CHAPTER II

LITERATURE REVIEW

I.Genus *Staphylococcus*

Staphylococcus is the only genus of medical importance in family **Micrococcaceae**. Staphylococci are gram positive spherical cell. They are nonmotile, nonspore-forming and grow well under aerobic and anaerobic conditions. Staphylococci can be naturally found among the normal flora of the skin and mucous membranes (Jawetz *et al.*, 1989). Their appearance on a gram stained smear is usually sufficient to distinguish them from the streptococci which belongs to the family **Streptococaceae**, since the streptococci are much more prone to form chains of cells. These two of genera can be easily separated on the basic that only staphylococci is capable of producing enzyme catalase. In addition, staphylococcal colonies are considerably larger than those formed by the streptococci.

Staphylococci ferment a wide variety of sugars to form acid, but no gas is formed. This characteristic permits a differentiation from the large number of avirulent but morphologically similar members of the genus *Micrococcus*. All micrococci are obligate aerobes, and,

although they oxidize many sugars, but they do not produce acid (Volk, 1982).

From more than 20 species of *Staphylococcus*, only three are clinical important: *S. aureus*, *S. epidermidis*, and *S. saprophyticus*. *S. aureus* is the most important pathogen for man, *S. epidermidis* has emerged as pathogens causing nosocomial bacteremia. Although, *S. epidermidis* is a relatively avirulent pathogen, it is associated with hospital-acquired infections, especially in patients whose susceptibility to the organism is increased and in whom there is a nidus of foreign material, such as a prosthesis or plastic catheter. *S. saprophyticus* can cause urinary tract infection in women (Joklik, 1992).

II. *Staphylococcus aureus (S. aureus)*

2.1 Morphology and characteristics

S. aureus can be easily differentiated from other species of staphylococci. *S. aureus* normally produces a light golden pigment colonies. The characteristics that are useful for distinguish *S. aureus* from other medically important *Staphylococcus* species are shown in Table 1. It possesses a species specific antigenic determinant call polysaccharide A. Polysaccharide A is a component of the cell wall consisting of ribitol-type teichoic acid which is esterified with N-acetylglucosamine. It can ferment mannitol and produce coagulase (an enzyme inducing the clotting of plasma) that is used to delineate this species.

Table 1. Selected characteristics distinguishing 3 medical important species of the genus *Staphylococcus*

	S.aureus	*S.epidermidis*	*S.saprophyticus*
:Coagulase	+	-	-
:Anaerobic growth	+	+	-
:Anaerobic fermentation of glucose	+	+	-
:Anaerobic fermentation of mannitol	+	-	-
:Heat-resistance endo-nucleases	+	-	-
:Novobiocin sensitivity	S	S	R

+, 90% or more strains positive
-, 90% or more strains negative
R = Resistant (MIC >2.0 μg/ml)
S = Sensitive (MIC <0.6 μg/ml)
(Modified from Zinser Microbiology 20th, p.402, 1992)

2.2 Natural habitat and pathogenesis

Natural habitat: The primary natural habitat of Staphylococci are mammalian body surfaces, where the organisms are found in large numbers. In their adaptation to parasitism, staphylococci have been among the most versatile and successful of the pathogenic bacteria. The skin and nares of infants are colonized by *S. aureus* within a few days after birth, but because of antibodies passively received through the placenta, the carrier rate drops during the first 2 years of life. By the age of 6, the child has acquired an adult carrier rate of approximately 30%. Persons who harbor staphylococci may be chronic or persistent carriers, however, most are intermittent carriers who harbor the organism for only a few weeks. *S. aureus* strains are most commonly found in the anterior nares on skin and mucous membranes. The nasal epithelium of

nares on skin and mucous membranes. The nasal epithelium of carriers may have a special affinity for *S. aureus*. Colonization of the nares leads to dissemination to body surfaces (Joklik, 1992). Nasal carriage of *S. aureus* occurs in 10 to 50% of human (Volk, 1982). The number of nasal carrier of *S. aureus* found in the nasopharynx may increase to a 50 to 70% carrier rate in a hospital setting. Such carriers provide the reservoir for the spread of staphylococcal infections, most frequently by hands.

Pathogenesis: Persons who are proved to serious staphylococcal infections include those whose self defense mechanism for instance ability to phagocytose and destroy the staphylococci is not completely developed, or is significantly inhibited. Such persons may include the newborn, surgical or burn patients, persons receiving immunosuppressive drugs, or those with immune deficiency diseases such as chronic granulomatous disease. Those with lower respiratory viral infections, such as influenza, measles, and diabetic patients, are also more susceptible to staphylococcal infections. *S. aureus* is always potentially pathogenic, but defensive mechanisms in man normally prevent them from causing diseases. *S. aureus* can caused varities of diseases include superficial infections of the skin, deep tissue infections, systemic infections and septicemia sometimes possibly threaten to death (Volk, 1982).

The characteristic feature of staphylococcal infection is abscess formation. This can occur in any part of the body, but in each area the basic lesion consists of inflammation, leukocyte infiltration, and tissue necrosis. In a fully developed lesion, there is a central necrotic core filled with dead leukocytes and bacteria separated from the surrounding tissue by a relatively avascular fibroblastic wall.

1. Skin infections

Staphylococcal infection of the skin is the most common bacterial infection in man. **Folliculitis** is an infection of the hair follicle. An extension into the subcutaneous tissue results in the formation of a focal suppurative lesion, the boil or **furuncle**. A **carbuncle** is similar to a furuncle but has multiple foci and extends into the deeper layers of fibrous tissue. Carbuncles are limited to the neck and upper back, where the skin is thick and elastic. In children, cutaneous lesions are less well localized than they are in adults.

In the newborn infant, pustules or impetiginous lesions are the most frequent staphylococcal skin manifestations. Staphylococcal **impetigo** also is common in young children, often occurring around the nose. It is characterized by the formation of encrusted pustules on the superficial layers of the skin. When crusts are removed, a red weeping denuded surface is exposed. The disease is

highly contagious and, when introduced into a nursery or school, spreads in an epidemic manner. In the United States, staphylococci appear to participate with streptococci in most common impetiginous lesions.

Scalded skin syndrome. The scalded skin syndrome comprises a spectrum of dermatologic diseases with a common etiology, the staphylococcal exfoliative toxin. All of the clinically recognizable features of this syndrome are attributable effects of the staphylococcal infection itself. The scalded skin syndrome primarily afflicts neonates and children under 4 years of age. Its relatively rare occurrence in adults, except in immunologically compromised patients, suggests the presence of neutralizing antibodies in the majority of the population.

