

Both phenotypic and genotypic methods have been used for detecting and typing of \textit{S. aureus} strains. The phenotypic methods, in general are simple, easy to perform, cost effective but less discriminatory as compared to genotypic methods. The latter however, technically more demanding, costly and more discriminatory. Also, it is possible to classify microorganism upto the species level with the help of genotypic methods (Mehndiratta \textit{et al.}, 2012). The utilization of competent and perfect epidemiological typing methods is prerequisite for monitoring and for
restricting the incidence and spread of epidemic clones within and between hospitals. So, typing system must discriminate between unrelated isolates. Also the isolates belonging to the same clonal lineage can be identified in order to determine whether epidemiologically related isolates have genetic relatedness or not (Singh et al., 2006). The criteria which form the basis of a good typing system include: typeability, reproducibility, stability, structural simplicity and epidemiological concordance i.e. obtaining similar results in different epidemiological studies with a given typing system (Struelens et al., 1996). The typing ability refers to the ratio of isolates which can be scored in a typing system and assigned to a particular type. Reproducibility refers to the ability of the typing system to assign the same type on repeat testing of the same strain. Stability is the biological features of clonally derived isolates to express constant markers over time and generations. Discriminatory power of typing system is defined as the average possibility that different genotypes will be assigned to two unrelated strains in the population of a given genus and can be calculated by using the formula of Simpson’s index of diversity (Hunter and Gaston, 1988). A phenotypic method utilizes: biochemical profiles, antimicrobial susceptibility profiles, bacteriophage types, and antigens present on the cell surface etc. for typing isolates. Genotypic strain typing methods are based on the analysis of differences in the chromosomal and extra chromosomal nucleic acid sequences between strains (Tenover et al., 1997). With reference to staphylococci, phenotypic methods include: testing growth and biochemical characteristics by conventional or commercial identification sets, biotyping (Devriese, 1984), phage typing (Blair and William, 1961) and whole-cell protein electrophoresis (Clink and Pennington, 1987). Genotypic methods focus on the characterization of chromosomal, plasmid or total genomic DNA extracted from a bacterial pathogen. The indirect genotypic methods include techniques either without DNA amplification (restriction analysis and DNA hybridization) or with DNA amplification by PCR and identifying conformation polymorphism of nucleic acids (Struelens, 1998). Different molecular approaches have been used for better understanding the epidemiology of S. aureus (Willems et al., 2011). Ease of interpretation and simplicity of the method are the key issues for many techniques, so that a particular technique can readily be adopted.

2.8.1 Genotypic methods

The important genotypic methods which are employed for typing of S. aureus isolates are discussed below:
**Restriction endonuclease analysis (REA)**

By this method, the genomic DNA extracted from isolate is digested with restriction enzymes and the fragments thus, generated are separated by agarose gel electrophoresis and visualized in ethidium bromide stained gels under a u.v. transilluminator. Restriction enzymes or restriction endonucleases PstI, EcoRI and PvuII have been used in Staphylococci (Etienne et al., 1990). Different strains of same species can have different REA profiles because of variation in their DNA sequences (Jordens and Hall, 1988). Another method known as small fragment restriction endonuclease analysis (SF-REA) has also been used. This method is very similar to REA but differs only in that polyacrylamide gel electrophoresis (PAGE) is used for separation of restriction fragments in SF-REA rather than agarose gels. DNA fragments of smaller molecular mass of < 1 kb can be analysed by this method. Several restriction enzymes like EcoRI, BamHI and HindIII are used for diagnosis and the resulting bands can be visualized by silver staining. SF-REA method has been used for the typing of *S. aureus* strains because of its high discriminatory power than conventional REA on agarose gel (Tveten et al., 1991).

**Southern blot analysis of RFLP and ribotyping**

In this technique, restriction fragments generated by digestion of DNA with restriction enzymes are separated by gel electrophoresis, transferred onto nitrocellulose membranes and their identity established by hybridisation with labeled DNA probes. Variations in terms of number and size in fragments often referred to as restriction fragment length polymorphism (RFLP) can be used for strain differentiation. This method is complex and patterns obtained are sometimes difficult to analyse (Schlichting et al., 1993). The most commonly used probe is 16s rRNA (ribosomal RNA) because most species have more than one chromosomal rRNA. This technique is known as ribotyping. The probes used in this technique are either labeled with radioisotopes ($^3$H, $^{125}$I, $^{32}$P-ATP) or non-radio isotopic probes (biotinylated, digoxigenin labeled) can be used. EcoRI has been found to be more useful as compared to other restriction enzymes because this enzyme produces fairly large number of bands. This technique is however, time consuming but reproducible and possesses comparatively lower efficiency as compared to PFGE in differentiating MRSA strains (Prevost et al., 1992).

