2.0 REVIEW OF LITERATURE

The literature pertinent to the proposed research has been reviewed in this chapter

2.1 General Features of S. aureus

*Staphylococcus aureus* is the leading cause of bloodstream, lower respiratory tract, skin and soft tissue infection in many developed countries, including the United States (Diekema, 2001). Infections caused by community-associated as well as hospital acquired, methicillin-resistant *S. aureus* (MRSA) have become a major public health threat (Martin and Henry, 2008). In the developing countries, as compared to diseases like malaria, tuberculosis and HIV infections due to *S. aureus* are perceived as less significant due to low morbidity. However, this organism can be regarded as a developing world pathogen because this organism occurs globally (Nickerson, 2009). *S. aureus* is a Gram-positive, spherical bacterium (*coccus*) 1µm–1.3µm in size. These organisms appear in clusters, like bunches of grapes due to division in three planes. The *Staphylococcus* genus has 32 species and 8 subspecies. These are non motile, non spore forming, facultative anaerobic that may grow in aerobic condition by fermentation. Best grow on bacteriological media under aerobic condition at an optimum temperature of 37°C but pigment production can be seen at room temperature (22°C-25°C). This organism produces round golden yellow pigmented colonies with entire elevation, smooth and opaque in nature (Kloos and Lambe, 1991). Cell wall of *S. aureus* is about 20-40 nm thick and made up of peptidogycan, the basic component of cell wall, teichoic acid and other components like surface protein, exoproteins etc. (Knox and Wicken, 1973). The presences special types of amino acids i.e. α, ε-di-aminopimelic acid and acetylmuramic acid in the *S. aureus* cell walls are unusual feature. This organism expresses exotoxins and enzymes which are able to lyse host cell as well these which help in invasion of host tissue. It can also produce super antigen toxins which directly interact with the immune system (Dinges *et al.*, 2000). Some strains of *S. aureus* can grow on foods and produce toxins in the food stuff which if ingested can result in acute gastro-intestinal disorder. *S. aureus* produces a heat stable enterotoxin which survives heating at 100°C for 30–70 min. Beside, food poisoning, *S. aureus* is implicated in a number of other disease conditions, *e.g.* wound infections, septicaemia, toxic shock and other conditions which have been discussed under *Staphylococcus* infections. *S. aureus* produces co-agulase and nuclease enzymes where as *epidermidis* does not. *S. aureus* is one of the major causes of community-acquired (CA) and hospital-acquired (HA) infections.

2.2 Genome of S. aureus and its organization
The whole genomes of two related *S. aureus* strains namely N315 and mu50 have been sequenced. The size of the genome of strain N315 is 2813641 bps (Kuroda *et al.* 2001). This is a MRSA that was isolated in 1982. Another MRSA strain, mu-50 was vancomycin resistant that was isolated in 1997 (Kuroda *et al.*, 2001). The details of the genome organization are presented in Fig 2.1. *S. aureus* genome is composed of a complex mixture of genes many of which seem to have been acquired by lateral gene transfer. Most of the antibiotic resistance genes are carried either by plasmids or by mobile genetic elements including a unique resistance island. Three groups of new pathogenicity islands have been identified in the genome: a toxic-shock-syndrome toxin island family, exotoxin islands and enterotoxin islands. The *S. aureus* strain has ability to acquire useful genes from various organisms which is based on complexity of the genome and evidence of lateral gene transfer. Repeated duplication of genes encoding super-antigens is responsible for capability of *S. aureus* to infect humans and eliciting severe immune reactions (Kuroda *et al.*, 2001). This organism produces a wide variety of exoproteins that contribute to its ability to colonize and consequently cause disease in humans. Nearly all strains secrete a group of enzymes and cytotoxins which includes four haemolysins (alpha, beta, gamma, and delta), nucleases, proteases, lipases, hyaluronidase, and collagenase. The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth. Some strains produce one or more additional exoproteins, which include toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins, exfoliative toxins and leukocidin (Dinges *et al.*, 2000).
This strain has less G+C content about (33%), total genome 2597 open reading frame (protein coding region) with several bacteriophage islands, pathogenicity island, transposons
and insertion sequence (IS) element distributed through genome and also contains number of plasmids with different antibiotic resistance gene. Some of the general feature of MRSA strains N315 (Kuroda et al., 2001) are presented in the Table 2.1

Table 2.1: General features of genome of MRSA *S. aureus* (N315) (Kuroda et al., 2001).

<table>
<thead>
<tr>
<th>Length of sequences</th>
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<tr>
<td><strong>G+C content</strong></td>
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<tr>
<td>Total genome</td>
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<tr>
<td>Protein coding region</td>
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<tr>
<td>RNAs</td>
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<td>Non coding region</td>
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<td><strong>Ribosomal RNAs</strong></td>
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<td>16s</td>
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<td>23s</td>
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<td>5s</td>
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<td>IS431</td>
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</tr>
<tr>
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<tr>
<td><strong>Transposons</strong></td>
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<tr>
<td><strong>SSCmec</strong></td>
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<tr>
<td><strong>Plasmid</strong></td>
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<tr>
<td>G+C content</td>
<td>28.7%</td>
</tr>
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</table>

(Adapted from Kuroda et al., 2001)

2.3 Clinical manifestations of *S. aureus*

*S. aureus* is implicated in a number of clinical conditions: toxic shock syndrome, food poisoning, meningitis as well as dermatological disorders ranging from minor infections such as skin pustules to scalded skin syndrome. Pneumonia and bacteremia are some of the serious consequences of MRSA infections, but intra abdominal infections, osteomyelitis, food poisoning and deep tissue infections are also important clinical conditions (Haddadin et al.,
2002). The most common life threatening manifestation of *S. aureus* infection is bacteremia, which may range from uncomplicated bacteremia to endocarditis. The mortality rate associated with *S. aureus* bacteremia remains significantly high in patients with complications (Tan et al., 2001). Another condition, meningitis caused by *S. aureus* is uncommon, accounting for only 1% to 9% of cases of bacterial meningitis (Jensen et al., 1993). Besides, other conditions such as renal abscess and gastroenteritis may result due to *S. aureus* infection (Kloos, 1986). *S. aureus* continues to be a prominent agent of nosocomial infections. MRSA strains have now emerged as a major epidemiological problem in hospitals throughout the world. Also, CA-MRSA infection among individuals without healthcare-associated (HCA) risk factors has now emerged as an epidemic that is responsible for rapidly progressive, fatal diseases such as necrotizing pneumonia, severe sepsis and necrotizing fasciitis (Boyle and Daum, 2007). Other species of the genus staphylococcus such as *S. epidermidis* and *S. saprophyticus* are opportunistic pathogens which can cause staphylococcal infections. *S. epidermidis* may associate with endocarditis, prosthetic heart valve endocarditis, bacteremia, surgical wound infections, intravascular catheters, postoperative endophthalmitis, conjunctivitis and keratitis. *S. saprophyticus* is often implicated as opportunistic pathogen in human urinary tract infections (UTIs), especially in young sexually active females. It is considered to be the second most common cause of acute cystitis or pyelonephritis in these patients. Colonized infected inpatients as well as colonized hospital workers, serve as carriers at risk of developing endogenous infection or transmitting infections. Some species of staphylococcus genus *S. hemolyticus*, *S. hominis*, and *S. lugdunensis* are usually found as contaminants of blood cultures but they could also be associated with a variety of infections (Martineau et al., 2001). The clinical conditions caused by *S. aureus* are described below in details.

**Bacteremia and endocarditis**

*S. aureus* and coagulase-negative staphylococci (CoNS) are ideal infective endocarditis causing (IE) pathogens in that they possess a number of virulence factors, which enable them to establish this condition. Acute endocarditis caused by *S. aureus* is a serious disease, which can cause mortality as high as (50%). Although patients with *S. aureus* endocarditis may initially have non-specific influenza-like symptoms, their condition can deteriorate rapidly resulting in disruption of cardiac output and peripheral septic immobilization. The patient's prognosis is poor unless appropriate medical and surgical intervention is instituted immediately. In addition, *S. aureus* continues to be an important
cause of both community acquired (CA) and hospital acquired (HA) bacteraemia. Commonly associated predisposing medical conditions include; diabetes mellitus and renal insufficiency. Bacteremia has an obvious primary focus of infection, usually an intravascular catheter. Overall mortality in patients with \textit{S. aureus} bacteremia remains high, particularly in the older age patients, underlying respiratory or cardiac disease, associated infective endocarditis (IE), and septic shock. MRSA is a growing challenge world wide (Allan and Sharon, 2004).

