1. Introduction

1.1. Classification

*Staphylococcus aureus* is an aerobic or facultative anaerobic, coagulase positive organism which colonises the skin, nasal passage and axillae of humans. It occurs in grape like clusters when viewed through the microscope and has large round golden yellow colonies often with beta hemolysis when grown on blood agar [Murray et al 2003].

<table>
<thead>
<tr>
<th>Scientific Classification of <em>Staphylococcus aureus</em></th>
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<tr>
<td><strong>Binomial name</strong></td>
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<tr>
<td><strong>Genus</strong></td>
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<td><strong>Family</strong></td>
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<td><strong>Order</strong></td>
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<td><strong>Class</strong></td>
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<td><strong>Phylum</strong></td>
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<td><strong>Kingdom</strong></td>
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1.2. History and Natural habitat

Staphylococci were first observed in human pyogenic lesions by Von Recklinghausen in 1871. Pasteur in 1880 obtained liquid cultures of cocci from pus and produced abscesses by inoculating them into rabbits. But it was Sir Alexander Ogston, a Scottish surgeon in 1880 who established conclusively the causative role of the coccus in abscesses and other suppurative lesions. He also gave the name *Staphylococcus* (Staphyle, in Greek meaning ‘bunch of grapes’: Kokkos, meaning a berry) due to the typical occurrence of the cocci in grape like clusters in pus and in cultures. Ogston had noticed that non-virulent staphylococci were also present on skin surfaces. Most staphylococcal strains from pyogenic lesions were found to produce golden yellow colonies, and the strains from normal skin, white colonies on solid media. In 1884, Rosenbach named them *Staphylococcus aureus* and *Staphylococcus albus* respectively. Later *S.albus* was renamed as *Staphylococcus epidermidis* which
were coagulase negative, mannitol nonfermenting and usually non pathogenic strains [Murray et al 2003; Humphreys 2002].

Staphylococci are widespread in nature although they are mainly found living on the skin, skin glands and mucous membrane of mammals and birds. They may be found in the mouth, blood, mammary glands, intestinal, genitourinary and upper respiratory tracts of these hosts. *Staphylococcus aureus* generally have a benign or symbiotic relationship with their host; however they may develop the lifestyle of a pathogen if they gain entry into the host tissue through trauma of the cutaneous barrier, inoculation by needles or direct implantation of medical devices. Infected tissues of host support large populations of staphylococci and in some situations they persist for long periods. The presence of enterotoxigenic strains of *S. aureus* in various food products is regarded as a public health hazard because of the ability of these strains to produce intoxication or food poisoning. *S. aureus* is a major species of primates, although specific ecovars or biotypes can be found occasionally living on different domestic animals or birds [Murray et al 2003].

### 1.3. Cultural characteristics

The cocci are spherical, approximately 1μm in diameter, arranged in grape like clusters. They may be found singly, in pairs or in short chains especially in liquid culture. They are non-motile and non-sporing and some strains possess microscopically visible capsules [Anathanarayan 2002].

They grow readily on ordinary media within a temperature range of 10-42°C. Optimum temperature is 37°C and pH 7.4-7.6. On nutrient agar a typical 24hr *S. aureus* colonies are pigmented, smooth, entire, slightly raised, translucent and hemolytic on routine blood agar. Small colony variants (SCVs) of *S. aureus* produce colonies that are pinpoint in size, non hemolytic and non pigmented. In liquid medium, uniform turbidity is produced. Selective media used for isolating *S. aureus* contain 8-10% NaCl like salt-milk agar, ludlam’s medium containing lithium chloride and tellurite [Bannerman 2003].
Fig 1: Gram positive cocci in clusters. *Staphylococcus aureus* from pus

Fig 2: Golden yellow pigmented colonies of *Staphylococcus aureus* on nutrient agar.

Fig 3: Structure of cell wall of *Staphylococcus aureus*

Fig 4: Cell surface antigens of *S. aureus*
1.4. Biochemical reactions of *Staphylococcus aureus*

They ferment sugar producing acid but no gas. Mannitol is fermented anaerobically only by *S. aureus*. They are catalase and urease positive. They reduce nitrates to nitrites, liquefy gelatin and are MR, VP positive but indole negative. They are lipolytic when grown on medium containing egg yolk. They produce phosphatase which can be demonstrated by growing on nutrient agar containing phenolphthalein diphosphate. In a medium containing potassium tellurite, tellurite is reduced and black colonies are produced [Humphreys 2002; Anathanarayan 2002].

**Coagulase Production**

The ability to clot plasma is generally accepted criterion for the identification of *S. aureus*. Two different coagulase tests are performed: a tube test for detecting free coagulase and slide test for bound coagulase or clumping factor. While the tube test is definitive, the slide test may be used as a rapid screening technique to identify *S. aureus*. Coagulase test is carried out using rabbit plasma containing EDTA [Bannerman 2003].

**Heat Stable Nuclease**

A heat stable staphylococcal nuclease (thermonuclease (TNase)) that has endo and exonucleolytic properties and can cleave RNA or DNA is produced by most strains of *S. aureus*. TNase can be demonstrated by the ability of boiled cultures to degrade DNA in an agar diffusion test or detected by using metachromatic agar diffusion procedure and DNase toludene blue agar.

**Acetoin Production**

Acetoin production from glucose or pyruvate is a useful alternative characteristic to distinguish *S. aureus*. This is done using a conventional Voges-Proskauer test tube method with an incubation time of 72hrs [Bannerman 2003].

1.5. **Laboratory Diagnosis**

One or more of the following specimen are collected to confirm a diagnosis.

1) Pus from abscesses, wounds, burns etc is much preferred to swabs.

2) Sputum from cases of Pneumonia e.g. post influenzal or ventilator associated pneumonia. Bronchoscopic specimens are increasingly used in critically ill patients.
3) Faeces or vomit from patients with suspected food poisoning or the remains of implicated food.

4) Blood from patients with suspected bacteremia, e.g. septic shock, osteomyelitis or endocarditis.

5) Mid stream urine from patients suspected of cystitis or pyelonephritis.

6) Anterior nasal or perennial swabs (moistened with saline or sterile water) from suspected carriers. Nasal swabs should be rubbed in turn over the anterior walls of both nostrils.

The characteristic clusters of gram positive cocci can be demonstrated by microscopy and the organism can be cultured readily on blood agar and most other media. Tube or slide coagulase is performed to distinguish *S. aureus* from coagulase negative staphylococci [Humphreys 2002].

### 1.6. Virulence factors and pathogenesis

*Staphylococcus aureus* typically produces 5 types of penicillin binding proteins (PBPs). The antibacterial activity of beta lactam antibiotics results from their covalent binding to the active sites of penicillin binding proteins, PBPs. PBPs are enzymes that catalyse transpeptidase reaction i.e. the cross-linking reactions between peptidoglycan polymers. Therefore, β- lactam antibiotics are potent inhibitors of cell wall synthesis. The 5 types of PBPs found in susceptible strains of *S. aureus* includes PBPs1, 2, 3, 3' and 4 with molecular weights of approximately 85,000, 80,000, 75,000, 70,000 and 45,000 daltons respectively, which functions as transpeptidases, endopeptidases and carboxy peptidases B lactam antibiotics are substrate analogs that covalently bind to the PBP active site serine, inactivating the enzyme at concentrations that are approximately the same as minimum inhibitory concentrations (MICs). [Chambers 1988; Chambers 1997]. Depending on the strain *S. aureus* is capable of secreting several toxins which can be categorized in to 3 groups . Many of these toxins are associated with specific diseases.

