3. Review of Literature

3.1. Classification

*Staphylococci, Micrococci and stomatococcus belongs to the family Micrococcaceae.* *Staphylococci* are gram positive cocci, aerobic or facultative anaerobic, which colonises the skin, nasal passage and axillae of humans. It occurs in grape like clusters when viewed through the microscope. The genus *Staphylococcus* contains 33 defined species and 20 species found in man. Species of *Staphylococci* are initially differentiated by the Coagulase test and are classified into two groups: the *coagulase-positive* and *coagulase-negative staphylococci* (CONS).

**Coagulase Positive Staphylococci**

Of the the 20 species found in man,*Staphylococcus aureus* is coagulase positive.

**Table A: Scientific Classification of Staphylococcus aureus**

<table>
<thead>
<tr>
<th>Domain</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
<td>Bacteria</td>
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<tr>
<td>Phylum</td>
<td>Firmicutes</td>
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<tr>
<td>Class</td>
<td>Cocci</td>
</tr>
<tr>
<td>Order</td>
<td>Bacillales</td>
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<tr>
<td>Family</td>
<td>Micrococcaceae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Staphylococcus</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>Binomial name</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
</tbody>
</table>
Coagulase-negative Staphylococci

There are 19 coagulase-negative staphylococci, *S. epidermidis* and *S. saprophyticus* are the most clinically significant species in this group. *S. epidermidis* has been known to cause hospital acquired infections, whereas *S. saprophyticus* is associated mainly with Urinary tract infection, predominantly in female adolescents and young women.

3.2. **History and Natural habitat**

Staphylococci were first reported in human pyogenic lesions by Von Recklinghausen in 1871. Louis 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 grapes 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 pigmentation, 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 *S. epidermidis* which were coagulase negative, mannitol nonfermenting and usually non pathogenic strains. [Murray *et al*. 2003; & Humphreys 2002].

*Staphylococci* are wide spread in natural world although they are living on the skin, skin glands and mucous membrane of mammals and birds. They may be found in the oral cavity, mammary glands, intestinal, genitourinary and upper respiratory tracts of these hosts. *Staphylococcus aureus* generally encompass a benign or symbiotic relationship with their host, however they may grow as the lifestyle of a pathogen if they get entry into the host body through distress of the outermost barrier which is skin, injury 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 of time. 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].

### 3.3. Cultural characteristics

The coci are spherical, approximately 0.5-1.0 μm 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 24hrs *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 haemolytic 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].
Figure 1: Gram Positive cocci in bunch. *Staphylococcus aureus* from clinical sample pus and from pure culture.

Figure 2: Beta hemolysis on Blood agar and Golden yellow pigmentation of *Staphylococcus aureus* on Nutrient agar Medium.

Figure 3: Cell wall of *Staphylococcus aureus*
3.4. Biochemical tests 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 toluene blue agar.

**Acetoin Production**

Acetoin production from glucose and 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 72 hrs [Bannerman 2003].

### 3.5. 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 in cases of Pneumonia e.g. post influenzal and ventilator associated pneumonia (VAP). Bronchoscope specimens (Bronchoalveolar lavage) are increasingly used in critically ill patients.
3) Faeces or vomitus from patients with suspected food poisoning or the remains of implicated food.
4) Blood from suspected bacteremic and septicemic patients, e.g. septic shock, osteomyelitis or endocarditis.
5) Mid stream urine from patients suspected of U.T.I(cystitis/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 bunch of gram positive cocci can be demonstrated by microscopy and the organism can be cultured readily on blood agar and most other media[figure 1 & 2]. Slide or Tube coagulase test is performed to distinguish *S. aureus* from coagulate negative staphylococci[Humphreys 2002].

### 3.6. Virulence factors and pathogenesis

*Staphylococcus aureus* typically produces five 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 five types of penicillin binding proteins found in susceptible strains of *S. aureus* includes PBP1, 2, 3, 3 and 4 with molecular weights of 85,000, 80,000, 75,000, 70,000 and 45,000 Daltons respectively, which functions as transpeptidases, endopeptidases and carboxy peptidases β-lactam antibiotics are substrate analogous 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]. Depending on the strain _S. aureus_ is capable of secreting several toxins which can be categorized into three groups. Many of these toxins are associated with specific diseases.

1. Pyrogenic toxin super antigens (PTS Ag S) 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 six serotypes of enterotoxins which cause diarrhoea 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 cracking or pilling in the skin observed.

3. Membrane damaging toxins include \(\alpha\) toxin, \(\beta\) toxin and \(\gamma\) toxin and the classical Panton-Valentine-Leukocidin (PVL) factor. PVL is a bi-component toxin associated with severe haemolytic 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 \(\alpha\) haemolysin 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. \(\beta\) haemolysin— is a sphingomyelinase, haemolytic for sheep cells. Gamma haemolysin is a bicomponent protein necessary for haemolytic activity. Delta haemolysin 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 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, hyaluronidases etc. Lipases or Lipid hydrolases help in infecting skin and subcutaneous tissues. Hyaluronidase break down the connective tissue. Staphylokinase biochemically fibrinolysin are fatty acid modifying enzymes and proteases that help in initiation and spread of infection.

2. **Surface factors that inhibit phagocytic engulfment:** 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 cocci to the host cell surface and protects them from complement mediated opsonisation. 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 present in the plasma, binds to Prothrombin, converting fibrinogen to fibrin.

4. **Membrane damaging toxins: hemolysins 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 T.J. 2004]
<table>
<thead>
<tr>
<th>Type of virulence</th>
<th>Selected factors</th>
<th>Genes</th>
<th>Associated clinical syndromes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Involved in attachment</td>
<td>MSCRAMMs (e.g., clumping factors, fibronectin-binding proteins, collagen,</td>
<td>clfA, clfB,</td>
<td>Endocarditis, osteomyelitis, septic arthritis, and</td>
<td>Patti et al. 1994; Foster, 1998.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fnbA, fnbB,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cna, sdr</td>
<td></td>
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<tr>
<td>Involved in persistence</td>
<td>Biofilm accumulation (e.g., polysaccharide intercellular adhesion), small-</td>
<td>ica</td>
<td>Relapsing infections, cystic fibrosis, and syndromes as described above for</td>
<td>Donlan, 2002; Arrecubieta, 2006.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>locus, hemB</td>
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<td></td>
<td>mutatio</td>
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<td></td>
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<tr>
<td>Involved in Evading &amp;</td>
<td>Leukocidins (e.g., PVL and á-toxin), capsular polysaccharides, protein A, soluble</td>
<td>lukS-PV,</td>
<td>Invasive skin infections and necrotizing pneumonia (CA-MRSA strains that cause</td>
<td>Foster, 2005; O'Riordan, 2005;</td>
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<tr>
<td></td>
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<td>, hlg, cap5</td>
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<td></td>
<td></td>
<td>spa</td>
<td></td>
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<tr>
<td>Involved in tissue</td>
<td>Proteases, lipases, nuclease, hyaluronate lyase, phospholipase C, and metalloproteases</td>
<td>V8, hysA,</td>
<td>Tissue destruction and metastatic infections</td>
<td>Projan, 1997</td>
</tr>
<tr>
<td>invasion &amp; penetration</td>
<td></td>
<td>hla, plc,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>sepA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Involved in Toxin</td>
<td>Enterotoxins, toxic shock syndrome toxin-1, exfoliative toxins A</td>
<td>sea-q (no</td>
<td>bulbous impetigo, and sepsis syndrome</td>
<td>Dinges, 2000; Timmerman, 1993</td>
</tr>
<tr>
<td>mediated disease</td>
<td></td>
<td>sef), tstH,</td>
<td></td>
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<td></td>
<td></td>
<td>eta,</td>
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<tr>
<td>With poorly defined role</td>
<td>Coagulase, ACME, and bacteriocins</td>
<td>opp-3</td>
<td></td>
<td>Baba, 2002; Diep, 2006</td>
</tr>
<tr>
<td>in virulence</td>
<td></td>
<td>cluster, bsa, arc cluster,</td>
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</tbody>
</table>
3.7. 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 faeces. 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. Fibronectin binding proteins (Fnβ PA and Fnβ 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 virus 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-toxins producing food poisoning strains into food. Infants may be colonized with *S. aureus* shortly after birth, acquiring the organism from people in their immediate surroundings.

Healthy carriers

*S. aureus* grows harmlessly on the moist skin of the nostrils in many healthy persons. This condition is referred to as colonisation. 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, HIV infected patients, hemodialysis 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. healthy 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 19.0 % and 34.0 %. Some carriers called shedders disseminate exceptionally large number of staphylococci, and transmission occurs through hand, clothing and dust consisting of skin squames and cloth fiber [Humphreys 2002 & Foster 2004].

**Clinical symptoms**

*S. aureus* is notorious for causing acne, pimples, boils, furuncles, carbuncles styes impetigo and other superficial skin infections in humans. It may also cause serious infections such as pneumonia, deep abscesses, endocarditis, phlebitis, osteomyelitis, mastitis and meningitis particularly in persons debilitating by chronic illness, traumatic injury, burns or during 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 throughout 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 umbilical cord. In this case skin turns blistery as a
result of the production of exfoliative toxin that peels away the skin to expose a red layer on skin.

Toxic 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. Toxic 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 (IL-1). 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 sometimes associated with surgical wound infections. In the year 1981 approximately 1000 cases of TSS were reported and in 1997 100 cases were reported.

S. aureus bacteraemia 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 bacteraemia afflicts intravenous drug users and otherwise healthy patients with infections at various sites. Approximately one third of the patients with bacteraemia 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 bacteraemia. Endocarditis is a complication of S. aureus bacteraemia. Endocarditis in patients with bacteraemia 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 bacteraemia. 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 
   pleuric chest pain is a common feature whereas cough and sputum 
   production are less likely. [Chamber 1997 & Fowler 2006].

### 3.8. 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.
Review of Literature

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 in to 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].
3.9. *MecA 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 1997].
Figure 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 SCCmec 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]

3.10. 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 (50µ g of methicillin per ml). Most of the cells are susceptible to relatively low, therapeutically achievable concentrations of drug (eg: 1-5mg of methicillin per litre). Thus heterogenous 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 50µ g 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 8mg/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 \textit{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 – \textit{femA}, \textit{femB}, \textit{femC}, \textit{femD}, \textit{femE} and \textit{femF} which map to numerous sites throughout the staphylococcal genome have been characterized [Chambers 1988].

\subsection*{3.11. Vancomycin Resistant \textit{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 \textit{Streptomyces orientalis}. Vancomycin never became the first line treatment for \textit{S. aureus} for several reasons:

1) The drug must be given intravenously because it is not absorbed orally.
2) β-lactamase resistant semi synthetic Pencillinins 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 glycosylated 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 \textit{Staphylococcus aureus} (VRSA) [Lowy 2003; Hiramatsu \textit{et al.} 2005].

\subsection*{3.12. Mechanism of Antimicrobial Drug Resistance}

Antimicrobial resistance is the ability of a micro organism 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 transporable 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.
### TABLE C: Mechanism of Resistance to commonly used antimicrobials

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3.13. 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].

Figure 6: The activity of β- lactamase gene blaZ in resistance to penicillin
Review of Literature

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 PBP s 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 blaI and blaR1. The sequence homology of MecI-MecR1 with blaR1- blaI 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 att. BSCC.