\textbf{Wound infections}

Staphylococcal infections of the skin can be minor or life threatening, depending on the integrity of the skin surface and the invasiveness of the bacterial strain. \textit{Staphylococci} are the most common species recovered from patients who develop wound infection after surgery. \textit{S. aureus} and the CoNS rank first and third respectively among aerobic bacteria associated with wound infections. In general, staphylococci that cause wound infections come from the flora of the patient or the personnel performing the procedure. Studies have shown that the patient's own flora serves as the source of the infecting \textit{S. aureus} in about one-half of cases of surgical infections (Fischetti \textit{et al.}, 2000).

\textbf{Catheter associated Infections}

Although intravascular catheters and vascular grafts have revolutionized the practice of medicine for the betterment of patients, unfortunately, the catheter and devices are associated with the marked incidence of infection. Staphylococci, both \textit{S. aureus} as well as CoNS, play a major role in these infections. Because of their unique environmental niche on the skin and mucus membranes of humans and their ability to adhere to biomaterials and cause infection, they will undoubtedly continue to cause significant morbidity and mortality as the use of prosthetic medical devices increases. From 20\% to 65\% of all infections of catheters and shunts are caused by CoNS as they are particularly well adapted for causing these infections because they can produce a polysaccharide slime that binds them to catheters and shunts and protects them from antibiotics and inflammatory cells. A persistent bacteremia is generally observed in patients with infections of shunts and catheters because the organisms have an easy and continuous access to the blood stream (Murray \textit{et al.}, 2002).

\textbf{Skin and soft tissue infections}

Staphylococci are normal inhabitants of the skin and mucous membranes of mammals and birds, where they exist saprophytically. Several, factors such as tight clothing, scratching
immuno-suppression or insertion of prosthesis can alter the host-parasite relationship and cause disease. Colonization, particularly by \textit{S. aureus}, leads to hand carriage; the organisms are frequently spread to other areas of the body from the hands. These organisms often follow a nose to hands to wounds route of infection (Zimakoff \textit{et al.}, 1996). The variety of enzymes and toxins produced by \textit{S. aureus}, result in a spectrum of diseases, but other virulence factors also operate. Purulent skeletal muscle infection (pyomyositis), folliculitis and furuncles, recurrent furunculosis, impetigo, botryomycosis and secondary cutaneous infections in patients with eczema are some of the skin infections due to \textit{S. aureus} (Crossley \textit{et al.}, 1997).

**Central nervous system infection**

The staphylococci are important pathogens of cerebrospinal fluid (CSF) shunt infections, acute purulent meningitis, brain abscess associated with trauma, spinal and cranial subdural empyema and epidural abscess. \textit{S. aureus} is one of the most common pathogens associated with these conditions. The \textit{Staphylococci} typically enter the shunt system from a contaminated wound or from the patient's skin surface at operation. In most cases of staphylococcus meningitis \textit{S. aureus} is the agent. This condition may develop as complications of neurological procedure.

\textit{S. aureus} also a common causative organism of brain abscesses that develops as a result of cranial trauma, either penetrating wounds of the brain or craniotomy. This organism is the common cause of spinal subdural empyema and 15 to 25 \% of cases of cranial subdural empyema. CoNS is however, rarely associated with these conditions. \textit{S. aureus} is also associated with spinal epidural abscess in 50 to 60 \% cranial epidural abscesses (Crossley \textit{et al.}, 1997).

**Diseases of the eye**

Staphylococcal diseases of the eye include both infections and expression of cell-mediated immunity (CMI) to staphylococcal antigens. Different animal models have been used to study eye infection due to Staphylococci for example guinea pig for studying staphylococcus keratitis (Davis \textit{et al.}, 1978), and rat model to see the effects of antibiotics and corticosteroid treatments on the progression of \textit{S. aureus} infection of the cornea (Badenoch \textit{et al.}, 1985). Radial incision of the rabbit conjunctiva followed by inoculation of \textit{S. aureus} resulted in purulent conjunctivitis and a model for testing antimicrobial treatments in this disease (Behrens and Begall, 1993).
Osteomyelitis and other infections

Colonization of the bones and joints by *S. aureus* may occur as a result of either haematogenous infection or it can be a secondary infection resulting from trauma or the extension of disease from an adjacent area (Murray *et al.*, 2002). Most cases of osteomyelitis were caused by strains of *S. aureus* during 1970s, but later on the incidence of osteomyelitis caused by gram-negative pathogens increased dramatically but, staphylococci still remains the most common pathogen in most types of osteomyelitis. *S. aureus* is the most frequent single pathogen in adults, accounting for 50 to 70% of cases of osteomyelitis. The estimated frequency of *S. aureus* in pyogenic osteomyelitis in all age groups may reach 80 to 90% (Crossley *et al.*, 1997). This organism is the primary cause of septic arthritis in young children and in adults who are receiving intra-articular injections or who have mechanically abnormal joints. Staphylococcal arthritis is characterized by a painful and erythematous joint. Infection is usually seen in the large joints such as shoulder, knee, hip, and elbow. The diagnosis in children is easily, but in adults, it depends on the nature of the underlying disease as well as the occurrence of any secondary infectious complications (Murray *et al.*, 2002).

Respiratory tract infections

*S. aureus* can cause respiratory disease which can develop after the aspiration of oral secretions or from the haematogenous spread of the organism from a distant site. Aspiration pneumonia is seen primarily in the very young, the aged, and patients with cystic fibrosis, influenza, chronic obstructive pulmonary disease. Haematogenous pneumonia is common for patients with bacteraemia or endocarditis. *S. aureus* is also associated with other conditions such as sinusitis, bronchitis, or pneumonia. However, the organism is isolated frequently from these conditions responsible for perhaps 20 to 30% of cases of nosocomial pneumonia (Murray *et al.*, 2002).

Urinary tract infection

Although staphylococci are not commonly involved in urinary tract infections (UTIs), *S. aureus* may cause UTIs. The infections range from asymptomatic infections to severe infections and associated with bacteraemia. *S. saprophyticus* has a predilection for causing UTIs in young, sexually active women and is rarely responsible for infections in other patients. It is also infrequently found as an asymptomatic colonizer of the urinary tract (Murray *et al.*, 2002).
Toxin-mediated syndromes

*S. aureus* cause a number of toxins mediated syndrome e.g. toxic shock syndrome (TSS), is caused by a toxic shock syndrome toxin type 1 (TSST-1). Staphylococcal toxic shock syndrome (TSS) is an illness that is the result of the combined effects of one or more toxins of *S. aureus* (Crossley et al., 1997). Staphylococcal TSS may manifest in two general forms, menstrual or non-menstrual. Menstrual TSS occurs in women whose vaginal/cervical mucosa are colonized by TSST-1 producing *S. aureus*. Use of tampon is a risk factor in menstrual TSS, and a correlation between tampon absorbency and risk of developing TSS has been established. TSS or a TSS-like syndromes can be induced in laboratory animals (Quinn et al., 2002), but a definitive model for this disease has not been developed so far. Non-menstrual TSS may result from *S. aureus* infection elsewhere in the body, either staphylococcal enterotoxins (SEs) or TSST-1 may mediate the non-menstrual form (Fischetti et al., 1993). TSST-1 in the bloodstream can trigger a massive release of cytokines that cause shock and death. Several staphylococcal enterotoxins (SEs) cause food poisoning. Exfoliative toxins result in staphylococcal scalded skin syndrome (SSSS) and other conditions e.g. staphylococcus enterocolitis.

Food Poisoning

Staphylococcal food poisoning, one of the most common food borne illnesses, is intoxication rather than infection. Disease is caused by bacterial toxin present in food rather than from a direct effect of the organisms on the patients. Some strains of *S. aureus* can also cause enterocolitis, with clinical signs of watery diarrhoea, abdominal cramps, and fever. Enterocolitis occurs primarily in patients who have received broad spectrum antibiotics, which suppress the normal colonic flora and permit the growth of *S. aureus* (Murray et al., 2002).

2.4 Virulence factors of *S. aureus*

*S. aureus* is equipped with several virulence factors which may be defined in a narrow sense substance that, when purified to homogeneity and introduced into a test animal, produces a pathogenic effect. Most of the virulence factors especially those involved in attachment and probably most of the degradative enzymes (e.g. lipases, proteases and hyaluronidase) would not fit in this definition and can not be considered as true virulence factors. A boarder sense those substances which are exported out of the cytoplasm either to
the cell surface or into the extracellular environment of the *S. aureus* can be considered as potential virulence factors. These factors may act in deleterious way affecting the host. At least some of these factors may actually assist the host in combating an infection (Crossley *et al.*, 1997). Several factors responsible for Pathogenicity of *S. aureus* have been shown to date to be either necessary or sufficient for the establishment of an infection, although the infective or lethal dose may vary. No strain has yet been identified that produces all the known toxins. *S. aureus* bacterium binds specifically to the endothelial cell via interactions between adhesions and host receptors, which prompt the cell to initiate phagocytosis of the organism and thus, adhesions act as virulence factor (Lowy, 1998). *S. aureus* produces a variety of toxins which are involved in different disease conditions. These toxins are discussed below.

