CHAPTER 2

REVIEW OF LITERATURE

2.1 MICROBIOLOGICAL STUDIES

2.1.1 Epidemiology

*Staphylococcus* was first observed in human pyogenic lesions by Von Reckling Hausen in 1871. Pasteur in the year 1880 obtained liquid cultures of the cocci from pus and he produced abscess by inoculating them into rabbits. Scottish surgeon Alexander Ogston established conclusively the causative role of the coccus in abscesses and other supralative lesions. The author also named it *Staphylococcus* due to its typical occurrence of the cocci in grape like clusters in pus.

*S. aureus* is the most prominent species of this genus in food microbiology because it can produce enterotoxin in humans and animals. *S. aureus* is also known to be the common cause of nosocomial community-acquired infections and surgical wound infections.

The cause of skin infections in United States has been due to *Staphylococcus* sp. Most of these infections are minor (such as pimples and boils) and many can be treated without antibiotics. However *Staphylococcus* can also cause serious infections such as surgical wound infections and pneumonia. In the past, most serious *S. aureus* infections were treated with antibiotics but during the years, the organism have become resistant to various drugs including penicillin and its related antibiotics. These resistant bacteria are called Methicillin resistant *S. aureus* or MRSA (CDC).
MRSA infection usually develop in hospitalized patients who are already very sick or who have an open wound (such as a bedsore) or a tube going into their body such as a urinary catheter or intravenous catheter (IV). MRSA infections acquired in hospitals and health care settings could be severe. In addition, certain factors can put some patients at higher risk for MRSA including prolonged hospital stay, receiving broad spectrum antibiotics, being hospitalized in an intensive care for burns, spending time close to other patients with MRSA or having recent surgery of carrying MRSA in the nose without developing illness.

Cases of MRSA diseases in the community have been associated with recent antibiotic use, sharing contaminated items, having active skin diseases, and living in crowded settings. Clusters of skin infections caused by MRSA have been described among injecting drug users aboriginals in Canada, New Zealand, Australia, Native Americans in the United States, incarcerated persons, players of close contact sports and other populations. Community associated MRSA infections are typically skin infections, but also cause severe illness as in the cases of four children who died from community associated MRSA (Sabat et al., 2000).

There was an initial success with penicillin in treating Staphylococcus aureus infections. But resistance to this drug began to emerge, now 70 to 80% of Staphylococcus aureus isolates are resistant to penicillin (Atkinson et al., 1984). Methicillin and other semi synthetic penicillins were successful in treating penicillin-resistant S. aureus infections until the 1980’s, when methicillin-resistant Staphylococcus became endemic in many hospitals. (Panlio et al., 1992). Since the emergence of methicillin-resistant S. aureus, the glycopeptide vancomycin has been the only effective treatment for Staphylococcal infections. In May 1996, Japan documented for the first time clinical infections due to S. aureus that acquired vancomycin resistance. The emergence of such resistance could produce morbidity
and mortality similar to that caused by *S. aureus* infections in the era before antibiotics became available (Hiramatsu *et al.*, 1997).

In 1963, USA introduced methicillin in clinical practice against naturally occurring strains of *S. aureus* resistant to penicillin and other antibiotics. They had not encountered resistance to methicillin even after the course of extensive tests. However, Jevons (1961) described that three strains among a total of 5,440 isolates tested, were showed increased resistance to methicillin (Sutherland 1964).

Methicillin Resistant *Staphylococci* are cross resistance to cloxacillin, nafeillin and ampicillin. It has been shown that the methicillin resistant organisms are protected from lysis by increasing the osmotic pressure of the medium. Growth in the presence of Methicillin is almost equal to that on antibiotic free medium. When a high concentration of Sodium Chloride (5%) is used to increase osmotic pressure the morphology of the organisms remains abnormal.

The mechanism whereby certain *S. aureus* strains resist the antibacterial effects of methicillin (and other β-lactamase-resistant penicillins) remain unknown even almost 20 years after these drugs were first introduced. Methicillin resistance or intrinsic resistance as it is sometimes termed does not appear to be due to enzymatic destruction of the antibiotic. Dyke could find no evidence for production of “methicillinase” activity by 108 epidemiologically distinct methicillin resistant *S. aureus*. Also β-lactamase, a negative variant of MRSA retains their resistance to methicillin (Dyke 1969, Seligman *et al.*, 1966).

The two pairs of MS and MR strains showed little difference in their cell wall phosphate levels, thus showing that there is not a simple correlation exist between cell wall teichoic acid content and methicillin resistance. All cells of a
heterogeneous MR strain have identical electro kinetic properties (Wilkinson et al., 1978).

The resistance pattern of 6,723 *Staphylococcal* isolates in the United States were described by Flower, Watters and Levy (1963) those of 1249 pure cultures of *Staphylococci* obtained from various hospitals in Japan from 1957 to 1962 were meta-analysed by Mitsuhashi (1962). The greatest percentage of cross resistance found was TC.SM.PC.SA (23.8%) followed by PC.SA (18.5 %) PC.TC.SA (14%) And SM.PC.SA (7.7 %) (Kawaharda et al., 1985).

Mitsuhashi et al., (1963) reported that the resistance of *Staphylococcus* to macrolide antibiotics was jointly eliminated by treatment with acriflavin suggesting that MAC resistance is controlled by a single genetic element which exist extra chromosomally.

The contribution of pencillinase activity to the methicillin resistance of *Staphylococci* has been controversial since pencillinase can inactivate methicillin (Seligman et al., 1966).

Stewart and Holt (1963a) stated that their strains consist of uniformly resistant organism, which could destroy the isoxazolyl penicillins (i.e., oxacillin and cloxacillin) but could not inactivate methicillin.

Strains of *Staphylococci* with multiple drug resistance are widespread. They appear and spread rapidly after the introduction of new antimicrobial agents. The strains isolated in Uppasala were all resistant to several antibiotics, but showed some variations in susceptibility to erythromycin, chloromphenicol. Several methicillin resistant *staphylococci* showed similar characteristics (Dornbusch et al., 1969).
Staphylococcus aureus is the most frequent infectious agent in chronic mastitis of cattle. On occasion it may also cause acute infections. Chronic mastitis with clinical or sub clinical manifestation is a serious economic problem all over the world. In Switzerland, S. aureus were recovered from 61.5% of Mastitic milk samples derived from 104 cows on 22 different farms. Furthermore, the spread of S. aureus suggest an endemic infection in cattle (Baumgartner et al., 1984).

The prevalence of community acquired methicillin resistant S. aureus (MRSA) infections increased at the University of Chicago Children’s Hospital (UCCH) from 10 per 1,00,000 admissions from 1988 - 1990 to 259 per 1,00,000 admissions from 1993 - 1995. Because this increase may have represented a one time occurrence or a limited disease out break of previous observations at UCCH was updated in 1998 and 1999 to see whether this trend had continued.

Twenty-three hospitalized children had an MRSA, isolated during the one year study period. Ten were community-acquired, equally distributed between children with predisposing risk factors and those without. The overall prevalence of community acquired MRSA was 208 per 1,00,000 admissions. (Hussian et al., 2000)

MRSA causes illness in persons outside the hospitals and health care facilities as well. Cases of MRSA diseases in the community have been associated with recent antibiotic use showing contaminated items. Having active skin disease and living in crowded settings.

Recent case reports of vancomycin treatment failures in the United States, Japan and France have prompted a retrospective analysis of 42 cases of septicemia caused by epidemic methicillin resistant S. aureus 15 (EMRSA-15) which is the most prevalent epidemic strain of methicillin-resistant Staphylococcus aureus in the
United Kingdom, between 1994 and 1988. Mortality was lowest (4%) in patients with rifampicin – isolates treated with vancomycin and rifambin. It raised to 38% in patients but in whom the organism became resistant to rifambin during therapy, and it reacted 78% in patients who had rifambin resistant isolates or in whom rifambin was contraindicated (Burnie et al., 2000).

A five-fold increase of about 11% to 58% in the prevalence of methicillin – resistance was observed in 1994 – 95 amongst clinical isolates of *S. aureus* in the state clinical hospital No.2 in SZCZECIN, one of the largest hospitals in Pomeranian region of Poland. (Bilska et al., 2000).

To investigate the possibility of *in-vivo* development of decreased vancomycin susceptibility, the vancomycin susceptibilities of 12 methicillin resistant *S. aureus* (MRSA) isolates serially recovered from six patients with vancomycin therapy were tested by MIC determination method and population analysis. While all of the MRSA isolates were susceptible to vancomycin standard method, population analysis showed the upward shifts indicating decreased vancomycin susceptibility among serial isolates from two patients. These bacteria with decreased vancomycin susceptibility could be selected by using vancomycin selection of pre-therapy isolated under laboratory conditions (Sugino et al., 2000).

Intravenous vancomycin was approved in 1991 in Japan and has been widely used for treatment of infection caused by methicillin resistant *S. aureus* (MRSA). They investigated whether vancomycin resistance had emerged in MRSA isolated from their hospital since the approval of the use of the Intravenous vancomycin. The median minimum inhibitory concentration of the 69 MRSA strains isolated in 1988 and the 74 isolates in 1998 was 0.75 µg/ml respectively. However, all the strains were classified in the susceptible group. None of them were MRSA heterogeneously
resistant to vancomycin (Hetero VRSA), which has been defined as a strain having a 1/10 or greater heterogeneously resistant subpopulation to vancomycin. In another set of investigations, no hetero VRSA strains isolated after intravenous administration of vancomycin for 14 to 77 days (Mori et al., 2000).

Toronto western hospital, University of Toronto, Canada, evaluated the efficacy of an ointment containing bacitracin, polymyxin B and gramicidin for the eradication of colonization by methicillin resistance *S. aureus* in medical patients, 19 (91%) of who had previously failed 1 week course of topical muporicin. Muporicin resistance was documented in 5 (45%) of 11 patients successful decolonization was achieved in 9 (82%) of 11 patients, (Fung et al., 2000).

Queensland Health, Brisbane, Australia, analyzed a historical cohort of 504 bacteremic patients (316 MSSA and 188 MRSA), examined factors associated with mortality risk factor for the development of MRSA which includes male gender, admission due to trauma, immunosuppression, presence of a central vascular line, an indwelling urinary catheter, and a past history of MRSA infection. An over all mortality of 22% was significantly greater in the MRSA group (Selvey et al., 2000).

Methicillin resistant *S. aureus* nasal colonization was investigated in patients with elective cardiovascular surgery, renal patients admitted for arteriovenous graft surgery, and patients transferred from other hospitals. Renal failure patients were significantly more likely to colonized and represent a potential source of MRSA to their institution (Price et al., 2000).

In Universite Paris Creteil, France, a study on MRSA was carried out, which was divided into two periods. During period A screening samples were obtained on admission for 30% of patients and 25 MRSA carriers were identified. During period B 90.5% of admitted patients were screened, and 26 MRSA carriers were detected
on admission. Over all the rates of imported and acquired cases were similar between the two periods (Girou et al., 2000).

The geographical distribution of 65 clinical isolates of methicillin resistant *S. aureus* (MRSA) recovered from 7 hospitals in Thailand were investigated. The presence of *mec A* gene in MRSA was identified (Wong et al., 2000).

An open study was conducted in patients with serious systemic infections with methicillin resistant *S. aureus* (MRSA). Patients with MRSA were recruited, for whom the treatment with glycopeptides had failed. Of 11 patients with MRSA infection, four were cured, six were failed with treatment and one was intermediate (Shetty and Wilson 2000).

Nursing staff workload may influence hospital acquired *Staphylococcal* transmission. Closure of wards to new admissions is used in some institutions as a part of methicillin- resistant *S. aureus* (MRSA) outbreak control. They postulated that by reducing staff workload, allowing more time for good infection control practices, workload pressure might raise during outbreaks if wards are not transferred to specialist isolation facilities (Fauington et al., 2000).

A total of 20 patients with MRSA infection and 79 colonized patients (with 94 admissions) were identified. These represent a rate of 2.9 MRSA cases per 1,000 admissions. The mean number of additional hospital days attributable to MRSA infection was 14, with 11 admissions having at least 1 attributable day. The total attributable cost to treat MRSA infections was $2,87,200, on an average of $14,360 per patient. The cost for isolation and management of colonized patients were $128,095, or $1,363 per admission. Costs for MRSA screening in the hospital were $109,813. Assuming an infection rate of 10% to 20%. They determined the costs associated with MRSA in Canadian hospitals to be $ 42 million to $59 million
 annually. The cost will continue to rise if the incidence of MRSA increases further (Kim et al., 2001).