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 Staphylococci 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 fluoroquiniolone 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 8.0-16.0μg/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].
Figure 7: Glycopeptide resistance mediated by synthesis of additional quantities of peptidoglycan

Figure 8: Vancomycin resistance mediated by conjugal transfer of \textit{vanA} operon from \textit{E. feacalis} which encodes an altered terminal peptide on the peptidoglycan

A] Conjugal transfer of VanA operon from \textit{Enterococcus feacalis} to \textit{Staphylococcus aureus}

B] Alteration of the terminal peptide from D-Ala-D-Ala to D-Ala-D-Lac in VRSA mediated by the \textit{VanA} operon.
5) Trimethoprim – sulfamethoxazole

Resistance to SXT arises from mutations in the enzymes Dihydrofolate reductase (DHFR), inhibited by these antibiotics. The mutant from 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 gene. 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 lincosamides (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 methylated 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 intra cellular 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 significant 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 50 S 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 housekeeping methyl transfer reactions.
11) Mupirocin

Mupirocin is a pseudomonic acid, a natural product of *Pseudomonas fluorescense*. 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 gentamycin resistance that renders mupirocin ineffective.

12) Fusidic acid

It is protein synthesis inhibitor, active invtro 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].

3.14. 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, dalfopristin 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 (e.g. 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 50 S 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 glycylicycline 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 one 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 polymorpho nuclear 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 \textit{S.aureus} [Collins2000].

3.15. Molecular typing methods for MRSA

For routine purposes \textit{S. aureus} is easily identified by demonstration of free coagulase enzyme. However \textit{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 short comings 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 \textit{S. aureus} isolates [Trinidade \textit{et al.} 2003].

\textit{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 R.E cleavage site on the rRNA gene operons. But in comparative trials ribotyping is found to be less discriminatory than *Sma*I macrorestriction patterns. [Trinidade et al. 2003 & Witte 2000]

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 *Sma*I 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; Trinidad et al. 2003; Olive 1999 & Witte 2000]

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 (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 poly
morphisms of the hypervariable region of staphylococcal methicillin resistance
gene (mec) for strain resolution. The DNA sequence between IS431 mec and mecA
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

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 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 drawback 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. [Trinidade 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 25/75mm 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.*
3.16. Incidences of Drug Resistance in Staphylococci
3.17 Neonatal Septicemia

Definition:
National Neonatal Forum of India has defined neonatal sepsis as follows: 3
Probable (Clinical) Sepsis: In an infant having clinical picture suggestive of septicemia, if there is the presence of any one of the following criteria:

- Existence of predisposing factors: maternal fever or foul smelling liquor or prolonged rupture of membranes (>24 hrs) or gastric polymorphs (>5 per high power field).
- Positive septic screen - presence of two of the four parameters namely, TLC (< 5000/mm), band to total polymorphonuclear cells ratio of >0.2, absolute neutrophil count < 1800/cumm, C-reactive protein (CRP) >1mg/dl and micro ESR > 10 mm-first hour.
- Radiological evidence of pneumonia. Culture Positive Sepsis: In an infant having clinical picture suggestive of septicemia, pneumonia or meningitis, if there is presence of either of the following:
  - Isolation of pathogens from blood or CSF or urine or abscess (es)
  - Pathological evidence of sepsis on autopsy. Classification Neonatal sepsis is of two types: Early onset Sepsis (EOS): Early onset sepsis presents within first 72 hours of life. In severe cases the neonate may be symptomatic in utero (fetal tachycardia, poor beat to beat variability). Clinically, the neonate usually presents as respiratory distress and pneumonia. Presence of the following risk factors has been associated with an increased risk of EOS: 6, 7
  - Low birth weight (24 hours).
  - More than 3 vaginal examinations during labor.
  - Prolonged and difficult delivery with instrumentation.
  - Perinatal asphyxia (Apgar score)
  - Prelacteal feeding
  - Ventilation
  - Aspiration of feeds
CLINICAL FEATURES

Manifestations of neonatal sepsis are vague and ill-defined. Alteration in established feeding behavior is common and early, but is a nonspecific symptom. Other features are hypothermia or fever (former is more common in LBW babies), lethargy, poor cry, poor perfusion i.e. prolonged capillary refill time (>2 seconds), hypotonic or absent neonatal reflexes, bradycardia or tachycardia, respiratory distress i.e. apnea or gasping respiration, hypoglycemia or hyperglycemia and metabolic acidosis.

System wise specific features are:

- **Central nervous system**: These are bulging anterior fontanel, blank look, high-pitched cry, excessive irritability, coma, seizures, and neck retraction. Presence of these signs should raise clinical suspicion of meningitis.
- **Cardiac**: The cardiac signs are mainly hypotension and poor perfusion. A recent study emphasized the value of early diagnosis of sepsis using analysis of heart rate characteristics on ECG monitoring. Griffin et al. found that abnormal heart rate characteristics such as reduced variability and transient decelerations occurred 24 hours prior to onset of symptoms in sepsis and sepsis like illness [Griffin MP et. al, 2007]. Another group found that sample asymmetry of RR interval increased in the 3-4 days preceding sepsis with the steepest increase in the last 24 hours. These tests may prove helpful in starting the therapy long before the baby shows signs of deterioration [Kovatchev BP et. al.2007].
- **Gastrointestinal**: These are feed intolerance, vomiting, diarrhea, abdominal distension, paralytic ileus, and necrotising enterocolitis.
- **Hepatic**: The common hepatic signs are hepatomegaly and direct hyperbilirubinemia (Infants with the onset of jaundice after 8 days of age or with direct hyperbilirubinemia were more likely to have urinary tract infection.)
- **Renal**: There may be acute renal failure.
- **Hematological**: Hematological signs are bleeding and petechiae, purpura.
- **Skin**: There may be multiple pustules, sclerema, mottling, umbilical redness and discharge. De Felice et al., 2002 used Colorimetric analysis of skin color for severity of sepsis. Color readings were taken from 10 different body sites using a portable tristimulus and color was expressed using the standard CIE.
3.18. Staphylococci at a glance from 1883 to 2008

Staphylococcus is a versatile organism with several virulent features and resistance mechanism and was first identified in pus by the famous surgeon Sir Alexander Ogston in Aberdeen of Scotland in 1883 [Ogston 1883 & Fowler Jr, 2006]. The first observation pointing to the endogenous supply of bacterial wound infection was made in 1915 by Sir Almroth Wright [Wright 1915]. Humans are a natural reservoir for S. aureus, and asymptomatic colonization is far more common than infection. Colonization of the nasopharynx, skin or perineum, particularly if the cutaneous barrier has been disrupted or damaged, may occur shortly after birth and may recur anytime thereafter. Multiple drug resistance is now the norm among the Gram-positive bacteria like pneumococci, enterococci and staphylococci. S. aureus is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of life-threatening infections in human, and its capacity to adapt to different environmental conditions. The evolution of increasingly antimicrobial resistant bacteria stems from a multitude of factors, including the strong selective pressure caused by the widespread and sometimes inappropriate use of antimicrobial agents, the increase in regional and international travel and the relative ease with which antimicrobial-resistant bacteria cross geographic barriers [Lowy et. al., 2003].

The resistance of Staphylococcus aureus to phenolics has been described as early as 1925 [Reddish et al 1925]. In the year of 1932, Panton and Valentine described leukocidin as a virulence factor. Panton Valentine leukocidin (P.V.L) is composed of LukS-PV and LukF-PV [Prevost et. al. 1995], It shows lytic activity against polymorphonuclear cells, monocytes, and macrophages in humans and rabbits [Cribier et al.. 1992]. In the year of 1935, Glenny and Stevens differentiated beta toxin from alpha toxin by antibody neutralization and showed that it lysed sheep, but not rabbit erythrocytes [Arbuthnott 1982]. It is also unique in its ability to cause hot-cold lysis. S. aureus beta toxin is encoded by the gene hlb [Projan et al. 1989]. Gamma-hemolysin was found to be produced by more than 99% of clinical S. aureus strains. It is able to lyse Red blood cells from a wide range of mammalian species. In addition, gamma-hemolysin has leukotoxic properties as it is able to lyse polymorphonuclear cells, macrophages and monocytes (Smith and Price, 1938). Beta toxin is a neutral sphingomyelinase and
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displays species dependent activity. In 1947, Williams and Harper [1947] proposed the existence of delta toxin which is unique since it is small, heat stable toxin, which has surfactive properties and is lytic towards many types of membrane from most animal species including those on Red blood cells, other cells, organelles and even bacterial protoplasts [Williams and Harper, 1947 and Arbuthnott 1982].

The first case of penicillinase producing S. aureus was published in 1940s, almost a year before penicillin was marketed for clinical use [Abraham 1940]. After the first therapeutic use of penicillin in 1941, beta-lactam antibiotics have been used extensively and successfully as antimicrobials in the treatment of bacterial infections. However, soon reports appeared of penicillin resistant S. aureus strains producing the enzyme β-lactamase (called penicillinase until 1960), which hydrolyses the β-lactam ring. The first strain of penicillin- G resistant S. aureus was isolated in London, England soon after the introduction of Penicillin- G into hospitals. When Kirby's first description of penicillinase-producing strains of S. aureus was published in 1944 [Kirby, 1944], resistance was infrequently encountered, with only a handful of strains. As with MRSA, penicillinase-producing strains first were isolated from hospitalized patients. The prevalence of penicillinase-producing strains of S. aureus within hospitals soon began to rise as penicillin became readily available after second World War. Afterward, most hospital isolates were resistant to Penicillin-G [Barber et al., 1948].

It has been found previously that penicillinase-producing strains of Staphylococcus aureus each harbor an extrachromosomal genetic element, which is also called as plasmid, which apparently carries all the genetic information necessary for penicillinase enzyme synthesis.

The earliest known Staphylococcus aureus plasmid encoding multidrug efflux was characterized from a clinical strain dating from 1951 by Paulsen I.T et al. Penicillinase stable Cephalosporins and semi synthetic Penicillins were introduced in the late 1950s. Macrolides, Lincosamides and Streptogramins were first introduced in the year 1952, are often referred to as M.L.S antibiotics. They inhibit protein synthesis by binding to the 50S ribosomal subunit on bacterial ribosomes. The life saving glycopeptide antibiotic named vancomycin was introduced clinically in the year, 1958 for the treatment of gram-positive bacteria. The first identified virulence factors of staphylococci were leucocidins that causes
formation of pores in the cell membrane [Gladstone 1957]. Approximately one half of all skin infections are caused by \textit{S. aureus} [Elek 1957].

In the early 1960s the introduction of methicillin promised to resolve one of the therapeutic problems associated with infections due to multiple drug resistant, penicillinase producing \textit{S. aureus}. But the first methicillin resistant strain was described in the U.K in 1961 [Jevons 1961]. Between 1960 and 1963 a moderate increase in the number of methicillin resistant strains on the order of 5% was noted. Homogenous populations replaced progressively heterogeneous populations of MRSA. Staphylococcal enterotoxin A was first produced in vitro in 1963 [Casman and Bennett 1963]. In Australia, no resistant strains had been identified as of 1964, but 17% of isolates were methicillin resistant in 1970. Between 1965 and 1969 the incidence of methicillin resistant strains increased from 11 to 28% in Zurich, Switzerland hospitals. In France, the incidence of methicillin resistant strains in nine Parisian hospitals was 12% in 1961, 19% in 1963 and 35% in 1966. In Denmark in 1967 about a quarter of Staphylococcal isolates were methicillin resistant. In the United States, a few strains were isolated in 1963. In fact no methicillin resistant strains were isolated in Boston, Massachusetts hospitals between 1960 and 1967. In 1967 however, 1.4% of \textit{S. aureus} isolates were methicillin resistant.