### 2.4.1 Toxin

**Beta toxin**

β toxin, also called sphingomyelinase C, is a 35 kDa heat-labile protein produced by *S. aureus*. This enzyme is toxic to a variety of cells, such as erythrocytes, leukocytes, macrophages, and fibroblasts. This toxin catalyses the hydrolysis of membrane phospholipids in susceptible bacterial cells. β toxin has species dependent activity. Erythrocytes of sheep, cow and goat are most sensitive, human erythrocytes are intermediate in sensitivity, whereas murine and canine erythrocytes are resistant. The degree of sensitivity of erythrocytes depends on membrane sphingomyelin content. The role of β toxin in human disease remains to be proved; however, together with a δ toxin, it is believed to be responsible for the tissue destruction and abscess formation (Beaman *et al.*, 2002).

**Delta toxin**

δ toxin is 3 kDa polypeptide produced by almost all *S. aureus* strains and the majority of other Staphylococci. The toxin has a wide spectrum of cytolytic activity, affecting erythrocytes, other eukaryotic cells, many other mammalian cells, organelles, and spheroplasts and protoplasts, as well as intracellular membrane structures. δ toxin acts as a surfactant disrupting cellular membranes by means of a detergent-like action. Thus toxin also been reported to be dermonecrotic and lethal in experimental animals when used in high concentration. The activity of delta-toxin is inhibited by phospholipids (Crossley *et al.*, 1997). δ toxin is a 26-residue long peptide encoded by the *Hlg* gene with peak production at
the end of the exponential growth phase and can be purified by different procedures. Different cells such as neutrophils, monocytes, lymphocytes, and erythrocytes have different affinity for this toxin (Alouf and Popoff, 2006). The toxin acts by formation of pores in the membrane, leading to the lysis of erythrocytes and other mammalian cells. At least two variants of S toxin exist; toxins expressed by human and canine strains of *S. aureus* are only 62% identical and are immunologically distinct (Emori *et al.*, 1993).

**Exfoliative toxins A and B**

Two exfoliative toxin A (ETA) and exfoliative toxin B (ETB) are responsible for Staphylococcal scalded skin syndrome (SSSS), usually seen in newborns. This syndrome is characterized by intra epidermal separation of layers of the skin at the desmosomes. The toxins are serologically distinct. Disease commences suddenly with a generalized erythema, often near the mouth and spreading over the entire body in the course of a few days. When the skin is lightly rubbed, the epidermal layer wrinkles irreversibly, there is no permanent scarring on areas of the skin, and the causative toxins are not themselves lethal to the host (Fitzgerald *et al.*, 2001).

**Staphylococcal enterotoxins**

Staphylococcus enterotoxin (SE), A through E and G have been identified (Balaban *et al.*, 2000). The enterotoxins are resistant to hydrolysis by gastric enzymes and stable at 100°C for 30 min. Staphylococcal food poisoning is the leading cause of food-borne microbial intoxication worldwide and is usually linked to improper storage of food (Holemberg, 1984). The SEs is stable in the gastrointestinal tract and indirectly stimulates the emetic reflex centre. Although the vagus nerve is involved, molecular events that take place are not clearly understood. There is mast cell activation and inflammatory mediators and neuro-peptide substance P are released upon SE activity in the gastrointestinal tract and elsewhere (Adel, 1996). Staphylococcal enterotoxins (SEs) belong to a large family of staphylococcal and streptococcal pyrogenic exotoxins (PT), sharing common phylogenetic relationships, structure, function, and sequence homology. The PT family is composed of SEs, streptococcal pyrogenic exotoxins (SPE).

**Gamma-toxin**

Gamma-toxin can lyse human, sheep and rabbit erythrocytes as well as human lymphoblastic cells. γ toxin, leukocidin, and other bi component toxins are a family of
proteins encoded by \textit{pvl} gene. All toxins in this family contain two synergistically acting proteins: one S component (LukS-PV, H1gA, or H1gC) and other F component (LukF-PV or H1gB) designated on the basis of their mobility (slow or fast) in ion-exchange chromatography. The prototype bi component toxins are the closely related Panton-Valentine leukocidin ((PVL) and gamma-toxin. The PVL S and F components are \textit{LukS-PV} and \textit{LukF-PV}. Gamma-toxin likewise contains S and F components designated H1gA and H1gB, respectively (Emori \textit{et al.}, 1993). Synergistic function involves sequential binding of the F and S components. The action of gamma-toxin on erythrocytes involves initial binding of H1gB (F) followed by H1gA (S) and subsequent generation of a pore (Dickinson \textit{et al.}, 1993).

**Leukocidin**

Leukocidin as the name suggest has the potential to kill leukocytes particularly polymorphonuclear leukocytes. Phagocytosis by leukocytes is affected adversely which is the main defence against infection due to \textit{S aureus}. Panton-valentine leukocidin (pvl) consists of two protein components, S and F. The S component is like the B part of an A-B toxin, in that it binds to GM\(_1\) gangliosides. However, both have enzymatic activity, involved in phospholipids and phosphatidyl inositol metabolism, respectively. Phosphatidyl inositol, an important signalling molecule in eukaryotic cells, controls a number of cellular processes. The action of this two components appears to alter phospholipid metabolism and cause disruption of normal cellular activities (Salyers \textit{et al.}, 2002). The two components of the PVL bind sequentially to human neutrophils, although most reports suggest that the S component binds first and subsequently forms pores (Colin \textit{et al.}, 1994).

**Toxic shock syndrome toxin-1**

Toxic shock syndrome is characterized by fever, hypotension, and rash followed by desquamation and the involvement of multiple organ systems. It is mediated through a toxin known as toxic shock syndrome toxin-1 (TSST-1), formerly called pyrogenic exotoxin C and enterotoxin F. This toxin is an exotoxin secreted during the growth of some strains of \textit{S. aureus}. TSST-1 has not been found in staphylococcal isolates from all patients with toxic shock syndrome (TSS), but most of these isolates not producing TSST-1 are reported to produce enterotoxin B. The role of this enterotoxin in TSS is not clearly understood. Coagulase-negative staphylococci can also cause this syndrome (Murray \textit{et al.}, 2003).

**2.4.2 \textit{S. aureus} Enzymes**
A number of enzymes are produced by *S. aureus* which contributes to the pathogenicity of this organism in the host. The important ones are described below:

**Coagulase**

Coagulase production is the main criterion used for the identification of *S. aureus* (Van *et al.*, 1994). *S. aureus* strains possess coagulase bound (also called clumping factor) as well as cell free coagulase. Coagulase bound to the staphylococcal cell wall can directly convert fibrinogen to insoluble fibrin and result in clumping of the bacteria. The cell free coagulase reacts with a globulin plasma factor (coagulase reacting factor) to form staphylothrombin which catalyses the conversion of fibrinogen to insoluble fibrin. Coagulase is used as a marker for the virulence of *S. aureus*. (Emori *et al.*, 1993).

**Catalase**

All *Staphylococci* produce catalase, which catalyses the conversion of toxic hydrogen peroxide to water and oxygen. Hydrogen peroxide can accumulate during bacterial metabolism or after phagocytosis (Murray *et al.*, 2003). Different toxic forms of oxygen are produced as inadvertent by-products during the reduction of $O_2$ to $H_2O$ in respiration. These reactive oxygen intermediates have many deleterious effects on living organisms, ranging from DNA strand damage to peroxidation of membrane lipids. *S. aureus* have evolved enzymes that destroy toxic oxygen products. The most common enzyme in this category is catalase, which attacks hydrogen peroxide (Madigan *et al.*, 2003).

**Fibrinolysin**

Fibrinolysin, also called staphylokinase, is produced by almost all *S. aureus* strains which can dissolve fibrin clots. Staphylokinase is distinct from the fibrinolytic enzymes produced by *Streptococci*.

**Hyaluronidase**

Hyaluronidase hydrolyses hyaluronic acids, the acidic mucopolysaccharides that hold together certain cells of the body, particularly cells in the connective tissue. This digesting action of this enzyme is thought to be involved in the tissue blackening of infected wounds
and to help the microorganism spread from its initial site of infection. More than 90% of *S. aureus* strains produce this enzyme (Tortora *et al.*, 2001).