The effectiveness of hand-cleansing agents in removing a hospital strain of methicillin resistant *Staphylococcus aureus* from artificially contaminated hands of five volunteers were studied (Guilhermetti et al., 2001). From a clinic population 30,010 children had positive sputum cough swab cultures for MRSA. Prevalence of MRSA raised from 0 in 1992-94 to 7 in 1998 (Miall et al., 2001).

The principal mode of transmission for MRSA is by the transfer of the organism from a carrier or infected patient to uninfected patients by the hands or clothing of staff. From January 16, 1976 to April 2 1997, five patients who had undergone open–heart surgery in a hospital located in Northern Taiwan, developed surgical wound infections in mediastinitis caused by MRSA (Wang et al., 2001).

University of Geneva hospital, Geneva, reported MRSA has become highly endemic. High endemicity is associated with increased hospital acquired infection, increased use of glycopeptides and subsequent risk of emerging antibiotic resistance Gram – positive bacteria and additional health care costs (Rubinovitch and Pittet, 2001).

Their determined risk factors associated with persistent carriage of methicillin resistant *S. aureus* among 102 patients enrolled in a double blind placebo – controlled trial of nasally administered muporicin ointment. MRSA decolonization was unsuccessful in 77 (79 %) of 98 patients who met the criteria for evaluation (Harbarth et al., 2000).

It has been widely published by the media which has added to people’s alarm. The screening treatment and resultant isolation affects all aspects of the
patients care and will also influence the management of any wounds. MRSA has been isolated in both acute and chronic wounds and so the management of these must be included in any infection control policy. (Dunford 1997).

Oral carriage of MRSA may serve as a reservoir for re-colonization of other body sites or for cross infection to other patients or health care workers. At least two cases have been reported of cross infection from a general dental practitioner to patients. Nursing homes are another important source of colonization and infection and two cases of acute parities caused by MRSA in elderly patients have been described (Smith et al., 2001).

Methicillin resistant S. aureus (MRSA) has been prevalent in hospital University Kebangsaan Malaysia (HUKM), in the last three years, Seventy one MRSA strains were collected between January and March 2000 from the various wards in HUKM were tested for antibiotic resistance. Most of the strains were isolated from ICU, surgical and medical wards (Alfizah et al., 2002).

In recent years the demographics of the Pacific Northwest dairy industry have been undergoing considerable change. The number of herd is decreasing while herd size is increasing. The National Animal Health Monitoring Systems (NAHMS) reported in 1996 that nearly half of United States dairy operations added animals to their herd. The majority of those additions were as breed heifers and lactating cows. Importing cattle into a dairy herd presents a threat to herd biosecurity, and therefore S. aureus, the most prevented contagious mastitis pathogen has the potential to be carried into a herd by imported cattle with S. aureus intramammary infection (IMI) presents an obvious risk for mastitis in uninfected resident cattle, a less obvious risk is replacement heifer (Middleton et al., 2002).
2.1.2 Isolation And Identification

*Staphylococcus aureus* are spherical cells about 1µm in diameter arranged in irregular clusters. Young cocci cells are strongly gram-positive; on aging many cells become gram-negative. *Staphylococci* are non motile and do not form spores. (Javetz *et al.*, 1989)

It is of both aerobe as well as facultative anaerobe. Temperature range favorable for the growth is between 10°C – 42°C, optimum 35°C- 37°C. Growth occurs in ordinary nutrient media. A uniform turbidity forms in nutrient broth cultures. Colonies are circular disks, relatively large after 24 hrs growth with a diameter of 2-4 mm. They are opaque and convex with a shining surface and may be pigmented white, yellow, golden-yellow or golden. Confluent growth appears like oil-“paint”.

*Staphylococci* will grow in the presence of 10-15 percentage of Sodium Chloride. The salt may be incorporated in media, making them highly selective for *S. aureus*.

On blood agar the colonies are similar to those on agar but somewhat larger. Marked zones of haemolysis appear on sheep blood or rabbit blood agar (Cruickshank 1972).

Nutrient agar containing one percentage of glycerol monoacetate has been used to enhance pigmentation and allow differentiation of *S. aureus* into groups showing different kinds of pigmentation. This can be of epidemiological value as strains showing multiple antibiotic resistance and belonging to certain well known phage-types usually produce yellow colonies, whilst strains of miscellaneous phage-types produce orange and buff colonies (Williams & Turner, 1963).
Another useful indicator medium containing low concentration of mercuric chloride (1 in 27,500) in peptone agar base. Strains resistant to this concentration tend to be resistant to antibiotics such as penicillin and the tetracycline, and include the well known “hospital” strains of phage group I and III (More, 1960).

Colonies of *Staphylococci* when growing on media containing a number of biological fluids such as milk or egg yolk may be surrounded by zones of opacity or clearing. Many coagulase-positive *Staphylococci* when grown in glucose egg yolk broth give a dense opacity due to lipolytic activity. Coagulase-negative strains do not give this reaction (Gillespie and Alter, 1952).

*Staphylococcus aureus* readily grows on ordinary media like nutrient agar at a temperature of 37°C and pH 7.4 to 7.6 in which it produces circular, convex smooth shiny opaque colonies. On Nutrient agar slope it produce oil paint appearance. On MacConkey agar medium, they produce small pink colonies due to its lactose fermentation.

It ferments number of sugars like Glucose, Mannitol, Maltose, lactose etc. They are positive for both MRVP test and coagulase test. They also produce β-haemolysis on 7% human blood agar (Ajuwape *et al.*, 1977).

Eighty-one strains of *S. aureus* that appeared to be tetracycline resistant on the basis of a preliminary disc-diffusion test were examined for resistance to tetracycline and to the semi synthetic tetracycline, minocycline (Ashewhow 1974).

Novick (1963) proposed the possibility that PC resistance in *Staphylococcus* is associated with a plasmid and is they inherited extra chromosomally.
Cultures of methicillin resistant *Staphylococcus* may inactivate more methicillin than do cultures of methicillin sensitive *Staphylococci*.

The rabbit plasma, tube coagulase test has been recommended as the reference method for the identification of *Staphylococcus aureus*. Because of its simplicity this test has been the method of choice in human medical bacteriology for many years. Its popularity remained even after the introduction of the clumping factors test the plate culture DNase test, the heat stable DNase reaction and the protein A test. In food microbiology only the tube coagulase and the heat stable DNase are recommended for use.

With the exception of the negative heat-stable and plate culture DNase reactions of many avian *Staphylococcus aureus* strains, the specific characters of *Staphylococcus aureus* strains of animal origin are similar to those of strains associated with man. In epidemiological investigations a distinction can usually be made between strain of human and animal origin with the help of a series of other test described by Hajek & Marsalek (1971) and Witte et al., (1977).

The strain DU 4916 does resemble other methicillin resistant *Staphylococci* in many properties and these findings are certainly consistent with the hypothesis of a single clone origin for these strains. MR.S. *aureus* can produce α-β-δ-hemolysins (Lacey 1974).

Methicillin resistant *Staphylococci* are almost always pencillinase positive. In addition, they are usually multiple resistant to other antibiotics and fall into a relatively restricted range of phage types. Methicillin resistance confers a selective advantage not only against methicillin but also against penicillins (Cohen and Sweeney 1970).
The MRSA strains to produce $\alpha$-$\beta$- and $\delta$ hemolysins. An association existed between methicillin resistance and production of enterotoxin B, since 37 out of 42 methicillin-resistant strains were enterotoxin B producers when methicillin-resistant strains were enterotoxin B producers when four methicillin-resistant strains were treated with acriflavin, conversion to methicillin susceptibility occurred in 2.1 to 12.5% (Dornbusch et al., 1969).

Two distinct strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from patients in a dermatology ward were also resistant to mupirocin strains isolated in St.Johan’s Institute of Dermatology St, Thomas Hospital, London (Pawa et al., 2000).

Lorenz et al.,(2000) reported that immunodominant structures which were expressed in-vivo during sepsis caused by MRSA. Using human sera they compared the immune response of humans with MRSA sepsis with immune response normal individuals and asymptatically colonized individuals. They identified and characterized four *Staphylococcal* specific antigenic structures (Lorenz et al., 2000).

Strains showing a negative reaction in test tube for coagulase constitute 10 to 20% of all *Staphylococcus aureus* isolated from patient of hospital of Infant Jesus in Warsaw. Most of them are MRSA (Mlynarczyk et al., 2000). In addition to hetero VRSA were found in that hospital.

Two hundred and three isolates if *S. aureus* were collected from 140 patients (Sadoyama et al., 2000). A Swedish tourist was admitted to Cuban hospital due to epileptic seizures caused by brain tumors. Upon return to Sweden and admission to our hospital, methicillin-resistant *Staphylococcus aureus* (MRSA) was isolated and later consider to be free of MRSA but then developed a brain abscess from which MRSA was isolated (Ahlm et al., 2000).
The methicillin resistant *Staphylococcus aureus* strains (MRSA) isolated from clinical samples in various hospitals during 8 years, with particular consideration of Gdansk area. The study was carried out on 225 strains of MRSA from which 95 were isolated in years 1990 to 1995 and 130 in years in 1997 to 1998. The drug susceptibility was determined by the disc diffusion method (Wisinieswska et al., 2000).

Until recently, therapeutic options were limited to vancomycin making the use of this drug widespread. Unfortunately, the continued application of this drug has led to the emergence of glycopeptides intermediate *Staphylococcus aureus* (GISA). GISA strains have demonstrated thickened or aggregated cell walls, an penicillin binding proteins and greater autolytic activity (Rybak and Akins 2001).

It is gram-positive *cocc* MR *Staphylococcus aureus* is positive for MRVP and coagulase test [both tube and slide test]. It can produce β-hemolysins on 7% human blood agar and it produces golden yellow co lour pigment on Mannitol Salt Agar with 6μg/ml of oxacillin. (NCCLS). MRSA are easily grown on 5 % NaCl containing medium. And frequently isolated from burns, wound, surgical wound, urine, and blood and Tracheal samples.

The Kirby – Bayer, disk agar diffusion test the most widely used anti microbial susceptibility testing systems employed in American hospitals. This technique was developed at the University of Washington in the early 1950s and in 1959 Bayer et al., reported the usage of this method for testing *Staphylococcus aureus*. These early evolutions demonstrated that the system was well suited for performing susceptibility tests on *Staphylococcus aureus* isolates. Methicillin was not included in these early studies because the drug did not become available until above 1960.
Between 1960 and 1966 when the Kirby-Bauer was undergoing further evaluation, methicillin resistant *S. aureus* were discovered in Britain and became an important nosocomial infection. A description of the standardized disk diffusion test was published in 1966 (Boyce, 1984).

Additional studies revealed that the resistance could be detected more easily if one or more of the following procedures were used, incubation of plates at 30°C for 18hrs or at 37°C for 48 hrs, use of a heavy inoculums, or use of agar containing 5 % NaCl (Annear 1968, Hewitt *et al.*, 1969).

In the early 1970’s two groups of investigators studied the ability to the standardized Kirby-Bauer method to detect MRSA strains (Drew *et al.*, 1972) (Thornsbery *et al.*, 1973). Those groups determined that incubation of plates at 35°C for 18 hrs or over night resulted in adequate detection of MRSA strains available at that time.

Varaldo *et al.*, (1984) decided to test the effect on the in vitro susceptibility to β-lactam antibiotics mostly used in diagnostic laboratories to enhance the expression of methcillin resistance in the presence of 5 % NaCl in the test medium.

The National committee for Clinical Laboratory Standards (NCCLS) recommended three manual methods for MRSA detection. They include screening on an agar plate containing oxacillin (6µg/ml) disk diffusion and tube macro micro dilution.

Rohani *et al.*, (2000) *S. aureus* were tested against 13 different antibiotics by disc diffusion method as recommended by the NCCLS.
2.1.3 Pathological Studies

*Staphylococcus aureus* produces a wide variety of pathogenic factors that contribute to its ability to colonize and cause infection. Hiramatsu *et al.*, 2001 classified the factors into four major categories, namely adhesions, exoenzymes, exotoxins, and others. They identified almost all known *S. aureus* pathogenic factors within the strains N315 and Mu 50 genomes as well as 70 new candidates (factors).