The nose is regarded as the major site of \textit{S. aureus} carriage from where the organism can spread to other parts of the body [White 1963 & William 1966]. Direct contact transmission involves contact of body surface to body surface and physical transfer of \textit{S. aureus} to the host from an infected or colonized person [Fekety Jr 1964].

Studies by Novick and Richmond in 1965 revealed that a certain amount of variability has been encountered among the penicillinase plasmids harbored by different staphylococcal strains. It was been found that: (i) there are at least three molecular variants of the enzyme itself; (ii) most, but not all, of the penicillinase plasmids carry a genetic determinant of resistance to mercuric ion; (iii) plasmids carried by a very small number of the strains bear a determinant of resistance to erythromycin; (iv) the plasmids determine the fraction of penicillinase excreted into the medium during growth, and this also varies from strain to strain. Jarlomen \textit{et al.} showed that Staphylophage 80, propagated on a hospital strain of \textit{Staphylococcus aureus} 80/81, could transduce antibiotic
resistance markers such as penicillin and tetracycline to a variety of staphylococcal recipient strains in vitro [Jarlomen et al. 1965].

In 1965 Gravenkemper et al. discovered two strains resistant to methicillin which were very active producers of penicillinase, and exhibited cross-resistance with other antistaphylococcal antibiotics. Resistant cultures showed resistance to methicillin only with large inocula, and consisted of a mixture of cells. The methicillin-resistant strains caused destruction of methicillin and oxacillin in vitro, but the rate of hydrolysis was slow. Antibiotic destruction was probably due to high concentrations of staphylococcal penicillinase, and not to another specific enzyme [Gravenkemper et al. 1965].

Weaver and Pattee examined the resistance of Staphylococcus aureus to erythromycin and it was found to possess the characteristics of an inducible enzyme. The induction of resistance to high concentrations of erythromycin in S. aureus occurred only after prior exposure to subinhibitory concentrations of erythromycin [Weaver and Pattee 1964]. Initial studies on staphylococcal plasmids were confined to those that gave resistance to penicillin G and other penicillins, but subsequently plasmids conferring resistance to erythromycin (Novick & Richmond 1965), neomycin (Lacey 1971), tetracycline, chloramphenicol (Chabbert et al. 1964), streptomycin (Grubb & O'Reilly 1971) and fusidic acid (Evans & Waterworth 1966) have all been described and methicillin resistance is a multifactorial process, one element of which is specified by a plasmid [Dornbusch et al. 1969]. In practice, it is rare to find more than one type of resistance determined by a single staphylococcal plasmid [Richmond 1972]. In 1969 the first clinical S. aureus isolate resistant to gentamicin was reported [Lacey & Mitchell 1969]. There was no systematic surveillance for antibiotic resistance among S. aureus isolates circulating within communities. The first comprehensive description and accurate assessment of the epidemiology of drug-resistant strains of S. aureus were published in 1969 by Jessen et al. [Jessen et al. 1969]. In the early 1970s, physicians were finally forced to abandon their belief that, given the vast array of effective antimicrobial agents, virtually all bacterial infections were treatable. Their optimism was shaken by the emergence of resistance to multiple antibiotics among pathogens like S. aureus.

The successful development of methicillin and cephem antibiotics in the 1960s was quickly followed by emergence and world wide dissemination of
methicillin and cephalosporin-resistant *S. aureus* in 1970. World wide epidemics of *S. aureus* diseases have been recognized over the years (Klimek 1976; Lacey 1973). But at the beginning of 1970s, a significant decrease in the number of methicillin resistant strains was reported. Although penicillinase-producing strains were universally present in hospitals by the early 1950s, community isolates of *S. aureus* were considered to be largely penicillin susceptible. Penicillin continued to be recommended as an effective anti-staphylococcal agent as late as the early 1970s [Weinstein 1975]. The first methicillin and aminoglycoside resistant strain was described in Australia in the mid 1970s and shortly afterwards throughout the world. Classical genetic experiments had shown that *mec* is not transferable between *S. aureus* strains by conjugation [Lacey 1972] but is transferable by bacteriophage mediated generalized transduction [Cohen and Sweeny 1970].

In 1976 methicillin and gentamicin resistant strains were described. However a resurgence of methicillin and aminoglycoside resistant strains were noted at the end of 1970s and the beginning of 1980s in France, Ireland, Greece, Australia and South Africa. No vancomycin-resistant staphylococci were reported in the first 20 years the drug was used. Then the first reports of vancomycin resistance in coagulase-negative staphylococci emerged in 1979 and 1983 [Siebert 1979; Tuazon and Miller 1983]. In Greece between 1978 and 1979 about 50% of the strains were methicillin resistant in Athens hospital. From the mid 1970s the number of resistant isolates had increased progressively and epidemics have been described in all regions of US. Vancomycin drug resistance among staphylococci was developed in laboratories even before the drug was in use clinically (Geraci 1956 & Ziegler 1956).

The disease Toxoid Shock Syndrome (TSS), mediated by toxic shock syndrome toxin (TSST-1) was first described in 1978 and designated TSS by Todd et al. (1978). Toxic shock syndrome is a staphylococcal illness characterized by acute onset of high fever above 104°F, diffuse erythematous skin rash, desquamation of the skin one to two weeks after onset, especially on the palmar and soles region; hyperemia of mucous membranes, hypotension, and involvement of multiple organ systems as evidenced by diarrhoea, cardiopulmonary dysfunction, thrombocytopenia, or a variety of other symptoms
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[Davis et. al. 1980 & Shands et. al. 1980]. Toxin mediated diseases caused by other staphylococcal toxins included scalded skin syndrome, are known to be infrequent in the United States, but there are a few data available on the prevalence of such diseases in other parts of the world. Ogawa et al. [1985] demonstrated that following adherence staphylococci are endocyted into membrane bound vacuoles in an endothelial cell mediated process. Following internalization there is a fusion of the phagosome with cellular lysosomes and limits any intracellular bacterial replication. Endothelial cells are ineffective phagocytes and Staphylococci are capable of prolonged intracellular survival. Bacteria are released into the medium, spread to the underlying extracellular matrix and cause progressive cellular damage, ultimately destroying the cell [Hamill et al. 1986; Lowy et al. 1988; Vann et al. 1987]. Virtually all strains of staphylococci of human origin are lipolytic. Genes encoding lipases have been cloned from S. aureus (Lee and Iandolo, 1986), and infections include folliculitis, cellulitis, furuncles, carbuncles, hydradenitis suppurativa, mastitis, pyodermas and pyomyositis. Impetigo which involves release of epidermolytic toxins, can range from mild recurrent infections to the more serious bullous impetigo characterized by blisters that continually break and become infected to the potentially life threatening Scalded skin syndrome [Swartz 1987]. It is the leading cause of both soft tissue and bone and joint infections [Maki DG and Ringer M 1987]. The toxin responsible for T.S.S, known as the toxic shock syndrome toxin (TSST-1) was first identified by Schlievert [Schlievert et. al. 1981] and the criteria for defining TSS cases were established in 1981 [Reingold et. al. 1982]. TSST-1 was found to be a potent super-antigen which elicit a variety of cytokines that along with tumour necrosis factor, contribute a variety of illness [Schlievert et al. 1981 & Uchiyama et al. 1994]. It is produced exclusively by S. aureus, and approximately 20% of natural isolates are producers. TSST-1, formerly designated enterotoxin F, was the first toxin shown to be involved in TSS and is accepted as the major toxin associated with this illness, whether menstrual or nonmenstrual, accounting for about 75% of all cases. It has also been described in association with many types of S. aureus infections some of which are quite minor [Parsonnet 1996]. Staphylococcal food poisoning is the leading cause of food borne microbial intoxication worldwide and is usually linked to improper storage of food especially meat products [Holmberg et al.. 1984].
VISA isolates were first found in nature more than twenty five years ago while investigators were screening isolates for vancomycin susceptibility [Watanakunakorn 1984]. Staphylococci were found to produce diseases in two ways: directly by invasion and subsequent tissue destruction whether locally or after having spread via the blood stream and through the effects of toxin [Sheagren 1984]. 

*S. aureus* can invade and survive inside epithelial cells including endothelial cells which may also allow it to escape host defenses particularly in endocarditis [Arrecubieta et al. 2006, Hamill 1986, Moreillon 2002, and Ogawa 1985;]. Time to onset of symptoms after consuming contaminated food averages four hours [Holmberg 1984].

Resistance to trimethoprim (Tp) was found to be mediated by a plasmid-encoded gene in staphylococci. The staphylococcal Tp gene codes for a single protein with DHFR activity that appears to be unrelated to DHFR genes that mediate Tp in members of the Enterobacteriaceae [Cougher et al. 1987]. The bifunctional enzyme AAC(6’)/APH(2’”) encoded by aac(6’)-aph(2’”) gene was found to inactivate a broad range of clinically useful aminoglycosides such as gentamycin, tobramycin, netilmicin and amikacin [Lovering et al 1988; Rouch et al 1987]. Genetic and biochemical properties of the tetracycline resistance element of the *Staphylococcus aureus* plasmid pT181 have been studied [Mojumdar and Khan 1988].

In Italy, 6% of isolates were methicillin resistant in 1981 and 6% were resistant in 1986. From 1988 a further increase in these strains was noted. This study also showed that these strains produced large quantities of penicillinase and were resistant to streptomycin and tetracyclines. At the time of their introduction fluoroquinolones showed good activity against methicillin susceptible and resistant *S. aureus* [Gahin- Hausen 1987]. However the sporadic emergence of resistance was soon reported in *S. aureus* isolated from the skin flora of patients during ciprofloxacin therapy [Humphreys 1985], as well as in the skin flora during the treatment of methicillin resistant *S. aureus* carriers [Mulligan 1987].
Studies by Janosi et al. 1990, revealed that the determinant for PMS phenotype was located on plasmids, which also encoded beta-lactamase production and cadmium ion resistance, but not arsenate resistance. Three types of plasmid with molecular size of 50 kilobases (kb), 23.8 kb, and 16.8 kb, were found among the strains with PMS resistance phenotype, and the 50 kb and 23.8 kb plasmids also encoded mercury resistance [Janosi et al. 1990].

Staphylococcus aureus has been pointed out as being the agent responsible for 19% of the nosocomial infections at the Hospital São Paulo, 60% of the isolates being MRSA [Wey et al. 1990]. Studies have proven that infection control measures should be accompanied by identification of carriers and subsequent elimination of S. aureus carriage [Working Party 1990]. One report mentions the complete control of an outbreak when only simple infection control measures were taken [Guiguet 1990], while several others found that only extensive modifications of local infection control practices were effective [Bitar 1987; Boyce 1981; Kluytmans et al. 1995, Murray-Leisure et al. 1990].

S. aureus has been found to be the second most prevalent organism causing intravascular device associated bacteremia. [Schaber et al. 1991]. Blood stream infections are defined as infections in which no other primary site can be discerned [Banerjee et al. 1991]. Regan et al. have shown that elimination of nasal carriage by using topical mupirocin also eliminates hand carriages [Regan 1991]. A survey conducted in Central Europe in 1991 showed that the incidence of oxacillin resistant strains of S. aureus ranged from 4 to 30.9% depending on the country. The proportion of ciprofloxacin resistant strains ranged from 26 to 47% but is greater than 70% in some countries. The proportion of MRSA rapidly increased from below 5% in the early 1980s to 29% in 1991 [Panlilio et al. 1992]. This study also showed that the prevalence generally increased with the size of the hospital. The interspecies conjugation between vancomycin resistant enterococci (VRE) and S. aureus has been known to occur since 1992, when Noble demonstrated it by experimental transfer of vancomycin resistance from VRE to S. aureus in the laboratory [Noble et al. 1992].