**Pulse field gel electrophoresis (PFGE)**
Pulse field gel electrophoresis is based on the digestion of bacterial DNA with restriction enzymes which recognize few sites along the chromosome, larger DNA fragments (30-800Kb) are generated in the process. This method is widely accepted method for typing MRSA strains and is modification of conventional agarose gel electrophoresis in which direction of the electrical field across the gel is changed from time to time, which facilitates separation of large fragments with respect to size, overlapping of fragments. Restriction enzyme SmaI has been widely used for analysis of MRSA strains by PFGE. Although this technique is highly reproducible and discriminatory because it can type all strains, it has certain disadvantages too. The method is technically more demanding, cost of reagents and equipments is high and the time required to perform the test is more (Bannerman et al., 1995). However, this technique is considered as “gold standard” for typing MRSA strains in order to achieve internationally comparable results (Cookson et al., 1996).

**Binary typing (BT)**

Binary typing of DNA is based on hybridization of genomic DNA with southern type hybridization and strain differentiating DNA probes. Strains from different geographical regions and diverse origins can be genotyped using strain specific DNA probes. These probes are produced by random amplification of chromosomal DNA followed by cloning (Shopsin et al., 2001). Specificity of probes is validated by testing against several MRSA isolates. Toxin genes, antibiotic resistance genes, SCCmec loci and phage derived open reading frames can be used as binary targets. This technique also has been used for studying clonal relationship among bovine MRSA strains and their routes of transmission (Zadoks et al., 2000).

**Plasmid profiling and restriction enzyme analysis of plasmids (REAP)**

Plasmids are variable components of many *Staphylococcus* genomes. Four classes of plasmids are responsible for antibiotic resistance (Novicks, 1990). For typing MDR-MRSA isolates, intact plasmid DNA is separated by agarose gel electrophoresis (Hall et al., 1989). Plasmid profiling is an important epidemiological tool. In REAP, the isolated plasmid DNA is digested separately with HindIII and EcoRI. Combination of these two enzymes is used for typing of *S. aureus*. The digest is then electrophoresed on agarose gel and pattern of restriction fragment for each enzyme is determined. The REA profiles of strains are then compared (Tenover et al., 1994). This method can be used for detecting a gene encoding antibiotic resistance using specific probe (Hartstein et al., 1995). Due to instability of the plasmid, the method shows moderate
reproducibility (Tenover et al., 1994), and additional typing method is therefore required for analysis.

2.8.2 Polymerase chain reaction (PCR) - based typing methods.

PCR based methods have played an important role in the diagnosis and epidemiology of infectious diseases. Three basic procedures are followed for analysis: i Polymerase chain reaction (PCR), ii the PCR-RFLP and iii sequencing of nucleotides of the genome or amplicons. PCR methods are simple and rapid for discriminating MRSA strains (van Belkum et al., 1993). Among these methods, arbitrarily primed- polymerase chain reaction (AP-PCR) or random amplified polymorphic DNA (RAPD) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) are valuable for typing of MRSA strains. These methods are discussed below:

Arbitrarily primed-polymerase chain reaction (AP-PCR) and random amplified polymorphic DNA (RAPD)

AP-PCR is based on the use of small oligonucleotide primers (8-12 oligomers) with an arbitrary sequence under less stringent PCR conditions (Welsh and McClelland, 1990). This technique is useful in screening the outbreak strains rapidly. This method, however, cannot be used as a reference method for typing MRSA strains (van Belkum et al., 1995) but it is comparatively less efficient as compared to typing of MRSA strains by PFGE (Saulnier et al., 1993).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP allows differentiation of strains. This method is simple, rapid and reproducible and only reasonably discriminatory. The coagulase gene (coa) RFLP and the staphylococcal protein (spa) gene RFLP have been broadly used to distinguish MRSA strains (Mehndiratta et al., 2009).