1. Pyrogenic toxin super antigens (PTS AgS) have super antigen activity that includes toxic shock syndrome (TSS). This group includes TSST-1, which causes toxic shock syndrome and staphylococcal enterotoxins which cause a form of food poisoning. They produce 6 serotypes of enterotoxins which cause diarrhea and vomiting when ingested.
2. Exfoliative toxins are implicated in the disease staphylococcal scalded skin syndrome (SSSS), which occurs most commonly in infants and young children. Exfoliative toxins have protease activity which causes peeling of the skin observed with SSSS.

3. Membrane damaging toxins include $\alpha$ toxin, $\beta$ toxin and $\gamma$ toxin and the classical Panton – Valentine Leukodicin (PVL) factor. PVL is a bicomponent toxin associated with severe hemolytic and necrotizing pneumonia in children. The genes encoding PVL components are encoded on a bacteriophage found in community associated methicillin resistant *Staphylococcus aureus* strains. $\alpha$ toxin- also called $\alpha$ hemolysin is a protein inactivated at 70°C but reactivated paradoxically at 100°C because at 60-70°C the toxin combines with a heat labile inhibitor which is denatured at 100°C. It is leucocidal, cytotoxic, dermonecrotic, neurotoxic and lethal only on rabbit erythrocytes. $\beta$ hemolysin- is a sphingomyelinase, hemolytic for sheep cells. Gamma hemosylin – is a bicomponent protein necessary for hemolytic activity. Delta hemolysin – has a detergent like effect on cell membranes of erythrocytes, leucocytes, macrophages and platelets. Leucocidin – Panton valentine leucocidin is a bicomponent toxin having membrane damaging toxins similar to gamma lysine [Humphreys 2002; Foster ; Foster 2004; Bohach 2000]

1. **Surface proteins that promote colonization of host issues:**- Invasins that promote bacterial spread in tissues which include enzymes like leucocidin, kinases, hyaluronidase etc. Lipases or Lipid hydrolases help in infecting skin and subcutaneous tissues. Hyaluronidases break down the connective tissue. Staphylokinase (fibrinolysin) are fatty acid modifying enzymes and proteases that help in initiation and spread of infection.

2. **Surface factors that inhibit phagocytic engulfinent:** - Protein A present on *S. aureus* has chemotactic, antiphagocytic and anticomplementary activities. It induces platelet damage and hypersensitivity. Teichoic acid, an antigenic component of the cell wall facilitates adhesion of the cocei to the host cell surface and protects them from complement mediated opronisation. Capsular polysauharide surrounding the cell wall inhibits opsonisation.

3. **Immunological disguises such as coagulase and clotting factor:** -Clumping factor is a surface protein called bound coagulase which is responsible for slide coagulase
test routinely used for the identification of *S. aureus*. Coagulase is an extracellular enzyme which along with coagulase reacting factor CRF present in the plasma, binds to prothrombin, converting fibrinogen to fibrin, calcium or other clotting factors are not required for coagulase action.

4. **Membrane damaging toxins such as hemolysins, leucotoxins and leucocidin.**

5. **Exotoxins such as sea-G, TSST and ET** that damage host tissues and provoke symptoms of disease.

6. **Inherent and aquired resistance to antimicrobial drugs** [Humphreys H 2002; Foster TJ 2004]

**Table 2: Virulence factors of *S. aureus***

<table>
<thead>
<tr>
<th>Type of virulence factors</th>
<th>Selected factors</th>
<th>Genes</th>
<th>Associated clinical syndromes</th>
<th>References</th>
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<tbody>
<tr>
<td>Involved in attachment</td>
<td>MSCRAMMs (e.g., clumping factors, fibronectin-binding proteins, collagen, and bone sialoprotein-binding proteins)</td>
<td><em>clfA</em>, <em>clfB</em>, <em>fnbA</em>, <em>fnbB</em>, <em>cna</em>, <em>sdr</em>, <em>bbp</em></td>
<td>Endocarditis, osteomyelitis, septic arthritis, and prosthetic-device and catheter infections</td>
<td>Patti et al 1994; Foster 1998.</td>
</tr>
<tr>
<td>Involved in persistence</td>
<td>Biofilm accumulation (e.g., polysaccharide intercellular adhesion), small-colony variants, and intracellular persistence</td>
<td><em>ica</em> locus, <em>hemB</em> mutation</td>
<td>Relapsing infections, cystic fibrosis, and syndromes as described above for attachment</td>
<td>Donlan 2002; Arrecubieta 2006.</td>
</tr>
<tr>
<td>Involved in evading/destroying host defenses</td>
<td>Leukocidins (e.g., PVL and á-toxin), capsular polysaccharides (e.g., 5 and 8), protein A, CHIPS, Eap, and phenol-soluble modulins</td>
<td><em>lukS</em>-PV, <em>lukFPV</em>, <em>hlg</em>, <em>cap5</em> and 8 gene clusters, <em>spa</em>, <em>chp</em>, <em>eap</em>, <em>pse</em>, á gene cluster</td>
<td>Invasive skin infections and necrotizing pneumonia (CA-MRSA strains that cause these are often associated with PVL) abscesses (associated with capsular polysaccharides)</td>
<td>Foster 2005; O'Riordan 2005; Tzianabos 2001. Wang 2007</td>
</tr>
<tr>
<td>Involved in tissue invasion/penetration</td>
<td>Proteases, lipases, nucleases, hyaluronate lyase, phospholipase C, and metalloproteases (elastase)</td>
<td><em>v8</em>, <em>hya</em>, <em>hla</em>, <em>plc</em>, <em>sepA</em></td>
<td>Tissue destruction and metastatic infections</td>
<td>Projan 1997</td>
</tr>
<tr>
<td>Involved in toxin-mediated disease and/or sepsis</td>
<td>Enterotoxins, toxic shock syndrome toxin-1, exfoliative toxins A and B, á-toxin, peptidoglycan, and lipoteichoic acid</td>
<td><em>sea-q</em> (no <em>sef</em>), <em>tstH</em>, <em>eta</em>, <em>eta</em>, <em>hla</em></td>
<td>Bullous impetigo, and sepsis syndrome</td>
<td>Dinges 2000; Timmerman 1993</td>
</tr>
<tr>
<td>With poorly defined role in virulence</td>
<td>Coagulase, ACME, and bacteriocin</td>
<td><em>arc</em> cluster, <em>opp-3</em> cluster, <em>bsa</em></td>
<td></td>
<td>Baba 2002; Diep 2006</td>
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1.7. Epidemiology

*Staphylococcus aureus* lives on people and survive on inanimate objects and surfaces (fomites), such as bedding, clothing and doorknob. Humans are the major reservoir for *S. aureus*. The organisms frequently colonise the anterior nares and are found in approximately 30% of healthy individuals. However studies of individuals overtime have found that up to 90% of the people are eventually colonized in the nares with *S. aureus* at some point in their lives. They can also be found transiently on the skin, oropharynx, vagina and in feces. They are well equipped to colonise the skin because they grow at high salt concentration and lipid concentration. They make enzymes, referred to as lipases and glycerol ester hydrolases that degrades skin lipids. The ability of *S. aureus* to colonise the skin and mucosal surfaces is associated with bacterial cell surface proteins that bind to a variety of extracellular matrix proteins. Fibronection binding proteins (Fnb PA and Fnb PB) have been identified on the surface of *S. aureus* which allows the bacteria to invade epithelial and endothelial cells and to attach to exposed fibronectin in wounds. [Humphreys 2002; Bannerman 2003; Tenover 2000]

Large numbers of staphylococci are disseminated in pus and dried exudates discharged from skin lesions, large infected wounds, burns and in sputum coughed from the lung of a patient with bronchopneumonia. Direct contact is the most important mode of spread, but air borne dissemination may also occur. *S. aureus* is an important secondary pathogen associated with patients recovering from influenza and para-influenza infections. Small discharging lesions on the hands of doctors and nurses are danger to their patients. Cross-infection is an important method of spread of staphylococcal disease, particularly in hospitals and scrupulous hand washing is essential to prevent the spread. Food handlers may similarly introduce entero-toxin producing food poisoning strains into food. Infants may be colonized with *S. aureus* shortly after birth, acquiring the organism from people in their immediate surrounding.