In 1992 a mechanism of fluoroquinolone resistance which is the NorA gene was characterized and cloned from chromosomal DNA of a quinolone-and methicillin-resistant Staphylococcus aureus strain conferring resistance to
hydrophilic quinolones such as norfloxacin, enoxacin, ofloxacin, and ciprofloxacin. The uptake of a hydrophilic quinolone, enoxacin, by S. aureus harboring a plasmid carrying the norA gene was found to be about 50% of that by the parent strain lacking the plasmid.

On the other hand, the uptake of a hydrophobic quinolone, sparfloxacin, was hardly affected by the norA gene [Yoshida 1992]. Aminoglycosides inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit. The major mechanism of aminoglycoside resistance in staphylococci, is drug inactivation by cellular enzymes such as aminoglycoside acetyltransferases (AAC), aminoglycoside adenyl transferases (also named aminoglycoside nucleotidyltransferases [ANT]), or aminoglycoside phosphotransferases (APH). A number of loci encoding these enzymes have also been identified in staphylococci (Shaw et al. 1993).

Lee et al. in 1993 reported the incidence of MRSA strains in South Korea. Between 1964 and 1968 no strain was resistant to methicillin. In 1980, 1985, 1990, 1991 and 1992 the percentages of methicillin resistant strains were respectively 14.1, 22, 43.4, 63 and 61%. Clindamycin, erythromycin, cyclines and Cotrimoxazole were inactive against 88, 97, 86 and 4% of MRSA respectively. In a Spanish survey conducted in November 1991, 15.7% of MRSA strains were resistant to ofloxacin. Use of vancomycin had increased dramatically over the years, because of the increasing prevalence of methicillin resistance in both coagulase-negative staphylococci and Staphylococcus aureus (Ena 1993). In Sweden in 1993, 76% Staphylococcus strains were resistant to piperacillin and between 4.5 and 15% were resistant to ciprofloxacin. Sader et al. [Sader et al. 1993] found a prevalence of approximately 70% MRSA among Staphylococcus aureus isolates in some hospitals of the metropolitan area of Sao Paulo. Mortality from S. aureus bacteremia ranges from 11 to 48%, a figure that has increased steadily for a number of years [Mortara et al. 1993].

Mulligan et al. states that indications for eradication of MRSA are elimination of an outbreak in a health care setting and prevention of recurrent infections in an individual. In settings where MRSA is recurrent elimination of carriage has not been found to be cost effective [Mulligan et al. 1993]. In an outbreak situation the first goal is to identify all carriers including patients and
health care workers. Then elimination of carriage should be achieved in all identified carriers [Coello et al. 1994 & Kluytmans et al. 1995]. Hand washing remains the single most important measure to reduce the risk of transmitting microorganisms from one person to another [Emori et al. 1993, Garner et al. 1996]. In addition to hand washing, gloves play an important role in reducing the risks of transmission of S. aureus in health care settings. Containment measures for patients with infections with S. aureus usually require standard precautions which include hand washing after touching body fluids or contaminated items whether or not gloves are worn. Restriction from patient care activities or food handling is indicated for personnel who have draining skin lesions with S. aureus until they have received appropriate therapy. No work restrictions are needed for colonized personnel unless they have been epidemiologically implicated in S. aureus transmission in a facility.

Until 1990s MRSA rarely caused infections among community members without exposure to health care settings. An outbreak of CA-MRSA infections occurred between 1989 and 1991 among indigenous Australians in Western Australia without healthcare contact [Udo et al. 1993]. Infections were also reported from neighboring regions [Gosbell et al. 1993]. The disease spectrum included abscesses, central nervous system infections, bacteremia, endocarditis, osteomyelitis, Urinary tract infections and a host of syndromes caused by exotoxins including bullous impetigo, food poisoning, Staphylococcal scalded skin syndrome and Toxic shock syndrome. Surgical site infections (SSIs) constitute approximately 15% of the infections reported to the NNIS system by hospitals that collected hospital wide surveillance data [Emori and Gaynes 1993; NNIS 1996; Nawas et al. 1998]. Transmission of S. aureus strains from health care workers to patients have resulted in mediastinitis [Gaynes et al. 1991], nosocomial toxic shock syndrome [Kreiswirth et al. 1986] and the spread of antimicrobial resistant strains within an institution [Domínguez et al. 1994]. The intracellular environment provides a potential sanctuary protecting staphylococci from host defence mechanisms as well as from the bactericidal effects of antibiotics [Balwit et al. 1994; Vesga et al. 1996]. Several studies have been devoted to identify the adhesion mechanisms of Staphylococcus aureus, which are the most frequent causes of prosthesis-associated infections. S. aureus is capable of expressing two fibronectin-binding proteins, FnBPA and FnBPB,
encoded by two closely linked genes (Jonsson et al. 1991). Recently, in particular for *Staphylococcus aureus*, considerable attention has been given to the host protein receptors as mediators for bacterial adherence. Fibrinogen binding protein is an important factor that promotes bacterial adhesion. *Staphylococcus aureus* has been shown to interact specifically with fibrinogen. Three different extracellular fibrinogen-binding proteins, two of which have coagulase activity, are produced by *S. aureus* strain. The role of these fibrinogen-binding proteins during staphylococcal colonization and infection has not yet been fully elucidated [Boden and Flock 1994]. As implanted biomaterial always gets coated by a fibronectin-layer, these proteins are suggested to be important in the promotion of foreign body infections (Greene et al. 1995).

In a multi centre European study, [Voss et al. 1994] collected 7333 strains of *S. aureus*, 12.8% of which were resistant to methicillin. The proportion varied according to the country or region, far less from 1% in Scandinavia to more than 30% in Italy, Spain and France. A high level resistant to ciprofloxacin was noted. This study revealed an interesting variation in the geographical distribution of MRSA in Europe according to which the prevalence of MRSA increases significantly from the northern to Southern countries. In India in some of the centres the isolation varied from 20-40 per cent [Geha et al. 1994].

In the Netherlands, where MRSA is rare and cross-transmission is therefore readily detected by simple procedures such as phage typing, there was 92% homology in MSSA postsurgical wound infections with the patients’ own nasal isolates, and 86% of MSSA infections were due to a unique strain [Kluytmans et al. 1995]. Gamma toxin, leukocidin and other bicomponent toxins are a family of proteins encoded by the hlg and luk PV loci. These toxins contain 2 synergistically acting proteins: one S component and one F component designated on the basis of their mobility. PVL S and F components are LukS-PV and LukF-PV. S and F components of gamma toxin are designated as HlgA and HlgB respectively [Prevost et al. 1995].

Morton et al. studied conjugative plasmids encoding high-level mupirocin resistance found in *Staphylococcus aureus* isolates from two geographic locations in the United States. Transfer genes on three mupirocin resistance plasmids with different restriction endonuclease profiles were found. The mupirocin
The resistance gene was flanked by two directly repeated copies of IS431/257 [Hodgson et al. 1994 & Morton et al. 1995].

Out breaks in neonates usually result in skin infections and bacteremia although more serious diseases such as osteomyelitis and meningitis can occur [Haley et al. 1995]. The nationwide use of third generation cephalosporins in Japan in the early 1980s coincided with the abrupt and dramatic rise in MRSA detection rates [Tanaka et al. 1995]. Although the presence of Oxacillin Resistant Staphylococcus aureus (ORSA) in the Asia-Pacific and South Africa (APAC) region has been well recorded, data on the true prevalence of ORSA in the region are limited. Longitudinal data from Australia have been published and show that in hospital isolates from the Eastern Seaboard, the percentage of S. aureus strains that were ORSA remained relatively constant at approximately 30% from 1986 to 1994 (Turnidge et al. 1996.).

Colonisation particularly of the nares by S. aureus leads to hand carriage and from the hands the organism are frequently spread to other areas of the body. Thus Staphylococci follow a nose to hand to wound route of infection. [Fekety Jr. 1964 & Zimakoff et al. 1996]. Contact transmission is the most important and frequent mode of transmission for S. aureus [Bolyard et al. 1998; Garner 1996].

In 1996 the three phenotypes of resistance to MLS in S. aureus has been studied. The first is mediated by 23S-rRNA methylases, encoded by three homologous staphylococcal determinants, *ermA*, *ermB* and *ermC*, which cause a target alteration of the ribosome, thus preventing antimicrobial agents from binding to their ribosomal target site. The second resistance type, mediated by *msrA/msrB* and *vga*, is based on the active efflux of the antimicrobial agent by an A.T.P dependent pump, thereby maintaining intracellular concentrations below the level required for binding to ribosomes [Allignet et al. 1996].

In India 32.8 per cent MRSA was reported in Mumbai, Delhi and Bangalore in 1996. *Staphylococcus aureus* strains isolated from pus or blood of patients during January 1993 to November 1994 at Vellore were tested for antimicrobial susceptibility and 24% were found to be MRSA. 97 per cent were resistant to trimethoprim- sulphamethoxazole; 85.5 per cent to gentamicin and 45 per cent to amikacin. While more than 90 per cent were resistant to norfloxacin and ciprofloxacin, only 53 per cent were resistant to ofloxacin [Mathur et al. 1994;
Pulimood et al. 1996]. In a surveillance study conducted simultaneously at three centres across India, Mehta et al. found that from a total 739 cultures of S. aureus, 235 (32%) were found to be multiply resistant with the individual figures for resistance being 27% (Mumbai), 42.5% (Delhi) and 47% (Bangalore) [Mehta et al. 1996].

Virtually any S. aureus infection can lead to bacteremia. S. aureus causes about 11 to 38% of community acquired bacteremia [Cockerill et al. 1997; Kauffman and Bradley 1997]. In a review of data on adult bacteremia from 3 hospitals by [Weinstein et al. 1997] S. aureus was the most common cause of clinically significant bacteremia (18.9%). Approximately 10 to 40% of community acquired cases of S. aureus bacteremia progress to endocarditis [Dajani et al. 1997; Karchmer 1992; Mortara and Bayer 1993]. Staphylococcal osteomyelitis is classified as either acute or chronic [Waldvogel and Vasey 1980]. Acute hematogenous osteomyelitis is usually a disease of children primarily neonates in whom it affects the long bones of the lower extremity [Rissing 1997].

Antimicrobial agents of the class of tetracyclines bind to the 30S ribosomal subunit, thus resulting in inhibition of protein synthesis. Staphylococci can become resistant to tetracyclines in two different ways. The first mechanism of resistance is based on ribosomal protection and is encoded by the tetA(M) gene. The second tetracycline resistance mechanism in staphylococci is active efflux mediated by the tetA(K) or tetA(L). Sulfonamide resistant S. aureus has been reported soon after the introduction of these antimicrobial antibiotics. The chromosomal determinant sulA is responsible for an increased production of p-aminobenzoic acid. The resistance mechanism encoded by plasmid determinant sulB is yet unknown. Chloramphenicol acts as a bacteriostatic antimicrobial agent that binds to the 50S subunit of bacterial ribosome, thus inhibiting the transpeptidation in protein synthesis. Although it has not been widely used as an antistaphylococcal agent, resistance has arisen and is due to the inactivation of the antibiotic by a chloramphenicol acetyltransferase enzyme (CAT), which acetylates the drug [Paulsen et al. 1997].