PCR-RFLP of Coagulase (coa) gene

The coagulase gene consists of three different regions i. the N-terminus containing the prothrombin- binding site, ii central region which is highly conserved and iii C-terminal region composed of 81-bp tandem repeat units each encoding 27-amino acid residues. The C-terminal repeated units comprised of four, five, six, seven or eight units of 81-tandem repeats (Schwarzkope et al., 1994). It is for this reason that the size of 3′ region of coa gene is variable in different MRSA strains. The heterogeneity in the number of tandem repeat units of the coa gene offers a potential target for typing of MRSA (Stranden et al., 2003). Using a panel of 10 antimicrobial agents, these workers obtained nine different biotypes out of which biotypes -1 and
accounted for 44.2% and 35.6% respectively. Coagulase gene typing of MRSA strains produced four different genotypes I, II, III and IV. PCR-RFLP generated four patterns A, B, C and D with AluI digested PCR products (Watanabe et al., 2005). Kobayashi et al., 1995 examined 240 isolates out of which 210 MRSA and 30 MSSA by AluI restriction enzyme digestion of PCR amplified 3'- end region of the coa gene including 81-bp repeated units. Himabindu and coworkers (2009) amplified the repeated units encoding hyper variable regions of the coa gene of S. aureus. Based on RFLP patterns, these workers classified the strains into 31 and 21 genotypes.

**PCR-RFLP of Staphylococcal proteinA (spa) gene**

A fairly good correlation has been observed between the clonal grouping of MRSA spa typing and other typing techniques (Koreen et al., 2004; Tang et al., 2000). Strommenger and co-workers (2008) determined the value of spa typing in combination with BURP (based upon repeat pattern) grouping analysis an excellent tool for national and international surveillance of S. aureus as well as for analysis of the short term epidemiology. It has been suggested that the number of 24-bp tandem repeat unit along the x-region of spa gene correlates with the virulence level of the strains. Mehndiratta et al., in 2009 characterized MRSA strains by molecular typing based on polymerase chain reaction-restriction length polymorphism (PCR-RFLP) of spa gene and demonstrated that spa genotyping was more efficient over bacteriophage typing in the discrimination of the strains. Bacteriophage typing is subjective and at times the strains are nontypeable. This needs alternate techniques such as spa typing to further distinguish these nontypeable MRSA strains. Walker et al., in 1998 analysed isolates of MRSA with known PFGE and bacteriophage types and observed that PCR-RFLP was additionally discriminatory and provided subtyping information.

**2.8.3 Direct DNA sequence analysis-based typing methods**

DNA sequence analysis is an objective genotyping method is highly portable, easily stored and can be analyzed in a rational database. Two different strategies are used i.e. Single locus sequence typing (SLST) and Multi locus sequence typing (MLST).

In *staphylococci*, most frequently ribosomal RNA (16S or 23S r RNA) genes are sequenced for identification (Sasaki et al., 1997). Since these genes have highly conserved nucleotide sequences, therefore, can be used for determining phylogenetic relationships (Takahashi et al., 1999).
Single-locus sequence typing (SLST)
This method is used to compare sequence variation of a single target gene. The selection of genes is generally of short sequence repeats (SSR) regions which are adequately polymorphic. Genes for proteinA \((\text{spa})\) and coagulase \((\text{coa})\) in MRSA strains having 24bp and 81bp tandem repeats which can be used to differentiate MRSA strains by analyzing the number of repeat sequence numbers within the x-region of \(\text{spa}\) genes (Tang \textit{et al.}, 2000).

Multi-locus sequence typing (MLST)
MLST is useful for studying clonal evolution of MRSA. This technique is based on the measurement of DNA sequence variation in a set of housekeeping genes (generally seven genes) whose sequences are unnatural because of the essential function of the protein encoded by them (Maiden \textit{et al.}, 1998). These genes are; \(\text{arcC, aroE, glpF, gmK, pta, tpi}\) and \(\text{yqiL}\). Diverse sequence of each house keeping gene is assigned as distinct allele, and each MRSA strain is denoted by the alleles of these genes (Enright \textit{et al.}, 2000).

Staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}) typing
Eight main types of SCC\textit{mec} (type I to type VIII) are well-known among MRSA strains. Each SCC\textit{mec} type encodes for resistance to different antibiotic (Zhang \textit{et al.}, 2009). It is well documented fact that healthcare associated-MRSA (HA-MRSA) strains contain type I, type II and type III SCC\textit{mec} cassette (Arakere \textit{et al.}, 2005). A combination of SCC\textit{mec} typing and MLST is recommended for reliable typing for multicentre surveillance, inter hospital and international transmission and further evaluation of MRSA strains (Struelens \textit{et al.}, 2009).

Toxin gene profile typing
The toxin gene profile of the strains can be used an important epidemiological marker for typing of MRSA strains. Several toxins are produced by MRSA strains which include: toxic shock syndrome toxin-1 (TSST-1), enterotoxins and exofoliative toxin. The genes for enterotoxins are carried by Staphylococcal pathogenicity islands and the toxin genes for Panton Valentine Leucocidin (PVL) are carried on bacteriophages which can easily be exchanged between strains of different lineages. The MRSA strains possess more toxin genes than MSSA strains. MRSA strains recovered from different geographical areas have shown to posses different toxin gene profiles (Yongwe \textit{et al.}, 2007). A strong correlation exists between toxin gene profiles and HA-
MRSA strains (Kim et al., 2006). Multiplex PCR technique is considered useful technique for toxin detection in MRSA. This method is a valuable for studying chromosomal diversity and evolutionary history of MRSA strains (Sharma et al., 2000).

2.9 Prevention and control of S. aureus infections

Staphylococcal infections have been responsible for high morbidity and mortality. Antibiotic therapy provides a selective advantage for such threatening infections, particularly within healthcare settings where antimicrobial therapy is most frequently used. Other control measures include; hygiene which includes patient’s care. Several approaches have been adopted for successful prevention and control of S. aureus infections and are discussed below:

2.9.1 Treatment of S. aureus infections with antibiotics

As discussed in section 2.5, MRSA strains have emerged as serious pathogens. Such strains are often multidrug resistant and MRSA infections do not respond to antibiotics. Consequently, treating such infections becomes very difficult. Glycopeptides such as vancomycin are widely used as drug of choice for serious MRSA infections. MSSA infections are not much challenging because wide range of antibiotics can be used for treating them, especially β-lactam antibiotics such as penicillins with β-lactamase inhibitors, e.g. amoxicillin/clavulanic acid or narrow-spectrum izoxazolill penicillins resistant to staphylococcal β-lactamases (cloxacillin). Moreover, MSSA strains seem to be susceptible to other groups of antibiotics, aminoglycosides (gentamycin, amikacin), fluorochinolones (ciprofloxacin, levofloxacin), lincomamides (clindamycin), macrolides (erythromycin), tetracyclines (doxycycline, minocycline) and glycopeptides (vancomycin, teicoplanin) (Gilbert et al., 2011). Currently, three recently introduced antibiotics are being used routinely for successful treatment of HA-MRSA infections: linezolid of the oxazolidinone group; quinupristin/ dalfopristin of streptogramin group and tigecycline from the glyyclycline group (Gilbert et al., 2011).

2.9.2 Alternative therapies for the treatment of S. aureus infections

Natural substances from bacteria, plants and animals which have antibacterial activity can be used as alternatives/adjunct to the antibiotics after their proper evaluation.

2.9.2.1 Antibacterial compounds derived from bacteria

S. aureus and S. epidermidis produce staphylococcins such as bacteriocins, antimicrobial peptide etc. Bacteriocins (auricin A53 and Pep5) produced by S. epidermidis exhibited wide spectrum of inhibitory activity (Nascimento et al., 2006). Gram-positive bacteria from the Lactococcus genus
produce polycationic peptide bacteriocins (lantibiotics) which are active against both MRSA and MSSA strains. *In vitro* experiments suggest that the future, bacteriocin preparations could be applied topically for treating *S. aureus* infections since they do not affect the host tissues (De *et al.*, 2009). Lysostaphin is an enzyme which was originally isolated from *S. simulans* it has the potential of being anti-MRSA agent. This enzyme, glycine endopeptidase has the ability to specifically cleave the cross-linking pentaglycine bridges in the cell wall of *S. aureus*. Lysostaphin is used in the form of a cream at 0.5% concentration. When compared to 2% mupirocin or 5% nisin this cream proved more effective (Kokai-Kun *et al.*, 2003). Dajcs *et al.*, (2002) observed good therapeutic effects of this compound when instilled as 0.3% lysostaphin into the eyes of experimental rabbits having keratitis.