**Healthy carriers**

*S. aureus* grows harmlessly on the moist skin of the nostrils in many healthy persons. This condition is referred to as colonization. Colonisation frequently precedes infection in susceptible patients. The anterior nares are the principal sites of colonization with three distinct patterns in the population: persistent carriers (20%),
intermittent carriers (60%), and non carriers (20%). Whereas, 10 -20% of healthy adults are persistently colonized with *S. aureus*, populations with higher colonization rates include patients with atopic dermatitis, surgical patients, hemodialysis patients, HIV infected patients and those with intra vascular devices. *S. aureus* is the leading cause of post operative wound infection and the second most frequent cause of nosocomial pneumonia and bacteremia. Health care workers who come in contact with patients colonized or infected with *S. aureus* have higher rate of nasal carriage and can serve as vehicles for transmission of *S. aureus* to patients. The morbidity and mortality rates of nosocomial and community acquired staphylococcal infections range between 19% and 34%. Some carriers called shedders disseminate exceptionally large number of staphylococci, and transmission occurs through hand, handkerchief, clothing and dust consisting of skin squames and cloth fibers [Humphreys 2002; Foster 2004].

**Clinical syndromes**

*S. aureus* is notorious for causing boils, furuncles, styes impetigo and other superficial skin infections in humans. It may also cause serious infections such as pneumonia, deep abscesses, osteomyelitis, endocarditis, phlebitis, mastitis and meningitis particularly in persons debilitated by chronic illness, traumatic injury, burns or immunosuppression. Small colony variants (SCVs) of *S. aureus* are a naturally occurring subpopulation which grows slowly and produces small colonies on routine media. This is most common in patient populations with unusual persistent infections such as cystic fibrosis or chronic osteomyelitis. Staphylococcal skin lesions such as pimples or abscesses are filled with a core of pus. Abscesses can progress to produce boils, which can develop into carbuncles. Carbuncles are larger, deeper, extremely painful and dangerous lesions since they can progress into systemic infections through out the body. A relatively common manifestation of staphylococcal infection is impetigo. This is a superficial infection of the skin and usually occurs around the mouth in the form of blisters that ooze a yellowish liquid. Impetigo occurs in very young children particularly following a runny nose, which sets up irritation in the surrounding tissues. It is not particularly serious but easily spreads from child to child.

Scalded skin syndrome (SSS) is another disease which tends to be more prevalent in children with infection of the stem of umbilical cord. In this case skin
becomes blistery as a result of the production of an exfoliative toxin that peels away the skin to expose a red layer.

Toxin shock syndrome (TSS) is a community acquired disease attributed to the infection or colonization with *S. aureus*. A single clone has been shown to cause the majority of cases. TSS was prevalent in young menstruating females using certain types of highly absorbent tampons. TSS associated non genital *S. aureus* has also been found in men and non menstruating women. TSS is associated with strains that produce and secrete the exotoxin. Toxin shock syndrome toxin (TSST-1) which is a member of the super antigen family has the ability to stimulate T cells, and induce tumour necrosis factor (TNF) and cytokine Interleukin 1 (IL1). Symptoms include high fever, nausea, vomiting peeling of skin (particularly on the palms and the soles) and a dangerous drop in blood pressure that leads to life threatening shock. It is some times associated with surgical wound infections. In 1981 approximately 1000 cases of TSS were reported and in 1997 100 cases were reported.

*S. aureus* bacteremia is classified in to hospital acquired, health care associated and community associated. They are related to risk factors such as intravascular devices and co morbid conditions. Community acquired bacteremia afflicts intravenous drug users and otherwise healthy patients with infections at various sites. Approximately one third of the patients with bacteremia develop complications which manifest within 48 hrs of diagnosis, which include septic shock, acute respiratory distress syndrome and disseminated intravascular coagulation. Metastatic complications occur in the joints, kidneys, central nervous systems, skin, intervertebral disk, lungs, liver/spleen, bone and heart valves.

Infective endocarditis (IE) is a complication of *S. aureus* bacteremia. Endocarditis is a complication of *S. aureus* bacteremia. Endocarditis in patients with bacteremia frequently involves normal cardiac valves. Because of the difficulty in clinically identifying *S. aureus* IE, the use of echocardiography has been advocated to evaluate patients with bacteremia. Despite early diagnosis and appropriate therapy, IE is often associated with devastating and life threatening sequale. Complication includes heart failure, paravalvular cardiac abscesses, neurological manifestations and systemic embolization.
Staphylococcus aureus is a significant etiological agent of nosocomial pneumonia. In addition to its role as a nosocomially acquired pulmonary pathogen, S. aureus has recently established itself as an emergent threat in the community. Necrotising pneumonia and sepsis caused by community acquired MRSA strains carrying PVL genes are being increasingly recognized. Afflicted patients are typically healthy individuals without any healthcare contact. These infections are characterized by multifocal involvement of various organs including lungs, brain, heart, liver and kidneys. The pathological feature in the lungs is extensive hemorrhagic necrosis of the pulmonary parenchyma. S. aureus pneumonia can present in different forms with distinct pathophysiological mechanisms:

1. Lobar pneumonia usually occurs as a result of aspiration. Patients are acutely ill with high fevers and productive cough. In severe infections empyema, abscess formation, cavitation and pneumatoceles may be present.
2. Diffuse interstitial pneumonia usually follows microaspiration and develops in conjugation with or following viral pneumonia.
3. Peripheral localized areas of pneumonia are noted with hematogenous seeding of the lungs from septic emboli, secondary either to right sided endocarditis or soft tissue or joint infection. In this type of pneumonia pleuritic chest pain is a common feature whereas cough and sputum production are less likely. [Chamber 1997; Fowler 2006].

1.8. Evolution and epidemiology of MRSA

Ever since the first use of penicillin, S. aureus has shown a remarkable ability to adapt. The first report of penicillin resistant strain of S. aureus was published in 1945 revealing its association with the penicillinase enzyme produced by the bacteria. Semisynthetic penicillinase resistant penicillin called Methicillin group of antibiotics were introduced in 1959. But methicillin resistant Staphylococcus aureus (MRSA) were identified within one year of introduction of Methicillin into clinical practice. MRSA was first reported in the UK and Europe in the early 1960s and in the US in 1968. The NNIS reports an increasing trend of MRSA. A 40% increase in resistance in 1999 was noted compared to 1994-1998 data. MRSA is now endemic in many hospitals and is one of the leading causes of nosocomial pneumonia and surgical site infection and the second leading cause of nosocomial blood stream infections.
The first line treatment for serious invasive infections due to MRSA is currently glycopeptide antibiotics (vancomycin and teicoplanin), but with several drawbacks mainly centered around the need for intravenous administration (no oral preparation available), toxicity and the need to monitor drug levels regularly by means of blood tests. There are also concerns that glycopeptides do not penetrate well into infected tissues particularly in meningitis and endocarditis. In 1999 MRSA treatment costs were estimated to be 6-10% more than treating an MSSA infection resulting from the high cost of vancomycin and costly isolation procedures. Because of the high level resistance to penicillins and because of the potential for MRSA to develop resistance to vancomycin, CDC has published guidelines for appropriate use of vancomycin. Yet recently vancomycin resistant strains have been reported. The first case of VISA was reported in Japan in 1996, but the first case of *S. aureus* truly resistant to glycopeptide antibiotic was reported in 2002. As of 2005, 3 cases of VRSA had been reported in the US [Ito et al 2001].