A review of 50574 S. aureus Hospital acquired infections reported to the NNIS system from 1987 to 1997 showed that 29% were resistant to the semi synthetic beta lactam agents oxacillin, nafcillin and methicillin [NNIS 1996].
Several previous reports suggest that the prevalence of *S. aureus* strains resistant to methicillin, nafcillin or oxacillin has increased in the United States and abroad [Aubry-Damon *et al.* 1997; Barrett *et al.* 1998; Irish *et al.* 1998; Struelens *et al.* 1994; Zimakoff *et al.* 1996] and such strains have the propensity to cause outbreaks [Dominguez *et al.* 1994; Haley *et al.* 1995; Roman *et al.* 1997].

AAD2’-aph6’’and is the most frequently encountered aminoglycoside resistance mechanisms among staphylococcal isolates [Busch- Sorensen *et al.* 1996; Martineau *et al.* 2000].

Overall for the decade 1987 to 1997 the percentage of MRSA isolated from patients in an ICU and patients in non critical care wards were 32% and 26.3% respectively. The development of vancomycin resistance among *S. aureus* isolates would dramatically impact the ability to control the spread of MRSA in health care settings [Barrett *et al.* 1998; CDC 1997].Fluoroquinolone resistance emerged very rapidly in HA-MRSA in the years after widespread utilization of agents of this class; at one institution, fluoroquinolone resistance increased from 7% before 1988 to 83% in 1990 [Hershow *et al.* 1998].

*S. aureus* is an unusual cause of community acquired pneumonia but is a common etiological agent of pneumonia in the hospital setting, frequently as a consequence of influenza [Lowy 1998; NNIS 1998]. In addition to being the leading cause of bacteremia in the United States Staphylococci also are among the four most common causes of food borne illness, surpassing even campylobacteriosis and listeriosis [Armstrong *et al.* 1998].Typically *S. aureus* pneumonia develops in elderly patients approximately 4 to 14 days following the onset of influenza. Chest pain, fever, shortness of breath and signs of pleural effusion are nearly always present [Lowy 1998]. Mortality is approximately 15 to 20%. The first clinical isolate of VISA was reported in 1995, which was from a French child who had been receiving vancomycin for MRSA infection (Ploy *et al.* 1998). The resistance to vancomycin was not easy to induce and so it was thought unlikely to occur in a clinical setting [Moellering 1998]. Though a cause for concern, reports of vancomycin resistance in coagulase negative Staphylococci did not generate a great deal of attention as CoNS are generally considered to be relatively nonvirulent organisms. A very different response greeted the first report of decreased susceptibility to vancomycin in *S. aureus* in 1997.
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(CDC 1997 & Hiramatsu 1997). The increase in multi resistant cocci is due to a significant increase in vancomycin use during the last two decades, which evidently served as a selective pressure for the emergence of VISA and VRSA in US hospitals [Kirst et al. 1998]. Given the enormous public health concerns regarding the dissemination of VISA and VRSA, the Hospital Infection Control Practices Advisory Committee of the CDC published infection control guidelines for all staphylococci for which the MIC of vancomycin is ≥8 μg/ml in 1997 [CDC 1997].

In *Staphylococcus aureus* strains resistance to beta lactams is determined by *blaZ* gene carried on a plasmid. In contrast to most *S. aureus* isolates in which the gene for staphylococcal beta-lactamase (*blaZ*) is plasmid borne, isolates typeable by group II bacteriophages frequently carry *blaZ* on the chromosome. Furthermore, the chromosomal gene encodes the type B variant of staphylococcal beta-lactamase. The type and amount of enzyme produced by phage group II isolates were studied by Voladri and Kernodle [1998]. They identified type B and type C enzymes each encoded on the chromosome and 21-kb plasmid respectively.

The distribution of fluoroquinolone resistance-associated point mutations in genes encoding the subunits of DNA gyrase and DNA topoisomerase IV was examined in clinical isolates of *Staphylococcus aureus* and point mutations were detected by polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis and mutations were characterized by sequencing of PCR products. Mutations at Ser84 of *gyrA* and Ser80 of *GrlA* were widely distributed among isolates exhibiting various degrees of fluoroquinolone resistance. *gyrB* and *grlB* mutants were rare. These studies found that *gyrA* and *grlA* mutations impart high levels of fluoroquinolone resistance in *S. aureus* clinical isolates [Takahashi et al. 1998].

Mupirocin resistance was correlated with the presence of plasmids in methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated in the Rio de Janeiro Federal University Hospital in Brazil, where topical mupirocin has been used extensively since 1990. Strains exhibiting high-level resistance carried a large and relaxable plasmid of about 35 kb. Mupirocin-sensitive derivatives, obtained by
growth at 42°C of a strain exhibiting high-level resistance, were devoid of the large plasmid [Bastos et al. 1999]. In 1997 a collaborative epidemiological survey carried out in all 19 European hospitals showed that 1.6% S. aureus showed high level mupirocin resistant [Schmitz and Jones 1997; Maniatis et al. 2002 & Udo et al. 1999].

The staphylococcal Panton-Valentine leukocidin (PVL) genes, [luk-S-PV-
lukF- PV], existing on the genome of a temperate bacteriophage phiPVL was isolated from mitomycin C-induced Staphylococcus aureus V8 (ATCC 49775) [Kaneko et al. 1997]. Gravet et al. characterized the staphylococcal bi-component leucotoxins family, LukE (32 kDa) and LukD (34.3 kDa) from Staphylococcus aureus strain Newman and found that LukE+LukD was as effective as the Panton-Valentine leucocidin for inducing dermonecrosis when injected in the rabbit skin, but not hemolytic and poorly leucotoxic compared to other leucotoxins expressed by Staphylococcus aureus [Gravet et al. 1998]. The Panton-Valentine leukocidin (Luk-PVL) belongs to the family of bicomponent toxins and Luk-PVL is associated with skin and soft-tissue infections as well as with more serious infections, e.g., severe necrotizing pneumonia [Diep et al. 2004 & Lina et al. 1999]. In 1996, a wound infection caused by VISA was reported in Japan in a child receiving vancomycin for an MRSA wound infection [Hiramatsu, Aritaka 1997 & Hiramatsu, Hanaki 1997]. A hVISA strain (Mu3) was first identified in 1996 in Japan in a 64-year-old patient with MRSA pneumonia whose condition responded poorly to vancomycin treatment [Hiramatsu 1997]. VISA strains were first reported in UK and France in 1998 [Howe et al. 1998; Ploy et al. 1998] and in the US in 1999 [Smith et al. 1999 & Rotun et al. 1999].

Studies by Bayles et al. reported staphylococcal internalization by bovine mammary cells. Following entry some bacteria escape the membrane bound vacuoles and induce cellular apoptosis [Bayles et al. 1998]. The changes resulting from internalization of staphylococci precipitate a series of cellular changes that contribute to the pathogenesis of life threatening diseases. The events include the ability of Staphylococcus aureus to cause (1) endocarditis by adhering to and invading damaged or undamaged valvular tissue and causing progressive local cardio vascular damage (2) metastatic infections by traversing endothelial or other epithelial cell barriers, elaborating proteolytic enzymes and spreading to adjoining tissues or (3) a sepsis syndrome similar to that produced by endotoxin resulting
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from endothelial cell injury with the expression of endothelial cell receptors and the release of cellular cytokines [Hamill et al. 1986; Ing et al. 1997; Lowy 1998].

The molecular epidemiology of MRSA in a university hospital in Italy was studied in a five-month period in 1996, during which all S. aureus isolated were collected. The study found that MRSA represented 32.3% of all isolates, with very high percentages from the intensive care units (adult and neonatal). [Villari et al. 1998]. Results for the United Kingdom show that the increase in MRSA proportions, reported from 1992 through 1998 (Speller et al. 1997, Reacher et al. 2000), continued until 2001. In Germany a national surveillance study carried out at regular intervals, reported an increase of MRSA from 2% in 1992 to 21% in 2001 (Kresken and Hafner1999). In India about 32% MRSA was reported in Rohtak and Mangalore in 1999[Verma et al. 2000].

Mutations of the rpoB gene conferring resistance to rifampin were analyzed in MRSA isolates obtained from six countries. The majority of clinical isolates showed multiple mutations within rpoB. Cross-resistance to rifampin, rifabutin, and rifapentine was demonstrated for all mutants identified. The level of resistance to rifamycins correlated with both the mutation position and type of amino acid substitution [Wichelhaus et al. 1999].

Since 2000, an increase in MRSA strains with low cefazolin MICs were observed. It was an important pathogen in human infections causing a wide variety of infections from mild skin infection to more serious and invasive infections [Tenover et al. 2000]. 50-70% MRSA was reported from Japan by Takeda et al. in 2000 and in Latin America by Gales et al.. [Takeda et al. 2000; Gales et al. 2000]. In other countries such as Tunisia, Malta, Algeria [Kesah et al. 2000], Sweden, Switzerland, the Netherlands (SENTRY participants group, 2001) it was low.

Many S. aureus strains produce one or more specific staphylococcal exotoxins, including staphylococcal enterotoxins (SEs), staphylococcal exfoliative toxins, and toxic shock syndrome toxin 1 (TSST-1). These toxins cause infections ranging from relatively mild involvement of the skin and soft tissue to life-threatening sepsis, necrotizing pneumonia, and TSS [Gillet et al. 2001; Lina et al. 1999; Lowy 1998; McCormick et al. 2001; Novick 2000]. In recent years, the existence of new types of SE genes (seg, seh, sei, sej, sek, sel, sem, sen,
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seo, sep and seq), which belong to the operon of the enterotoxin gene cluster (egc), has been reported [Jarraud et al. 1999; Jarraud et al. 2001; Nilsson et al. 1999; Omoe et al. 2002]. In addition, SE-like proteins R, S, T, and U have been identified but remain poorly characterized. In Poland about 15% of S. aureus were resistant to methicillin and more than 60% were resistant to cyclines but >90% remained susceptible to aminoglycosides. 31% MRSA was observed in Kenya and Cameroon [Kesah et al. 2000]. In Australia the frequency of methicillin resistant strains varied according to geographical location. 25.2% in Queensland and 0.4% in the western region of Australia during 1993. Of interest is the significant proportion of ORSA isolates from Australia that were non multi drug resistant. 83% of these isolates were found in a single Western Australian institution. NORSA strains emerged from the remote Kimberly region of Western Australia in the mid-1980s. Since that time, the epidemiology of these strains has been further characterized. They are most commonly found in remote Central and Western Australian Aboriginal communities, where skin colonization rates were as high as 42% and skin sepsis is the most frequent clinical manifestation. While they typically caused community-acquired infections, they have been responsible for hospital outbreaks [Turnidge and Bell 2000; Wadtvogel 2000].

MRSA is an important cause of infection in hospitalized patients throughout the APAC region. The proportion of S. aureus strains resistant to oxacillin in the APAC region (45.9%) was high (ranging from 5.0% in the Philippines to 79.5% in Hong Kong). This proportion was higher than those reported from other geographic regions over the same time period: Latin America (34.9%), United States (34.2%), Europe (26.3%), and Canada (5.7%) (Diekema et al. 2001). A small number of strains, mostly from Western Australia, were nonmultidrug-resistant (NORSA). 33% MRSA was reported from Jeddah, Saudi Arabia [Madani et al. 2001] and a much lower prevalence of 12% was reported from the eastern province [Bukharie 2001].