**Lipases**

*S. aureus* produces several lipases. These enzymes hydrolyse lipids, an essential function to ensure the survival of staphylococci in the sebaceous areas of the body. Lipases break down fats to their fatty acid and glycerol components. Each component is then metabolized separately. These enzymes facilitate invasion of cutaneous and subcutaneous tissues by the organism and for superficial skin infections such as furuncles (boils), and carbuncles to develop (Tortora *et al.*, 2001).

**Nuclease**

A thermostable nuclease (TNase) is produced by nearly all strains of *S. aureus* and has been used as a diagnostic criterion for this species. TNase hydrolyses single and double stranded DNA and RNA at the 5’ position of phosphodiester bonds by a calcium-dependent mechanism. The role of this enzyme in the pathogenesis of infection is not exactly known (Murray *et al.*, 2003).

### 2.5 Emergence of drug resistance among *S. aureus* strains

The history of the antibiotics began with the discovery of first antibiotic penicillin, in 1929, based on the observation by Sir Alexander Fleming. A series of different antibiotics were quickly discovered after penicillin was introduced into the market. Penicillin was regarded as magic bullet during 1940 to 1960. Resistance to this antibiotic was reported in United Kingdom (U.K.) in 1961 which presented new challenge to treat infections due to *S. aureus*. In fact, penicillin was the drug of choice for many types of gram-positive bacterial infections especially streptococcal infections. The discovery and introduction of streptomycin, chloramphenicol and tetracycline, in late 1940s and early 1950s changed the era of antibiotic chemotherapy. These antibiotics were effective against a wide range of bacterial pathogens. The resistant *S. aureus* strains caused major problems in many hospitals. Multi drug resistance to antibiotics such as penicillin, tetracycline, and streptomycin was reported soon after. The multi drug resistance was regarded as the ‘Hallmark of dangerous strains’. Sir Williams further correlated that resistance was often clonal associated with particular strains and particular phage types. The resistant strains often colonized the noses of hospital staff, which could be the source of transmission and spread of these strains, similar
observations have been repeatedly noticed again in the current situation of MRSA (Livermore et al., 2001). The first cephalosporins, cephalothin and cephaloridine, were developed primarily for their stability to staphylococcal penicillinase. This was possible because of the discovery of how to replace the 6′ phenylacetyl group benzyl penicillin with other acyl substituents. This discovery provided the synthetic route for methicillin and the oxacillin. These compounds have the bulky 6′ acyl groups that sterically hinder attack on the β-lactam ring, thus allowing activity to be retained against penicillinase-positive S. aureus. Staphylococci secrete their β-lactamase extracellularly thus protecting the entire bacterial population, therefore little or no subsequent selection of strains with more potent β-lactamase variants was observed (Livermore and Williams, 1996). In addition to the development of penicillinase-stable β-lactams, gentamicin was introduced in early 1960s. The latter had better anti-staphylococcal activity and less toxicity than earlier aminoglycosides. S. aureus strains resistant to penicillinase-resistant β-lactam drugs, such as methicillin, were reported in hospitals in early 1960s, (Jevons, 1961). MRSA quickly spread around the world and is now a major nosocomial pathogen. Even though large number of antibiotics has been introduced into the market, microorganisms are capable of developing resistance at a very rapid pace. The new strains that are resistant to almost all antibiotics are called Multi-Drug Resistant (MDR) strains. These strains spread world wild quickly making the treatment of infectious diseases a challenging task for the clinicians (Hiramatsu et al., 2001).

2.6 Mechanisms of antimicrobial resistance to antibiotic and their genetic basis

The important factors that contribute towards the development of resistance in bacteria against antibiotic include: mutations in cellular genes or acquired genes that alter antimicrobial target sites or affect gene expression, the ability to exchange genes, and the acquisition and expression of new DNA by horizontal gene transfer. The ability of the bacteria to evolve these changes amongst themselves has led to bacterial adaptation or resistance to various antibiotics. The important mechanisms by which the bacteria develop resistance are as follows:

1. The production of an enzyme that inactivates the antimicrobial agent: the most common cause of resistance to β-lactam drugs is enzyme mediated antibiotic degradation. β-lactamase
is the commonest that have been identified. These enzymes can hydrolyze β-lactam antibiotics, which are produced by some Gram negative bacteria. But the β-lactamases produced by Gram negative bacteria are usually concentrated in the periplasmic space while those produced by Gram positive bacteria predominantly by \textit{S. aureus} are distinct from those produced by Gram negative bacteria.

2. The production of an alternative enzyme that is inhibited by antimicrobial agent \textit{e.g.} major cause of trimethoprim resistance is the production of an altered (plasmid encoded) dihydrofolate reductase that lacks the capacity to bind trimethoprim.

3. A mutation in the antimicrobial agent’s target: A mutation in the DNAs is the most important mechanism for resistance to this important new group of antibiotics in both Grams positive bacteria and Gram negative bacteria. MRSA strains mediated through the production of penicillin binding protein (PBP2 or PBP2A) which having lower affinity for methicillin (Laura \textit{et al.}, 1991).

4. The reduced uptakes of the antimicrobial agents by the bacteria. Aminoglycosides are hydrophilic molecules that require active transport to gain entry into bacteria. Diminished drug uptake is one of the mechanisms of resistance to aminoglycosides (Ahmad \textit{et al.}, 1980).

5. Macrolides resistance among coagulase negative Staphylococci (CoNS) are usually due to presence of \textit{erm} C methylase, which is responsible in methylation process. A significant number of strains are resistant as a result of active efflux of the drug (Eady \textit{et al.}, 1993).

6. Overproduction of the target of antimicrobial agents. Over production of the normal (chromosomally encoded) dihydrofolate reductase is an additional mechanism of trimethoprim resistance particularly in \textit{E. coli} and \textit{Klebsiella pneumoniae} (Burchall \textit{et al.}, 1982).

Bacteria often acquire the ability to use more than one of these resistance mechanisms through genetic mutation and transfer. Mutations can occur naturally within any species of the bacteria. An element in the environment which is not suitable to bacteria creates a selective pressure on it as a result of which the bacteria undergo mutational changes. Bacteria with the mutation will also have the advantage of the additional nutrients and the space left by the wild phenotype which has been eliminated from the environment. The greater the selective pressure exerted on bacteria, due to greater or more frequent exposure to the antibiotics the greater the potential advantage for development of beneficial mutations in the
bacteria (Walsh, 2000). This selective advantage caused by mutations is from parent bacteria to offspring (vertical transmission). The gene mutations also can be transferred horizontally through plasmids or transposons, which are extra chromosomal genetic elements. Plasmids are circular double-stranded DNA that are located within the cell apart from the chromosomal DNA of the bacteria. In bacteria, the chromosomal DNA encodes for general characteristics of the cell as well as metabolic process and repair mechanisms. The plasmid DNA usually encodes for additional functions such as virulence and resistance (Kaye and kaye, 2000). These elements are small pieces of DNA capable of moving between chromosomal DNA and plasmids. Transposons are able to detach themselves from one part of DNA and attach themselves to another part. Transposons are able to carry resistance genes within themselves from one DNA to another. These mobile genetic elements are able to transfer resistance genes horizontally between the same species of the bacteria and also between different species within the genus (Walsh, 2000). The genetic elements can move from one cell to another by conjugation, transduction or transformation. During the conjugation process, the genetic material is exchanged between bacteria through the conjugation tube while bacteriophages can transfer small amounts of bacterial DNA from one bacterium to another by the process of transduction. Some bacteria can go in competence phase. At this phase, the bacteria can take up naked DNA from the environment in a process called transformation. This DNA can be incorporated into their own genome. This additional new piece of DNA can harbour the genes that may encode for antibiotic resistance; therefore, the recipient strain will acquire a new resistance profile.