*S. aureus* became methicillin resistant by acquiring a ‘mecA gene’, usually carried on a larger piece of DNA called a Staphylococcal Cassette Chromosome (SCC). It has been possible to trace the evolution and dissemination of methicillin resistance within the genus *Staphylococcus* but the origin of SCCmec is still unclear. The expression of mecA yields PBP2a (penicillin binding protein) with reduced binding for β-lactam antibiotics. PBPs are necessary for correct synthesis of bacterial cell wall and when they are blocked by penicillin, the cell wall is incorrectly formed and the cells are liable to lyse. The presence of PBP2a allows the bacterium to synthesize cell wall normally even in the presence of inhibitory concentrations of penicillin or methicillin.

Some strains of *S. aureus* over express β-lactamase and thus appear resistant to oxacillin and rarely methicillin despite being mecA negative. β-lactamase is an enzyme that cleaves the penicillin molecules at its cyclic ring and second generation penicillins like methicillin were specifically designed to resist the β-lactamase activity [Arakere et al 2005; Yoko et al 2007; Bressler et al 2005].

1.9. *Mec Associated DNA*

β-lactam resistance in MRSA is caused by production of a variant Penicillin binding Protein designated as PBP2a or PBP2’. Unlike the intrinsic set of PBPs (1-5),
PBP2a has a remarkably reduced binding affinities to β-lactam antibiotics, so that even in the presence of normally inhibitory concentrations of β-lactam antibiotics, MRSA can continue cell wall synthesis solely depending upon the uninhibited activity of PBP2’, encoded by mecA gene located on the chromosome of MRSA.

mecA gene is a 2.4 kb chromosomal determinant encoding the PBP2’ protein which is not subjected to dissemination among staphylococcal strains via plasmid spread. Expression of PBP2a is under the control of negative regulation elements mecI and mecRI. These genes regulate the mecA response to β-lactam antibiotics in a fashion similar to that of blaZ gene by blaR1 and blaI. BlaI is a DNA binding protein that represses β-lactam gene transcription. mecI and mecRI perform analogous regulatory roles for mecA. Mec is always found near the pur-nov-his gene cluster on S. aureus chromosome. mecA, mecR1 and mecI are encoded by approximately 5kb of DNA that itself is located within 25 to 50kb of additional DNA that way contain upto 100 open reading frames. Transposons and insertion sequences are present including Tn554 which contains ermA, the gene encoding inducible erythromycin resistance located 5’ of mecA and one to four copies of IS431, at least one of which IS431 mec, is located 3’ of mecA. The region between mecA and IS431 mec is highly variable containing varying number of direct repeat units (DRUs) due to deletion, rearrangement and recombination events that may occur in this region. This is known as hyper variable region (HVR). IS431 is an extremely common insertion sequence in the staphylococcal chromosome associated with a host of resistance determinants including mercury, cadmium and tetracycline. The ability of IS431, elements through homologous recombination to trap and cluster resistance determinants explains the multiple drug resistance phenotype that is characteristic of methicillin resistant staphylococci [Chambers 1988; Chambers 1997].

**Fig 5: Downstream mecA arrangement of S. aureus ATCC49476 showing HVR region. The scale is shown in kilobases [Senna et al 2002].**
**Staphylococcal cassette chromosome (SCC mec)**

*mecA* gene is a part of a mobile genetic element found in all MRSA strains known as the staphylococcal cassette chromosome or SCC *mec*. Four different SCC *mec* elements ranging from 21 to 67 kb have been described. Three types of SCCs were originally described in hospital acquired MRSA strains (HA-MRSA) most of them isolated before 1990. A fourth type was recently described (type IV), first in community acquired MRSA (CS-MRSA) isolates and then in several MRSA backgrounds.

SCC *mec* carries a set of unique recombinase gene *ccrA* and *ccrB* that are specifically involved in recombination events (integration and excision) of SCC *mec* with the *S. aureus* chromosomes. Since the 1960s spontaneous loss of *mecA* gene has been observed during the storage or long term cultivation of MRSA strains in antibiotic free medium. Deletion of a large chromosomal region is identified in such *mecA* deletion. The deletion starts precisely from the left boundary of IS431 *mec* and extends leftwards for various distances beyond the *mecA* gene. *mecA* is transferred from cell to cell as a part of the SCC *mec* across staphylococcal species. Methicillin sensitive strains have been shown to become methicillin resistant by acquisition of a staphylococcal cassette chromosome *mec* element carrying the *mecA* gene [Chongtrakool et al 2006]. But so far no transducing phage capable of transferring genetic information across the staphylococcal species barrier has been described. Hence transmission by phage transduction has not been confirmed. There are also no reports of existence of other genetic transfer systems specific for movement of SCC *mec*. SCCs are found to show great geographical variation which makes cassette chromosome typing essential for complete characterisation of MRSA. Future elucidation of the mechanism of regulation of SCC*mec* excision may lead to the attractive possibility of the development of novel therapeutic measure to aid in antibiotic chemotherapy against MRSA infection by converting MRSA strain in vivo into MSSA strains against which many antibiotics are effective. [Ito et al 2001]

**1.10. Properties of methicillin resistance**

Methicillin resistant strains show 2 types of resistance – heterogeneous and homogenous. In heterogeneous resistance only rare cells (1 in $10^4$ to $10^8$) express the resistance trait and grow in the presence of high concentrations of drug (50µg of
methicillin per ml). Most of the cells are susceptible to relatively low, therapeutically achievable concentrations of drug (e.g., 1-5 mg of methicillin per litre). Thus heterogeneous strains consist of 2 populations – relatively susceptible cells and highly resistant cells. The homogenous minorities of cells are uniform in expression of resistance and can grow in high concentrations of the drug. Hartman and Tomasz has classified resistant strains into homogenous and heterogeneous categories based on efficiency of plating defined as the number of colony forming units (CFUs) on drug containing agar plates multiplied by 100% at a concentration of 50 µg of methicillin per ml in tryptic soy broth agar, pH 7.0 at 37°C after 72 to 96 hrs of incubation. For homogenous strains 1% or more CFUs grow and for heterogeneous strains <1% do so.

Most of clinical isolates exhibit this heterogeneous pattern of resistance under routine growth conditions, such as growth in hypertonic culture medium supplemented with NaCl or sucrose or incubation at 30°C. Addition of EDTA or incubation at 37°C to 43°C favours heterogeneous pattern. These changes with varying culture conditions are phenotypic. Passage of heterogenous strains in the presence of β-lactam antibiotics alters the resistance phenotype by selecting for highly resistant mutant clones.

Another type of methicillin resistance is the borderline resistance characterized by MICs at or just above the susceptibility break points (e.g., oxacillin MICs of 4 to 8 mg/l) borderline strains are divided into two categories based on presence or absence of mecA. Borderline strains that contain mecA are extremely heterogeneous and the resistance of mecA negative strains is attributed to the hyper production of staphylococcal β-lactamase.