### 2.9.2.2 Plants derived antibacterial compounds

A number of plants derived compounds or even crude plant extracts have been shown to possess antimicrobial activity. Stilbenoids isolated from *Stemona japonica* showed higher activity towards *S. aureus* as compared to known antibacterial compounds such as bakuchiol and magnolol (Filipowicz *et al.*, 2003). The antibacterial activity of aqueous extract of *Enantia chloranthastem* bark has been shown to have inhibitory activity against many microorganisms. The highest antibacterial activity of this plant extract was seen against *S. aureus* (Adesokan *et al.*, 2007). Flavonoids are heterocyclic compounds present in leaves, flowers and fruits of certain plants. The bactericidal activity of these compounds has been reported against several bacterial species, including *S. aureus* (Ozcelik *et al.*, 2011). However, the mechanism of this activity is poorly understood (Cushnie and Lamb, 2011). Inhibitory activity of leaf extract of green tea (*Camelia sinensis*) and curry (*Murraya koenigii*) plants has been used demonstrated against *S. aureus* in our laboratory (Batta *et al.*, 2012).

### 2.9.2.3 Antibacterial compounds derived from animals

Some compounds derived from animals possess antibacterial activity e.g. renalexin, a 20-residue peptide isolated from skin of *Rana catesbeiana* (bullfrog) showed activity against clinical MRSA strains (Giacometti *et al.*, 2000). A native thermostable antibacterial factor isolated from larvae of *Lucilia sericata* was found effective against MRSA and MSSA (Bexfield *et al.*, 2004).

### 2.9.3 Anti-virulence therapy

Anti-virulence therapy is one of the attractive approaches for controlling MRSA infections. This involves the use of agents against virulence determinants of MRSA strains. Such therapy would
have several advantages over the use of antibiotics. i. there would be no selective pressure on other non pathogenic and commensal bacteria. ii. certain adverse effects of antibiotics such as allergic reactions, nephrotoxicity etc. can be avoided. iii. limited use of antibiotics would reduce the chances of developing drug resistance. A combination of the conventionally used antibiotics and antivirulence therapy has the ability to manage MRSA infections (Shoham, 2011). Several anti-virulence strategies have been developed. Statins could enhance clearance of \textit{S. aureus} by phagocytes. This process is mediated through the production of antibacterial DNA based extracellular traps by neutrophils, macrophages and monocytes. Nano materials have also been reported to prevent biofilm production (Taylor and Webster, 2011).

\textbf{2.9.4 Vaccines}

The effective anti \textit{S. aureus} vaccine is still a challenge for researchers. The potential specific target is PVL toxin for CA-MRSA. Antibody against PVL is under investigation for its protective potential. Antibody levels against PVL in children with PVL-positive MRSA infection, had protective neutralizing antibody against PVL but it did not protect against primary or recurrent CA-MRSA skin infection (Hermos \textit{et al.}, 2010). EP67 is effective in limiting the infection by promoting levels of cytokines as well as increase in neutrophil influx (Sheen \textit{et al.}, 2011). Peptidoglycans, which consists of approximately 50\% of cell wall mass of \textit{S. aureus} is another potential component for vaccine development. Peptidoglycan based vaccine against \textit{S. aureus} A170PG was protective against some MRSA strains (Capparelli \textit{et al.}, 2011). Future vaccines need to contain multiple antigens like surface protein, toxoids and capsular polysaccharides. Also, for the development of successful vaccine, the biological role of cell-mediated immune response to MRSA infections needs better exploration (Patti, 2011; Daum and Spellberg, 2012). In animal models, aerosol vaccination with \textit{S. aureus} endotoxins has shown good results but required further clinical trials. DNA vaccination against the clumping factor A and passive immunisation with monoclonal antibodies have been tried to develop new strategies (Michie, 2002). DNA immunization against adhesions might prove valuable in combating \textit{S. aureus} infections (Brouillette \textit{et al.}, 2002).