Adhesins

Advance *S. aureus* cells to host molecules are a prerequisite for tissue colonization and overt disease. In addition to the wall bound MSCRAMM protein super family conferring adhesin, cellular invasion and virulence other adhesive *Staphylococcus aureus* proteins and cell secreted and in part rebinding to bacterial surface structures via mechanisms not yet defined (Hussain *et al.*, 2001).

*S. aureus* express various surface proteins, some of which are adhesions that act as specific receptors for the extra cellular matrix proteins of the host tissue. Production of collagen binding protein is strongly associated with pathogenesis of osteomyelitis and septic arthritis, and sialo proteins binding proteins is also associated with initiation of bone infection, (Hiramatsu *et al.*, 2001). Coagulase, extra cellular fibrinogen (E8b) binding protein, Extra cellular adherence protein (Eap) have been bound to fibrinogen (fg), (Hussain *et al.*, 2001). Extra cellular matrix binding protein Emb initiate subacute bacterial endocarditis by mediating bacterial attachment to the extra cellular matrix of he damaged heart valve. The new proteins EbhA, EbhB would be investigation as possible causative agents for *S. aureus* endocarditis.
**Exoenzymes**

Many exoenzymes secreted by *S. aureus* degrade and digest organic compounds and macromolecules of human tissue.

*Staphylococci* are the most important cause of prosthetic joint infections. *S. aureus* is a significant pathogen in acute arthritis, affecting both native and prosthetic joints. Recent study investigated oral carriage of *Staphylococci* in patients with rheumatoid arthritis, and showed that a significantly light proportion (56%) of these patients carried oral *Staphylococcus* than control (Levy et al., 1990).

*S. aureus* resistance with different regiments of pre operative prophylaxis wound isolates was obtained from 15 Hospitals in 14 cities across the United States (Kernodle, et al., 1998).

In recent reviews teichoic acid and protein A are described as the most important surface structures which interfere with the defense mechanism of the host. It is stated that a few strains are encapsulated, but encapsulation may be more common among freshly isolated clinical *S. aureus*. Teichoic acid mediates the adherence of *S. aureus* to the nasal mucosal cells, but the possibility that capsules may interfere with the contact between teichoic acid and the mucosal lining was not mentioned. (Sompolinsky, et al., 1985).

The ecological distribution of the *Staphylococci* in human, the cut skin surface hair follicles, sweat glands, lymph nodes, and lymph channels are all potential sources of microbial patients have been shown to acquire *Staphylococcal* skin population that exhibit multiple anti microbial resistance. A change in the microbial ecology of the skin, which includes the acquisition of organisms expressing multiple resistance or enhanced virulence amplifies the risk of infection.
to patients receiving prosthetic implant (Levy et al., 1990). Historically graft infections most often present as acute episodes involving pyogenic microorganisms such as *E. coli* or *S. aureus* (Levy, et al., 1990).

Between late 1985 and early 1991, a total of 273 isolates of *Staphylococcus aureus* associated with deep surgical wound infections, were identified (Kernodle, et al., 1998).

Staphylococcal exo proteins such as coagulase, enterotoxin B, and nuclease were also produced optimally in limited conditions.

The vagina is basically an anaerobic environment. The insertion of a tampon was reputed to change temporarily the vaginal micro-environment from anaerobic to aerobic. The subsequent decrease in oxygen tension may create a suitable microenvironment for TSST – 1 synthesis (Sarafian and Morse, 1987).

*S. aureus* is the most frequent infections agent in chronic mastitis of cattle on occasion it may cause acute infection. Chronic mastitis with clinical or sub clinical manifestation is a serious economic problem all over the world. In Switzerland *Staphylococcus aureus* were recovered from 61.5 % of mastitis milk samples derived from 104 cows on 22 different farms. Furthermore, the spread of *Staphylococcus aureus* in some farms suggests, an endemic infection (Baumgartner, et al., 1984).

In rabbits *S. aureus*, many manifest as fatal septicemia. However if the rabbits survive, there may be clinical presentations of pneumonia septicemia (pod dermatitis) with a high incidence of mastitis and in some cases, mastitis and abortion may occur. Many litters exhibited abscess at two to three weeks of the age. The spread *S. aureus* from rabbits to the animal technician has also been reported,
suggesting that close association enhances spread of *Staphylococcal* strains among livestock and attendants through contact or aerosol (Ajuwape and Aregbesola, 1997).

The *Staphylococcal* enterotoxins are a series of biologically active proteins known to be responsible for *Staphylococcal* food poisoning. Evidence has accumulated to indicate that the enterotoxins may play a role in some cases of toxic shock syndrome (T.S.S). Furthermore, animal studies have showed that intravenous infection of enterotoxins A and B into rhesus monkeys can produce many of the signs and symptoms associated with TSS.

*S. aureus* isolated from 55 patients, who had either confirmed or probable non-menstrual TSS and found that eight of the isolates did not produce toxic shock syndrome toxin-1 (TSST-1), but these strains produced *Staphylococcal* enterotoxins B. *S. aureus* strains may produce several exoenzymes and toxins with a considerable range of biological activities; including *Staphylococci* scalded skin syndrome and T.S.S have been related to toxin production.

The incidence of enterotoxin producing among 196 *Staphylococcus aureus* strains isolated from blood cultures was examined. The isolates from the patients with haemotogenous osteomyelitis of arthritis produces enterotoxins less frequently than these from the other patients. (Roder, *et al.*, 1995).

Risk factors for the development of MRSA include male gender, admission due to trauma, Immuno suppression, presence of a central vascular line, Immuno suppression, presence of a central vascular line or an indwelling urinary catheter, and a past history of MRSA Infection. Overall mortality was 22%. Death due to bacteraemia was significantly greater in the MRSA Group (Selvey, *et al.*, 2000).
Patel et al., (2000) reported that Ninety patients with significant underlying medical illness were treated at 63 centers in five countries. The most common indication was bone and joint infection (44.44%) and skin structure infection (16.4 %). The most common non venous adverse events related to study medication were anthralgias (10.8%) myalgias (8.6%) and nausea (8.6 %).

MRSA in children can be community acquired and can cause otitis externa, otitis media with otorrhea or acute mastoiditis intravenous therapy that includes vancomycin is necessary for resolution (Santos, et al., 2000).

A Swedish tourist was admitted to a Cuban hospital due to epileptic seizures caused by brain tumors. Upon return to Sweden an admission to their hospital, methicillin-resistant S. aureus. He was later considered to be free of MRSA but then developed a brain of abscess from which MRSA was isolated (Ahlm, et al., 2000).

MRSA is a bacterial pathogen of clinical importance because, (i) a single strain may be transmitted hospital via nurse-patient-physician contact (ii) it may cause life threatening diseases such as pneumonia and endocarditis (iii) S.aureus is the most frequently isolated organism found in post operative wound infections . (Welch and Southern, 1984).

Staphylococcus aureus is also the leading cause of intramammary infection in ruminants which is the most economically important disease to the dairy industry in the United States, with approximately one half of dairy cows afflicted with some form of mastitis. This disease accounts for approximately 70 % f the total expenses for dairy farmers and results in the loss of billions of dollars each year (Bayles, et al., 1998).
Peacock et al., (2002) stated that the development of multilocus sequence typing (MLST) represented a major advance in relating the organism on the basis of nucleotide sequence of fragments of concerned housekeeping genes. MLST of *Staphylococcus aureus* was described by et al., in methicillin resistant strains. They compared MLST with pulsed filed gel electrophoresis. There were no differences between PFGE and MLST among the isolates tested. They suggested that these two methods had similar discriminatory abilities.

Thankluong et al., (2002) studied that the regulation of *S.aureus* capsular polysaccharide expression by *agr* and *sar A*. They analyzed CPS production Caps Specific mRNA synthesis and blaze reporter gene activities of the transcriptional and translational fusions in strain beck and its *agr* and *sar A* isogenic mutants during different phases of bacterial growth. In the wild type strain, caps mRNA was undetectable until the mid-logarithmic phase of growth, where as CPS production was undetectable until 2 hours later, at the onset of stationary phase. By comparing CPS production and Caps transcription, they observed that serA affected CP-5 production both transcriptionally and post translationally. They showed that agr was a major activator for cap gene expression not only in type 8 strains Becker but also in strains representing the four-agr groups.

Sara et al., (2001) reported that *agr* expression precedes escape of internalized *S.aurerus* from the host endosome, presented a new reporter gene method to assess intracellular growth of *S.aureus* in MAC-T cells that utilizes a gfp-lux ABCDE reporter operon under the control of *Bacillus megaterium* xyl A promoter, which in *S.aureus*. This facilitates assessment of the growth of internalized bacteria in a non-destructive assay. The dual gfp-lux reporter cassette was also evaluated as a reporter of agr expression and used to monitor the temporal induction of agr during the MAC-T-internalization process. The data obtained
suggest that agr induction occurs prior to endosomal lysis and that agr-regulated exoproteins appear to be required for the release and replication of \textit{S.aureus} within the infected MAC-T cells.

Judithneneth and Lee (1995) in their study antibodies to capsular polysaccharide are not protective against experimental \textit{S.aureus} endocarditis. Capsular Ab’s were induced either by active immunization with killed \textit{S.aureus} or by passive immunization with hyper immune rabbit antiserum to \textit{S.aureus}. Control rats were injected with phosphate-buffered saline Animals with indwelling catheters were challenged intravenously with $5 \times 10^4 - 4 \times 10^6$ CFU of the homologous \textit{S.aureus} strain. Both immunized and control rats developed \textit{S.aureus} endocarditis. They determined whether the presence of an indwelling catheter interfered with antibody –mediated protection against \textit{S.aureus} endocarditis, catheters were removed 2 hours after insertion in additional group rats. Inoculums of $10^8$ CFU of strain. Reynolds was needed to provoke endocarditis in rats characterized for 2 hour, compared with $5 \times 10^5$ CFU for rats with in dwelling catheters passively transferred capsular antibodies were not protective since both immunized and non immunized animals developed endocarditis animals developed endocarditis. Their findings of study indicate that antibodies to the capsular polysaccharide are not protective in the rat model of experimental \textit{S.aureus} endocarditis.

Walter et al., (1998) in their work on capsular antibodies induce Type-specific phagocytosis of capsulated \textit{S.aureus} by Human polymorph nuclear leukocytes, capsular types 5 and 8, which account for about 70% of \textit{S.aureus} strains isolated from the blood of patients, resisted in vitro phagocytosis by human polymorpho nuclear leukocytes (PMN).
Antisera and monoclonal antibody to type 5 and 8 capsular polysaccharides induced type-specific *in vitro* phagocytosis of capsulated organism by PMN. Antibodies directed against the O-acetyl moiety of the type 8 cps were more effective in inducing phagocytosis of type 8 organisms by PMN. Either type-specific antiserum or monoclonal Ab reactive with native o-acetylated type 8 cps was most effective in inducing in vitro phagocytosis of type 8 organism by PMN. Their results provide further evidence that cps of *S.aureus* are associated with host immunity to this organism.

Lee *et al.*, (1999) during their studied the effects of *in-vitro* and *in vivo* growth conditions on expression of type 8 capsular polysaccharide by *S.aureus*. Study was performed to identify growth conditions that would optimize the production of CP8 and to determine to identify growth conditions that would optimize the production of CP8 and to determine whether enhanced CP8 expression would influence *Staphylococcal* virulence. Their results indicate that staphylococci grown on surface, both *in vivo, in vitro* produce larger quantities of cell associated CP8 than those grown in liquid cultures. No differences were observed in 50% lethal dose for mice of stain Becker grown on solid medium or in liquid medium.

Marisa *et al.*, (2002) their studied induction of cell-mediated immunity to *S.aureus* in the mouse mammary gland by local immunization with a live attenuated mutant. Their aimed at evaluating whether Ima immunization with la-*S.aureus* can induce cell-mediated immune responses to the pathogen with in the mammary gland. Their result demonstrated that Ima immunization with la- *s aureus* induced primed lymphocyte populations legible of responding against staphylococcal antigens during *in vitro* stimulation, as well as during in vivo infection by *S.aureus*. CD4⁺ and CD8⁺ T-cells appear to be the main lymphocyte subpopulations involved in this
response. It is suggested that IFN-γ production induced by Ima immunization play a vital role in eradication of intracellular *Staphylococci*.

Kevin *et al.*, (1999) studied in the development and characterization of a *S.aureus* nasal colonization model in mice. They inoculated mice intra nasally with *S.aureus* Reynolds the majority of mice inoculated with $10^8$ CFU of *S.aureus* maintained nasal carriage for at least 20 days. Nasal colonization rates were similar for inbred and out bred mice colonization was not affected by mouse passage of strain Reynolds.