Actual MRSA prevalence is subject to wide geographical variation. In Europe MRSA rates as high as 58% in Italy and 54% in Portugal have been reported [Fluit et al. 2001]. In India studies by Vidhani etal reported sensitivity to macrolide group of antibiotics like Erythromycin (17.7%) in MRSA. Amongst the aminoglycosides, maximum sensitivity was seen with amikacin and 26.6% MRSA
were sensitive to the same. 67% of MRSA were found to be sensitive to fluoroquinolone group i.e. ofloxacin. All S. aureus isolates (MSSA and MRSA) were found to be uniformly sensitive to vancomycin [Vidhani et al. 2001].

The reporting of methicillin resistance for Staphylococcus aureus bacteraemia was made mandatory in England from April 2001 precisely because it was seen as a marker of the prevalence of MRSA infection, which in turn was seen as a marker of infection control practice and rates of hospital-acquired infection. [PHLS Communicable Disease Surveillance Centre. 2001] In a survey conducted in the UK and Ireland 2001–2002 by the BSAC found a resistance rate of 42.0% to oxacillin. Oxacillin-resistant S. aureus showed a high prevalence of resistance to ciprofloxacin (95%) and erythromycin (84%). 23% were resistant to clindamycin. There was some resistance to gentamicin (10%, mostly with MICs of 64 mg/L) but little to tetracycline (<2%), and none to vancomycin, teicoplanin or linezolid. 30% resistance to trimethoprim was found.

The high prevalence of VRE in US hospitals paved the way for emergence of vanA gene transfer to MRSA. Rapid and steady rise in the number of MRSA and VRE in US hospitals in the past two decades is another contributing factor increasing the chance of encounter of two species in patient’s bodies [Farr et al. 2001]. The significant rise in MRSA and VRE in US hospitals is correlated with the generous use of broad spectrum cephalosporins such as ceftriaxone and ceftazidime [Harbarth et al. 2001].

S. aureus has an enhanced ability to produce biofilm on inert polystyrene surfaces and to adhere to and invade epithelial airway cells. The detection of the genes governing the production of extracellular polysaccharide involved in biofilm formation, in particular, the icaA, the icaC and the icaD genes were studied by Aricola et al. [2002].

Now MRSA is prevailing both inside and outside hospitals, gradually replacing methicillin susceptible S. aureus (MSSA), which is a part of the normal flora of healthy humans [Okuma K. 2002]. Bacteraemia reports to the Health Protection Agency documented a rapid rise in methicillin resistance in S. aureus during the 1990s, from under 1.7% of S. aureus isolates in 1990, through 4% in 1993, 21% in 1996 and 34% in 1998 to reach 42% in England and Wales in 2000 [Reacher et al. 2000; PHLS.2002; Johnson et al. 1997]. 42% in 2001
and 43% in 2002 was also reported [Health Protection Agency. 2002, Health Protection Agency.2003.]. Sentinel data from the European Antimicrobial Resistance Surveillance System (EARSS, http://www.earss.rivm.nl) also report a steady level (44%) of methicillin resistance in the UK in 2001 and 2002. *S. aureus* had become the most frequent cause of nosocomial pneumonia [Chastre 2002]. Occasional isolates of *S. aureus* with reduced susceptibility to vancomycin have been reported in the UK in recent years, [Howe et al. 1998, Hamilton-Miller 2002.]. In 2002, the first fully vancomycin resistant clinical *S. aureus* strains were isolated in US from a diabetic patient in Michigan, US. Vancomycin resistant *S. aureus* (VRSA) strains contained plasmids carrying vanA gene complex carried on a transposons which had been transferred by conjugation from vancomycin resistant enterococci (VRE) that coexisted in the patient’s body [Bartley 2002].

Between April 1998 and December 1999, in the APAC region Oxacillin resistance was detected in 43.1% of blood isolates, 56.9% of respiratory tract isolates, 40.5% of wound isolates, and 57% of urine isolates, the majority of which were multi drug resistant. Multidrug resistance (MORSA) was defined as resistance to penicillin and oxacillin plus three or more of the agents such as erythromycin, clindamycin, rifampin, ciprofloxacin, gentamicin, trimethoprimsulfamethoxazole, and chloramphenicol. Considerable variation was found in resistance profiles between countries in ORSA strains. The predominant antibiogram for Australia, Singapore, and Taiwan included resistance to erythromycin, clindamycin, ciprofloxacin, gentamicin, tetracycline, and sulfamethoxazole-trimethoprim. The most common profile for Japan, Hong Kong, and mainland China included resistance to erythromycin, clindamycin, ciprofloxacin, tetracycline, and gentamicin for Hong Kong and China, as well as a proportion of Japanese strains (56.5%) [Bell et al. 2002]. The incidence and trends of MRSA during a 12-year (1989-2000) period at a university teaching hospital was carried out and the relationship between strain distribution studied by antibiogram and molecular typing. The studies showed the establishment of a dominant MRSA clone (PFGE type A group) in the intensive care, medical, and surgical units and the appearance of a new MRSA strain in 1995 (PFGE type B), which partly explained the rise in incidence of MRSA cases and a disproportionate rise in MRSA bacteremia from 1995 to 2000 in Hong Kong [IP et al. 2004]. Surveillance of MRSA in Europe during the period 1999 to
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2002 by Tiemersma et al. included 50,759 isolates from 428 laboratories serving approximately 500 hospitals. Overall, 20% of these isolates were reported as methicillin resistant. The lowest MRSA prevalence was reported in northern Europe and highest prevalence in southern Europe, Israel, the United Kingdom, and Ireland. MRSA proportions varied almost 100-fold, with the lowest proportion in Iceland (0.5%) and the highest proportion in Greece 44%. Increases in MRSA proportions were significant in Belgium (from 22% in 1999 to 27% in 2002), Ireland (39%–45%), Germany (9%–19%), the Netherlands (0.4%–1%) and the United Kingdom (31%–45%). The proportion of MRSA decreased significantly in Slovenia only, from 22% in 2000 to 15% in 2002. Vancomycin resistance did not occur. Intermediate susceptibility of Staphylococcus aureus (VISA) was only reported for five isolates from France in 2001 [Tiemersma et al. 2004]. Studies by Howe et al. showed that reduced susceptibility to vancomycin has emerged in many successful epidemic lineages with no clear clonal disposition [Howe et al., 2004].

In India Krishnan et al. studied the predominance of one or more epidemic strains within an endemic situation. A representative selection of 65 isolates of MRSA over a period spanning five years (1994-1998) was subjected to antibiogram comparison, phage-typing and pulsed field gel electrophoresis (PFGE) using Smal. The antibiogram comparison was not a discerning method of strain discrimination. At least 11 different phage-types were seen among these 65 isolates; 35.4% belonged to phage-types A and SM-A. The study showed that the isolates from India were diverse and distinct from strains of MRSA such as EMRSA reported from many studies in the UK [Krishnan et al. 2002].

Linezolid is an important therapeutic option for treatment of infections caused by glycopeptide- and beta-lactam-resistant gram-positive organisms. Linezolid resistance was found to be caused by mutations within the domain V region of the 23S ribosomal RNA (rRNA) gene, which is present in multiple copies in most bacteria. Pillai et al found that after serial passage on antibiotic-free medium, the isolate maintained resistance to high concentrations of linezolid and compared with linezolid- susceptible Staphylococcus aureus isolates, the linezolid-resistant Staphylococcus aureus isolate demonstrated no significant differences in in vitro growth characteristics [Pillai et al. 2002]. Since 1997 the potential benefits of
macrolides therapy in cystic fibrosis patients have been promoted and resistance reached 53% in cystic fibrosis patients compared to 38% in the same geographical area in the general population [Prunier et al. 2003].

In 2003 Salgado et al. reported that according to the NNIS survey MRSA is currently the most commonly identified antibiotic-resistant pathogen in US hospitals and 50% of hospital acquired infections in the ICUs in the USA were due to MRSA [Salgado et al. 2003, NNIS 2003]. In North America Hoban et al. reported 43.2% MRSA [Hoban et al. 2003]. In Brazil, the frequency of isolation of Staphylococcus aureus and its role in nosocomial infection has been greater than in other countries [Trindade et al. 2003]. 33% MRSA was reported in Saudi in 2003 [Austin et al. 2003]. Several studies have established that the transmission of MRSA between patients within hospital settings occur to a great extent through health care workers (Berthelot et al. 2003 & Blok et al. 2003).

Studies by Orwin et al. identified a novel enterotoxin-like protein that is a member of the new subfamily (group V) of pyrogenic toxin superantigens (PTSAgs) and examined its biochemical and immunobiological properties. They demonstrated that the PTSAg had many of the biological activities associated with SEs, including superantigenicity, pyrogenicity, and ability to enhance endotoxin shock, but lacked both lethality in rabbits when administered in subcutaneous mini osmotic pumps and emetic activity in monkeys [Orwin et al. 2002]. Omoe et al. identified and characterized a novel staphylococcal enterotoxin-like putative toxin, which is named SER [Omoe et al. 2003].

Analysis of the natural population dynamics and expansion of pathogenic clones of S. aureus provided evidence that essentially any S. aureus genotype carried by humans can transform into a life-threatening human pathogen but that certain clones are more virulent than others (Melles et al. 2004).

Characteristics of Staphylococcus that help it evade host immune systems has been reviewed by Foster [Foster 2005]. Several epidemiological studies have found increased morbidity and mortality from nosocomial MRSA compared with those from MSSA [Cosgrove et al. 2003; Gastmeier et al. 2005; Engemann et al. 2003 & Reed et al. 2005]. Outbreaks have been reported in variety of settings including hospitals (Mallaval et al. 2004), long term care facilities (Larsen et al.
2005), outpatient clinics (Lee et al. 2005), as well as in the community (Rihn et al. 2005). Community MRSA strains have several distinguishing characteristics that may enable them to more readily colonize and infect otherwise healthy hosts [Nour et al. 2005].

It was believed that food poisoning in Osaka in 2000 was due to small amounts of staphylococcal enterotoxin A (SEA) in reconstituted milk. Studies by Ikeda et al. in 2005 indicated that SEH was also present in the raw material of reconstituted milk, indicating that the food poisoning was caused by multiple staphylococcal enterotoxins [Ikeda et al. 2005]. Al Haj Hussein et al. [2005] reported a prevalence of 31% MRSA in Saudi in 2005. In Japan nearly 70% of S. aureus blood stream isolates in 2001 were methicillin resistant (Boyce et al. 2005). On the other hand Scandinavian countries have consistently reported lower rates of MRSA. In 2005 MRSA prevalence was generally reported to be high in North America (43.7% and 43.2%) [Kuehnert 2005 & Bryskier 2005].

Over the past decade, the changing pattern of resistance in S. aureus has underscored the need for new antimicrobial agents. Glycopeptide antibiotics, vancomycin and more recently teicoplanin, often are the last option for treatment of MRSA. They exert their antibacterial effects by inhibiting synthesis of the S. aureus cell wall. Prevalence of heterogeneous vancomycin-intermediate S. aureus (hVISA) and vancomycin-intermediate S. aureus (VISA) MRSA strains has been reported in Europe, Asia, and the United States [Appelbaum 2006]. MRSA with reduced vancomycin susceptibility (VISA) has been reported from many countries. In subsequent years, close to 100 cases of S. aureus with reduced susceptibility to vancomycin have been reported, with some strains responsible for life-threatening systemic infections [Sancak et al. 2005 & de Lassence et al. 2006].