Several chromosomal genes physically distinct from mec, that are necessary for full expression of resistance has been identified. These ‘fem’ (factors expression for methicillin) factors or auxiliary factors are present in both susceptible and resistant strains. Six fem genes – femA, femB, femC, femD, femE and femF which map to numerous sites throughout the staphylococcal genome have been characterized [Chambers 1988].

1.11. Vancomycin Resistant Staphylococcus aureus (VRSA)

Until recently vancomycin was the only antibiotic effective against MRSA. Vancomycin is not a drug recently developed for treatment of MRSA, but is an old drug discovered in 1956. It was first isolated by EC Kornfield from a soil sample
collected from the interior jungle of Borneo by a missionary. It is produced by the organism *Streptomyces orientalis*. Vancomycin never became the first line treatment for *S. aureus* for several reasons:

1. The drug must be given intravenously because it is not absorbed orally.
2. β-lactamase resistant semi synthetic penicillins such as methicillin were subsequently developed.
3. Early trial using impure forms of vancomycin were found to be toxic to the ear and to the kidneys.

These findings led to vancomycin being relegated to the position of a drug of last resort.

It is a branched tricyclic glycosilated non ribosomal peptide produced by fermentation. It inhibits proper cell wall synthesis in gram positive bacteria, that is, it specifically prevents incorporation of N-acetyl muramic acid (NAM) and N-acetyl glutamic acid (NAG) peptides into peptidoglycan matrix. The dramatic increase in use of vancomycin to treat infections caused by methicillin resistant staphylococci led to the emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) [Lowy 2003; Hiramatsu et al 2005].

1.12. Mechanism of Antimicrobial Drug Resistance

Antimicrobial resistance is the ability of a microorganism to withstand the effects of an antibiotic. Antibiotic resistance evolves naturally via natural selection through random mutation but can also be engineered. Once such a gene is generated, bacteria can transfer the genetic information in a horizontal fashion by plasmid exchange. If a bacterium carries several resistance genes, it is called multiresistant or informally a superbug. Antibiotics whether made in the laboratory or in nature by other microbes are designed to hinder metabolic processes such as cell wall synthesis, protein synthesis or transcription. The phenomenon of antibiotic resistance may in some cases be innate to the microorganism or due to chromosomal mutation in which case it is termed as vertical evolution meaning that the spread occurs through bacterial population growth. The most common method by which resistance is acquired is through the conjugation transfer of R plasmids also called horizontal evolution. Apart from plasmids another method is the transfer due to transposable elements on either
side of a pathogenicity island which are a group of genes that appear on the DNA and carry the codes for several factors which make the infection more successful.

There are four major mechanisms by which microorganisms exhibit resistance to antimicrobials:

1) Enzymatic inactivation of the drug.
2) Alterations to the drug target to prevent binding.
3) Alteration of metabolic pathway affected by the drug or a bypass mechanism.
4) Reduced drug accumulation by decreasing drug permeability or increasing active efflux of the drug [Foster].

TABLE 3: Mechanism of Resistance to commonly used antimicrobials

<table>
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<th>Antimicrobial</th>
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<th>Genetic basis</th>
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</table>
Commonly used antibiotic and the Resistance Mechanisms

1) Penicillin

Penicillin was introduced in early 1940s and improved the prognosis of patients with Staphylococcal infection. By late 1960s more than 80% of both community and hospital acquired Staphylococcal isolates were resistant to penicillins. Resistance to penicillins and other β-lactams were attained by the enzymatic inactivation of the drug by β-lactamase an enzyme that cleaves the β-lactam ring and renders the antibiotic inactive. The gene for β-lactamase is a part of transposable element located on a large plasmid with additional antimicrobial resistance genes. β-lactamase is encoded by the blaZ gene. BlaZ gene is under the control of 2 adjacent regulatory genes, the anti repressor blaR1 and blaI. On exposure to β-lactams, BlaR1, a transmembrane sensor-transducer, cleaves itself. The cleaved protein functions as a protease that cleaves the repressor BlaI, directly or indirectly (an additional protein BlaR2 may be involved in this pathway) and allows blaZ to synthesis enzyme. [Lowy 2003].

Fig 6: The activity of β-lactamase gene blaZ in resistance to penicillin
2) Methicillin

Methicillin introduced in 1961 was the first semisynthetic penicillinase resistant penicillin. The gene responsible for methicillin resistance is chromosomally localized mecA gene which synthesizes PBP2a, a 78kDa protein. PBP2a substitutes for the other PBPs because of its low affinity for all β lactam antibiotics. The repression of resistance to methicillin is regulated by homologues of the regulatory genes for blaZ. These genes mecI and mecR1 regulate the mecA response to β lactams in a fashion similar to blal and blar1. The sequence homology of MecI-MecR1 with blar1- blal gene results in the induction of mecA expression from this leaky alternative system. Deletion or mutation in mecI or the promoter region of mecA results in constitutive expression rather than variable expression of mec. An additional series of genes, the fem (factor essesntial for methicillin resistance) plays a role in cross linking peptidoglycan strands and also contribute to the heterogeneity of expression of methicillin resistance. mecA gene is a part of a mobile genetic element SCCmec which contains additional genes for antimicrobial resistance. SCCmec contains 2 recombinases ccrA & ccrB from the invertase / resolvase family that are responsible for site specific integration and excision from the chromosome at a region near the origin of replication attBSCC.

3) Fluoroquinolones

Pefloxacin, ciprofloxacin and ofloxacin have sufficient activity against staphylococci to be considered for the treatment of serious infections by these organisms. The primary target of fluoroquinolones in Staphylococi is topoisomerase IV, which separates concatenated DNA strands. Unlike in E-coli DNA gyrase, which relieves DNA supercoiling is the secondary target in Staphylococci. Quinolone resistance among S. aureus emerged quickly more prominently in methicillin resistant Staphylococci. Fluoroquinolone resistance develops as a result of spontaneous chromosomal mutation in topoisomerase IV or DNA gyrase or by the induction of multidrug efflux pump. When quinolones are used to treat infections caused by other bacterial pathogens, subjects colonized with S. aureus are exposed to sub therapeutic antibiotic concentration and are therefore at risk of becoming colonized with resistant
mutants, which become the reservoir for future infections. Amino acid changes in critical regions, Quinolone resistance determining region (QRDR) of the enzyme-DNA complex reduce quinolone affinity for both its targets. The parC subunit (grlA in *S. aureus*) of topoisomerase IV and gyrA subunit in gyrase are the most common sites of resistance mutations. Single amino acid mutations are sometimes sufficient to confer clinical resistance but mutations can accumulate in the QRDR region, increasing levels of resistance. Another mechanism of fluoroquinolone resistance in *S. aureus* is the induction of *NorA* multidrug efflux pumps. Increased expressions of this pump can result in low level quinolone resistance.

4) **Vancomycin**

Vancomycin has been the drug of choice for treatment of infections caused by methicillin resistant Staphylococci. But the increased use of vancomycin to treat infections caused by methicillin resistant Staphylococci led to the emergence of VRSA. Two forms of vancomycin resistance have been identified in *S. aureus*. One form is found in vancomycin intermediate (VISA) strains with MICs to vancomycin 8 – 16µg/ml. The reduced susceptibility to vancomycin appears to result from changes in peptidoglycan biosynthesis. VISA strains synthesis additional quantities of peptidoglycan the result in irregularly shaped thickened cell wall. There is also decreased cross linking of peptidoglycan strands which leads to the exposure of more D-Ala – D-Ala residues. As a result there are more D-Ala – D-Ala residues to bind and trap vancomycin and the bound vancomycin then acts as a further impediment to drug molecules reaching their target on the cytoplasmic membrane [Lowy 2003].