Lower inoculums doses resulted in reduced colonization after 7 days. However, mice given streptomycin in their drinking water developed long-term carriage of *S.aureus* and they were colonized with inocula as low as $10^5$ CFU, *S.aureus* recovered from the rare of experimentally colonized mice expressed high levels of capsule, and the ability of a capsule-defective mutant to persist in the nares was reduced in comparison to that of the parent strain.

Intracellular *S.aureus* escapes the endosome and induces apoptosis in epithelial cells was shown by Kenneth *et al.*, in their study. Invasion of an established bovine mammary epithelial cell line (MAC-T) by a *S.aureus* their result suggest that after internalization *S.aureus* escapes the endosome and induces apoptosis in non professional phagocytes.

Nancy *et al.*, (1984) studied that the monoclonal antibodies to *Staphylococcal* enterotoxins B and O. cross-reactivity and localization of epitopes on tryptic fragments. They made murine monoclonal ab’s to react with staphylococcal enterotoxins B and C, of nine Ab’s only 3 reacted C SEB and 2 reacted with SEB by SEC1. 3 reacted with all subtypes of SEC, and one reacted only with SEC2 and CEC 3. All of the Ab’s reacted protein blotted on to
nitrocellulose from electrophoresis gels which corresponded to the enter toxin band with the immunoblot technique four of the five antibodies reactive with SEB reacted with the tryptic fragment of mol.wt 1-17,000 and the five Ab’s reactive with the tryptic fragment of mol.wt: - 1-14,000.

William et al., (1998) carried out a work the identification and characterization of the Put P proline permease that contributes to In vivo survival of S.aureus in animal molecule. A signature tagged mutagenesis (STM) system was adapted for studying the genes required for in vivo survival for S.aureus An STM library was created. Pools of mutants were screened for growth attenuation after in vivo passage. One of the mutant that was identified displayed marked attenuation following large-pool screening in all there animal models, sequence analysis of the entire open reading frame showed a 99% identify to the high –affinity proline permease gene characterized in another strain of S.aureus. In wound and murine abscess infection models, the put P mutant was approximately 10-fold more attenuated than was wild-type strain. The reduced viability of the bacteria possessing the mutation in the S.aureus high-affinity proline permease suggest that proline scavenging by the bacteria in important for in vivo growth and proliferation and that analogs of proline may serve as a potential anti staphylococcal therapeutic agents.

Monajthakker et al., (1998) during their study in S.aureus Serotype 5 capsular polysaccharide is antiphagocytic and enhances Bacterial virulence in a murine bacteremia model. They compared the mouse virulence of S.aureus Reynolds and capsule –defective mutant strains cultivated under conditions of high/low capsule expression. Their studies indicate the optimal expression of capsule enhances bacterial virulence in the mouse model of bacteremia, probably by rendering the organism resistant to opsonal phagocytic killing by leukocytes.
Emily et al., (2001) compared the *Staphylococcus aureus* and *Salmonella enterica* serovar Dublin induce tumor Necrosis factor- related Apoptosis- inducing ligand expression by normal mouse and human osteoblasts. They examined the ability of *S.aureus* and *Salmonella* serovar Dublin to induce the production of tumor necrosis factor-related apoptosis-inducing ligand by normal osteoblasts suggest a mechanism where by bacterial pathogens mediate bone destruction via osteoblast apoptosis.

Sordelli et al., (2000) reported a capsule expression on by Bovine isolates of *S.aureus* from Argentina. Genetic & Epidemiological analyses. They serotyped 195 *S.aureus* strains isolated between 1989-1997 from the milk of mastitis cows in Argentina. Only 77% were serotypes and all recovered between 1989 and 1992. The remaining 168 isolates were non reactive with CP 5 or CP8 specific Ab’s. PFGE performed with 127 of the 195 *S.aureus* isolates and revealed that most 86% strains belonged to one of form major PFGE groups. Results of their study underscore the variability in capsule expression by *S.aureus* strains isolated from different geographical regions and cast doubt on the rotes of CP5 by CP8 in the pathogenesis and immune prophylaxis is of bovine mastitis in Argentina.

Shuter et al., (1996) studied on *S.aureus* binding to human nasal mucin”. They characterize S.aureus adhesin to nasal mucosa invitro and investigate the interaction of *S.aureus* C human nasal mucin. They suggest that *S.aureus* binding to mucin may be critical for colonization of nasopharyngeal mucosa.

Peglar et al., (1988) worked an adherence of *S.aureus* to endothelial cells. Influence of capsular polysaccharide, Global regulator age and bacterial growth phase. They examined the influence of type 5 capsular polysaccharide CP5 production, the global regulator agr, and the bacterial growth phase on *s.aureus*
adherence to EC. Where as S.aureus Newman. Showed maximal adherence to EC in the logarithmic phase of growth, an isogenic agr mutant showed maximal adherence in the stationary growth phase. S.aureus adherence to EC by CP5 expression was negatively correlated a mutation in the agr rocks diminished CP5 production and led to increased adherence. Likewise, induction of CP5 expression by addition of NaCl to the growth medium resulted in reduced S.aureus. Adherence to EC. S.aureus Newman cells that adhered to EC did not express CP5. A Newman Cap50 mutant was a capsular and showed significantly greater adherence to EC than the parental strain did these data are consistent with the post exponential and agr independent expression by S.aureus of at least one putative EC adhesion, whose binding domain may be marked by CP5.

Martaportoles et al., (2001) studied in S.aureus cap50 has UDP-ManNAc. Dehydrogenase activity and is essential for capsule expression. They demonstrated that Cap5P, a 2-epimerase, catalyzes the conversion of UDP-N-acetyl glucosamine to UDP-N-acetyl glucosamine. They showed that UDP-Man Nacdehydrogenase encoded by S.aureus cap50. They expressed cap50 in E.Coli and purified the recontrinant protein. The UDP-ManNac dehydrogenase activity of purified cap50 was assessed by incubating cap58 and UDP-olc Nac together with Cap50 NAD⁺ and reducing agent. Enzymatic activity measured by the increase in absorbance at 340 nm resulting from NADH formation A cap50 mutation created by rendering its CP5 negative. Results indicate that S.aureus cap50 is essential for capsular production and that capsule promotes the S.aureus virulence in mouse models of abusers formation.

Malcolm et al., 2001 conducted a study on Per R controls oxidative stress resistance and Iron storage and is required for virulence in S.aureus. S.aureus encodes three ferric uptake regulators (Fur) homologous: Fur, Per R, Zur. To
determine the exact role of PerR, they inactivated the gene by allelic replacement using a Kanamycin cassette, creating strain M7H001. PerR was found to control transcription of the genes encoding the oxidative stress resistance proteins catalase, alkyl hydroperoxide reductase, bacterio ferritin co migratory protein and thioredoxin reductase. Furthermore PerR regulates transcription of the genes encoding the iron storage proteins ferritin and the ferritin-lir DPS homologue, MogA. Transcription of PerR was auto regulated, and PerR repressed transcription of the iron homeostasis regulator. Fur which is a positive regulator, of catalase expression, PerR functions as a manganese- dependent transcriptional repressor of the identified regulator. The Per-R regulated Kat A gene encodes the sole catalase of S.aureus, which is an important starvation survival, determinant but is surprisingly not is surprisingly not required for pathogenicity in a murine skin abscess model of infection. In contrast, PerR is not necessary for starvation survival but is required for full virulence in this model of infection. Per R of S.aureus may act as a redox sentinel proteins during infection, analogous to the in vitro activities of oxy R and Per R of E.coli and B.subtilis, respectively. However it differs in its response to the metal balance within the cell and has the added capability of regulating iron uptake and storage.

2.2 BIOTECHNOLOGICAL STUDIES

2.2.1 Protein Analysis

2.2.1.1 SDS - Page-immunoblotting

Julie et al. (2002) studied the conservation, surface exposure and in vivo expression of the Frp family of Iron-regulated cell wall proteins in S.aureus. SDS-PAGE identified 2 conserved, immunogenic S.aureus cell wall proteins, of 40 and 87KDa, expressed under iron-restricted growth conditions. In vitro and in vitro N-terminal sequencing and subsequent genome analysis showed that these proteins
are encoded by adjacent monocistronic open reading frames designated frp A and frpB, respectively. Studies with and S. aureus for mutant confirmed that expression of these proteins in different iron-restricted in vitro. FrpA and Frp B share shone amino acid sequence homology with each other and with a putative S. aureus membrane protein FrpC. Antisense mutagensis and bioassays showed that Frp A and FrpB are not required for growth of S. aureus under iron-restricted conditions in vitro and do not appear to be involved in the transport of iron from siderophores or in binding of hemin. Further analysis suggested that FrpA may be involved in adhesion of S. aureus to plastic in vitro-binding of S. aureus to micro titer wells was found to be iron regulated and iron-restricted S. aureus containing antisense frpA or frpAB but not frpB constructs showed reduced binding compared to vector construct controls.

Stahlberg et al. (1987) during their study in immuno blot analysis of human. IgM, IgG and IgA responses to plasmid- encoded antigen of Y. enterocolitican serovar 03. The Ab’s observed were directed against several plasmid – encoded polypeptides. Immunoblotting of sera from patients of Yersinia triggered arthritis did not reveal any Ag’s that were involved additional. However IgA mediated recognition of certain antigens of mol.wts 26, 34, and 52.5 ended to persist longer in the arthritic patients.

Jones et al. (1986) in their studied of antibody to the gastric Campylobacter-like organism-clinical correlations and distribution in the normal population. Antibodies to GCLO detected in sera by CFT and ELISA patients with endoscopically visible gastritis had a higher frequency of Ab. In a normal population, Ab was uncommon in individuals below 20 years old, but the prevalence of Ab was increased with age there was little evidence to support an important pathological role for GCLO in disorders of the upper gastro intestinal tract, although
the possibility that it may a C0-factor in the pathogenesis of gastric ulcer cannot be excluded.

Gaston et al. (1988) during evaluation of electrophoretic methods for typing methicillin-resistant *S.aureus* strains plasmid profiles, whole-cell protein profiles and immunoblotting profiles were evaluated and compared with phage typing and it was concluded that both WCPP and IP provide valuable epidemiological data on MRSA and IP was the easiest of three methods to interpret.

Clink and Pennington (1987) studied about *Staphylococcus* whole cell polypeptide evaluation as a taxonomic and typing tool analysis. WCP obtained by SDS-PAGE complete concordance of results from both techniques was achieved. Visual analysis of the polypeptide patterns and comparison by use of co-efficient of Dice showed minor differences in band patterns between same sp strains. Their results suggest that although SDS-PAGE can be used to identify *S. aureus sp*, this type of analysis will not readily provide the basis for a typing method.

Narendranathan et al. (1988) studied in antigens involved in resistance to mucosal association by *Vibrio cholerae*. Resistance to growth of *V.cholerae* at the mucous of blind intestinal loops developed in rats after intestinal exposure to live organisms serological studies suggested that neither serum vibriocidal activity nor intestinal mucus Ab’s are likely provide or direct test of antibacterial immune status. This immunity has not been attributed to LPS antigen and goes not appear to involve flagella-associated antigens separation of monomeric sub units of outer membrane proteins by hydrophoretic interaction high-pressure liquid chromatography revealed. Significant quantitative differences and may be sufficient to explain the better protection observed when homologous serotypes were used for immunization and challenge in the long-term resistance model.
A series of 133 isolates of MRSA was finger printed by immunoblot technique by Woel Lee and J.P Burnie, 1988 their typing method confirmed the existence of an epidemic strain that accounted for 102 of the isolates. Remaining 31 isolated were grouped in to a further seven types which correlated with results of phage typing and anti biograms.

Kathryn et al. (1988) in their studied and reported that antibody response to outer-membrane antigens of *P.aeruginosa* in human burn wound infection. OMP’s were investigated and compared C those of cells cultivated in the laboratory. Their result indicated that the responses observed were directed against OMP’s and not against contaminating LPS.

Kawahrdia et al. (1985) described a method for typing *S.aureus* capsular polysaccharide that is based on direct bacterial cell agglutination and immunoprecipitation of cell extracts with mono specific antisera encapsulated strains were identified by their in agglutinability with teichoic acid antisera the typing sera reacted specifically with extract of eight proto type stains.