2.6.1 Methicillin resistance

The first MRSA was discovered in the year 1961, when methicillin was introduced into the market for the first time (Jevons 1961). MRSA isolates have been reported as both nosocomial and community-acquired pathogens (Maltezou and Giamarellou, 2006). Originally, S. aureus contained three penicillin-binding proteins, PBPs1, 2, and 3 to catalyse cross-linking of peptidoglycan. The MRSA isolates have an additional component, PBP 2 or 2a, which has low affinity for β-lactams (Georgopapadakou and Liu, 1980). MRSA isolates are resistant to all β-lactams. The structural gene for methicillin resistance, meca, encodes a novel penicillin binding protein PBP2, which has much lower affinity for β-lactam antibiotics. It is absent in methicillin susceptible strains (Hiramatsu. et al., 1997). This gene is carried on a genetic element, Staphylococcal chromosomal cassette (SCC) mec, which inserts precisely into the S. aureus chromosome (Hiramatsu et al., 2001). SCC mec is a novel genetic
element containing two recombinants (ccrA and ccrB) and mecA and its regulatory genes (Ito et al., 2001). mec-A encoded PBP2 which has low affiliating for β-lactam antibiotics. This gene is present on SSC mec (Chongtrakool et al., 2006). The details of various SCC mec elements are presented in Figure 2.2. SCC mec is found in other staphylococcal species from which it is presumed to have been transferred; however, the original donor of mecA to staphylococci is unknown, as the element has not yet been identified outside this genus. S. sciuri has an intrinsic PBP that shares 87.8% amino acid homology with PBP2, and it has been suggested that this may be a precursor to its homologue in S. aureus (Wu et al., 1996). mec A gene has approximately 30 to 50 kb additional chromosomal DNA which is present only in methicillin resistant strains (Hiramatsu et al., 1997). Expression of mecA is either constitutive or inducible by some β-lactam antibiotics, but not by methicillin or oxacillin, or heterogeneous, with only a few cells in a population expressing the gene. The expression mecA may be regulation dependent. Presence of the mecA gene in staphylococcal isolates is considered synonymous with oxacillin resistance. Low level resistance is generally the result of β-lactamase over production, increased levels of intrinsic PBPs or reduction of their binding affinity. High level resistance is always dependent on the expressions of PBP2. Regulation is complex: mec encodes a repressor for mecA, and expression of methicillin resistance thus demands that gene is inactivated by mutation or deletion; transcription of mecA is further modulated by interactions involving the chromosomal fem genes, which encode peptidoglycan modifying enzymes (Adapted from Chongtrakool et al., 2006)
These multiple interactions explain the variable expression of methicillin resistance among mecA-positive lineages. Some MRSA have homogeneous resistance to β-lactams, with all the cells in a population expressing resistance; others have heterogeneous resistance, with resistance only expressed by a small minority of the cell population unless salt is added or the incubation temperature is lowered (Chambers, 1997). The heterogeneous nature of methicillin resistance is an inherent limitation to the accuracy of susceptibility testing. The detection of mecA gene by PCR as a rapid method of identification of MRSA is been well established (Martineau et al., 2000)

2.6.2 Glycopeptide Resistance (Vancomycin Resistance)

Vancomycin resistance was first described in enterococci in 1988, but the first clinical isolate of S. aureus found to be resistant to vancomycin was not reported until 2002, but in India Tewari reported in year 2006 (Tewari and Sen, 2006). Resistance in S. aureus developed due to the exchange of genetic materials with enterococci. Vancomycin does not appear to penetrate the cell membrane or interact with cellular proteins but functions by complexation of peptidyl-DAla-D-Ala termini on the bacterial cell surface (Barna & Williams, 1984). This arrangement prevents transglycosylation and cross-linking of the
peptidoglycan layer (Reynolds, 1994). Vancomycin resistance is associated with production of a 38-kDa membrane-associated protein \textit{van-A}, whose amino acid sequence has been determined (Dutka-Malen \textit{et al.}, 1995). Sequence similarity was found with Gram-negative D-Ala-D-Ala ligases, which are cytoplasmic enzymes responsible for synthesis of the D-Ala-D-Ala dipeptide for peptidoglycan assembly (Walsh, 1993). \textit{Van-A} has been purified, and shows D-Ala-DAla ligase activity, but has substantially modified substrate specificity, compared with Gram-negative D-Ala-D-Ala ligases (Bugg \textit{et al.}, 1991). \textit{Van-A} is able to synthesize a number of mixed di-peptides including D-Ala-D-Met and D-Ala-D-Phe in preference to D-Ala-D-Ala, suggesting that its cellular role may be synthesis of a D-Ala-X di-peptide, which is incorporated into peptidoglycan and is able to be cross-linked but is not recognized by vancomycin.

\textbf{2.6.3 Aminoglycoside resistance}

The main mechanism of aminoglycoside resistance in \textit{Staphylococci} is drug inactivation by cellular aminoglycoside modifying enzymes. Several distinct gene loci encoding such modifying enzymes have been characterized in \textit{Staphylococci}. Plasmid mediated aminoglycoside modifying enzymes of all three classes (aminoglycoside phosphotransferases, acetyltransferases, and nucleotidylyltransferases) have been found in \textit{Staphylococci} (Shaw \textit{et al.}, 1993). Transposon \textit{Tn}4001 like element is widely distributed in both \textit{S. aureus} and CoNS. In early 1980s, the endemic MRSA strains carrying multiple resistance determinants were reported to cause worldwide nosocomial infections. These multi-resistance phenotypic patterns were observed in the majority of MRSA strains until the mid-1990s. Hence, most of the tests that were developed focused on the detection of only \textit{mecA} gene which gives a proper clue for choosing glycopeptides as alternative therapeutic agents (Strommenger \textit{et al.}, 2003).

\textbf{2.6.4 Resistance to Quinolones}

Resistance to quinolones has been a problem ever since nalidixic acid was introduced into clinical medicine long back. For a time, the greater potency of the fluoroquinolones, compared with that of older quinolones, permitted complacency regarding their use, but successful treatment outcomes of quinolones led to increased use of these drug, which, in turn, led to an escalating rate of resistance. In the 1990s, the use of fluoroquinolones in the United States increased by \sim40\%, with a doubling in the rate of resistance to ciprofloxacin among gram-negative bacilli isolated from the intensive care units of hospitals (Nehauser \textit{et
In the United States, 10% of enteric bacteria (e.g., Enterobacter cloacae, Morganella morganii and Serratia marcescens) were resistant to ciprofloxacin. Striking associations between resistance to quinolones and oxacillin among S. aureus resistance to vancomycin among enterococcus species and production of extended spectrum β lactamase (ESBL) among Klebsiella pneumoniae. Higher rates of resistance to quinolones have been reported from other parts of the world (Wenzel et al., 2003). During 1997–1999, ~60% of Escherichia coli strains isolated from hospital-acquired infections in Beijing was resistant to ciprofloxacin (Wang et al., 2001). Although respiratory pathogens (e.g., Moraxella catarrhalis, Haemophilus influenzae, and Streptococcus pneumoniae) are generally susceptible to quinolones, resistance does occur, and localized out breaks have been reported. The resistance has led to clinical failures (Davidson et al., 2002). Resistance has also become a problem while treating gonorrhoea with fluoroquinolones (Newman et al., 2004) and in some parts of the world, can be a problem for the treatment of enteric infections due to Salmonella, Shigella or Campylobacter species (Lowy et al., 2003).