Until 2006 prevalence of MRSA was low in Australia (14.9%) [Diekema et al. 2004 & Nimmo et al. 2006]. In a study by Baddour et al. [2006], the prevalence of MRSA among S. aureus isolates in Saudi hospitals varied from one hospital to another and ranged from 12% to 49.4%. Studies on antibacterial resistance in Kuwait hospitals from 1994 to 2004 by Udo et al. revealed an increasing fusidic acid resistance from 22% in 1994 to 92% in 2004. Erythromycin resistance increased from 66% in 1994 to 88% in 2004 and ciprofloxacin resistance increased from 53% to 92% in 2004 after a peak of 96% in 2002. On the contrary over the period of 10 years trimethoprim resistance declined from 86% to 27% and
chloramphenicol decreased from 25% to 2% in 2004. The proportion of isolates resistant to gentamycin decreased from 98% to 77% in 2001. Plasmid DNA was detected in all MRSA strains ranging from 2kb to 38kb [Udo et al.2006].

Rajaduraipandi et al. reported 31.1% MRSA from clinical samples and 37.9% from carrier samples in Tamil Nadu. They found 63.6% multidrug resistant strains [Rajaduraipandi et al. 2006]. These studies revealed that MRSA is emerging to be a significant problem pathogen in the surgical setting with vancomycin probably the only reliable choice for these infections. VRSA was first reported in India by Tiwari and Sen in 2006. They isolated 2 strains with MICs to vancomycin 32µg/ml and 64µg/ml [Tiwari and Sen 2006].

Outbreaks of staphylococcal food poisoning (SFP) are very common across the world; however, there is hardly any report of SFP from the Indian subcontinent. An outbreak occurred in the state of Madhya Pradesh (India) after the consumption of a snack called "Bhalla" made up of potato balls fried in vegetable oil. More than 100 children and adults who ate the snack suffered from the typical symptoms of SFP and required hospitalization. Studies by Nema et al found that the food and clinical samples were found to contain a large number of enterotoxigenic Staphylococcus aureus. All enterotoxigenic isolates produced a combination of SEB and SED enterotoxins and were sensitive to oxacillin and vancomycin. Isolates were characterized by molecular biology tools, viz., SDS-PAGE, amplified ribosomal DNA restriction analysis (ARDRA), randomly amplified polymorphic DNA (RAPD) and nucleotide sequencing of seb, sed, and 16S rDNA genes. Results of these studies suggested that the isolates, irrespective of their isolation from food or clinical samples, were clonal in origin. Representative isolates from food and clinical samples, were also found to be highly heat resistant [Nema et al. 2007].

Central venous catheters have been found to be a major cause of nosocomial bloodstream infections and different attempts have been made to incorporate antimicrobial agents into catheters, particularly directed at the surface-coating of devices. To facilitate the antimicrobial adsorption, various cationic surfactants, which however showed several problems, have been used. On the other hand, impregnated catheters with only antimicrobials have demonstrated a short-term duration due to the difficulties to deliver the drug slowly. Ruggeri et al synthesized modified polyurethanes to introduce different
functional groups. Polymers were loaded with two antibiotics, cefamandole nafate and rifampin (RIF). It was seen that antibiotics released from various formulations inhibited the bacterial growth and exerted a synergistic effect when both were present. In particular, PEG10000-containing polymer was active against the RIF-resistant S. aureus strain up to 23 days [Ruggeri et al. 2007].

Genotyping of seh gene was recently carried out by Ruzickova V et al. in 2008. SEH-producing S. aureus isolates are of high prevalence in staphylococcal food poisoning cases. Given the unique epidemiological characteristic of these isolates, SEH and SEA probably are responsible for food poisoning [Sakai et al. 2008]. PCR primers specific for SE like superantigens SEN, SEO, SEP, SEQ, SER, and SEU genes had been developed. The complete SE sequences and their expression potential for strains positive to sen, seo, sep, seq, ser, and seu specific primers were also determined [Chiang et al. 2008]. Staphylococcal enterotoxin-like genes S and T (SES and SET), were identified in plasmid pF5, which is harbored by food poisoning-related Staphylococcus aureus strain Fukuoka 5 implicated in a food poisoning incident in Fukuoka City, Japan, in 1997 [Ono et al. 2008]. The structural characterization of alpha hemolysin monomer was also recently carried out by molecular modeling [Meesters et al. 2008]. A recent study reported a new group of hetero-MRSA strains genetically distinct from those dominant in the same hospital in the early 1980s might have emerged in the community and started invading the university hospital, a phenomenon caused by the change in the pattern of antibiotic use [Kishii et al. 2008].

Prevalence of MRSA was extremely high Southern European countries [Voss et al. 1994; EARSS 2002], Malaysia [Hanifah et al. 1992], Ethiopia [Geyid et al. 1991] and Sri Lanka [Hart et al. 1998]. Several reports from India suggest increasing incidence of MRSA [Pulimood et al. 1996; Mathur et al. 1994; Pal et al. 1991]. In India a significant part of nosocomial infections are now caused by MRSA and the percentage of resistant strains are ever increasing. Nosocomial MRSA is known to be multidrug resistant and thus difficult to treat [Krishna et al. 2007; Shome et al. 2008; Rallapalli et al. 2008]. Hence accurate and rapid identification of MRSA in a clinical specimen is essential for timely decisions on isolation procedures and effective antimicrobial therapy [Kohner et al. 1999; Murakami et al. 1991; Sakoulas et al. 2001; Unal et al. 1994 & Mulla et al. 2004].
The geographic spread of several MRSA clones between countries and continents has been reported and proven by molecular evidence.

**Typing and molecular characterization**

The purpose of speciation is identification of a microorganism as belonging to a basic taxon having a particular clinical significance. Typing is an important epidemiological tool for tracing the spread of particular strains and for identifying the routes of transmission and reservoirs. Typing systems for studying the epidemiology of *Staphylococcus aureus* infections have been investigated intensively. The ideal typing system should be rapid, inexpensive, technically simple, and readily available. Currently, there is no single definitive typing system for distinguishing individual strains of MRSA [Tenover et al. 1995]. Classical phenotypical typing methods include biotyping, phagetyping, serotyping and antimicrobial susceptibility testing. Comparative characterization (molecular typing) of isolates within a bacterial species is one of the major problems in microbiology and epidemiology. It is even more difficult to correlate data obtained in various laboratories, because traditional, including molecular, methods employed in typing pathogenic microorganisms cannot be standardized. Due to the disadvantages of phenotypic methods, *S. aureus* typing has recently been dominated by molecular biology techniques based on the variation of DNA sequences in bacterial isolates. A selection of the techniques used in *S. aureus* typing includes RAPD (randomly amplified polymorphic DNA) analysis, PFGE (pulsed-field gel electrophoresis), MLST (multilocus sequence typing), *spa* typing, *coa* typing, *agr*-group typing, MLEE (multilocus enzyme electrophoresis) and MLVA (multiple-locus variable-number tandem repeat analysis). Genotyping procedures can lead the way to elucidating the molecular genetics of colonizing *S. aureus* populations.

Studies by Stobberingh had revealed that DNA restriction and modification are the most common mechanism of variation [Stobberingh 1977]. A poorly understood mechanism of genetic exchange named mixed culture transfer or phage mediated conjugation has also been identified in staphylococci. [Lacey 1980]. The mechanism of resistance to methicillin was uncovered in 1981 with the identification of reduced- affinity penicillin binding proteins in MRSA [Hartman and Tomasz 1981].

Phage typing of *S. aureus* has been used since the 1940s and it has been
quite valuable in investigating epidemiology of staphylococcal infections [Parker 1983]. But a number of MRSA strains are not typeable with routine phage panels [Archer and Mayhall 1983; Cristino and Pereira 1989; Kerr et al. 1990; Khalifa et al. 1989; Richardson et al. 1988], although use of supplementary phage panels may be helpful. Unfortunately most laboratories are not equipped to handle phage typing and this technique can be used successfully only if it is performed in an experienced reference laboratory [Maslow 1993]. Plasmid profile analysis has been used to identify the reservoir and mode of transmission of S. aureus infections especially in hospitals [Cohen et al. 1982]. The phenotypic assays such as antibiogram typing and biotyping are also unreliable. Berger Bachi et al. in 1983 showed that additional chromosomally located genes not linked to the mec are essential for expression of methicillin resistance [Berger Bachi B 1983; Kornblum et al 1986]. The use of PFGE was introduced in 1984 by Schwarz and Cantor. Meanwhile, PFGE has become by far the most widespread molecular typing tool in developed countries. It is considered to be the method of choice for DNA fingerprinting of S. aureus.

Most methicillin resistant Staphylococcus aureus isolates contain a DNA segment (greater than 30kb) not present in methicillin sensitive strains. This additional segment of chromosomal DNA is known as mecDNA [Beck et al. 1986; Skinner et al. 1988]. Although DNA can be introduced into staphylococci in the laboratory via transformation, transduction and conjugation, the latter two are found to be significant mediators of natural genetic exchange. Studies have proved that transformation is very inefficient requiring a curious co factor that can be satisfied by a component of phage 55C and is thought to be limited by extracellular nucleases or restriction systems encoded by staphylococci [Lyon 1987 & Novick 1990]. Studies by Trees and Iandolo reported that mec could be mobilized from the chromosome to a penicillinase plasmid pI524 and suggested the possibility that mec may comprise a part of transposable genetic element [Trees and Iandolo 1988].

Berger-Bachi et al. cloned and characterized a chromosomally determined gene which encodes a factor essential for the expression of methicillin resistance (femA) in S. aureus. femA mapped in chromosomal segment number 18, genetically very distant from the methicillin resistance determinant (mec). The
product of femA was a protein of an apparent size of 48 kDa. Tn551 mediated inactivation caused a decrease in peptidoglycan associated glycine content of femA. Although FemA was needed for cell growth in the presence of beta-lactam antibiotics, they found that it had no influence on the synthesis of the low affinity, additional penicillin-binding protein (PBP2') encoded by mec and known to be essential for cell wall synthesis in the presence of inhibitory concentrations of methicillin. The nucleotide and amino acid sequence of femA showed homologies with ORF419, suggesting that these genes arose by gene duplication. Down stream and adjacent to femA lies another factor femB which also affects methicillin resistance levels although to a lesser extend than femA [Berger Bachi et al. 1989 Maidhof et al. 1991].

Speciation in staphyloccoci was first based on a data set of morphological characteristics, physiological properties and chemical composition of cell wall [Kloos 1991]. Later DNA-DNA hybridization studies have shown that staphylococci form a well defined genus which can be subdivided into several species group [Schliefer 1990]. Multilocus Enzyme Electrophoresis is extremely useful for showing clonal relatedness of S. aureus producing toxic hock syndrome toxin1 and also to study the clonal diversity of MRSA [Musser 1990]. The mecA gene, which controls the production of PBP2a, not only confers resistance to b-lactams but also mediates cross- resistance to fluoroquinolones, aminoglycosides, tetracyclines, macrolides, and trimethoprim-sulfamethoxazole. [Chambers 1990; Hiramatsu et al. 1992].

Apart from femA and femB genes other fem factors were also found in both methicillin resistant and methicillin susceptible Staphylococcus aureus. Different types of alterations in muropeptide patterns were observed in Tn551 mutants selected for femC genes. femD inactivation results in disappearance of unsubstituted disaccharide pentapeptide monomer from the cell wall [de Jonge et al. 1992]. High- level, homogeneously resistant revertants selected by passage of femD mutants in methicillin occur without reversing the femD biochemical defect. FemD mutants produced by Tn551 insertion were found to be deficient in muropeptide1 and muropeptide8 [Jolly et al. 1997]. The transposon insertion may be in a regulatory gene controlling peptidoglycan precursor formation. The femF mutation results in a heterogeneous pattern of resistance [Ornelas-Soares et al.
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1994]. Inactivation of *femF* causes a block in peptidoglycan precursor synthesis at the lysine addition step.