Fey et al. (1984) compared form versions of ELISA for their suitability for detecting *Staphylococcal* enterotoxins. The sand, which with labeled antibody, proved to be the best.

Shiley et al. (1990) during their study explained bacterium detected by lysis direct plating in a neonatal intensive care unit. The density of bacteraemia was determined in 787 neonatal blood specimens by using the 1.5 ml isolation for microbial tube. Coagulase-negative *Staphylococci* were the organism isolated most frequently from both true –positive cultures and contaminated cultures. Based on the first positive culture in an episode of sepsis, there were no cases of coagulase-negative *Staphylococcal* sepsis associated with the majority of the episode of sepsis.
The distribution of pathogens causing sepsis in this neonatal population was similar to the distribution of microorganisms associated with cannula-related sepsis in other hospitalized patients.

Krikler et al. (1986) reported about typing of strains of *S. aureus* by western blot analysis of culture supernates. Extra cellular proteins produced by *S. aureus* strains were examined by western blot analysis with blood donor plasma as some of antibodies comparison of epidemiologically related strains showed strong concordance between plot pattern and phage type.

Monoclonal antibodies to *S. aureus* capsular polysaccharide types 5 and 8 were used in an ELISA to serotype 74 and 42 coagulase-negative isolated from cow and goat milk, respectively by Pouri et al., (1990) 18 isolates were typable 13 *S. haemolyticus*, 1 *S. hyicus*, 1 *S. simalans* and 1 *S. warneri* from bovine origin and 2 *S. lentus* from caprine origin. Type 5 was predominant accounting for about 87% of typable isolates reactivity with monoclonal ab’s varied considerably according to isolates.

Ruthmathews et al. (1988) studied the characterization and cellular localization of the immuno dominant 47-kda antigen of *C. albicans*. Immuno-electron microscopy with an affinity-purified antibody to the 47-kda antigen showed that it was present in the cytoplasm and cell wall of both yeast and mycelical cells. In the cytoplasm it was usually near the cytoplasmic membrane and occasionally appeared in vesicular area. It was not detected in the nucleus/mitochondria. The 47-kda Ag did not bind to concavalin A and antigen city was lost after protease digestion. Peptide mapping suggested that the antigen was highly conserved between different stains of *C. albicans*.
Feaeri et al. (1988) studied in the urease enzymes of *C. pylori* and related bacterium areas enzyme of *C. pylori* was studied and compared with that of a related spiral-shaped bacterium isolated from the rodent ileum. Both bacteria possessed constitutive urease enzyme with activities up to 20-70 times that of *P. vulgaris*, activity was retained on SDS-PAGE. A catalytic subunit of mol.wt.300000 was located for all six strains of *C. pylori* subjected to SDS-PAGE. The response to *C. pylori* urease was not strain-specific but no cross-reactivity was detected between the *C. pylori* enzyme and that of St1. Possession of glutamate dehydrogenase activity by both organisms suggests that one role of the urease may be to assimilate the available urea nitrogen. Modification of the local environment to facilitate long-term colonization is another possible function. Protection from acid is unlikely to be a primary role as the natural habitat of the organism St1 is the non-acid-secreting tissue of the small intestine.

Shard et al. (1988) submitted the use of immuno blot detection of serum antibodies in the diagnosis of chronic *P. aeruginosa* lung infection in cystic fibrosis. They compared the results with determinations of precipitating antibodies in serum by crossed immune electrophoresis. The number of precipitins is a sensitive and specific indication of infection.

Sarafian (1987) in their study on environmental factors affecting toxic shock syndrome toxin 1 synthesis. The production of TSS T-1 was studied in batch and continuous culture of *S. aureus* strain 1169 in a carbohydrate free chemically defined medium. In continuous culture oxygen and arginine limitation were required for steady state TSST-1 synthesis. Aeration suppressed toxin synthesis. The Mg$^{++}$ ion concentration had no effects on the specific-toxin in anaerobic conditions. In aerobic conditions, specific toxin increased c-23 fold as the Mg$^{++}$ ion concentration
increased to 0.4mM. Further increased in the Mg\textsuperscript{++} ion concentration resulted in a reduction in specific toxin.

Catherinebranger et al. (1987) reported about esterase electrophoretic polymorphism of Methicillin sensitive and methicillin resistant strains of \textit{S.aureus}. Three kinds of esterase bands designated A, B and C was defined by their ranges of activity toward five synthetic substrate and their resistance to di-isopropyl fluorophosphates. Five allozymes of esterase A, 4 of esterase B and form of esterasic. Two major lymotypes were represented by 35 and 19 respectively; whereas other lymotypes were represented by one or at most seven strains. Most of the methicillin resistant strains are represented by the 2 major lymotypes, which differed from each other by the electrophoretic behavior of the three esterases. Their results indicate that, on the basis of esterase electrophoretic polymorphism, methicillin resistance is expressed in genetically different strains.

William et al. (1988) studied about the penetration of immunoglobulin through the \textit{Klebsiella} capsule and their effect on cell-surface hydrophobicity. Treatment of capsule or non-capsulate strains with the respective autologus antiserum resulted in a marked increased in surface hydrophobicity antisera raised against a rough non-capsulate (K\textsuperscript{O}+) strain had little effect on the surface hydrophobicity of either of the capsule strains K\textsuperscript{+}O\textsuperscript{+} and K\textsuperscript{2+}O\textsuperscript{+}, or of the non-capsulate K\textsuperscript{O}+ strain. The \textit{Klebsiella} capsular polysaccharide does not appear to present a permeability barrier to immunoglobulines although failure to detect outer-membrane proteins in the immune complexes of the capsule strains or of the K\textsuperscript{O}+ strain suggests that the O antigen may prevent access of Ab’s to these Ag’s.

Poxton et al. (1984) study that immunochemical fingerprinting of \textit{Clostridia difficile} strains isolated from an outbreak of antibiotic-associated colitis and diarrhea.
EDTA extracts of the organism were examined by AGE, PAGE and blot transfer. They concluded that PAGE and blot transfer. They concluded that PAGE-electroblot technique greatly aid in investigation into the epidemiologist of *C. difficile* infections.

### SDS-PAGE- Principle

Many important biological molecules such as amino acids, proteins, nucleotides and nucleic acids possess ionizable groups and can be made to exist in solution as electrically charged species either as cations or anions, molecules which have a similar charge will have different/charge ratio when they have difference in their molecular weights. These differences from a basis for a differential migration when the ions in solution are surfaced to an electric field. This principle made use of in electrophoresis (Upadyay, *et al.*, 1973).

The first recorded measurement of electrophoretic phenomenon was performed in 1891 by Quincke. Tiselius (1937) by describing his moving boundary apparatus become instrumental in popularizing the utility of electrophoresis to the biochemists proteomes are charged as a pH other then their isoelectric point (pI) and thus will migrate in an electric field in a manner dependent on their charge density Xie *et al.*, (1989) observed that the proteins of pathogenic and non-pathogenic strains were possessing obvious differences in isoelectric point (pI).

In early days electrophoresis was carried in disc electrophoretic apparatus. Gels were prepared by mixing cyanogens and N, N, N, N, tetra methylene diamine as accelerator in glycine hydrochloride containing urea and were polymerized at room temperature by the addition of ammonium persulphate as catalyst (Naumea *et al.*, 1969). Electrophoresis was accomplished at 8mc/tube (at constant current) for 1
to 6 hour at 50°C. Gels were removed from the glass tubes stained with amido black for 1 hour at room temperature as described by Davis (1964).

Recently the SDS-PAGE (Lameth 1970) slab gel is routinely used for *Leptospiral* protein antigen analysis (Masuzawa *et al*., 1989; Mendoze and Prescott 1992) and nucleic acid analysis (McDonald *et al*., 1977). Proteins are quantified (Lowery *et al*., 1951) are now widely used for analytical purposes.

Buffers used are formate, acetate, phosphate, barbiturate, EDTA, Tris and borated and also diluted sulphuric acid with a pH ranging from 10-11. The most commonly used buffer system for SDS-PAGE is that derived by Laemmli (1920) based on discontinues buffer system as of antigen and Davis (1964) with the addition of 0.1% W/V SDS. For the preparation of final sample buffer (FSB). Zingates (1984) method was found satisfactory.

Gels can be stained with commassie brilliant blue (Nicholson and Prescott, 1993) or fluoro graphed (Stamm *et al*., 1983) for protein analysis and silver stained (Brown 1983) for oligosaccharides and polysaccharides (Masuzawa *et al*., 1989).

**Western blot**

Polyacrylamide gel electrophoresis is an extremely powerful tool for the analysis of complex protein mixtures. Although the value of this method cannot be questioned, it is restricted in that the separated proteins remain buried with in the defuse gel matrix and are not readily available for further investigation (Gooderham 1983).

A number of methods have been developed in order to try and overcome this problem, for example, the elution of proteins from excised gel slices (Stedman *et al*.,
1982) and gel overlay techniques. Unfortunately all of these methods have serious drawbacks in the case of protein elution and in situ peptide mapping techniques, the resolution and the number of bands that can be processed is restricted where as the gel overlay techniques are generally time consuming and intensive (Stedman, et al., 1982).

Recently a new technique-protein blotting has been developed that promises to overcome many of these problems (Gooderham, 1983). Using this method proteins are transferred out of the gel and on to a filter (Erlich, et al., 1979) or membrane (Towbin, et al., 1979) forming an exact replica of the original protein separation but leaving the transferred proteins accessible to further study.

This technique (western blot) is superior to other method for characterizing antibody-antigen interactions, such as double diffusion studies because it is more sensitive. It is not dependent on preparation of antibodies and it permits, much finer resolution since a complex mixture of proteins is fractionated electrophoretically before reacting with the antibodies (Burnett, 1981). The latter worker modified the Towbin technique and stressed the usage of the western blotting” for the transfer of proteins. Although nitrocellulose filter are widely used by many markers some are still using different solid supports like nylon filters (Gooderham, 1984) diazophenythoether (DPT) paper diazotizable acrylamide cellulose paper, diazobenyloxy methyl paper (DPM) paper (Alwin, et al., 1977, Seed, 1982) diethylamino ethyl anion exchange (DEAE) paper (Danner, 1982 ). These papers are particularly usefull where the transferred proteins need to be very tightly bound to the filter (as in some immunoblotting experiments) or possibly in facilitated active transfer experiments (Erlich, et al., 1979).
The DBM and DPT paper have many shows comings like they have short half-lives and they should only be used in electro blotting buffer should be replaced with either a phosphate (Bittner, et al., 1980) or borate buffer. The reactive diazo group will bind not only proteins but also free amino acids (Gooderherm, 1984). The DEAE papers and membranes are available from a number as supplies and have been successfully employed in DNA blotting and not for protein blotting (Danner, 1982).

Now western blotting are routinely came either by direct electro blotting (Towbin, et al., 1979) or passive diffusion blotting transfer efficiency is approximate are tenth to one Lundredth of that seen in electro blot transfers (Gooderherm, 1984). A part from these the methods, there are a number of alternative transfer techniques such as southern blotting (Southern, 1975) and vacuum blotting (Perferoen, et al., 1982) performed for some applications but they are not yet widely used.

Recently published a simple method designed to improve the transfer of high molecular weight proteins. A wide range of probes including antibodies (Burnette, 1981) DNA (Jack, et al., 1987) RNA (Alwin, et al., 1977) have been used in protein blotting experiments and the potential applications this method are only limited by the available of suitable probes and assay system.

The potential application of protein blotting are very wide-ranging to quote some are immuno blotting (Tsang, et al., 1983). DNA binding proteins (Tridon, et al., 1982); glycoprotien (Hawkes, 1982) and receptors proteins (Oblas, et al., 1983).

In western blotting the samples to be assayed are solubilized with detergents and reducing agents, separated by SDS-PAGE and transferred in to a solid support (usually a nitrocellulose filter) which may then be stained. The filter is subsequently
exposed to unlabelled antibodies for the target protein the bound antibody is detected by one or several secondary immunological reagents ($^{125}$I-labelled protein-A antiimmunoglobulins or alternative to using radio labeled proteins, a high sensitivity silver staining method has recently been developed for use on protein blots (Yuee 1988). Although in principal this is a very useful technique the method is unfortunately very time consuming.