2.7 Epidemiology of methicillin-resistant S. aureus (MRSA)

Since 1961, MRSA have spread in hospitals and other healthcare facilities worldwide. MRSA is now the most commonly isolated antibiotic resistant pathogen in many countries. The widespread emergence of MRSA, especially in various types of nosocomial infections, is a serious clinical problem world wide. The incidence of methicillin resistance among nosocomial isolates of S. aureus is higher than 70% in some Asian countries such as Taiwan, China, and Korea (Ko et al., 2005). Very limited data are available with regard to the evolution and population genetics of MRSA isolates in this region. Dissemination of specific clones is responsible for the rapid emergence of MRSA in many parts of the world (Ko et al., 2005). Widespread occurrence of MRSA in 1980s resulted in empiric therapy using vancomycin in many healthcare institutions. The use of this antibiotic in United States (US) also increased during this period because of the growing numbers of infections in healthcare institutions with Clostridium difficile and coagulase negative staphylococci (CoNS). The resulting selective pressure was established that eventually led to the emergence of strains of S. aureus and other species of staphylococci with decreased susceptibility to vancomycin and other glycopeptides. Vancomycin-resistant S. aureus (VRSA) was first isolated in 1997, in
Japan (Hiramatsu et al., 1997). Later on the vancomycin resistant strains were reported from USA and other countries (Srinivasan et al., 2002). The introduction of VRSA in medical settings is based on the presumption that vancomycin resistance had come from vancomycin-resistant enterococci (VRE), especially after it was reported that the vancomycin resistance gene van A could be transferred from E. faecalis to S. aureus in vitro (Noble et al., 1992). Therefore, the term, vancomycin-intermediate S. aureus (VISA), has come into use. The Clinical and Laboratory Standards Institute (CLSI) recommended the criteria for vancomycin susceptibility and resistance of S. aureus in 2006. The earlier criteria had established that S. aureus with minimum inhibitory concentrations (MICs) of vancomycin of < or =4 microg/ml, 8 to 16 microg/ml, and > or =32 microg/ml were vancomycin-susceptible, -intermediate-resistant and -resistant, respectively. But according to CLSI recommendation, the bacteria showing vancomycin MICs of < or =2 microg/ml, 4 to 8 microg/ml, and > or =16 microg/ml are susceptible, intermediate resistant, and resistant, respectively (Hanaki et al., 1998). Strains of vancomycin-intermediate S. aureus (VISA) with vancomycin MIC of 8µg/ml have been reported from Japan, USA, France, UK and Germany. Most of these isolates appear to have developed from pre-existing MRSA infections. Until recently vancomycin resistance among gram-positive bacteria had been thought to be uncommon but the confirmed reports of vancomycin resistance in Enterococcus spp; S. aureus and CoNS have been reported from various parts of the world. Widespread use of vancomycin to treat infections caused by MRSA and other gram-positive cocci has led to the emergence of vancomycin resistance (Tiwari and Sen, 2006). MRSA in the hospital setting were established as the strictly nosocomial pathogen but latter on it was isolated from the community. The deaths due to severe CA-MRSA in children was reported from US (Wijaya et al., 2006), in need for treatment of such infections was realized. Sporadic cases of community acquired MRSA were also reported from West Australia and other part of world and the term CA-MRSA was coined for infection without the risk factor for nosocomial infections (Hsu et al., 2006). With the emergence of CA-MRSA strains, MRSA should no longer be regarded as a strictly nosocomial pathogen (Maltezou and Giamarellou, 2006). The terms community-acquired or community-associated have been used to classify CA-MRSA. Since it is difficult to exactly establish the origin of MRSA, the Centre for Disease Control and Prevention (CDC) now prefer the term community-associated and most MRSA outbreaks among athletic teams have been linked to community-associated strains. These strains are distinct from other MRSA strains with regard to molecular characteristics, clinical spectrum, epidemiology and susceptibility to antibiotics (Beam and Buckley, 2006). CA-MRSA has been spreading in
epidemic proportions in parts of the USA where conditions of overcrowding and poor sanitation prevail. Recently, MRSA has also emerged in the community setting in Asian countries (Ko et al., 2005). The concept that CA-MRSA initiated from nosocomial isolates that had somehow managed to spread in the community was reflected by various studies which demonstrated differences in clinical presentations and antimicrobial susceptibilities, SCCmec types and genetic heritage.

Five major structural types of SCCmec have been described. Of these, three types are found among hospital-acquired MRSA (HA-MRSA) isolates: Type I (34 kb) was identified in a 1960s isolate (strain NCTC10442); type II (53 kb) was identified in a 1982 isolate (strain N315) which is ubiquitous in Japan, Korea, and the United States; and type III (67 kb) was identified in a 1985 isolate (strain 85/2082) which is prevalent in Germany, Austria, India, and other South Asian and Pacific areas. Type IV (20 to 24 kb) is generally carried by CA-MRSA isolates; four subtypes of this type have been reported. In 2004, type V was reported in an isolate of CA-MRSA, in which the only difference was the presence of a restriction-modification system composed of the ccrC gene and the surrounding open reading frames (Yang et al., 2006). Presently, these elements were horizontally transferred into MSSA at different time points by distantly-related bacterial species. Epidemic clones from five major lineages of MRSA have spread worldwide and are responsible for practically all healthcare-associated MRSA infections. The majority of CA-MRSA isolates have been shown to possess geographically-related genetic backgrounds, SCCmec IV and panton-valentine leukocidin (pvl) genes, although this is not necessarily true of CA-MRSA described from all countries. The recent hospitalization and chronic illness requiring health care visits were most common risk factors for acquisition of MRSA (Salgado et al., 2003) other factors include: nursing home admission, antibiotic exposure, and close contact with a person with risk factor.

2.8 Genetic relationship and molecular study of MRSA and MSSA

As discussed earlier, the antibiotic resistance has emerged due to indiscriminate use of antibiotics and close evolutionary relationship between bacterial species. This is reflected by the fact that isolates collected before the use of antibiotics did not harbour resistance genes (O’brien 2002). Many strains of S. aureus show resistance to multiple drugs. The transfer of drug resistance genes among genomically related bacteria is highly probable. β-lactam antibiotics, such as penicillin and cephalosporin based medicines, staphylococci which have target peptidogycin present in the cell wall by interacting with its synthesis. These antibiotics
contain β-lactam rings that interfere with the enzymes responsible for cell wall construction (Robinson 1998). Strains of staphylococcus resistant to β-lactam antibiotics have a common resistance gene. This gene, denoted as mecA, is involved in the normal process of cell wall synthesis and does not contribute to resistance in the wild type. However, overexpression of the gene has been linked to increased antibiotic resistance. Isolates of resistant strains acquired in hospitals have much larger regions of this gene and tend to show more resistance to the antibiotics. The cellular mechanism, by which S. aureus is capable of withstanding the inhibitory effects of all β-lactam antibiotics, including the semi synthetic ones such as methicillin, is based on acquisition of the mecA gene. The exact mechanism responsible for mecA transfer is not known, but evidence supports horizontal transfer of mec gene between Staphylococcal species and of the mecA gene between different gram-positive bacteria. The assumption is that the ccr and mec genes were brought together in coagulase-negative staphylococci (CoNS) from an unknown source, where deletion in the mecA regulatory genes occurred, before the genes were transferred into S. aureus to generate MRSA (Jansen et al. 2006). S. epidermidis is a reservoir of antibiotic resistance genes that can be transferred to S. aureus as reflected by in vitro and in vivo studies. The transfer of mecA from S. epidermidis to S. aureus has been shown to occur in vivo, suggesting that mecA may be transferred more frequently to MSSA (Wielders et al., 2001). A high degree of diversity has been observed among MSSA strains. However, Schlichting et al. (1993) noticed that epidemiologically unrelated MSSA strains with similar PFGE patterns were found in different geographical regions, suggesting that some genotypes are ubiquitous. Multi-locus enzyme electrophoresis demonstrated the presence of clones in natural populations of S. aureus and, in particular, the presence of a clone responsible for most cases of toxic shock syndrome. In contrast to MSSA, MRSA strains have been shown limited number of genotypes. According to Fitzgerald et al., (2001) the mec gene has been horizontally transferred into distinct S. aureus chromosomal backgrounds, demonstrating that methicillin-resistant strains have evolved several times independently. Khan et al, 2000 utilized a PCR-based method used in combination with the selective antibiotic screening method to study the direction and mechanism of resistance transfer between poultry and human staphylococcal isolates. The DNA microarray technology has helped in detecting mecA in at least five divergent lineages, emphasizing that horizontal mecA transfer has played a fundamental role in the evolution of MRSA (Fitzgerald et al., 2001). Knowledge of the underlying genetic structure is important in understanding the epidemiological relationship between the strains. The molecular techniques can help to study underlying genetic structure and relationship between sensitive and resistant strains. The
Molecular typing plays an important role in epidemiological studies of nosocomial infection, such as MRSA infection. Pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing are considered the most discriminatory and reliable methods of typing, but they are technically complex, time consuming, and expensive. PCR-RFLP has been reported as a preliminary and simple screening method for molecular typing of *S. aureus* isolates. Coagulase serotyping is widely used in some Asian countries (Japan) in addition to conventional and genetic methods for distinguishing *S. aureus* strains. Numerous allelic forms of *S. aureus* coagulase exist and each isolate may produce one or more of different variants of this enzyme. The 3’ coding region of the coagulase gene contains a series of repeats that are well conserved but these repeats differ by the presence of *Alu*I restriction sites. Therefore, the *Alu*I and *Hae*II, *Sma*I restriction enzyme digestion of amplified PCR products produces fragments of different lengths. The *S. aureus* isolate can be discriminated by Restriction Fragment Length Polymorphism. Acquired resistance to antibiotics occurs either by mutations (point mutations, deletions, inversions etc. in bacterial genome) or by horizontal transfer of resistance genes located on various types of mobile DNA elements. The escape of resistance genes to mobile DNA fragments (plasmids) is enabling the process of transfer of antimicrobial resistance not only between bacteria of the same population, but between different genera of bacteria. In bacterial populations (plasmid bearing or not), the generation of antibiotic resistance depends on the rate of emergence of resistant mutants. Mutation frequencies to resistance can vary dramatically depending on the mechanism of resistance and whether or not organism exhibits a mutating phenotype.