All bacterial genomes harbour repetitive sequences that can be used for epidemiological typing. For *S. aureus* intragenic repetitive regions have been found in the coagulase (*coa*) and protein A (*spa*) genes. The coagulase protein is an important virulence factor of *S. aureus* coa has a polymorphic repeat region that can be used for differentiating *S. aureus* isolates. The variable region of *coa* is comprised of 81-bp tandem short sequence repeats (SSRs) that are variable in both number and sequence, as determined by restriction fragment length polymorphism analysis of PCR products [Goh *et al.* 1992].

In 1993 microlitre techniques for plasmid isolation was introduced [Goering 1993]. The value of plasmid profile analysis is limited however since plasmids can be spontaneously lost or acquired. Some MRSA strains lack plasmids and are therefore nontypeable by plasmid profiling. Restriction enzyme analysis of chromosomal DNA identifies the epidemic strains, but due to the numerous overlapping bands that are produced, it is difficult to interpret small variations in the restriction profiles. Application of repetitive extragenic palindromic PCR and enterobacterial repetitive intergenic consensus sequence analysis for the discrimination of MRSA strains have proven to be useful [Lessing *et al.* 1995].

The transformation of colonizing MSSA to MRSA has been found to occur very rapidly (i.e., 24–48 hours) in hospitalized patients and this strong evidence against importation and cross transmission of MRSA [Chetchotisakd *et al.* 1994], reveals that antibiotic selective pressure might play a larger role in the genesis of endemic MRSA than previously suspected. Thus MRSA is not often imported. Rather, it is typically homegrown. Improved typing systems are important for implementing appropriate infection control measures and for the clinical management of MRSA infections, particularly in evaluating the efficacy of therapy for infected or colonized patients [Emmerson 1994].

Although the polymorphism of *coaA* gene leads to amplimers of different length it has not been as discriminative as *smal* macrorestriction patterns [Schwarzkopf 1994]. In contrast *spa* gene shows more sensitivity than necessary and generates more than one pattern within a set of obviously clonal
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strains. While spa- typing does not have the resolving power of PFGE subtyping, it is fast, easy to use and interpret, and compatible for building relational databases. Most importantly, DNA sequence analysis of the proteinA repeat region provides an unambiguous, portable dataset that simplifies information sharing between laboratories and facilitates creating a large-scale database for studying global and local epidemiology [Freney et al. 1994].

PFGE is a molecular typing technique that does not have the same limitations as bacteriophage typing, since the bacterial genome is more stable than most protein markers. PFGE offers advantages over other DNA-based S. aureus strain-typing techniques (Prevost et al. 1992; Tenover et al. 1994). For example, plasmid typing offers only moderate reproducibility, restriction fragment length polymorphism typing requires the analysis of complex banding patterns, and ribotyping is highly labor-intensive and time-consuming. To date, all S. aureus isolates are typeable by PFGE. Because of its great discriminatory power, high degree of specimen typeability, excellent reproducibility and its good correlation with epidemiologically linked data, it is still accepted as the gold standard for the molecular typing of S. aureus isolates [Nawas et al. 1998]. Ichiyama compared genomic DNA fingerprinting patterns among isolates of methicillin-resistant Staphylococcus aureus (MRSA) by using pulsed-field gel electrophoresis (PFGE). Chromosomal fragment profiles digested with Sma I was found most suitable for discriminating between isolates which could not be differentiated by phenotypic methods. [Ichiyama S 1994].

Data published by Kluymans et al. [Kluymans et al. 1995] from an analysis of strains from an outbreak examined by PFGE and PCR-based fingerprinting of bacterial DNA with five different primers have revealed that both PFGE and arbitrarily primed-PCR techniques were equally good in discriminating outbreak-related strains. The majority of these strains came from a point source food-borne outbreak, and therefore, only a limited range of types was to be expected. PFGE identified more subclones than did PCR typing. The most common application of RFLP is ribotyping. Although ribotyping was found to be less discriminative than SmaI macrorestriction patterns it was successfully used for the discrimination of MRSA from different continents and can also be used for speciation [Hiramatsu 1995].

Although generically termed mec region, it was found that there
is considerable variation in both composition and size (20-60kb) of mec regions from different strains. Nucleotide sequencing has revealed the presence of terminal repeats at the end of mec region and confirmed an identical insertion site in different S. aureus strains [Hiramatsu 1995]. The mec region acts as a chromosomal hotspot for the insertion of additional antimicrobial resistance determinants in association with transposable elements. The expression of cell wall and extracellular proteins in Staphylococcus aureus is controlled by global regulatory systems, including sar and agr. sarA and the adjacent upstream DNA are essential to the expression of a DNA-binding protein(s) with specificity for the RNAII promoter, thereby controlling agr-related transcription [Heinrichs et al. 1996]. The two recombinase genes were found mediating the excision and circularization of the cassette structure and also mediating site and orientation specific insertion into the chromosome of a plasmid carrying them and a mec region attachment sequence.

mecA gene is seen as a part of SCCmec flanked by cassette chromosome recombinase genes (ccrA/ccrB or ccrC) that permit intra and inter species horizontal transmission of SCCmec. Study of early isolates of MRSA showed that the key genetic component responsible for resistance, mecA, is not native to the S. aureus genome. The ubiquitous carriage of mecA homologue by Staphylococcus sciuri has led to the suggestion that this represents the origin of mec determinants found in other species [Wu et al. 1996]. Evidence suggests that the mec regions have been introduced into S. aureus on a limited number of occasions [Hiramatsu et al. 1996]. Couto et al. suggested that the mecA gene may have arisen in Staphylococcus sciuri or a related commensal of wild animals [Couto et al. 1996].

DNA typing procedures either PCR mediated or PFGE of large DNA restriction fragments have frequently been applied and proven quite valuable for assessing the clonality among many given strains of S. aureus. [Van Belkum 1995, Cookson BD 1996]. Pulsed-field gel electrophoresis (PFGE) has been recommended as a highly discriminatory method for typing MRSA isolates because it can distinguish among several concurrent epidemic strains although it is a time-consuming and expensive typing method not well suited for screening large number of isolates by a diagnostic laboratory. PFGE has been used for the investigation of MRSA and has been compared with other methods in several
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studies [Bannerman et al. 1995; Chiou et al. 2000; Prasanna Kumari et al. 1997; Van belkum et al. 1995; Struelens et al. 1996; Tenover et al. 1996; Yoshida et al. 1997] and has been successfully been used to study the epidemiology of *S. aureus* nosocomial infection and methicillin resistance [Kumari DN et al. 1997; Yoshida T et al. 1997]. For *S. aureus* the enzyme SmaI has been most useful, yielding 8 to 20 fragments ranging from 8-800 kb in size. Staphylococcal isolates are regarded as different strains if their SmaI macrorestriction patterns differ in four or more bands [Tenover et al. 1997].

In 1997 the *femAB* operon was described which encodes two closely linked, cytoplasmic 49-kDa proteins that are required for formation of the pentaglycine interpeptide bridge that serves as the cross-link of peptidoglycan [de Jonge et al. 1993; Maidhof et al. 1991]. *femB* mutants produced cross links with only three glycines. *femA* mutants do not incorporate the second and third glycines into the bridge. Disruption of these genes reduces the level of resistance to nearly susceptible levels. Production of PBP 2a and other PBPs is unaffected. A study in which the entire *femAB* operon was eliminated by allelic replacement resulted in mutants which produced peptidoglycan with muropeptide subunits carrying only monoglycyl substituents. They were fully viable but showed morphological abnormalities, decreased growth rate and radically reduced methicillin resistance [Standen et al 1997]. Although homologous recombination is considered to play a role in the integration of *mec* region in some strains the studies also indicate that the *mec* region can behave as a site specific mobile genetic element. The size and features of the *mec* region suggests that it could be considered as a resistance island [Hacker et al. 1997]. A complete characterization of MRSA requires not only identification of the genetic background of the bacteria but also identification of the structural types of Staphylococcal Cassette Chromosome *mec* element (SCC*mec*), which carries methicillin resistance determinant *mecA*.

In 1998, Maiden et al. proposed multilocus sequence typing (MLST); through which alleles of several housekeeping genes are directly assessed by nucleotide sequencing, each unique allele combination determining a sequence type of a strain. The advantages of this approach was that the culturing of pathogenic microorganisms is avoided, as their gene fragments are amplified directly from biological samples, and that the sequencing data are unambiguous,
easy to standardize, and electronically portable. MLST is a highly discriminatory method of characterizing bacterial isolates on the basis of the sequences of internal fragments of seven housekeeping genes. For each gene fragment, the different sequences are assigned as distinct alleles, and each isolate is defined by the alleles at each of the seven housekeeping loci. It was first developed by using *Neisseria meningitidis* as the model species (Enright and Spratt, 1999). The MLST approach is too labor-intensive, time-consuming, and costly to use in a clinical setting. More than 2,500 bp must be compared for each isolate. In addition, for certain recent subpopulations, such as MRSA, genetic variability in the housekeeping targets will likely be limited and discrimination will be restricted [Maiden et al. 1998].

However, a single-locus target, if discriminating, provides an inexpensive, rapid, objective, and portable genotyping method to subspeciate bacteria. Using a single target depends on finding a region for sequencing that is sufficiently polymorphic to provide useful strain resolution. Loci with short sequence repeat (SSR) regions may have suitable variability for discriminating outbreaks [Van Belkum et al. 1998]. Two *S. aureus* genes conserved within the species, protein A (*spa*) and coagulase (*coa*), have variable SSR regions constructed from closely related 24- and 81-bp tandem repeat units, respectively. In both genes, the in-frame SSR units are degenerative, variable in number, and variable in the order in which repeat units are organized.

In 1999 a previously unrecognized penicillin binding protein (PBP) gene, *pbpF*, was also identified in *S. aureus* encoding a protein of 691 amino acid residues with an estimated molecular mass of 78 kDa,a molecular mass is very close to that of *S. aureus* PBP2 (81 kDa), and the protein was tentatively named PBP2B. The purified rPBP2B was shown to have penicillin binding activity [Komatsuzawa et al. 1999]. The protein PBP2a is encoded by *mecA* gene and the PBP2a enzyme restores cell wall biosynthesis in the presence of beta lactams. The *mecA* gene expression was found to be controlled by 2 regulatory proteins encoded by the upstream genes *mecR1* and *mecI* [Hiramatsu et al. 2001].

Recently, some new genotyping methods, such as the microarray technique, degenerate high-performance liquid chromatography, real-time PCR, TaqMan assay, and Invader assay, have been developed [Cooksey et al. 2000; Fang et al. 2003]. MLST was successfully adapted to *S. aureus* in 2000 (Enright et al.
MLST makes it possible to generate an expandable global database for each species at an Internet site, in order to use it for the purposes of genotyping pathogenic bacteria and other infectious agents [Platonov et al. 2000]. The earliest strain of MRSA in which SCCmec type IV has been identified was isolated in 1981 [Ito et al. 2001]. As in the work of Enright et al. & Crisostomo et al. [Crisostomo et al. 2001] identified probable recipient MSSA strains for early MRSA strains in another collection of isolates.