The second form of vancomycin resistance results from probable conjugal transfer of the VanA operon from a vancomycin resistant *Enterococcus faecalis*. VanA plasmid also encodes a sex pheromone that is synthesized by *S. aureus*, suggesting a potential facilitator of conjugal transfer. Resistance in these isolates is caused by alteration of the terminal peptide from D-Ala – D – Ala to D-Ala – D – Lac. Synthesis of D-Ala – D-Lac occurs only with exposure to low concentration of vancomycin [Lowy 2003; Hiramatsu et al 2005].
Fig 7: Glycopeptide resistance mediated by synthesis of additional quantities of peptidoglycan

Fig 8: Vancomycin resistance mediated by conjugal transfer of vanA operon from *E. feacalis* which encodes an altered terminal peptide on the peptidoglycan

A] Conjugal transfer of VanA operon from *Enterococcus feacalis* to *Staphylococcus aureus*

B] Alteration of the terminal peptide from D-Ala-D-Ala to D-Ala-D-Lac in VRSA mediated by the VanA operon.
5) Trimethoprim – sulfomethoxazole (SXT)

Resistance to SXT arises from mutations in the enzymes Dihydrofolate reductase (DHFR), inhibited by these antibiotics. The mutant form of the enzyme no longer binds the antibiotic with a higher affinity than its natural substrate. Mutations conferring resistance to sulfonamides or trimethoprim occur frequently but double mutations conferring resistance to both types of antibiotics occur only rarely and so a combination of these antibiotics is used [Murray et al 2003; Humphreys 2002].

6) Rifampin

Rifampin is a potent bactericidal anti staphylococcal agent with MICs of 0.05µg/ml or less. It blocks protein synthesis by inhibiting RNA polymerase. Rifampin penetrates well into tissues and abscesses, which are poorly penetrated by most other anti staphylococcal agents. Provided that MRSA is susceptible to both a fluoroquinolone and rifampin, emergence of fluoroquinolone resistance can be prevented by using these drugs in combinations. High level resistance to rifampin occurs when used alone due to point mutation in the β-subunit of RNA polymerase target that reduce the affinity of the enzyme for the antibiotic [Chambers 1997].

7) Aminoglycosides

Gentamycin, netilmicin and toloramycin are the most active aminoglycosides against staphylococci. They are not useful as single agents because resistance emerges. Tobramycin in particular is likely to be ineffective because the aadD gene encoding tobramycin resistance is present within mec. The main mechanism of aminoglycoside resistance is the inactivation of the antibiotic. MRSA produce aminoglycoside modifying enzymes (AMEs) which inactivate the antibiotic by adding phosphoryl, adenyl or acetyl groups to the antibiotic. Plasmid encoded resistance to gentamycin is also common.

8) Macrolides, Streptogramin and lincoasamides (MSL Group)

An enzyme that methylates an adenine on 23S rRNA and mediates a wide spread type of resistance to MSL group of antibiotics is called RNA methylase. The methalated adenine lies within a region that serves as a binding site for all 3 classes of antibiotic. Thus acquisition of a single resistance gene confers resistance to 3 structurally distinct class of antibiotic. The genes involved are ermA, ermB, ermF and ermG. Resistance to streptogramins is achieved by acetyl transferases that inactivate
streptogramins encoded by vat and sat genes. Another mechanism is an ATP dependent efflux system that pumps macrolides and streptogramins out of the cell.

9) Tetracyclines

Many antibiotics currently in use inhibit protein synthesis. These antibiotics enter the cell cytoplasm and accumulate to a concentration sufficient to allow them to bind the ribosome. A bacterial strategy that prevents antibiotics from reaching a high concentration in the cytoplasm is to pump the antibiotic out of the cytoplasm as rapidly as it is taken up. These protein pumps are called efflux pumps and the first efflux mechanism to be discovered mediated resistance to tetracyclines. The resistance protein is a cytoplasmic membrane protein that catalyses energy dependent transport of tetracycline out of the bacterium. Since tetracycline is removed as quickly as it is taken up, the intracellular concentration of tetracycline is too low to inhibit protein synthesis. Genes encoding tetracycline resistance in the gram positive bacteria are tetK, tetL. Apart from efflux the most widespread type of resistance is target protection. An enzyme has been discovered that uses chemical modification to inactivate tetracycline. The nature of modification is not known but it requires oxygen and thus works only in aerobically growing bacteria. Genes encoding this resistance is tetX. Another clinically important type of resistance is called ribosome protection conferred by a protein which when present in cytoplasm prevents binding to tetracycline to the ribosome. Genes encoding this type of resistance includes tetM, tetO and tetQ [Hiramatsu et al 2005].

10) Chloramphenicol

It inhibits protein synthesis by irreversibly binding to the peptidyl transferase component of 50S ribosomal subunit and prevents the transpeptidation reaction process of peptide chain elongation. The mechanism of resistance to chloramphenicol is an enzyme that adds an acetyl group to chloramphenicol, thus inactivating it. The enzyme is called acetyl transferase and the acetyl group is transferred from s-adenosyl methionine, a compound used in many house keeping methyl transfer reactions.

11) Mupirocin

Mupirocin is a pseudomonic acid, a natural product of Pseudomonas fluorescence. Mupirocin inhibits and kills staphylococci by inhibiting the enzyme, isoleucyl tRNA synthetase. It is available only for topical application and is indicated
for eradication of nasal carriage of MRSA. Low level resistance is due to point mutation in the gene of the target enzyme. High level resistance is due to the presence of an isoleucyl tRNA synthetase gene, located on a conjugative plasmid encoding gentamicin resistance that renders mupirocin ineffective.

**12) Fusidic acid**

It is protein synthesis inhibitor, active invitro against methicillin sensitive and methicillin resistant strains of Staphylococci. Resistance develops if fusidic acid is used alone and so it must be administrated with a second drug to which the strain is susceptible [Humphreys 2002; Bannerman 2003].

**1.13. Treatment and prevention**

*S. aureus* is inherently sensitive to many antimicrobial agents. MRSA resistant to all β lactams, aminoglycosides and fluoroquinolones are an increasing infection control problem and therapeutic challenge. Measures for the control of Staphylococcal infections in hospitals include:-

1. Isolation of patients with open Staphylococcal lesions.
2. Detection of Staphylococcal lesions among surgeons, nurses and other hospital staff and keeping them away from work till lesions have healed.
3. Strict aseptic techniques in hospitals, operation theatres and proper sterilization of devices such as catheter and other intravascular devices.
4. The oldest, simplest and most effective method of preventing cross infection at hospitals is scrupulous hand washing.

Glycopeptides such as vancomycin and teichoplanin are the agents of choice in the treatment of severe infections suspected to be caused by MRSA. Strains of MRSA with reduced susceptibility to glycopeptides have been found in several countries. In such cases, the infections are treated with agents such as quinupristin, dalfo pristin or linezolid. In addition it is often necessary to remove an infected source such as an intravascular catheter. Removal of pus may require surgical drainage and infected prostheses (eg: hipjoint) or intra vascular lines usually require removal if antibiotic treatment is to be successful. Life threatening toxin mediated disease such as toxic shock syndrome requires major medical support such as intravenous fluids to prevent multi organ failure, often best provided in an intensive care unit (ICU).
**Novel therapies for MRSA**

The increasing prevalence of MRSA and treatment failure of *S. aureus* bacteremia and *S. aureus* pneumonia treated with vancomycin has kindled great interest in new treatment options for MRSA.