### 2.9 Plasmid Profiling

The importance of plasmids in antimicrobial resistance is due to their role in gene transfer among Staphylococci. The transfer of genes seems more important in the spread of resistance than in the evolution of new antibiotic resistance gene. The plasmids have essential role in gene transfer, as resistance gene can migrate from a plasmid to chromosome and then back to plasmid that confers drug resistance to other bacteria. A close relationship between plasmid content and antibiotic resistance in *S. intermedius* cultures has been observed by Schwarz *et al.* (1989). These workers also found that chloramphenicol resistance in *S. intermedius* was plasmid-borne. Plasmids were used as typing tools by many researchers. Plasmid analysis, antibiotic susceptibility profile, bacteriophage typing and aminoglycoside modifying enzyme determinants have been used to study dissemination of MRSA in hospitals.
Greene and Schwarz (1992) evaluated antibiograms and plasmid profiles of *S. intermedius*, compared to the plasmids of other Staphylococci and found marked structural homologies between them.

### 2.10 Bacteriophage Typing

Phage typing is a conventional epidemiological tool which was first employed for typing *S. aureus* strains in England as early as 1940. This method is widely used even now since it is considered as ideal method of typing (Ayliffe, 1997). Typing of Staphylococci is important in epidemiology, when it is needed to find the similarities and differences of the strains obtained from different sources, to determine epidemic strains of *S. aureus*, and to evaluate the importance of different strains for human infectious pathology (Akatov *et al.*,1983).

*Staphylococcus aureus* bacteriophages

**Phage Twort**

*S. aureus* bacteriophage Twort belongs to the family *Myoviridae*. There are two genes in phage Twort which are required for the host cell lysis, holTWand plyTW (Weerakoon and Jayaswal, 1995). Several features of the holin protein families are shared by HolTw. These include: i. two membrane spanning domains ii. a β-turn linker separating the anti parallel transmembrane domains iii. a charged C-terminal domain (Young and Blasi, 1995). The holT is a 185 amino acid protein and overlaps with plyTW. The plyTW encodes endolysin by 35 bps but in a different reading frame, suggesting translational coupling of the two genes. This phage shares significant homology to the LytA amidase of Φ11 (42% identity). The C-terminal domain of PlyTW shares high degree of homology with the C-terminus of lysostaphin. HolTw complements a λ S gene mutation (Loessner *et al.*,1998).

**Phages Φ11, Φ12 and Φ13**

Phages Φ11, Φ12 are dsDNA phages which utilize a unique holin-endolysin system to achieve host cell lysis. The endolysins encoded by both Φ11 and Φ12 are comprised of three domains. The N-terminal region comprises the endopeptidase domain (CHAP), cleaving the D-alanyl-glycyl linkage. The central region of the endolysin harbours the amidase domain which cleaves the N-acetyl-muramoyl-L-alanine linkage. The C-terminal region contains the cell wall binding motif which comprises a SH3b domain (Baba and Schneewind, 1996; Sass
and Bierbaum, 2007). Phage \( \Phi_{13} \) is a dsRNA temperate phage with a genome of 42.7 kb and belongs to serogroup F (Carroll et al., 1995). Phage \( \Phi_{13} \) mediates a negative effect on \( \beta \)-haemolysin gene expression and a positive effect on staphylokinase production. The negative effect on \( \beta \)-hemolysin expression is achieved by phage integration into the host attB site, which is located within the \( \beta \)-hemolysin gene, hlb. Phage \( \Phi_{13} \) carries a gene for staphylokinase. The expression of this gene contributes to the formation of a phenotype with increased staphylokinase production and thereby to an increased virulence to the host (Zou et al., 2000).

**Phage 187**

This phage differs from all other \textit{S. aureus} phages in its host range, distinctive virions and its DNA restriction pattern (Loessner et al., 1999). It recognizes a distinctive teichoic acid (galactosamine ribitol teichoic acid). The holin gene of phage 187 is fully embedded in the 5’ region of the endolysin gene ply187 in contrast to other phages where it overlaps with the endolysin gene. The N-terminal domain of phage187 endolysin, Ply187, harbours the muralytic active domain and is thought to act as an N-acetylMuramoyl-L-alanine amidase due to its significant homology to LytA of phage \( \Phi_{11} \).

**Phage 68**

Phage P68 is the smallest ds DNA phages and belongs to the family \textit{Podoviridae} (Tao et al., 1998). It consists of an isometric head with a short non-contractile tail of ~ 40 nm in length. The pre-neck appendage of phage 68 is a hallmark of ‘\( \Phi_{29} \)-like phages. The total G+C content of the genome of this phage is 29.3%. Identical inverted terminal sequence repeats with a length of 210 bps were found at the end of the DNA. There are 22 open reading frames (ORF) within the genome of phage 68, out of which 12 are organized in an opposite direction. The lysis gene cassette of phage 68 comprises ORF15, which encodes the holin and ORF16, encoding the endolysin. Interestingly, the holin gene is almost completely embedded (overlap of 253 bps) within the endolysin gene (Vybiral et al., 2003).

**2.11 Laboratory diagnosis and molecular study of \textit{S. aureus}**

For diagnosing the infections caused by \textit{S. aureus}, both phenotypic and genotypic methods are employed. The conventional methods utilize the analysis of the sample obtained from the patients in the laboratory by culturing sample on suitable media and identification of the organism by cultural characteristics, Grams staining and biochemical tests. The isolates
can be tested for their susceptibility to different antibiotics prior to commencement of the therapy and early diagnosis an susceptibility patterns would help the clinicians for managing infections caused by *S. aureus*. For performing conventional antimicrobial susceptibility testing, the bacteria in pure culture are needed. These tests are relatively simple to perform. It may routinely take at least 24-48 hrs to obtain an antimicrobial susceptibility profiles. Interpretation of susceptibility is based on break points of different values determined by the pharmacokinetic properties of the drug, inherent characteristics of the organism and the sites of infections. Since these values are constantly changing, therefore, the simple phenotypic tests have their limitations. The susceptibility testing are highly dependent on experimental conditions and often more than one method would need to be performed to obtain an accurate susceptibility profile. To increase the rapidity and accuracy of susceptibility testing, the application of alternative identification methods that can be more reliable have recently been suggested. The development of rapid genotypic assays is an attractive approach for determining resistance profile targeting specific genes. The nucleic acid–based detection systems that can offer rapid and sensitive methods to detect the presence of resistance genes are becoming more accessible. Several commercial systems for rapid identification and determination of antimicrobial resistance have been developed as an alternative to the classical identification and detection protocols. The advantages of genotypic methods for the detection of antibiotic resistance include: i. detecting the presence or absence of a particular resistant gene ii. Being independent of categories involved in phenotypic expressions such as susceptibility, intermediate susceptibility and resistance for which breakpoints may vary between countries iii. Determination of low-level resistance iv. Can be performed directly on clinical specimens thus reducing the detection time and v. early interpretation leading to earlier therapeutic predictions. On the other hand, the genotypic methods do have their limitations: i genotypic methods detects resistant determinants whereas decision making in antimicrobial therapy is preferably based on detection of susceptibility ii some silent genes can cause false positive results and iii the presence of mutations in primer binding sites can affect PCR amplification, generating false-negative results (Sundsfjord *et al*., 2004).

Genetic assay complement conventional phenotypic test in the diagnosis of *S. aureus*. They can confirm the result of confirmatory tests for the detection of specific resistance mechanisms by phenotypic assays and are more sensitive and can be used to detect MRSA strains exhibiting heterogeneous low level expression pattern that cannot be detected by culture-based methods. Differentiation between MRSA and borderline oxacillin-resistant *S.
*aureus* strains is very difficult using phenotypic tests (Louie *et al*., 2000). They can be used for rapid detection of resistance determinants in clinical specimens; can thus assist early intervention in infection control strategies. Finally genetic assays can be utilized for molecular epidemiological purposes to analyze the spread of specific resistant pathogens (Kirkland *et al*., 1999). The application of genetic assays for detection of antimicrobial resistance is dependent upon total cost per test and user-friendly format of the particular test for training the lab technicians. The validation and quality assurance in the performance of the genotypic tests at diagnostic microbiology laboratory are crucial. The in-house developed PCR methods need to be validated before they can be used for clinical diagnostic purposes. This also applies to published methods as they may not have undergone rigorous testing. Commercial kits that have been properly validated can help to overcome some of the problems associated with “in house” tests but this depends on costs associated with them (Sundsfjord *et al*., 2004). The recent developments in multiplex and real-time PCR assays have fulfilled the clinical acceptance of genetic tests in laboratories. This would certainly lead to increased applications of genotypic tests at diagnostic microbiology laboratories (Espy *et al*., 2006). Genetic methods for the detection of antimicrobial resistance genes and their expression take advantage of the development of nucleic acid amplification and hybridization techniques. The available genetic information in large databases is used to design oligonucleotide primers complementary to the target of interest and labelled single-stranded nucleic acid probes for amplification of the particular gene. The available information on conserved and variable regions within the antimicrobial resistance gene is of fundamental importance for the analytic sensitivity and specificity of the amplification methods. The nucleic acid primers and probes may be specific for a defined gene or single nucleotide polymorphism or universal for a group of related resistance determinants. Alignment of multiple nucleotide sequences from the target of interest that are available from public databases can be used for primer or probe selection. Several commercial programs as well as free software are available for DNA sequence analysis on the internet that can be used for designing amplification primers. Selected primers and probes should be carefully checked to exclude potential cross reacting sequences (Sundsfjord *et al*., 2004).