2. Respiratory tract infection

Staphylococcal **pneumonia** is an important disease because of its high mortality rate (up to 50%). It may be a fulminant process in all age groups, but it is relatively rare except during epidemic periods of influenza. Infants less than 1 year of age appear to be the most susceptible and account for about 75% of the cases. Primary staphylococcal pneumonia is most often seen in patients with impaired host defense: children with cystic fibrosis or measles, influenza patients, or debilitated, hospitalized persons being treated with antimicrobial agents, steroids, cancer chemotherapy, or

immunosuppressants. Necrosis, with formation of multiple abscesses, is characteristic of the infection. The pneumonia usually is patchy and focal in nature.

3. Bone and joint infection

S. aureus is the women cause of primary **osteomyelitis.** This disease occurs primarily in male children under the age of 12 years, and in most cases, it follows hematogenous spread from a primary focus, usually a wound or furuncle. The organisms localize at the diaphysis of long bones, probably because the arterial circulation in this area consists primarily of terminal capillary loops. As the infection progresses, pus accumulates and emerges to the surface of the bone, raising the periosteum and producing a subperiosteal abscess. Clinical symptoms of acute osteomyelitis include fever, chills, pain over the bone, and muscle spasm around the area of involvement. When the infection occurs near a joint, staphylococcal pyarthrosis is a common complication. Approximately 50% of all cases of bacterial arthritis are caused by *S. aureus*. Staphylococcal **pyarthrosis** may occur after orthopedic surgery, in conjunction with **osteomyelitis** or local skin infections, or as a result of direct inoculation of staphylococci into the joint during intraarticular injections, especially in patients with rheumatoid arthritis who are receiving corticosteroids. Staphylococcal infection of joint destroys the articular cartilage and may result in permanent joint deformity.

4. Bacteremia and endocarditis.

Bacteremia may occur with any localized staphylococcal infection, but infections of the skin, the respiratory tract, or the genitourinary tract provide the primary focus for most of these lesions. Approximately 50% of staphylococcal septicemia are acquired in the hospital. A frequent complication is endocarditis, which is usually acute and malignant, with heart valve destruction within a few days. In spite of appropriate antibiotic treatment, *S. aureus* endocarditis has a high mortality, varying from 40% to 80%, depending on the underlying medical problem, the age of the patient, and resistance of the infecting strain to penicillin.

5. Metastatic staphylococcal infections.

One of the characteristic features of *S. aureus* bacteremia is the production of metastatic abscesses. The most frequent sites of the metastatic abscesses are the skin, the subcutaneous tissues, and the lungs. Internal abscesses of the kidneys, the brain, and the spinal cord are not uncommon.

6. Food poisoning.

In the United States, staphylococcal food poisoning is the most common form of bacterial food poisoning. It is caused by the ingestion of food that contains the preformed toxin elaborated by enterotoxin-producing strains.

7. Toxic shock syndrome (TSS).

TSS-1 toxin producing strains of *S. aureus* have been implicated in most cases of TSS, a multisystem disease that primarily afflicts young women. Most cases occur in menstruating women who use tampons; however, nonmenstruating women, children, and men with boils or staphylococcal infections of wounds can also have TSS. Clinical features include fever, marked hypotension, diarrhea, conjunctivitis, myalgias, and a scarlatiniform rash followed by fine desquamation.

2.3 Virulence factors

a. Extracellular enzymes

Beta-Lactamase: Beta-lactamase are responsible for many highly resistant strains, including penicillin-resistant staphylococci obtained from patients. In the development of the semisynthetic penicillins and cephalosporins, one of the primary goals was to find compounds that are insensitive to beta-lactamase activity. Unfortunately, most strains of *S. aureus* produce beta-lactamase. These enzymes spilt the beta-lactam ring of the penicillins and cephalosporins between the C and N atoms to form inactive compounds. The major basis for bacterial resistance to beta-lactam antibiotics, whether occurring naturally or acquired *in vivo*, is the inactivation of the drug by beta-lactamases. In the case of *S. aureus*, the gene on the plasmid can be derived from that on the chromosome via a transposon. Beta-lactamase production in *S. aureus* is

mediated mainly by a plasmid gene that can be easily transferred to other susceptible organisms by bacteriophages.

Coagulase: The plasma of many animal species is readily clotted by the extracellular or free coagulase of *S. aureus*. The production of coagulase is the marker for the species, although very few wild type strains of *S. aureus*, as well as mutant, may be coagulase negative. Although the correlation between coagulase production and pathogenicity provides a virulence marker, there is no definite evidence that coagulase is directly involved in pathogenicity.

Staphylokinase (Fibrinolysin): Staphylokinase is one of the proteolytic enzymes of staphylococci that has fibrinolytic activity but is antigenically and enzymatically distinct from the streptokinase of the streptococci. The determinant for the staphylokinase production is dependent on a phage genome and is expressed during lysogeny. In the dissolution of clot by the staphylococcal enzyme, the proenzyme plasminogen is converted to the fibrinolytic enzyme plasmin. Although produced by most strains of *S. aureus*, there is little evidence that it is a major factor in pathogenicity.

Nuclease: The elaboration of a heat-resistant nuclease appears to be uniquely associated with *S. aureus*. The enzyme, which is present in, at, or near the cell

surface, is a compact globular protein consisting of a single polypeptide chain. Heating at 65°c causes structural disruptions, but the changes are rapidly and completely reversible. The nuclease is a phosphodiesterase with both endonucleolytic and exonucleolytic properties and can cleave either DNA or RNA.

Lipase: Staphylococci produce several lipid hydrolyzing enzymes collectively referred to as lipases. The lipases are active on a variety of substrates, including plasma, fats and oils that accumulate on the surface area of the body. The utilization of these materials has survival value for the organism and may be the explanation of the intense colonization of staphylococci in the sebaceous areas of greatest activity. The production of lipase apparently is essential in the invasion of healthy cutaneous and subcutaneous tissues. In primary human isolates, there is a close correlation between *in vitro* production of lipase and the ability to produce boils. The decreased virulence of hospital strains of staphylococci observed during the last 20 to 30 years was parallel to the decrease in the number of staphylococcal isolates that produce large amounts of the enzyme. The decrease apparently is due to the presence of a prophage that blocks lipase production by insertional inactivation.