Most problems that arise with western blotting in practice can be solved by designing adequate controls. Those include the use of

1) Antibodies (preimmune sera or irrelevant monoclonal antibodies) that should not react with the target protein and

2) Control preparations that either contain known amount of target antigen or lack it altogether. If as is usually the case, monoclonal and polyclonal antibodies have been raised against native target protein it will be necessary to verify that they react with epitopes that either (i) resist denaturation with SDS and reducing agents

3) Are created by such treatment. This can be done by using denatured target antigen in a solid-phase radio immuno assay or in western-blot (Sambrook et al., 1989).

### 2.2.2 Genomic Analysis of *Staphylococcus aureus*

Comprehensive genomic analyses of the important human pathogen *S.aureus* was done by Ji and achieved by a strategy involving antisense technology in a regulatable gene expression system. In addition to known essential genes, many genes of unknown or poorly defined biological functions were identified. (Ji et al., 2001).
Quantitative titration of the conditional growth effect was performed either in bacterial culture or in an animal model of infection. This genomic strategy offers an approach to the identification of *Staphylococcal* gene products that could serve as targets for antibiotic discovery (Ji, *et al.*, 2001).

### 2.2.2.1 PFGE on *Staphylococcus aureus*

The pulsed field gel electrophoresis apparatus originally designed by Schwartz and Cantor, (1984) used alternately pulsed, perpendicular oriented electric fields and linear electrodes.

To arrange the physical orders of the fragment generated by digestion with one enzyme, probes were prepared by nonspecific primary and polymerase chain reaction using individual fragments of the other enzymatic digest as a template.

Probes were used for southern hybridization to the PFGE separated fragment distributions of the infrequent cleaving enzymes (*Sma* I and *Csp* I) using probes generated from four *Sma* I fragments and five *Csp* I order of *Csp* I fragments of the genome of *S. aureus* ISP 8 had been determined in relation to a previously published *Sma* I map of *Staphylococcus aureus* genome (Poddar and Mecelland, 1992).

Chiou *et al.*, (2002) reported that the pulsed field gel electrophoresis and coagulase gene restriction profile (CRP) analysis technique were used to analyse seven *S. aureus* isolates recovered from mite food borne disease outbreaks. Twenty two PFGE profiles and 11 CRPs were identified with discrimination indices of 0.86 and 0.72 respectively in addition, the variable regions of the coagulase genes of 29 isolates were sequenced and showed extensive identity, indicating that this was not an efficient alternative for the molecular typing of *S.aureus*. 
To determine the stability of PFGE patterns of MRSA in the nosocomial setting Blanc et al., (2001) analyzed isolates from long-term carriers and from patients involved in well-defined nosocomial infections. The number of fragment showed 0 to 6 fragment differences between the first isolates and subsequent isolates in long term carriers and the other group having 14 to 24 fragment differences.

Typing those isolates with another molecular method showed that isolates of the first group were related, whereas the second group could be considered genetically different.

Among long term carriers with clonally related isolates, 74 of 84 (84%) of consecutive isolates showed indistinguishable patterns, whereas 10 of 84 (12%) showed related patterns differing by one to six fragments. Moreover the frequency of related pattern was higher when the time between the first and the subsequent isolates was longer. During seven nosocomial epidemics lasting from 1 to 15 months, only 2 of 120 isolates (1.7) showed a pattern which was different although related from the predominant one involved in each of those outbreaks (Blanc, et al., 2001).

Pulsed field gel electrophoresis (PFGE) was the current method of choice for S. aureus strain typing. However the method was laborious and required expensive equipment (Oliveira and Ramos, 2002), in their study of the genomic 16S-23S rRNA region for genotyping were tested to determine the size of amplicons produced and to obtain better discrimination with agar gel electrophoresis and ethidium bromide staining.

The resolution of the typing system was determined by using sets of bacteria obtained from tertiary care hospital. These included DNA from three samples
obtained from bacteremic patients, six strains with known and diverse PFGE patterns and 88 strains collected over a 2-months period in the same hospital.

Amplification patterns obtained from samples from the patients were identical and PFGE from samples were known to be different and produced three genotypes. (Olivera and Ramos, 2002).

Olivera et al. (2001) said that the objectives of their study was to characterize patterns of the Brazilian endemic clone of methicillin resistant *S. aureus* (MRSA) from hospital through Brazil, they studied 83 MRSA public and private hospital in 19 cities located in 14 Brazilian states from September, 1995 to June 1997. The MRSA strains were typed using antibiograms, bacteriophage typing and pulsed field gel electrophoresis (PFGE). The analysis of genomic DNA by PFGE showed that 65 isolates indicated the presence of an endemic MRSA clone widely disseminated throughout Brazilian hospital. Use of molecular mapping was an important tool for monitoring the spread of potentially dangerous microbes.

Senna et al. (2002) reported that two hundred and fifty four methicillin resistant *S. aureus* (MRSA) strains typed by pulsed-field gel electrophoresis (PFGE) tested for the *mec A* associated hyper variable region (HVR-PCR) to determine their number of direct repeat units (DRUs) five different group of repeats were found among the MRSA strains and compared to 28 genotypes showed different numbers of DRUs HVR-PCR was rapid. Easy to perform and reproduction and has the ability to obtain an unambiguous positive result for each isolates analyzed. However this technique showed a discriminatory power inferior to that of PFGE concluding that PFGE was a more reliable method of typing MRSA than HVR-PCR.

Nosocomial infections caused by methicillin resistant *S.aureus* [MRSA] are one of the most serious problems in pediatric wards. The PFGE patterns of 56
isolates were classified into nine types including Type A, its subtype A1 and A2 and type B through to G. Seventeen isolates were identified from 50 of 56 isolates belonged to type A or its subtype. The predominant strain of MRSA isolates in the pediatric ward was a certain strain that have been originated from the same clone cross-infection control. (Murono, et al., 2002).

Since vancomycin-intermediate S. aureus (VISA) was first reported in Japan in 1997, there had been great concern that heterogeneous vancomycin–intermediate S. aureus (Hetro-VISA) was the putative precursor of VISA (Kim, et al., 2002). To investigate the prevalence, clinical significance and molecular epidemiology of S. aureus with reduced susceptibility of vancomycin, all consecutive isolates of S. aureus isolated from the clinical specimens from December 1998 to August 1999 as Asian medical centers were screened for VISA and hetero-VISA by using Brain heart infusion agar containing 4 µg of vancomycin / ml (Kim, et al., 2002).

Weist et al. (2002) reported that to determine the percentage of cross transmission in an intensive care unit (ICU) with high nosocomial infection rates according to the data of the German nosocomial infection surveillance system, Pulse field gel electrophoresis was performed for typing of S. aureus strains and arbitrary primed polymerase chain reaction was applied for other pathogens.

The presence of two indistinguishable strains in two patients was considered as one episode of cross transmission. Because of the method of this study, the percentage of NIS due to cross transmission was identified for this ICU. In reality the number of cross-transmission and thus the number of avoiding infections may have been higher. However it was difficult to assess whether the percentage of the NIS due to cross transmission identified for their ICU, may be the original
explanation for the relatively high infection rate in comparison to other surgical ICU
(Weist et al., 2002).

Lange et al. (1999) studied sixty – six isolates of *S. aureus* obtained from milk of dairy cows differing from sub clinical mastitis with different molecular typing methods and the comparison of pulsed field gel electrophoretically separated genomic small fragment patterns.

Among the 56 isolates of *S. aureus* from 30 outbreaks, 15 distinctive RFLP types were found by the use of the restriction enzyme as Sma I. A total of 32 isolates from patients’ food stuffs and cooks on six occasions had the same RFLP type derived from sporadic cases and phage types causes food borne outbreak (Suzuki, et al., 1999)

Peacock et al. (2002) conducted multilocus sequence typing (MLST) of *S. aureus* to study the global or long term epidemiologist but its role in local epidemiology had not been defined. The present study had compared MLST with PFGE by using *S. aureus* isolates associated with carrier in a busy renal unit. One hundred forty four patients were prospectively peritoneal dialysis.

Three nasal swab specimens were obtained one month on entering the study. A nasal swab was positive for *S. aureus* for at least one out of 50 patients (35 %) (Peacock, et al., 2002) typing of the 104 carrier isolates demonstrated 21 PFGE types and 21 sequence types.

Thirty one carriers had two or more positive nasal swabs; of these, the isolates all swabs from a given carrier had identical PFGE types for countries; the isolates in all of the same 29 swabs had identical sequence typings. Carriage strain in
two patients charged both PFGE type and sequence typings during the period of swabbing.

### 2.2.2.2 meca gene amplification and 16Sr RNA

PFGE of Sma I digest were transferred and hybridized with the meca probe. There were six transient clones, the PFGE pattern of which were quite different from those in the most frequent clonal types. These six transient MRSA clones were represented by single isolates of which three were unrelated. MRSA isolates showed to provide high level of methicillin resistance. MRSA strains had been showed to provide stable and reproducible phenotypic fingerprints. The identification of the phenotypic properties of MRSA clones.

Teineira *et al.* (1995) tested *S. aureus* for their antibiotic resistance patterns and genetic backgrounds meca gene specific DNA probes were examined by a variety of molecular fingerprinting techniques. The nature of the meca polymorph and Tn 554 attachment sites and RFLP of genomic DNA, after Sma I restriction and separation of digested DNA by PFGE. The overwhelming majority of the isolates shared a common PFGE pattern and carried meca polymorph III which indicated the presence of a single MRSA clone.

Tenover *et al.* (1995) interpreted the DNA fragment patterns generated by PFGE and transformed them into random genetic events that can alter the patterns. Most commonly random genetic elements including point mutations and insertions and deletions of DNA altered, PFGE patterns when fewer bands are detected, and the robustness and discriminatory ability of the criteria are unknown.

PFGE can be considered reliable for typing a given bacterial species. The techniques must be validated by demonstrating that when restriction fragments
patterns are analyzed by PFGE, generated unique fragment patterns. Discriminatory power and reproducibility are important attributes in any typing system for most of the common bacterial pathogens the validity of PFGE for molecular typing was well established.

Strulens et al. (1995) reported the accurate detection of methicillin resistance among *S. aureus* isolates. Detection of the *mec A* gene by PCR and/or DNA probe hybridization was reliable and rapid method. The observation described, identified a multi resistant MRSA clone with a high level of methicillin resistance.

Vannuffel et al. (1995) implicated that methicillin resistance in *S. aureus* and coagulase negative sp. was primarily mediated by the over production of PBP 2a, an additional altered penicillin – binding protein with extremely low affinities for β-lactam antibiotics. The *mec A* gene the structural determinant encoding PBP 2a had very high levels of homology in MRSA. The *mec A* gene was therefore considered a useful molecular marker of methicillin resistance in all *Staphylococci*. PCR appeared to be a rapid, sensitive and specific assay for detection compared with southern blot hybridization, macro restriction, finger printing.

Other chromosomally determined factors such as the *fem A-fem B* operon that act as regulator genes were essential for the expression of methicillin resistance in *S. aureus*. The cooperation of both *fem A* and *mec A* determinants seemed to be required, but the methicillin was not well understood. *Fem A* appeared to be a unique feature of *S. aureus*. Therefore simultaneous detection of the fem A and mec A genes in the same PCR tube had the advantage of identifying both the species and its phenotypic resistance. A multiplex PCR strategy that uses three set primers specific for *mec A, fem A* and the *Staphylococcal Insertion element* (IS) is 431. A probe corresponded to the 3’ end of the amplified fragment of the fem A
determinant hybridized to the PCR products of DNAs of *S. aureus* isolates, confirming the fem A gene origins of these amplified products the identification of *S. aureus* strains or for intrinsic methicillin resistance on the basis of fem A and mec A gene amplification.

A multiplex PCR approach associated amplifications of the mec A and 16S rRNA; gene had also been reported as a valuable tool for the identification of MRSA with the latter gene used as an internal control for sample DNA extraction. Three genetic markers that characterized the species, the antibiotic resistance mechanisms and a consensus sequence, amplification of mec A, fem A and is 431 genes in same PCR tube allowed to detect *S. aureus* with methicillin resistant phenotype.