Quinupristine and dalfopristine are streptogramin class of antibiotics. They are bactericidal and when combined they act in synergy on the 50S ribosomal submit to inhibit protein synthesis. They have been found active against both MRSA and MSSA but the cost, the requirement for administration by central catheter and the side effect profile have all limited the use of this antibiotic.

Linezolid is an Oxazolidinone antimicrobial agent that binds reversibly to the bacterial 23S ribosome, thereby inhibiting protein synthesis. As a result of reversible inhibition, linezolid exhibits bacteriostatic activity against *S. aureus* a major advantage is the oral bioavailability of the antibiotic. It has been approved for treatment of nosocomial pneumonia but not for MRSA endocarditis.

Daptomycin is a cyclic lipopeptide with rapid bactericidal activity against MRSA. It inserts itself into the bacterial cell membrane of killing the cell but the mode of action is not known. Tigecycline is a newly introduced glycylocycline derivative structurally similar to tetracycline. It has broad spectrum antimicrobial coverage including MRSA and acts by binding to the 30S ribosomal sub unit.

Dalbavancin is a semi synthetic glycopeptide characterized by a long half life (9 -12 days) that allows once – weekly administration. It exerts potent activity against MRSA via inhibition of cell wall synthesis. It has not yet been approved by FDA for human trials. Telavancin is an experimental lipoglycopeptide molecule characterized by two mechanism of action, inhibition of bacterial peptidoglycan synthesis, and alteration of bacterial cell membrane permeability and depolarization. Telavancin exhibits invitro bactericidal activity against *S. aureus* isolates including MSSA, MRSA and VISA isolates [Fowler 2006].

**Immuno therapy**

Since microbial adherence is important for the initiation and spread of *S. aureus*, the MSCRAMM (Microbial surface components recognizing adhesive matrix molecules) family of bacterial surface adhesion proteins represents an excellent target
for the development of novel immunotherapies. Tefibazumab is a humanized IgG monoclonal antibody with high affinity to clumping factor A, on MSCRAMM protein common to all *S. aureus* strains. It interferes with the adherence of *S. aureus* to extracellular matrix proteins in vitro and may enhance opsonophagocytosis of *S. aureus* by polymorphonuclear leukocytes [Fowler 2006].

**Vaccination**

*Staphylococcus aureus* Polysaccharide conjugate vaccine named ‘Staph Vax’ is an investigational polysaccharide conjugate vaccine that can be used to prevent *S. aureus* infections. It consists of type 5 and type 8 capsular polysaccharides.

Several cell surface antigens have been tested for efficacy as vaccine. Four cell surface proteins, with the strongest immune response were studied. Two of these proteins, IsdA and IsdB help the microbe acquire needed iron from the host’s red blood cells. The other two sdrD and sdrE are thought to be involved in bacterial adhesion to host tissues when tested alone. When tested alone as a vaccine, each of the four proteins provided only partial protection in mice. But when vaccinated using a combined vaccine, complete protection was observed against two strains including the virulent community associated strain [Fowler 2006].

Since these vaccines are still under trial and has not been tried on human volunteers, no vaccine is currently available to combat staphylococcal infections. Hyper immune serum from human volunteer donors or humanized monoclonal antibodies directed towards surface components such as capsular polysaccharide or surface proteins can prevent bacterial adherence and also promote phagocytosis of bacterial cells. This prototype vaccine is based on capsular polysaccharide from *S. aureus* and can be given to patients in hospital before surgery [Foster 2004].

An ideal vaccine candidate should induce responses that prevent bacterial adherence promote opsonophagocytic killing by leucocytes and neutralize secreted toxic proteins. Such a vaccine has so far not been developed for *S. aureus* [Collins 2000].

**1.14. Molecular typing methods for MRSA**

For routine purposes *S. aureus* is easily identified by demonstration of free coagulase enzyme. However *S. schleiferi* also exhibits clumping activity when suspended in human plasma and certain epidemic strains of MRSA do not. So the
accuracy of slide agglutination test is not high. The shortcomings of phenotypically based typing methods have led to the development of typing methods based on the microbial genotype or DNA sequence which minimizes problems with typability and reproducibility [Olive 1999]. The increase in frequency of MRSA as the causative agent of nosocomial infection and the possibility of emergence of resistance to vancomycin demands quick and trustworthy characterization of isolates and an investigation of clonal spreading within hospitals, so that enough information is generated to permit the implementation of appropriate measures for the control of these infections allowing for the containment of an outbreak. MRSA isolates from numerous geographical locations seems to be derived from only a small number of strains and hence belong to genetically restrict group. Currently, numerous typing techniques are available for discrimination of S. aureus isolates [Trinidade et al 2003].

i. **Plasmid Profile analysis**

Analysis of bacterial plasmids was the first molecular technique used for epidemiologic investigation of MRSA. It involves extraction of plasmid DNA and subsequent separation by electrophoresis in agarose gels. It is an easily executed and interpreted technique but with several limitations, especially inherent to the fact that plasmids are mobile extra chromosomal elements that can be spontaneously lost or readily acquired by bacteria. Consequently epidemiologically related isolates can display different plasmid profiles. Moreover many plasmids carry resistance determinants contained in transposons that can be readily lost or acquired quickly altering the composition of plasmid DNA. Hence the reproducibility and discriminatory power of plasmid is poor [Witte 2000].

ii. **RFLP (Random Fragment Length Polymorphism)**

RFLP is based on randomness of distribution of restriction endonuclease cleavage sites on the bacterial genome, which is reflected by fragment length. Following digestion with a high frequency restriction endonuclease, chromosomal DNA is separated into a series of fragments of different sizes. It is often difficult to interpret due to the large number of fragments generated. But visualization and interpretation can be facilitated by Southern blot hybridization where the fragments separated by electrophoresis are transferred to a nylon membrane and hybridised using specific probes. The most common application of this technique is ribotyping S.
aureus} possess up to 8 copies of the rRNA operons having length polymorphisms with regard to the location of RE cleavage site on the rRNA gene operons. But in comparative trials ribotyping is found to be less discriminatory than \textit{SmaI} macrorestriction patterns. [Trinidade et al 2003; Witte 2000]

\textit{iii. PFGE (Pulsed field Gel Electrophoresis)}

This technique developed by Schwarz and Cantor is based on the digestion of bacteria at DNA with restriction endonucleases that recognize few sites along the chromosome generating large fragments of DNA (10-800kb) that cannot be separated effectively by conventional electrophoresis. PFGE requires intact DNA and special care is taken to avoid mechanical breakage by incorporating the sample into low melting point agarose. The isolated DNA is then subjected to restriction digestion. In PFGE the orientation of electric field across the gel is periodically changed or pulsed, allowing DNA fragments in the order of mega base pairs to be effectively separated according to size. All bacteria can be typed by PFGE and the results are highly reproducible.

PFGE has been used for the investigation of MRSA and has been compared with other methods in several studies. Even though a number of restriction endonucleases have been tested none has shown better performance than \textit{SmaI} which yields 8-20 fragments ranging from 8-800 kb in size. The discriminatory power of PFGE is superior to phenotypic techniques as well as ribotyping, RAPD and PCR-RFLP. Hence PFGE has many of the characteristics attributed to an ideal typing technique and has been proposed as the gold standard for MRSA typing. The limitations include long time intervals until the final results are obtained and the high cost of reagents and specialized equipment used for this technique. [Senna et al 2002; Trinidade et al 2003; Olive 1999; Witte 2000]

\textit{iv. Techniques involving PCR}

PCR gives rise to a variety of techniques with many applications for discrimination between bacterial isolates. Typing techniques involving PCR can be divided into four main groups: PCR-RFLP, PCR-ribotyping, AP-PCR/RAPD and Rep-PCR. PCR-RFLP and PCR-ribotyping are no longer used for MRSA-typing. The arbitrarily primed PCR is a variation of classic PCR and was proposed by Williams et al and by Welsh and McCleland for genetic analysis of microorganisms. This
technique involves the random amplification of segments of target DNA using a small primer (of 10 bases) with an arbitrary sequence of nucleotides which has no known homology with a target sequence. During PCR this primer leads to amplification of one or more sequences of DNA generating a set of fragments that work as genetic markers. The number and size of these fragments are the basis for the typing of an isolate. It has good reproducibility and is simple and fast. When a single primer is used the discriminatory power is low. Using three or more primers increases the time required for carrying out the technique yet the discriminatory power is lower than PFGE.