Hyaluronidase: More than 90% of *S. aureus* strains produce hyaluronidase. This enzyme hydrolyze the hyaluronic acid present in the intracellular ground substance of connective tissue, thereby facilitating spread of the infection. Since inflammation antagonizes the spreading action by hyaluronidase, its importance in staphylococcal infections is limited to the very early stages of infection.

b. Toxins.

Cytolytic toxins: A number of bacteria produce toxins that cause physical dissolution of mammalian or other cells *in vitro*. Most of these are proteins, extracellular, and induce the formation of neutralizing antibodies. There is considerable diversity, however, in the manner in which the various cytolytic toxins interact with the cell surface. The hemolysins and leukocidin elaborated by *S. aureus* are among the best define of the cytolytic toxins. Four distinct hemolytic toxins (alpha-, beta-, gamma-, and delta-hemolysins) are produced by *S. aureus*, although different strains may vary in the levels that they express.

Alpha toxin (α-Hemolysin): This toxin is the principle hemolysin of human strains of *S.aureus*. It lysed rabbit red blood cells (RBCs), but can not lyse human RBCs However, human platelets and tissue culture cells are affected by this toxin. Alpha-Hemolysin also causes leakage

of ions from artificial liposomes. Alpha-Hemolysin exhibits a wide range of biologic activities, including the hemolytic, lethal, and dermonecrotic effects observed after the injection of broth culture filtrates. Alpha-Toxin disrupts lysosomes and is cytotoxic for a variety of tissue culture cells. In experimental animals, it causes dermal necrosis after of local infection and is lethal when given systematically. The main effect of the toxin appears to be the spasm of vascular smooth muscle. Its mode and site of action on cell membrane are unknown.

Beta toxin (Staphylococcal sphingomyelinase): This toxin is produced commonly by animal strains but it is produced by only 10 to 20% of strains isolated from humans. The β-hemolysin is a sphinomyelinase C, that is activated by Mg++ but not by Ca++. The toxin is an enzyme with substrate specificity for sphingomyelin (and lysophosphatides). Sphingomyelin degradation is the membrane lesion that leads to hemolysis when the cells are chilled. Erythrocytes from different animal species exhibit impressive differences in their sensitivity to β-toxin. A correlation exists between toxin sensitivity and content of sphingomyelin, most of which is located in the outer leaflet of the lipid bilayer of the erythrocyte membrane and thus is accessible to exogenous toxin.

Gamma toxin: This toxin consists of two basic proteins acting in concert, both being essential for hemolysis and toxicity. Rabbit, human, and sheep RBCs are susceptible to this toxin while horse and fowl RBCs are not. Agar and other sulfated polymers inhibit gamma-hemolysin, and so it is not active on blood agar plates. Cholesterol and many other lipids are also inhibitory. Because of these interaction, the existence of gamma-hemolysin was long debated. This toxin has pronounced hemolytic activity, but its precise mode of action is not known.

Delta toxin (δ-Hemolysin): It is produced by most human strains of *S. aureus*. Activity is blocked by phospholipids present in normal serum, but specific antibodies are also produced. δ-Toxin is a relatively thermostable surface active toxin whose strong detergent-like properties are responsible for its damaging effects on membranes. It exhibits a high degree of aggregation and is electrophoretically heterogeneous. Toxicity for animals is minimal. δ-Toxin exhibits a broad spectrum of biological activity and displays no pronounced specificity for cells of a particular species; erythrocytes, macrophages, lymphocytes, neutrophils, and platelets are all damaged.

Panton-Valentine (P-V) leukocidin: This toxin is produced by most *S. aureus* strains. It acts synergistically, in the presence of Ca++, to cause

degranulation of human and rabbit polymorphonuclear leukocytes (PMNs) and macrophages, which are the only susceptible cell known. The toxin is composed of two protein components (S and F) that act synergistically to induce cytolysis. Both components of leukocidin are highly antigenic and can be used as a toxiod.

Exfoliatin (epidermolytic toxin): Exfoliatin causes a variety of dermatologic lesions and is particularly responsible for scalded skin syndrome. It is produced by approximately 5% of random isolates of *S. aureus*. The toxin acts by cleaving the stratum granulosum of the epidermis, probably by splitting desmosomes which link the cells of this layer.

Enterotoxin: Approximately 50% of *S.aureus* strains produce enterotoxin. There are at least six different enterotoxin. They are a major cause (especially types A and D) of food poisoning, which is due to ingestion of preformed toxin in foods contaminated with *S.aureus*. In addition, enterotoxin B is often associated with staphylococcal pseudomembranous enterocolitis.

Toxic shock syndrome Toxin-1 (TSST-1):

S. aureus is associated with toxic shock syndrome (TSS), a severe and often fatal disorder characterized by multiple organ dysfunction. In most cases of TSS associated with menstruation and in approximately 50% of nonmenstrual

cases, TSST-1 producing strains of *S. aureus* are involved.

2.4 Resistant mechanisms of *S. aureus* to Beta-lactam antibiotics

S.aureus has gained on increased resistance to antibiotics for quite some times (Brumfitt and Hamilton, 1989). In the early 1940s, when benzylpenicillin was first introduced, staphylococcal infections were treatable with this drug. However, by 1948, penicillin-resistant strains were prevalent. During the 1950s, *S. aureus* developed resistance to other antimicrobial available agents such as streptomycin, erythromycin, tetracycline, chloramphenicol, and various other aminoglycosides. All of these resistance traits were found encoded on plasmids and several of them are known to be encoded on transposon (Goering and Duensing, 1990, Lyon and Skurray, 1987). However, it was shown that when either erythromycin, chloramphenicol, or tetracycline was used as the primary antibacterial agent in closed setting such as hospital units, a marked increase in resistance to the agent occurred, and resistant staphylococci quickly became predominant over susceptible staphylococci (Murray and Moellering, 1978).

Currently, there are no known strains of *S. aureus* resistant to vancomycin. Ciprofloxacin, has been efficacious in treatment of some staphylococcal infections. However, resistance has emerged during therapy of complicated infections and appears to be due to chromosomal mutation (Piercy *et al.*, 1989, Schaefler, 1989 and

Chambers, 1988). Future quinolones more potent against *S. aureus* are anxiously awaited.