Polymerase chain reaction (PCR) is the most commonly used nucleic acid amplification technique for detection of antimicrobial resistance genes. The PCR assays which utilize single primer pairs are simple to perform, inhibition may be observed in few instance. The addition of a second primer set as internal control is favourable (Fluit *et al*.,
Multiplex PCRs have been developed that can give simultaneous information by amplifying a number of genes together in the single assay. This technique was to amplify simultaneously multiple loci in the human dystrophin gene (Chamberlain et al. 1988). This method utilizes inclusion of two or more sets of primer pairs specific for different targets in the PCR assay. Quantitative PCR is a variation of multiplex PCR which can be used to quantify the amount of target DNA or RNA in a specimen (Tang et al., 1997). The PCR has problem of contaminating the sample with ‘carry-over products’. This problem has been largely removed by the advent of real-time PCR which enables us to monitor the amplified product during amplification process. The accumulating amplicon can be monitored based on labelled primers, oligonucleotide probes and/or fluorescing amplicons producing a detectable quantitative signal related to the amount and specificity of the amplicon (Sundsfjord et al., 2004). Several typing methods using PCR based on the microbial genotype or DNA sequence have been developed. Also, the differences indicate strain nucleotide sequences variation can be studied. PCR-based locus-specific Restriction Fragment Length Polymorphism (RFLP) is useful on this content. The specific locus to be examined is amplified with gene-specific primers and subjected to RFLP analysis. The DNA fragments are separated on an agarose or small polyacrylamide gel, and the digestion patterns are visualized following ethidium bromide staining. Locus-specific RFLP has been used in epidemiological studies of hepatitis C virus (HCV) by Davidson et al., 1995. Arbitrary primed PCR which is known as random amplified polymorphic DNA of molecular typing (Williams et al. 1990; Welsh McClelland 1990). RAPD assays are based on the use of short random sequence primers, 9 to 10 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of the bacterial genome. If two RAPD primers anneal within a few kilobases of each other in the proper orientation, a PCR product with a molecular length corresponding to the distance between the two primers results. The number and location of these random primer sites vary for different strains of a bacterial species. Thus, following separation of the amplification products by agarose gel electrophoresis, a pattern of bands which, in theory, is characteristic of the particular bacterial strain results. A number of studies have reported success in using RAPD assays to distinguish bacterial strains among diverse species, for example in A. baumannii (Rebolli et al., 1994) and S. aureus (Saulnier et al., 1993).

**Identification of S. aureus strains using Matrix-assisted laser desorption/ ionization time of flight mass spectrometry**
Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is recent methodology which is for bacterial identification (Walker et al., 2002). This technology was applied to selected isolates of *S. aureus*. These studies reflected using distinct protein peaks. It is possible to indentify bacteria. These experiments differed with regard to the matrix used for MALDI-TOF MS, the protein-extraction protocols and the bacterial culture conditions. Moreover, the use of different proteomic fingerprint ranges makes it difficult to compare the published data. For the Biotype 2.0 identification approach, protein peaks in the mass-to-charge ratio of 3000–15 000 KDa were used, most of them thought to represent ribosomal protein peaks. Three studies with differences with regard to the study design have been described applying MALDI-TOF MS technology to more than 68 strains of *S. aureus* (Rajakaruna et al., 2009). In these studies, the rate of correct identification seemed to be dependent on the underlying database and algorithm used for the identification, characterization of the strain collection as well as the investigated proteomic fingerprint range.

### 2.12 Prevention and control of *S. aureus*

The determination of antimicrobial susceptibility pattern of bacterial pathogens is one of the most important steps in treatment of the infections. For treatment of infectious diseases, the accumulated susceptibility testing data at local, regional or national level would be helpful not only in therapeutic management of *S. aureus* infections but also to monitor the spread of resistant organisms or resistance genes throughout the hospital and community. A critical strategy to minimize spread of antimicrobial-resistant bacteria in healthcare institutions is detection of colonized patients and healthcare workers to initiate efficient infection control measures. The global emergence and spread of antimicrobial resistance poses a major risk for human health due to the impact on morbidity, mortality, and healthcare costs (Sundsfjord et al., 2004). The treatment of serious MRSA infections presents a great challenge to clinicians, particularly bacteremia and infective endocarditis, for which bactericidal therapy is essential to maximize successful clinical outcomes. Vancomycin has been the preferred antimicrobial agent to treat such MRSA infections. However, the clinical efficacy of this glycopeptides has become more limited (Sader et al., 2007). Resistance to this drug has emerged due to its widespread use to treat infections caused by MRSA and other gram-positive cocci. Linezolid and quinupristin-dalfopristin are alternative treatment options for serious MRSA infections; however, these compounds also have certain limitations. Quinupristin-dalfopristin, a streptogramin combination, requires a central venous access to be administrated and may lead
to certain adverse reactions such as arthralgia and myalgia (Sader et al., 2007). The long term treatment with linezolid, an oxazolidinone may lead to hematologic toxicity. Also, bacteriostatic in nature against *staphylococci*, this drug is not indicated for the treatment of endocarditis and serious infections in immune suppressed patients. The currently, β-lactam antibiotics are used to suspect CA-*S. aureus* infection in the community along with removal of foci of infection, e.g., abscess drainage. Severe community-acquired infections such as pneumonia or septicaemia are treated with broad-spectrum intravenous β-lactam antibiotics in majority of the cases. Uncomplicated cutaneous abscesses caused by CA-MRSA are often cured by incision and drainage along with appropriate antibiotic therapy (Lee et al., 2004). In regions in which the incidence of CA-MRSA is high, intravenous vancomycin is now given empirically for selected septicaemia patients in some part of world (Murray et al., 2003) and oral clindamycin and trimethoprim/ sulfamethoxazole are considered first-line drugs for the treatment of cutaneous infections in parts of the USA (Buescher, 2005). CA-MRSA infections in contrast to the majority of HA-MRSA are generally susceptible to most non-β-lactam antibiotics (Vandenesch et al., 2003) but the former may easily acquire resistance to various older non-β-lactam antibiotics. Widespread use of clindamycin had resulted in a small but significant rise in resistance within 2 years in some part U.S.A. (Braun et al., 2005). The newer and more recent antibiotics such as linezolid, daptomycin, tigecycline and newer glycopeptides have proved effective for CA-MRSA (Sader et al., 2007). The knowledge of biochemical mechanisms involved in development of antimicrobial resistance is essential for improving antimicrobial susceptibility testing and therapeutic interpretation of resistance phenotypes (Livermore et al., 2001). The detection of methicillin resistance in staphylococci allows reporting of resistance to other β-lactams that have not been tested because the resistance mechanism predicts treatment failure. Qualified interpretation requires accurate bacterial speciation and careful selection of indicator drugs that are best able to discern certain resistance mechanisms. This notion is illustrated by the use of oxacillin to screen for reduced susceptibility to penicillin in Pneumococci. Rapid and accurate identification of MRSA in clinical samples is of considerable importance for the institution of early correct therapy and to reduce the work load associated with MRSA control and surveillance. Numerous molecular methods have therefore been developed to confirm phenotypically suspected MRSA and to reduce the detection time of MRSA in clinical samples, including blood culture (Francois et al., 2004).
**Prevention and effective control of CA-MRSA**

CDC (2003) has suggested some measures to control CA-MRSA such effective surveillance, appropriate treatment, draining of abscess whenever required, education and awareness among athletes to keep their wounds clean and covered, good personal hygiene among athletes and the staff. Athletes are responsible for transmission of such organism as per CDC 2003. Athletes should not be allowed to use common water facilities. Use of hand gels and limiting sharing of personal items such as towels etc are some of the important measures to prevent the spread of *S. aureus* infections. Adequate precautions should be taken by the healthcare workers while handling HA-MRSA so that they don’t transmit these organisms to patients or other workers.