In the repetitive palindromic Extragenic Elements PCR (Rep-PCR) primers based on short sequence of repetitive elements which are dispersed throughout the prokaryote kingdom is used. These elements are conserved within several bacterial genera and species. The differences in the band size in the molecular profiles obtained represent polymorphism in the distances between repetitive elements of different genomes. This technique has been employed for the discrimination of isolates of numerous bacterial species including *Staphylococcus aureus*. It was first described by Versalovic et al.

Another PCR based technique for typing MRSA is the microsatellite hyper variable region (HVR) typing. This technique relies upon the length of polymorphisms of the hypervariable region of staphylococcal methicillin resistance gene (*mec*) for strain resolution. The DNA sequence between IS431 *mec* and *mec*A is called the HVR because of the length of polymorphisms of different *Staphylococcus* isolates. HVR is composed of direct repeat units (DRU) elements each of 40bp. In spite of the fact that HVR-PCR method appears to be reproducible, rapid, easy to perform and capable of demonstrating differences between MRSA strains, it exhibits lower discriminatory powers than PFGE method. [Senna et al 2002; Yoko et al 2007; Olive 1999; Tenover et al 1994].

v. **Multilocus Sequence Typing [MLST]**

This is a technique derived from Multilocus Enzyme electrophoresis (MLEE), a phenotypic typing technique involving the electrophoresis of proteins that can be selectively stained. The proteins extracted from an organism are electrophoresed, stained and the position of each band generated reflects the expression of the protein’s
genotype according to the mobility of the protein. Two bands of the same protein (locus) in different positions would reflect two different proteins with different conformations and thus two alleles of the same gene. An obvious drawback is that the base sequence or genotype of a particular locus cannot be directly inferred based on the analysis of the expression because two different base sequence could express the same protein or even two proteins with the same electrophoretic mobility could be detected as the same band in MLEE.

To resolve this problem MLST was developed by Maiden et al (1998). In MLST instead of analyzing the expression of genes, the genes themselves are analysed by nucleotide sequencing. Different sequences are considered as being district alleles of a gene. A number of loci are chosen for each species usually an internal fragment of housekeeping genes yielding sequences of approximately 500bp for each locus. Housekeeping genes are selected since they are always present in a given species and still with sufficient variation within the species.

For *Staphylococcus aureus*, seven loci representing the internal fragments of housekeeping genes were chosen. Each locus is amplified by PCR and the PCR products sequenced. Approximately 30 alleles per locus have been described for *S. aureus*. The loci used are: Carbamate Kinase (arcC), Shikimate dehydrogenase (aroE), Glycerol Kinase (glpF), Guanylate Kinase (gmk), phosphate acetyl transferase (pta), triose phosphate isomerase (tpi) and Acetyl co enzyme A acetyl transferase (yqiL). The draw back of MLST is its high cost and the need for the equipment necessary to execute it. This restricts MLST to large centers involved in global epidemiology studies. MLST has satisfactory discriminatory power and ease of interpretation but due to wider acceptance PFGE still remains the gold standards for MRSA typing. [Trinidad 2003; Mathema 2004]

**vi. Microsatellite analysis**

Satellite DNA refers to repeat units of >100bp not common in prokaryotes. Prokaryotes contain micro satellite and mini satellite DNA which has repeat units ranging in size from 10 to 100bp commonly referred to as Variable Number Tandem Repeats (VNTR). PCR based amplified fragment length polymorphisms and direct DNA sequencing are both molecular methods to locate and analyse chromosomal regions for repeat units. When genetic differences in multiple independent targets is
analysed it is referred to as multiple locus VNTR analysis. In monomorphic species, the repeat units are highly conserved. So strain discrimination is based on copy number differences at multiple loci. But in species with greater heterogeneity such as *S. aureus* the microsatellite regions are generally heterogeneous and therefore it is the content of the array and not the array size that provides a high degree of discrimination.

There are currently two inframe microsatellite target in *S. aureus* that have been studied as genotyping tools: 24bp repeat unit in 3’ region of protein A (spa) and an 81bp repeat unit in the 5’ region of coagulase (coa) gene. In both cases the variable repeat regions within the genes are flanked by highly conserved sequences ensuring high fidelity in generating PCR amplification product for sequence analysis. Protein A and coagulase are separated by over 110kb on the *S. aureus* chromosome and so would not be co-inherited in a transfer event. DNA sequence analysis of proteinA repeats has revealed both accuracy and reproducibility of this single microsatellite region.

Genetic alteration in *coa* VNTR target occurs at a lower clock speed than the changes in the smaller *spa* microsatellite. So together, these genotyping targets can be used to address both short term and long term epidemiological questions. Another microsatellite region particularly studied in MRSA is the hyper variable region (HVR) located between IS431 and *mecA*. In addition to its use as a marker, the number of repeats in the region X of *spa*, has been related to the dissemination potential of MRSA, with higher number of repeats associated with higher epidemic capability.

PFGE is the gold standard for typing MRSA due to its high discriminatory power and excellent reproducibility. But it is slow time consuming procedure that requires trained personnel and sophisticated equipment. So PCR based methods of microsatellite analysis is rapid, less expensive and reliable. [Mathema 2004]

**vii. DNA Microarray**

Microarray technology permits researchers to analyse the expression of thousands of genes simultaneously in a single experiment. Such a complex analysis is facilitated by robotics and the minimization of both the assay and the sample size, and has an enormous impact on the understanding of many aspects of microbial gene expression and the host cells infected by the microorganism. Microarrays are made on
25 by 75mm glass slides. One of the most important applications of microarray technology is the molecular diagnosis and prognosis of human infection and inflammatory diseases. Due to the presence of highly conserved nucleotide sequence between closely related bacterial species, microarrays developed for one organism can be applied to the identification of novel genes of related species. Steps in microarray analysis include sample purification, amplification, hybridization and detection which can be carried out on a single chip. Thus microarrays are most suited for detection of organisms, discriminating among strains or for detection of host responses generated in the context of infect. [Stover 2004]

viii. Bioinformatics tools

With the introduction of bioinformatics tools to extract information from databases, it is now possible to search entire genomes for specific nucleic acid or protein sequences in seconds. Such database search tools are integrated with other tools and databases to predict the functions of the protein products based on the occurrence of specific functional domains or motifs. This work involves complete sequence analysis, structure determination, and modeling studies to explore how structure governs function [Liebler 2002].

The process of subtyping is epidemiologically important for recognizing outbreaks of infection, detecting the cross transmission of nosocomial pathogen, determining the source of the infection, recognizing particularly virulent strains of organisms and monitoring therapeutic strategies. Vancomycin is often given as a second line therapy against MRSA. But resistance to this antibiotic has been reported as well. MRSA is associated with higher mortality than MSSA strains. Drug resistance among this species has become so prevalent that the term MRSA can now refer to multidrug resistant S. aureus.