The first penicillin-inactivating enzyme was discovered not in *S. aureus*, but in *Escherichia coli* (Abrahum, 1940). The discovery of penicillin-inactivating enzyme in *S. aureus* was made somewhat later (Spink and Ferris, 1945). The increasing in the numbers of penicillin-producing staphylococci progressively limited the usefulness of penicillin G in infections caused by this organism.

It has been accepted that there are three types of resistance of staphylococci to the beta-lactam antibiotics. The best known type of resistance is drug inactivation due to beta-lactam inactivating enzymes properly defined as beta-lactamases. The second type of resistance is intrinsic, due to some mechanisms other than the inactivation of the antibiotics. The third type is tolerance to the killing action of beta-lactam antibiotics (Sabath, 1982).

a. Drug inactivation due to beta-lactamase(s)

An organism produces the beta-lactamase(s) that inactivates the antibiotic before the antibiotic has created irreversible changes in the bacterial cell. The beta-lactamase is most often an inducible enzyme in *S. aureus*, although in some instances it is a constitutive (Richmond, 1965). The dynamics of staphylococcal beta-lactamase interaction with inducible strains have been

called a race between the antibiotic's ability to initiate irreversible changes in the organism, and the organism's ability to synthesize and secrete beta-lactamase in sufficient amounts to inactivate the penicillin in its microenvironment (Sabath, 1982).

Beta-lactamase was shown to hydrolyze the cyclic amide bond in beta-lactam molecules, resulting in the lose of their antibacterial activity. Principle for detection of this enzyme was the hydrolysis of the beta-lactam ring of the chromogenic cephalosporin nitrocefin {3-(2,4-dinitrosthyryl)(6R,7R)-7-(2-theinylacetamido)-cephem} that caused a color change from yellow to red. The reaction often occurred very quickly and usually takes no longer than 10 minutes. For very low levels of beta-lactamase or when nitrocefin was a poor substrate, the reaction could proceed for a longer time, but a control tube inoculated with a beta-lactamase negative organism (*S. aureus* ATCC 25923) should be used (O'Callaghan *et al.*, 1972).

The cellular target for beta-lactam antibiotics are the enzymes that catalyze synthesis of the cross-linked peptidoglycan of the cell wall. The higher molecular weight molecules have been thought to be essential for bacterial function. These proteins correspond to penicillin-binding proteins (PBPs). Penicillin and other beta-lactam antibiotics have been proposed to act as analogs of acyl-D-alanyl-D-alanine, a position postulated from the structural similarities of steromodels of the different molecules. Under natural

conditions, the enzymes performing the transpeptidation and/or carboxypeptidation reactions, react with acyl-D-alanyl-D-alanine to form an acyl-D-alanyl enzyme complex, with the elimination of the terminal D-alanine. The complex would then interact with a free amino group on another peptide side chain, resulting in cross-linking of the two chains and release of the free enzyme. Treatment of the bacteria with a beta-lactam antibiotic would interfere with this process.

Although it is widely known that there are many different types of beta-lactamase produced by gram negative bacilli (Richmond *et al.*, 1971), many physicians and microbiologists are unaware of the fact that there is more than one kind of beta-lactamase produced by *S. aureus*. The work has shown that there are three different types of beta-lactamase produced by *S. aureus* (Richmond, 1965). It has been shown that these differences were based on four kinds of evidence: immunologic studies, difference(s) in inoculum effect (Sabath *et al.*, 1975), difference(s) in substrate profile, and difference(s) in inhibitory profiles (Laverdiere *et al.*, 1977).

In the 1960S, the beta-lactamase enzyme in *S. aureus* was shown to be encoded on plasmids that could be transferred from strain to strain by phage transduction. In addition, these plasmids were indicated to encode the resistance to heavy metals such as mercury (Lyon and Skurray, 1987). When techniques for physical mapping of

plasmids became available, it was recognized that certain regions around the beta-lactamase gene were sometime inverted, and the suggestion was made that this might be a mobile genetic element (Lyon and Skurray, 1987). However, it is now conclusive that the beta-lactamase gene was indeed on a transposon (e.g.,Tn 4201) and the plasmid which encodes it is not one of the transducible plasmids which encode metal resistance, but is a conjugative plasmid that also mediates resistance to gentamicin (Weber and Goering, 1988).

It was found that the beta-lactamase gene was inserted into a fragment of the gentamicin-resistance plasmid (Jaffe *et al.*, 1982) and also had been found in the chromosome of some strains of staphylococci (Lyon and Skurray, 1987). Unlike some of the transposons in gram negative bacteria, the staphylococcal beta-lactamase transposon does not appear to transpose as readily and has only been documented in a limited number of sites, perhaps due to a requirement for a specific insertion sequence.

b. Intrinsic resistance

The intrinsic resistance of staphylococci to beta-lactam antibiotics is paralleled in samples of other species that owe their resistance to beta-lactam antibiotics not to drug inactivation, but to a mechanism that as yet remains relatively ill defined. The term intrinsic resistance refers to all forms of increased minimum inhibitory concentration not due to drug

inactivation (Sabath, 1982). The examples of clinically important intrinsic resistance to the penicillins are seen in *Neisseria gonorrhoeae*, methicillin-resistant *S. aureus* (MRSA), methicillin-resistant coagulase-negative strains of staphylococci, carbenicillin-resistant *Pseudomonas aeruginosa*, many gram negative bacilli, enterococci, *Haemophilus influenzae*, and penicillin-resistant pneumococci.

Although studies have been done to determine the basis of intrinsic resistance in each of these species, the possibilities of detailed studies of intrinsic resistance are perhaps best in the study done by Sabath (1982) on the beta-lactamase negative segregants of methicillin-resistant strains of *S. aureus*.

c. Tolerance to the killing action

Tolerant staphylococci are those with a dissociation between inhibitory and killing action; strains in which the ration of minimum bactericidal concentration (MBC) divided by the minimum inhibitory concentration (MIC) is equal to or greater than 32. In practice, most normal strains of staphylococci show a ratio of MBC to MIC to be in the range of 1 to 4. Relatively few strains show a ratio around 32. Most tolerance strains will have ratios considerably in excess of 32. The problem with the study of tolerant staphylococci is the ability to show this trait in the laboratory. Because of the instability, the origin of tolerant strains appears to be in the patient, or possibly

animals, yet on prolonged study in the laboratory, the tolerance appears to disappear. In 1980, Bradley had presented evidence that a bacteriophage might play a role in transferring the tolerance trait from one strain to another.

Evidence indicates that each of the three forms of resistance is present in both *S. aureus* and coagulase-negative staphylococci. The features of the three forms are shown in Table 2.