Mazurek *et al.* (1996) studied an array of typing methods. Each typing method attempted to identify unique attributes of a strain, which differentiate it from other strains. The utility of a particular typing method can be judged by assessing its discriminatory powers, range of application, reproducibility and ease of performance. RFLP had been extremely used number of fragments produced by typical restriction enzyme was large. Southern blotting and hybridization with labeled probes reduced the number of visible fragments. This approach required construction of sequence specific probes and these probes had a limited range of application. An alternative approach which limited the number of restriction fragments used infrequently cutting restriction enzymes. The large restriction fragments produced are separated by PFGE. The resulting electrophoretic patterns were highly discriminatory for a wide range of organisms. Large amounts of high molecular weight DNA were needed infrequent – restriction site PCR finger printing method was needed Infrequent Restriction site PCR finger printing method was used IRS-PCR template was produced by double digestion of genomic DNA with a restriction enzyme that cuts recognition sequence double stranded adaptors were
lighted to the cohesive ends of restriction fragments. Both adaptors consisted of a long oligonucleotide and short oligonucleotides. These oligonucleotides annealed at low temperatures, allowed efficient ligation of the adaptor. The patterns generated by IRS-PCR were reproducible. The patterns generated with multiplex MAI isolates from the same patient were essentially identical.

Enright et al. (2000) developed a multilocus sequence typing (MLST) had schemed for *S. aureus*. The sequences of internal fragments of seven housekeeping genes were obtained. Multilocus sequence typing was a highly discriminatory method of characterization of bacterial isolates on the basis of the sequence of 450 bp internal fragments for each gene fragment; the different sequences were assigned as distinct alleles. Isolates that were identical by MLST had either identical fragment patterns or patterns that differed at two to five fragments. PFGE patterns that had less than four fragment differences were considered to be the same strain. MRSA clones were therefore expected to be identical or very closely related in allelic profile to their MRSA ancestors. An alternative possibility was that some of the MRSA clones contained isolates in which the mec genes had been lost or inactivated.

Booth et al. (2000) stated that distinct DNA fingerprint patterns or SALS were observed in *S. aureus*. The MRSA genetic determinant was also associated with individual isolates of lineages other than SALW supported the suggestion that mec A had spread horizontally *S. aureus* secreted more than 30 cell surface proteins many of which had been cloned, sequence and described potential roles in pathogenesis, PCR was employed to identified genetic determinants for known *Staphylococcal* virulence traits among prevalent and sporadic lineages. Detection of genetic determinants by PCR might may be limited by primer specificity for individual alleles.
Specificity of PCR may highlight subtle allelic variation in virulence genes which played a role in the expansion of certain prevalent lineages. Recombination among capsular biosynthetic operons resulted in intralineage shifting capsular polysaccharide serotype had been identified. Anti *staphylococcal* vaccines consisted of CP5 and CP8 were currently under investigation for their protective officially against *S. aureus* infection. The production of virulence factors that enhanced colonization, persistence and invasion at the infected site as well as factors that permitted their wide spread dissemination and evasion of host responses.

Grady *et al.* (2000) subjected MRSA isolates to genotype analysis by fluorescent amplified fragment length polymorphism (FAFLP) and by macro restriction PFGE. FAFLP was based on selective amplification by PCR of a subset of restriction fragments from a digest of a whole bacterial genome. The DNA of an organism was digested with a restriction endonuclease with a six base recognition sequence followed by digestion with a four base recognition sequence endonuclease site specific double stranded adaptors were lighted to the ends of the fragments to generate primes binding sites. A subset of these restriction fragments amplified by PCR was separated on an automated sequencer. One of the site specific primers was fluorescent labeled. Only fragments cut with the corresponding enzyme were seen by the laser detector of the sequencer. Because a differentially labeled internal size standard was run in each gel track, all fragments were sized with precision. FAFLP this offered. FAFLP offered a valuable new approach to high resolution genotyping of MRSA. Phage type EMRSA 15 generated reproduced DNA fingerprints resolved isolates as members of a clone complex. FAFLP was used to examine the genetic diversity. Multiprimer RAPD-PCR typing was carried out and they were also subjected to PFGE analysis. FAFLP detected differences between all isolates and strains in these two tight clusters. There were seven FAFLP defined clone
complexes. High resolution FAFLP genotyping assisted in mapping the short term evolution and dissemination of MRSA clone. All polymorphic amplified fragments which act as anonymous markers of genotype were sized to within one base pair, and novel feature in the definition of a clone. Amplified fragments can readily be cloned for nucleotide.

Blanc et al. (2001) determined the stability of PFGE patterns of MRSA. The mutation rate, including point mutations genetic rearrangements and horizontal transfer of mobile DNA elements such as phages and transposons were different from one bacterial species to another. The in vivo conditions of micro organism multiplication and transmission, conditions that might influence the mutation rate of the organism and investigations have also performed. Analysis of MRSA isolate with two different typing methods strongly suggested that Staphylococcus aureus isolates producing PFGE patterns differed with different fragments considered genetically different. Udo et al., (2001) stated that muporicin resistance in S.aureus was increased with increasing use of the antibiotic to control the spread of MRSA. The determinants for low level muporicin resistance were located on the bacterial chromosome. High level muporicin resistance was located on the bacterial chromosome. High level muporicin resistance was mediated by a mup A gene that was previously reported exclusively on muporicin resistance plasmids. Plasmid borne high level muporicin resistant MRSA had identical or similar PF patterns to the muporicin susceptible MRSA isolated in the unit before the detection of muporicin resistant. They harboured 3-5 plasmids with sizes ranging from 2.8 kb to 38 kb plasmid was present only in the high level muporicin-resistant isolates. Clones resistant to muporicin carried a 38 kb plasmid. The 38 kb plasmids encoded high level muporicin resistance and genomic DNA from the isolates used in PCR experiments to the amplified mup A DNA genomic DNA from isolates IBN 287
cured high level muporicin resistance. A PCR product of 1.6kb corresponded to the mup A gene was detected in all the 38 kb plasmids and from genomic DNA of isolates IBN 287, but not from the low level muporicin resistant isolates and XU320 the 3.0 and 2.8 kb plamids were cotransferred with the 38 kb plasmids, the strains carried them were resistant only to muporicin.

Anthonisen et al., (2002) said that the β-lactamase and que A genes from S. hemolyticus have 799.9 % identities at the nucleotide level with the same genes from S.aureus demonstrating that various Staphylococcal species able to colonize animal and human hosts can exchange the genetic elements involved in resistance to antibiotics and disinfectants. The use of antibiotics and disinfectants in veterinary practice and animal husbandry may also contribute to the selection and maintenance of resistance factors among the Staphylococcal species.

Different parts of the 12kb section analyzed had high degrees of nucleotide identity with regions from several other different S.aureus plasmid. This suggested the contribution of interplasmid recombination in the evolutionary makeup of this 12-kb section involving plasmids that can intermingle between various Staphylococcal species.

The lateral spread of resistance genes between various Staphylococcal species was probably facilitated by the generation of large multiresistance plasmids and h subsequent interspecies exchange of them. (Anthonisen, et al., 2002).

A total of 128 MRSA isolated from a burns unit in 1992-1997 was studied by plasmid analysis and PFGE. All the MRSA isolates produced β-lactamase and high MIC5 to methicillin. All MRSA gene isolates from both years carried mec A gene in Sma I fragment.
A banding pattern consisting of a readily discernible number of discrete bands usually about 2000 less in number was preferable. Fragments in the 1-10 kb sized range enabled short separation times while larger fragments were desirable if interpretation was complicated by the presence of plasmid bands (Jordon, 1991).

One restriction enzyme map of *S. aureus* bacteriophage Phi II DNA was established by reciprocal double digestions with the enzymes *EcoR I, Hae II* and *Kpn I* (Lofdahl et al., 1981).

The sequential order of the *EcoR I* fragments was established by a novel approach involving blotting of DNA partially cleaned with *EcoR I* and probing the blots with nick translated terminal fragments.

A circular map of the *Phi II* DNA was established and the phage genome was circularly permuted based on the failure to end label mature viral DNA, restriction map of replicating DNA and finally; homoduplex analysis in the electron microscope. A restriction enzyme map of the prophage from of *Phi II* DNA was obtained by analysis of chromosomal DNA from a lysogenic strain (Lofdahl et al., 1981).

Skulinck *et al.* (1992) studied a PCR based test was developed for the detection of *mec A* in *Staphylococci*. Primers based on the DNA sequence of the *mecA* gene from *S. aureus* were used in PCR to screened for the presence of *mec A* genes. Detection of *mec A* by DNA hybridization had provided a sensitive method for identifying MRSA and MSSA strains. *In vitro* amplification of DNA by PCR was rapid and sensitive for the detection of specific DNA sequences and required fewer than DNA hybridization protocols. In addition the detection of genes for enterotoxin, exfoliative toxin, TSS from *S. aureus* had also been reported.
Two approaches were employed to verify the 1.8 kb amplified DNA fragment representing the \textit{mec A} gene. A nested PCR was performed on an alignment of the initial PCR with primers internal to the primers. A positive result was indicated by amplification of a 1.1 – kb DNA fragment. No amplification of \textit{mec A} fragment was observed in initial or nested PCRs contained template or Methicillin susceptible strains. Hybridization analysis was performed with a radio labeled sample of the 1.8kb amplified fragment as hybridization probe. The \textit{mec A} gene in \textit{S. aureus} had been located on an approximately 4.0kb \textit{Hind III} restriction fragment. The 1.8kb PCR generated probe, as anticipated hybridized to an approximately 4.0kb \textit{Hind III} restriction fragment of DNA obtained from MRSA. The initial 1.8kb fragment, amplified in the initial PCR, was designed from \textit{mec A}. The presence of \textit{mec A} is indicative of a strain with the genetic potential to express methicillin resistance.

A positive PCR result was indicated by the presence of 1.8kb DNA fragment in PCRs with primers. The \textit{mec A} gene characterized as essential for methicillin resistance, had been cloned and sequenced from \textit{S. aureus}. Primers specific for \textit{fem A} were synthesized according to the published sequences and used in PCRs to survey various Staphylococcal isolates. \textit{mec A} was characterized as essential for methicillin resistance.

Genetic elements were also involved in expression of methicillin resistance phenotype. The \textit{mec A} gene product was essential for high level expression of methicillin resistant in \textit{S. aureus}. The relationship suggested that fem A might be used for detection of methicillin resistance by PCR. The PCR survey performed by using fem A as molecular target confirmed that this gene was present not only in MRSA.
Kim et al. (2002) identified MRSA with enzymatic detection of PCR (ED–PCR). ED – PCR was based on the capture of amplified products via biotin-Streptavidin affinity and the detection of an incorporated hapten in amplified products with an enzyme – linked antibody. MRSA mec A gene was targeted for ED-PCR. After PCR was performed with a pair of Biotin and dinitrophenol 5’ – labeled primers, the reaction mixture was applied to micro titer well precoated with streptavidin. Bound PCR products were detected with alkaline phosphatase conjugated anti – dinitrophenol antibody.

The RFLP pattern resulted from variable location and copy number of this insertion sequence in the genomes of different strains. The large amount of input DNA for genomic RFLP analysis and need more sensitive techniques had led to the development of PCR-RFLP Technique. A specific region of the genome was amplified and then restriction analysis of PCR product was used to determine micro heterogeneities of a single locus. RFLP analysis was based on the heterogeneous distribution of a genetic marker throughout the genome.

Three basic concepts had been developed for amplification of genomic DNA flanking known sequences by PCR: inverse or circular PCR, anchor PCR and single site PCR. PCR similar to anchor PCR mixed linker PCR used one primer specific for IS 6110 ligated to the restriction and a second primer specific for a synthetic linker ligated to the restriction fragments. The mixed linker approach allowed specific elimination of target of linker primer by treatment with UNG template. Initial denaturation of the hot start technique presented unspecific re-creation of complementary linker strand by filling of the resulting 3’ recessed ends by tag polymerize. The efficient ligation of a double stranded linker as demonstrated for closing purposes or whole genome PCR could be combined with the high specificity of a single stranded single site PCR. The main advantage of mixed – linker PCR
fingerprinting over the traditional RFLP method was its independence from *Mycobacterial* growth. The high sensitivity of mixed – linker PCR allowed generation of an almost completed fingerprint of genomic DNA.

Richard *et al.* (1994) founded the presence of a low affinity penicillin binding protein encoded by the *mec A* gene was the main factor responsible for Methicillin resistant in *Staphylococci*. Phenotypic expression of methicillin resistance was strain specific and was often heterogeneous. Detection of *mec A* gene by DNA hybridization or PCR had been proved useful for identification of MRSA.

The mechanisms of resistance had been associated with this phenotype *mec A* encoded resistance with this phenotype *mec A* -encoded resistance, overproduction of penicillinase and modification of PBPs. The Invariable presence of *mec* genes in MRSA provided an accurate method for identification of MRSA independent of environmental conditions that may affect the phenotypic expression of resistance.