Table 2**. A comparison of three types of beta-lactam antibiotic resistance of *S. aureus*

Characteristics	Type of beta-lactams Resistance		
	Beta-lactamase Mediated	Intrinsic	Tolerance
Minimum inhibitory concentration	Very high	High	Normal
Minimum bactericidal concentration	Very high	High	High
Limited to beta-lactam antibiotics	Yes	Yes	No
Approximate phenotypic expression	99.9%	$1/10^{n}$ (n=5)	$1/10^{2}$
Rate of growth of culture	Rapid	Slow	Rapid
Stability of resistance	Stable	Stable	Unstable*
Possible occurrence in hospital	80%-90%	1%-8%	44%
Clinical importance	Yes	Yes	Yes
Phage types	Many	Few	Many
Protein A	Common	Low	Not tested

* Storage of organisms at 4°C causes gradual resistance (tolerance), over a period from 4 to 12 months

** Modified from Sabath *et al.*, 1977

III. Methicillin-resistant *S. aureus* (MRSA)

Historical perspective

In 1950s, penicillinase-producing strains of *S. aureus* were so common that penicillin was becoming useless against staphylococcal infections. The introduction of methicillin, the first of the penicillinase-resistant semisynthetic penicillins, into clinical practice in 1959 and 1960 solved this problem, for the time.

Eventhough, strains of *S. aureus* resistant to methicillin were identified almost immediately after the use of this agent (Jevon, 1961), but these were generally regarded as laboratory curiosities of dubious clinical significance. In 1961 the first clinical failure caused by a methicillin-resistant strain scarcely was noted. In that study, the first three methicillin-resistant isolates of *S. aureus* were among 5,440 strains screened for methicillin resistance. Their methicillin minimum inhibitory concentrations (MICs) ranged from 3.1 to 25 μg/ml. These isolates also were resistant to antibiotics chemically unrelated to methicillin (Dowling, 1961).

The circumstances surrounding isolation of these three strains were noteworthy because they were typified those associated with outbreaks of methicillin-resistant staphylococci even today. The first isolate was from a patient with eczema who had been treated with penicillin. Two subsequent isolates came from an infected finger of a nurse and from the wound of a surgical patient whom she had tended. This occurrence of a multiple resistant strain in

a carrier recently treated with a beta- lactam antibiotic and subsequent nosocomial transmission literally at the hands of hospital personnel has become a familiar story (Chambers, 1988).

Resistance to methicillin in these original strains was heterogeneous. Only rare cell in the population expressed the resistance: 1 cell in 10^8 grew on agar containing 250 μg of methicillin per ml. The proportion of cells expressing this high level of resistance could be increased several fold by a single passage in methicillin. Unlike susceptible strains that had been selected for resistance to methicillin in the laboratory, which grew poorly and were avirulent, these naturally resistant strain showed normal growth and virulence (Know and Smith, 1961).

In 1963, the first major nosocomial epidemic of a methicillin-resistant strain of *S. aureus* was described (Stewart and Holt, 1963). The strain was isolated from an infant who had been treated with penicillin. Later on MRSA strains were also isolated from one nurse and 37 children in eight wards. Virulence of MRSA was reported in the case of the infant who became infected with the strain and died. Unlike previous three strains, this strain displayed more uniform growth in the presence of methicillin and was cross-resistant to cephalosporins, oxacillin, and cloxacillin.

Institutional outbreaks occurred in many European hospitals soon after methicillin was introduced in 1960. The responsible strains frequently became established as

significant endemic nosocomial pathogens, sometimes accounting for 30% to 50% of *S. aureus* isolates. In the same period, outbreaks of MRSA infection in hospitals in the United States were uncommon. However, between 1976 and 1981, outbreaks in 18 medical centers representing all geographic regions of the United States had been reported. Such reports indicated that MRSA was widely dispersed throughout the nation and that its prevalence as an endemic nosocomial pathogen was increasing (Thompson *et al.*, 1982).

In 1980, Peacock and co-workers reported the introduction and spread of a strain of MRSA at the University of Virginia Medical Center. During the 6 months after the index-care patient was admitted, 30 patients acquired the same strain of MRSA. Twenty-two of the cases were epidemiologically linked to a surgical intensive care unit.

Properties of methicillin resistance of MRSA

Heterogeneous resistance: The majority of MRSA strains are heterogeneous (Matthews and Stewart, 1984). It has been shown that only rare cells (1 in 10^4 to 10^8) expressed the resistance trait and grew in the presence of high concentrations of drug (e.g., 50 μg of methicillin per ml) and most cells appeared to be susceptible to relative low, therapeutically achievable concentrations of drug (e.g., 1 to 5 μg of methicillin per ml). Thus, heterogeneous strains could be considered to be composed of two populations of cells: relatively susceptible cells and

highly resistant cells (Seligmen, 1966). The characteristic of MRSA studied by Sabath (Sabath and Wallace, 1971 and Sabath *et al*, 1968) were shown that MRSA were slow growing, vary in size (somewhat larger than other staphylococci), heterogenicity of resistance (phenotype), frequent multiple drug resistance, usually penicillinase positive, dimorphic morphology (on solid medium), and difficult to phage type. MRSA may cause serious disease and death, cross-resistant to all beta-lactam antibiotics and be difficult to phage type.

Homogeneous resistance: A minority of the strains are homogeneous. The cells are uniform in expression of resistance and can grow in high concentration of drug. Thus, homogeneous strains are composed of a single population of cells, all of which tend to be highly resistant (Sutherland and Rollison, 1964).

In 1986," Hartman and Tomasz" have classified resistant strains into homogeneous and heterogeneous categories based on efficiency of plating, defined as the number of the colony-forming units (CFU) on drug-containing agar plates divided by the number on drug-free agar plates multiplied by 100%, at a concentration of 50 μg of methicillin per ml in tryptic soy agar, pH 7.0, at 37°c after 72 to 96 hours of incubation. For homogeneous strains, 1% or more of CFU grow; for heterogeneous strains, < 1% do so. Alterations in growth conditions may influence the pattern of resistance expressed by a strain.

There were some evidences that gene encoded for methicillin resistance of *S. aureus* might be encoded on a transposon (Trees and Iandolo, 1988). Strains that display this property produce a penicillin binding protein (PBP), designated PBP 2a or 2', that has low-affinity for beta-lactams. The demonstration that MRSA is on a new gene is another example of how molecular techniques can be used to study resistance. This was accomplished by cloning the resistance gene and the using it as a probe to show that methicillin-susceptible strains do not show any homology to this probe (Tesch *et al*, 1988 and Beck *et al*, 1986).

Clinical isolates of MRSA contain the methicillin resistance determinant *mec*, which confers an intrinsic resistance to all beta-lactam antibiotics. The *mec* determinant is located on additional DNA of unknown origin and nature and integrates into a specific site in the chromosome of *S. aureus*. The *mec* determinant codes for *mec A*, the structural gene for PBP 2a. This PBP 2a adds to existing PBPs of the cell and is the biochemical correlated to methicillin resistance (Ryffel *et al*., 1994).

Epidemiology and clinical role of MRSA infections

Numerous aspects of the clinical epidemiology of MRSA have made this organism a frequent focus of epidemiologic studies. In particular, MRSA is a common nosocomial isolate that can be readily transmitted from person to person and can establish an asymptomatic carrier state. As a clinical pathogen, MRSA can cause persistent

and recurrent bacteremia associated with occult sites of infection (Mulligan and Arbeit, 1991).

Since 1981, there has been a marked increase in the number of reported outbreaks of hospital acquired infections due to MRSA (Thornsberry, 1984, Boyce *et al*, 1981 and Schaefler *et al*, 1981). A notable feature of these strains is the case with which they spread, and so they are usually regarded as epidemic methicillin-resistant *S. aureus* (EMRSA). The outbreaks are most often confined to hospitals that have teaching affiliations with medical schools. This type of hospital has patients who are particularly prime to MRSA, they often receive numerous antibiotics and are treated in overcrowded contitions. In addition, there are special care units such as burn, surgical, pulmonary and trauma units in the hospital (Hunt *et al*.,1978).

S. aureus accounted for approximately 10% of all nosocomial infections and from 10 to 40% of all surgical infections (Cohen, 1986). Myers and Linnemann (1982) reported that MRSA accounted for 30% of all isolates of *S. aureus* from bacteremia and represented 50% of all nosocomial bacteremia due to *S. aureus*. There was a 60% mortality rate in patients with bacteremia, of which 44% of the death were directly attributed to the MRSA.

Immunocompromised, debilitated patients, or those with open wounds, are particularly at risk from infection with these strains and in some outbreaks death have

occurred, e.g., from septicaemia and endocarditis (Hone *et al.*, 1981). A detailed example of a recent study on MRSA outbreak was given by Boyce (1981) who described an epidemic strain of MRSA originating in a burn unit that rapidly spreaded throughout the hospital. A burn unit is certainly a fertile environment for MRSA. The open wound and the frequent dressing changes which often necessitate a dressing team or multiple persons; the simultaneous use of intraluminal devices such as intravenous catheters, endotracheal and nasogastic tubes, and foley catheters; plus the inherent immunosuppression of the burn patient, are all conductive to MRSA colonization. Obviously, nosocomial infection was related to the longer days of hospital infections, the resulting morbidity and mortality. The cost of care is always greater than that from the patients with underlying disease alone. Furthermore, the control of these outbreaks usually requires extraordinary measures involving closure of special units or surgical wards, and sometimes greatly disrupts the normal function of the hospitals.

Among the different sites of staphylococcal carriage in the human body such as nose, perineum, throat, skin and intestines, only the first two are considered to be primary sites for colonization and multiplication of those bacteria (Solberg, 1965). For practical purposes, the carriers are divided into three classes 1) persistent carriers, 2) persistent non-carriers, and 3) transient or intermittent carriers (Leedom *et al.*,1965 and Williams,

1963), which indicate a duration of the carriage. Although the persistent carriers are potentially more effective dispersals of the bacteria in hospital environment than the transient carriers, who easily change types of harbored staphylococci and easily disperse them, but both carriers are equally effective as a source of infection (Heczko *et al.*, 1973). The percentage of persistent non-carriers in normal populations estimated by several authors during the 1950-1960 such as predominance of *S. aureus* in hospital flora (Miller *et al.*, 1960, Duncan *et al.*, 1957 and Berquist, 1950) varied from 10 to 38 but never exceeded 40.

A little is known about the factors that make one person become liable to carry staphylococci repeatedly while another is never a carrier. There have been several speculations on the possible importance of human factors in determining carriage. Jacobs *et al.* (1961) reported that a nurse with minor deformities of the nose had more tendency to become carrier than nurses with no such abnormality. Heczko *et al.* (1973) indicated that some characteristics that influenced the carrier state were smoking habit and a presence of drying secretion in the nose. On the other hand the carriage was not related to age, sex and environmental factors.

Transmission of MRSA

Severely debilitated patients (especially surgical and burn patients) are most susceptible to MRSA infection. Factors associated with an increased risk of colonization and infection include prolonged (or prior) hospitalization, (previous) antibiotic therapy, invasive procedure, increased age, and the severity of concomitant diseases (Preheim *et al.*, 1987). MRSA has been introduced into hospitals via infected and/or colonized patients being transferred between health care facilities, particularly long-term care facilities, from community acquired organisms gaining entry into hospitals and by interhospital transport by physicians in academic training programs (Dickson and Czurylo,1988). The most important route of patient-to-patient transfer appears to be from the contaminated hands of hospital personnel to susceptible patients. Common sources of infection include the purulent discharge from actively infected lesions and nasal secretions from asymptomatic carries (Jawetz *et al.*, 1989). MRSA organisms remain viable on the hands of the health care workers for more than 3 hours but can be eliminated by simple handwashing (Standiford, 1987). MRSA has remained a major nosocomial pathogen in over 85% of the hospitals in the United States (Bacon *et al.*, 1987).

Controlling spread of MRSA

Ideally, all tests and procedures should be performed at the patient's bedside. A draped cart with

only essential items should enter the patient's room. All personnel in contact with the patient during the examination should wear protective barriers. Gloves are always required. Masks are required in cases of MRSA pneumonia or avoidance of coughs in the face by known carriers, and gowns are required when body contact may occur. If it is essential that a nonbedside examination be performed, the patient should be covered with a clean gown and/or sheet for transport,wear a mask (if indicated) and have all wounds covered. Information regarding the infection, its site and precautions should precede the patient to the procedure site. The prime controls for nosocomial transmission of MRSA are barrier precautions and strict hand washing after each patient contact (Shoop, 1991).