Geha *et al.* (1994) analyses a multiplex PCR array for detection of *Staphylococcal mec A* gene [The structural gene for PBP2a] which indicated methicillin resistance. They developed multiplex PCR array that used a specific set of primers for *mec A* gene and a 16s r RNA universal target sequence. PCR was chosen because of its high sensitivity for identification of small number of resistant organisms.

For *S. aureus* the PCR array was compared to the standard susceptibility methods in identifying intrinsic methicillin resistance. Its utility with this group of organisms was to characterize the border line resistant strains. The technique was applied in that multiplex PCR system as an internal control mechanism. Amplification of that portion of the 16s gene, which was common to all eubacteria, controlled for DNA extraction as well as for failure of the PCR to amplified target
sequences. The product resulted at the position expected for the 16s RNA product was interpreted as a sample extracted. Depending on the length and G-C content of the amplification products the amplicons are inactivated.

Dominguez et al. (1994) analyzed the chromosomal background of the mec A gene. PFGE after restriction with Sma I resolved chromosomal DNA of MRSA isolates. PFGE profiles founded to be stable during numerous passages in vitro.

Grithuyen et al. (1999) amplified 298 – bp fragment of the mec A gene with the primers 5′ – GTT GTA GTT GTC GGA TTT GG – 3′ (upstream) and 5′ – CTT CCA CAT ACC ATC TTC TTT AAC – 3′ (down stream) specific for the mec A gene.

A second set of primers was included in each reaction mixture to amplify a polymorphic region of the coagulase gene that varied between approximately 350 to 600 bp. The coagulase primers specific for the coagulase gene were 5′ – CTG GTA TCC GTG AAT A – 3′ (upstream) and 5′ – TTG TAT TGA CTG TAT GTC TTT GGA – 3′ (downstream). The later primers provided on internal control to check for the presence of S. aureus DNA and for the absence of PCR inhibitors. MSSA isolates yield two PCR products. The co A amplicon and the 298 – bp mec A amplicon.

In cloning and nucleotide sequence determination of the entire mec DNA of pre methicillin resistant S. aureus N 315 Ito et al., (2001) used nested PCR in which the first round of PCR was carried out for 30 cycles and 1 to 2 ml of the reaction mixture obtained was used as the substrate for the second round of PCR which was carried out for 10 cycles. Long PCR was performed by using expand Taq polymerase. Three microlitres of the reaction mixture was subjected to agarose gel electrophoresis to detect amplified DNA fragments.
XiaoXueMa and his coworkers amplified DNAs encompassing the entire SCC *mec* sequence by long-range PCR with several sets of primers. The region from the left extremity to the CCR genes. (L-C region) was covered by primer sets $\alpha_5$ and cLs1 (CA05) or CL 26(8/6-3p). Primers $\alpha_6$ and mc R8 were used to 1-R region. (Ito *et al*., 1999 and Katayama 2000) Ito *et al*., (2001) spanned a region from *Tn* 554 to the right extremity of SSC *mec* which was amplified by long range PCR with two sets of primers, *Tn* A 1016 and mN13 and cR1.

By using these DNA fragments amplified by long range PCR, the nucleotide sequence of entire SCC of 85/2082 was determined. For structural comparison of three types of *Staphylococcal* cassette chromosome *mec* A integrated in the chromosome in methicillin resistant *S.aureus*.

### 2.2.2.3 Genome sequencing

In order to understand the evaluating relationship between C- MRSA, the entire nucleotide sequence of the SSC *mec* elements integrated into the chromosome of two C-MRSA clinical strains was determined. Strain CA05 (JCSC1968) was isolated from the joint fluid of a patient with septic arthritis and osteomyelitis and strain 8/6-3p (JCSC1978) was isolated from the perineum of another patient (Hussain *et al*., 2000). Baba and his coworkers established the whole genome sequence specific of two MRSA strains, N315 and Mu50, both of which are strains associated with health care. Two further hospital acquired MRSA strains have been sequenced by others (COL, E-MRSA-16\[strain252\]).

In this study they sequenced the whole genome of the community acquired MRSA strain MW2 and aimed to investigate the genetic basis for this strain high virulence by comparison of its genome with those MRSA strains that are associated with health care. Ito and his coworkers (2001) designed the primer mN12 and mN13
on the basis of the nucleotide sequence of the right extremely of SCC mec of 85/3907 during their research work in structural comparison of three types of Staphylococcal cassette chromosome mec integrated in the chromosome in methicillin resistant *S. aureus*.

### 2.3 PHYTOCHEMICAL STUDIES

#### 2.3.1 Extraction and assay

Plants belonging to the leguminosae are abundant in flavanoids and phytoalexins, which include flavanone derivatives that are structurally analogous to anti MRSA flavanones. Tsuchiya *et al.*, 1996 reported that anti MRSA active components such as flavanones were isolated from leguminosae.

The use of medicinal plants in the world, especially in the South America contributes significantly to primary healthcare. In Brazil, essential oils obtained from the aromatic plants were used in the form of crude extracts, infusions and plasters to treat common infections without any scientific evidence of pharmacological studies.

Antibacterial activities of organic and aqueous extracts of fifteen Palestinian medicinal plants were performed against eight different species of bacteria. Of the fifteen plants tested, eight showed antibacterial activity unique for particular bacterial species. The most active antibacterial plants against both gram positive and gram negative bacteria were *Thymus vulgaris* and *T. origanum*. The organic extract from the same plants showed the same or greater activity than the aqueous extract. (Essawi and Srour., 2000).

A series of thirty Indian folklore medicinal plants used by tribal healers to treat infections, were screened for antibacterial properties at 10 mg per ml
concentration by using disc diffusion method against *B. subtilis*, *E. coli*, *K. aerogens* and *S. aureus*. Twenty plant species showed antibacterial activities against one or more species of bacteria used in this assay. Among them the leaf extracts of *Cassia occidentalis* and *C. auriculata* exhibited significant broad spectrum activity against *B. subtilis* and *S. aureus* (Samy and Ignacimuthu 2000)

The anticough activity of *Psidium guajava* Linn leaf extract was evaluated in rats, guinea pigs. *S. aureus* and *Streptococcus* group A were inhibited by water, methanol and chloroform extracts of dry *Guava* leaves. The LD 50 of Guava leaf extracts was more than 5g/Kg. These results suggested that *Guvava* leaf extract is recommended as a cough remedy. (Jairaj et al., 1999).

Digrak et al. (1999) reported that, the antimicrobial activities of several parts of various trees grown in the kahramanmaras region of turkey were investigated by the disc diffusion method.

The extracts of six plants selected on the basis of folk medicine reports were examine for their antibacterial effects against eight pathogenic bacteria. The results showed that n-butanol extract of *Calotropis procera* proved to be the most effective against the bacteria tested using the paper disc diffusion method. (Larhsini et al., 1999).

The combination of a 4 % tea tree oil nasal ointment and 5 % tea tree oil body wash and 2 % mupirocin nasal ointment and triclosan body wash for the eradication of Methicillin resistant *S.aureus* carriage. The tea tree oil combination appeared to perform better than the standard antibiotic combination, although the difference was not statistically significant due to the small number of patients. (Caelli et al., 2000).
Medicinal plants had been the topic of several analyses in several scientific meetings in Brazil. However, this issue is still polemic, especially when the questions of pharmacological studies involving analysis refer to the strategies to be employed in this kind of study the extracts to be prepared, the animal models to be used, the reproducibility of these studies and finally, the posology employed by folk medicine.

Although a consensus on the answers to these questions has not been reached, there is one aspect with which everybody agrees, if the selection of plants is made on the grounds by their traditional use, the chances for research success is greater. Pharmacological investigation of medicinal plants has provided important advances for the therapeutic approach to several pathologies as well as extremely useful tools for the theoretical study of physiology and pharmacology.

*Azadiracta indica juss* is one of the medicinal plants. *Azadiracta indica juss* comes under Meliaceae family, consisting of about 50 genera and 800 species. The members of this family are found to be distributed in tropical region.

The plants of Meliaceae family are mostly trees or shrubs. The wood of this family emits characteristics smell. The roots are tap and branched. The stems are branched, solid woody with characteristics smell. The leaves are usually alternate, petiolate, pinnately compound or rarely simple and estipulate.

The inflorescence is cymose type, in many cases the auxiliary cymose panicle are present in *Azadirachta indica juss*. The people in many parts of the world are just becoming aware of what Indians have known for centuries. The *Azadiracta indica juss*, its origin is of north east India, it is a member of meliaceae family resembles an English Oak in appearance. It can grow up to 30 meters in height and can grow about 40 meters height in certain countries.
All parts of the neem tree, leaves, fruits, seeds, bark, roots and the oils derived from these, have every specific application in medicine, toiletries, cosmetics, public health, livestock production, health, agriculture and pest control. In Sanskrit, the neem tree is known as “ARISTHA”, which meaning relives of all diseases. Indian often refers the tree as “village pharmacy” All parts of this plant are used as an antibiotic. (www.neem.com).

*Duranta pulmeneri jacq* family, almost entirely tropical or subtropical in verbena, it includes 80 species, which are mainly distributed in the tropical and extra tropical regions of America. In our country, the family is represented by several genera. The *Duranta pulmeneri jacq* leaves are used as cure for snakebite. The family has most important economic value. It consists of several important ornamental plants.

Fourteen extracts from Brazilian traditional medicinal plants which are used to treat infectious diseases were assayed for potential antimicrobial activity against multi drug resistant bacteria of medical importance. *S.aureus* strains were susceptible to extracts of *Punica granatum* and *Tabebysia avellanedae*. The minimum inhibitory concentrations (MICs) of the total extracts and of additional fractions of these plants were determined by employing methicillin resistant and methicillin sensitive *S. aureus* (MRSA and MSSA). (Machado et al., 2003).

The most interesting among the extracts tested was that of the fruit rind of *Punica granatum*, which is a waste product, this nontoxic extract was very effective against a broad range of intestinal pathogens. (Fadula, 1975).

*Punica granatum* Linn is a shrub or small tree native to Asia (Jafri et al., 2001). Where its several parts have been used as an astringent, haemostatic as a
remedy for diabetes, as antihelmintic specifically against tapeworms and diarrhea and dysentery. (Das et al., 1999).

In Brazil, the fruit known as “roma” is used for the treatment of throat infections, coughs and fever. There are several commercial phytopreparations in Brazil containing extracts from Pomegranate. For the validation of such products it is necessary to desire the chemical markers, substances that are present in the preparations attest their quality (Gunther et al., 1996).

Although many reports on the antimicrobial activity of pomegranate exist in the literature, none of them relates such activity with its chemical composition. Our research work delineates the isolation and identification of the tannin responsible for the activity against a bacterium of medically important pathogenic strain, MRSA in both hospital and the community.

2.3.2 Thin Layer Chromatography

TLC is a simple, quick and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the Rf of a compound is compared with the Rf of a known compound.

Thin Layer Chromatography (TLC) is a solid liquid technique in which the two phase are solid (stationary phase) and a liquid (mobile phase). (Pomeranz and Meloan 1987).
2.3.3 High Performance Liquid Chromatography (HPLC)

Preparative HPLC refers to the process of isolation and purification of compounds. Important is the degree of solute purity and the throughput, which is the amount of compound produced per unit time. This differs from analytical HPLC, where the focus is to obtain information about sample compound. The information that can be obtained includes identification, quantification and resolution of a compound.

Chemical separations can be accompanied using HPLC by utilizing the fact that contain compounds have different migration rates for a given a particular column and mobile phase. Quantification of compounds by HPLC is the process of determining the unknown concentration of a compound in a known solution. It involves injecting a series of known concentration of the standard compound solution on to the HPLC for detection. The chromatograph of these known concentrations will give a series of peaks that correlate s to the concentration of the compound injected. (Williard et al., 1998).

2.3.4 Nuclear Magnetic Resonance (NMR) Detectors

Certain nuclei with odd numbered masses including $^1$H and $^{13}$C spin about an axis in a random fashion. However, when placed between poles of a strong magnet, the spins are aligned either parallel or anti parallel to the magnetic field with the parallel orientation favored since it is slightly lower in energy. The nuclei are then irradiated with electromagnetic radiation, which is absorbed and places the parallel nuclei in to a higher energy state; consequently, they are now in “resonance” with the radiation. Each H or C will produce different spectra depending on their location and adjacent molecules are surrounded by electron clouds, which change the encompassing magnetic field and thereby alter the absorption frequency (Mc Clure 1994).