Active measurement follow to control epidemic cause by MRSA include

(1) isolation of all colonized patients.

(2) surveillance cultures to identify unrecognized colonized patients.

(3) surveillance cultures of personnel associated with infected and colonized patients.

(4) treatment of patients with infection using an active antistaphylococcal drug.

(5) treatment of the carrier state, both in patients and personnel,with the application of a topical antistaphylococcal antiseptic solution to the axilla and perineum.

and (6) at discharge, the charts of infected patients are flagged for identification so if they are readmitted, culture surveillance is performed immediately (Hunt *et al.*, 1988).

Treatment of MRSA infections

Treatment of MRSA infections should include the use of appropriate antibiotics, isolation of infected patients,the protection of high-risk patients from exposure to MRSA, and, if indicated, the identification and treatment of carriers. Infection control depended on the complete treatment of infections with adequate doses of effective antibiotics for a time sufficient to eliminate the strain. Staphylococci are difficult to eradicate and are notorious for developing resistance to antimicrobial drugs. Vancomycin,rifampicin and trimethoprim-sulfamethoxazole have all been used to treat MRSA infections. Each drug generally limits the infection but may not eradicate the organism, and there have been cases reported of resistance developing to each drug. Many different topical medication have been used with varying success to treat nasal carriers,including bacitracin ointment, hexa-chlorophane showers, chlorhexidine cream, vancomycin, and most recently mupirocin ointment (Cederna *et al.*, 1990).

IV. Epidemiological study of MRSA

Eventhough, a variety of techniques have been used to perform epidemiologic studies, only few have been extensively evaluated. Many epidemiologic analyses consist of demonstrating that the outbreak isolates are the same or similar for a particular characteristic. Some studies have further shown that the epidemic strain differs from a set of randomly obtained control isolates (Selander *et al.*, 1987). Since there is considerable genetic diversity among microorganisms, it is expected that small numbers of epidemiologically unrelated isolates would differ in numerous characteristics, not of which will be appropriate for more extensive studies. A much more stringent evaluation of a technique is to compare isolates that are epidemiologically unrelated but have been proven to be indistinguishable by one or more other approaches (Arbeit *et al.*, 1990 and Lawrence *et al.*, 1989). Epidemiologic typing systems for differentiating among strains of MRSA are phenotypic and DNA-based study.

4.1 Phenotypic study

Phenotypic characteristics, such as antimicrobial susceptibility and bacteriophage type, have been used widely in epidemiologic studies of MRSA. Phenotypic assays are inherently limited by the capacity of all microorganisms to alter unpredictably expression of the underlying genes (Wachsmuth,1985). Thus, isolates of the same genotype can exhibit either positive or negative

phenotype for a given characteristic (Mulligan and Arbeit, 1991).

a. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing is easy, rapid, readily available, and has been successfully employed to detect the introduction of a distinctive strain of MRSA (Archer and Mayhall, 1983). However, susceptibility patterns (which have almost no discriminatory power for methicillin-resistant *S. aureus*) have only limited utility in analyzing MRSA. Because many MRSA are similarly resistant to multiple antibiotics. Susceptibility patterns may vary among epidemic isolates because many antibiotic resistances are plasmid-borne and can be gained or lost by a strain during course of an outbreak (Gillespie *et al.*,1984). Nevertheless, antimicrobial susceptibility patterns may provide the first clue to the simultaneous presence of multiple different strain of MRSA (Mulligan and Arbeit, 1991).

The results from methicillin susceptibility test indicated that the variation in incubation temperature (30,35 and 37°C) had little effect on MICs and MBCs against MRSA (Peacock *et al.*,1981). However, the growth conditions used to propagate a heterogeneous population could affect the level of resistance observed. Growth of the organism at 30°C or in elevated sodium chloride levels enhanced the frequency of resistant cells, while growth at higher temperature (43°C) or at low pH suppressed the resistant

phenotype (Lorian, 1991). The level of methicillin resistance is influenced by osmolarity, temperature, growth medium, other external factors, and growth phase. These phenotypic variations, however, have to be clearly separated from resistance expression of highly resistant subclones or mutants formed by MRSA (Ryffel *et al.*, 1994).

b. Bacteriophage typing

Bacteriophage typing has been a mainstay in the epidemiologic analysis of *S.aureus* (Blair and Williams, 1961). The procedure is technically demanding and typically performed only by health departments and other reference laboratories. Because the assays can be poorly reproducible, the results are most reliable and useful when all isolates to be compared are tested simultaneously and detailed reports indicating both strong and weak reactions are provided. In many studies, a substantial percentage of MRSA strains have been showed to be the routine phage panels, and even among reaction strains, the procedure frequently has a poor discriminatory power, with many epidemiologically unrelated isolates having similar types. In 1989, Khalifa reported that by using this typing technique there was a significantly high percentage of nontypeable virulent strains of MRSA was isolated from patients and nasal carriers.

c. Capsular serotyping

The capsular polysaccharide serotyping system for *S. aureus* developed by Karakawa and Vann (1987) has not been proven to be useful in epidemiologic studies because 85 to 90% of all clinical isolates belong to only 2 of 11 capsular types. Most MRSA are capsular type 5 (Branger *et al.*, 1990 and Arbeit *et al.*, 1984).

d. Electrophoretic protein typing and immunoblots

The proteins and other bacterial products expressed by MRSA can be analyzed using widely available procedures based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The materials in the gel can be detected directly by staining (Gaston *et al.*, 1988) or indirectly by preparation of immunoblots (Mulligan *et al.*, 1988). Alternatively, the proteins can be internally radiolabelled and detected by autoradiography (Stephenson *et al.*, 1986). All strains are typeable by these approaches, which typically identify variation among independent isolates and thus provide good discriminatory potential. However,due to the complexity of the patterns resolved, comparisons among multiple strains can be difficult and significance of small differences is uncertain (Gaston *et al.*, 1988).

The various electrophoretic implementations may provide different result due to differences in the methods for extracting the materials from the organisms,

the methods detecting proteins or the choice of antibody for developing immunoblots (Mulligan and Arbeit, 1991).

e. Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MLEE) detects changes in the electrophoretic mobilities of individual soluble metabolic enzyme. Such variations, referred to as electromorphs, identify allelic alterations in the chromosomal genes encoding the enzymes. The distinctive combinations of electromorphs are designated as electrophoretic types (ETs). The evaluation of multiple, constitutive metabolic enzymes ensures that all isolates are typeable. Because of the specialized technical procedures involved, MLEE has been confined to a limited number of research laboratories.

4.2 DNA-based study

The variability inherent in discriminating among strains by phenotypic assay has stimulated interest in DNA-based typing system. It is increasingly apparent that there are considerable complexities in using these tools to address epidemiologic questions.

a. Plasmid DNA analysis

It is difficult to define the epidemiology of outbreaks with MRSA by antibiotic susceptibility testing because many different strains of MRSA were suceptible to similar antibiotics. Therefore, it is necessary to search

for other markers to define unique strain. Phage typing has been used traditionally to define patterns of transmission of *S. aureus*, but this method is time consuming and may not differentiate strains (Zierdt *et al.*, 1980). Plasmid profile analysis has been represented as the first DNA-based technique to be used in epidemiologic studies of *S. aureus* (McGowan *et al.*, 1979). Plasmid DNA can be isolated rapidly and the electrophoretic systems involved are now commonplace. Several investigators have suggested that this technique may be appropriate for many clinical laboratories. Plasmid profiles of MRSA isolates have been proven to be useful in a number of studies,but the approach has a limitation in the study on MRSA that contain one or no plasmid (Kozarsky *et al.*, 1986 and Archer and Mayhall, 1983).

More refined plasmid analyses have been obtained by digesting the plasmids with restriction enzymes and resolving the resulting restriction fragments by agarose gel electrophoresis (Zuccarelli *et al.*, 1990 and Coia *et al.*, 1988). This approach clearly provides greater reproducibility and discriminatory power, but still suffers from the variability related to the extra chromosomal nature of the plasmids (Mulligan and Arbeit, 1991).

b. Restriction enzyme analysis of chromosomal DNA

In conventional restriction enzyme analysis (REA), chromosomal DNA is digested with restriction enzymes

that have frequent recognition sites , and consequently generates a large number of relatively small restriction fragment. Typing profiles are obtained by analyzing the digests using constant field agarose gel electrophoresis, which separates such restriction fragments by size (Mulligan *et al.*, 1990). Bacterial isolates of the same species can have different profile because of nucleotide sequence variations that alter the distribution of restriction sites along the chromosome.

A limitation of this technique is that the profiles represent hundreds of unresolved and overlapping bands and are thus exceedingly intricate. Consequently, distinguishing and interpreting small variations in restriction profiles is often difficult and may be unreliable (Van Ketel, 1988). A recent study of coagulase negative staphylococci demonstrated that differences in the plasmid content of the isolates caused variations in the REA profile of the total cellular DNA (i.e., chromosomal plus plasmid). To develop a reproducible classification of isolates, the investigators used an automated gene scanner to subtract out the plasmid bands and determine the specific chromosomal DNA patterns. This issue may be relevant to studies of MRSA, which frequently carry plasmids.

c. Southern blot analysis of chromosomal DNA

The DNA restriction fragments separated by electrophoresis can be transferred from the agarose gel

onto a nitrocellulose membrane, to which the DNA binds tightly (Southern, 1975). The membrane, referred to as a Southern blot, can be hybridized with specific DNA probes to identify those restriction fragments carrying sequences complementary to the probe. Variations in the number and size of such fragment(s) are referred to as restriction fragment length polymorphisms (RFLPs) and reflected variations in the nucleotide sequences within or flanking the loci of interest. All strains carrying the sequence of interest can be typed by this technique and the profiles detected are highly reproducible and can be accurately compared. However, the technique involves considerable technical effort and currently remains restricted to research laboratories (Mulligan and Arbeit, 1991).

Ribotyping is based on Southern blot analysis of the RFLPs associated with the ribosomal RNA operons (Stull *et al.*, 1989). All bacteria carry these operons and are, therefore, typeable. The approach has been successfully used in recent epidemiologic studies of several gram-negative species (Altwegg *et al.*, 1989). Since bacteria typically have multiple, separate copies of the ribosomal RNA operons, the RFLP profiles are comprised of multiple distinct bands and provide relatively good discriminatory power. Nevertheless, most studies have observed that epidemiologically unrelated isolates occasionally have the same profile.

Southern blot analyses of the RFLPs associated with other chromosomal loci, including virulence factors and antimicrobial resistance genes, also have been used for epidemiologic studies of MRSA (Storrs *et al.*, 1988 and Mulvey *et al.*, 1986). However, the variations in Southern blots hybridized with probes derived from virulence factors should be interpreted with caution.

d. Pulsed field gel electrophoresis of chromosomal DNA

Pulsed field gel electrophoresis (PFGE) is derived from conventional, constant field agarose electrophoresis but can resolve molecules orders of magnitude larger (Smith *et al.*, 1986). Restriction endonucleases that cut the chromosomal DNA only infrequently can be used to digest the bacterial genome into a limited number of relatively large fragments. Thus, the *S. aureus* chromosome can be digested in approximately 20 fragments,all of which can be resolved by PFGE as discrete bands,thereby providing a distinct restriction profile representing the entire chromosome in a single gel (Patel *et al.*, 1989). All isolates are typeable by PFGE, and the profiles are highly reproducible. The preparation of suitable DNA is more time consuming and technically demanding than for conventional REA studies, and some but no all implementations of PFGE require expensive specialized equipment (Carle *et al.*, 1986 and Chu *et al.*, 1986). In each of these studies, the reproducibility and

discriminatory power of the technique was excellent, typically exceeding other phenotypic or genotypic assessment.

The comparative characteristics of the typing systems for MRSA has been shown in Table 3.

Table 3* Characteristics of typing systems for MRSA

Typing system	Proportion of Strains Typeable	Reproducibility	Discriminatory Power	Laboratory Avaiability
Phenotypic Methods				
-Antimicrobial susceptibilities	All	Good	Poor	Clinical
-Bacteriophage typing	Variable	Fair	Poor	Service
-Whole cell electrophoresis	All	Good	Unknown	Research
-Immunoblot	All	Excellent	Good	Research
-Multilocus enzyme electrophoresis	All	Excellent	Unknown	Research
Genotypic Methods				
-Whole plasmid analysis	Most	Fair	Fair	Service
-Plasmid restriction enzyme analysis	Most	Excellent	Good	Service
-Chromosomal restriction enzyme analysis	All	Good	Unknown	Research
-Ribotyping	All	Excellent	Unknown	Research
-Pulsed field gel electrophoresis	All	Excellent	Unknown	Research

* Modified from Mulligan and Arbeit, 1991