CHAPTER III

LITERATURE REVIEW

1. Staphylococcus

The genus is named after the Greek term, 'staphyle'', that means a bunch of grapes to describe these grape-like cocci (21). This name is appropriate because the cellular arrangement of these gram-positive cocci resembles a cluster of grapes. According to Bergey's Manual of Systemic Bacteriology, the genus *Staphylococcus* consists of gram-positive cocci , 0.5-1.5 μm in diameter occuring singly , in pairs and in tetrads , and characteristically dividing in more than one plane to form irregular clusters. The cell wall contains peptidoglycan and teichoic acid. Cell wall teichoic acids of staphylococci maybe poly(polyolphosphate) , poly(glycerolphosphate-glycosyl phosphate) or poly(glycosylphosphate) , depending on the species. Glycerol or ribitol or both occur as typical components of poly(polyolphosphate) teichoic acids. Substituents of these teichoic acids may include N-acetylgalactosamine , glucose and N-acetylglucosamine. Most strains grow in the presence of 10% NaCl and in a temperature range from 18°C to 40°C.

Metabolism is respiratory and fermentative. Carbohydrate and amino acids are utilized as carbon and energy sources. The main product of glucose fermentation is lactic acids and in an aerobic condition the main products are acetic acid and CO_2. Most species require an organic source of nitrogen (amino acid and certain B group vitamins). Others can use $(NH_4)_2SO_4$ as a sole source of substrate nitrogen. Staphylococci are susceptible to lysis by lysostaphin but are resistant to lysis by lysozyme under standard condition (20). Lysozyme is an enzyme that cleaves the glycan strands between N-acetylmuramic acid and N-acetylglucosamine that both found in the cell wall of staphylococci and micrococci. At certain concentration of lysozyme , most micrococci are susceptible , whereas , staphylocci are resistant that

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the reason can not clearly defined. Lysostaphin is an enzyme that lyses glycine-glycine bonds. Staphylococci have the glycine-containing interpeptide linkages that make them susceptible to lysostaphin , but micrococci do not have these linkages. Members of the genus staphylococci have a DNA G+C content of 30 to 39 mol%. The respiratory chain of staphylococci contain only a- and b- cytochromes , except *S. caseolyticus* , *S. lentus* , *S. sciuri* and *S. vitulus* that contain a-, b- and c- type cytochromes. And staphylococci contain unsatured polyoprenoid side chains in their menaquinones (21).

A total of 27 species and 7 subspecies are currently recognized in the genus, with 14 species and 2 subspecies found on human (20) (Table 3-1) *S. aureus* is the most commonly associated with human infections and is the most virulent and best-known member of this genus. *S. epidermidis* and *S. haemolyticus* also commonly found on human. However, *S. aureus* is the only species that found in human that produces the enzyme coagulase and is referred as coagulase-positive staphylococci, thus all other species are commonly referred to as "coagulase-negative staphylococci" (20)

Staphylococcal physiology and structure

Staphylococcal structure and function are outlined in Table 3-2 and Figure 3-1.

1. Capsule: Staphylococcal capsule is a loose-fitting, polysaccharide layer that occasionally found *in vitro*, but is believed to be more commonly present *in vivo*. The capsule is antiphagocytic that interfering with the interaction between underlying teichoic acid-peptidoglycan complex and complement, which is activated the alternative pathway (22). It is useful for bacteria to adhere to the catheters and other synthetic materials (example; graft, prosthetic valves and joints and shunts). This property is important for avirulent coagulase-negative staphylococci. The capsule also increases the ability of the organism to spread to enhance the virulence of the pathogen in tissue infection (22).

2. Peptidoglycan: The peptidoglycan layer is the major structural component of the staphylococcal cell wall. It composed of peptide cross-linked glycan chains (Figure 3-2). The glycan chains are built wiith approximately 10 to 12 alternating subunits of N-acetylmuramic acid and N-acetylglucosamine. N-acetylmuramic acid are attached by tetrapeptide side chains , and glycan-linkage are then cross-linked with peptide bridges between the side chains. This layer has endotoxin-like activity, can attract polymorphonuclear leukocytes (abscess formation), and can activate complement.
3. Protein A: The surface of most *S. aureus* strains but not the coagulase-negative staphylococci is uniformly coated with protein A. This protein is covalently linked to the peptidoglycan layer and is an immunologically active substance in the cell wall. It has the unique affinity to bind the Fc receptor of immunoglobulin IgG_1, IgG_2 and IgG_4, thus effectively preventing the antibody-mediated immune clearance of the organism. Extracellular protein A can also binds to and aggregates IgG molecules, formingly immune complexs with subsequent consumption of complement.
4. Teichoic acid: Teichoic acids are complex, phosphate-containing polysaccharides bound to the peptidoglycan and cytoplasmic membrane. These polysaccharides are species-specific. In *S. aureus*, these are ribitol teichoic acid with N-acetylglucosamine residues (polysaccharide A), but glycerol teichoic acid with glucosyl residues (polysaccharide B) are present in *S. epidermidis*. Staphylococci use cell wall teichoic acid to attach the mucosal surface, because of their specific binding to fibronectin . Although the teichoic acids are poor immunogens , a specific antibody response is stimulated when they bound to peptidoglycan. The monitering of this antibody response has been used to detect systemic staphylococcal disease.

5. Clumping factor: Most strains of *S. aureus* contain clumping factor of bound coagulase in the outer membrane. This protein binds fibrinogen and causes clumping or aggregating of the staphylocci.
6. Cytoplasmic membrane: The staphylococcal cytoplasmic membrane contains a complex of protein, lipid and a small amount of carbohydrate that forms an osmotic barrier for the cell and provides an anchor site for the cellular biosynthesis and respiratory enzymes.

Natural habitats

Staphylococci are widespread in nature. Though they are mainly found living on the skin, skin gland and mucous membrane of mammals and birds. Sometimes they are found in the mouth, blood, mammary gland and intestinal, genitourinary and upper respiratiry tracts of these hosts (21).

Several staphylococci species preferentially colonizes specific niches of the human body. *S. aureus* prefers the anterior nares. From 20% to 40% of the general human population carry this species in their nares; in the hospital personnel, it is carried by 50%, 70% and 90% of physicians, nurses and ward attendants, respectively (19).

The other staphylocci found on human and other primates include *S. epidermidis*, *S. capitis*, *S. caprae*, *S. hominis*, *S. lugdunensis*, *S. saccharolyticus*, *S. warneri*, *S. pasteuri*, *S. haemolyticus*, *S. auricularis*, *S. sapprophyticus*, *S. cohnii*, *S. xylosus* and *S. simulans* (20).

Colonization of neonatates with *S. aureus* is more common in the anterior nasopharynx. Adherence to the mucosal epithelium is regulated by receptors for staphylococcal teichoic acids. Approximately 15% of normal healthy adults are persistent nasopharyngeal carriers of *S. aureus*, with higher incidence of carriage reported in hospitalized patients, medical personnel, individuals with eczematous skin diseases, and in individuals who regularly use needles illicitly (drug abuser) or for

medical reasons (example: insulin-dependent diabetics , patients receiving allergy injections or those undergoing hemolysis).

Because staphylococci are carried on the skin surface and in nasopharynx, shedding of the bacteria is common and is responsible for many hospital-acquired infections. Staphylococci are susceptible to high temperature as well as to disinfectants and antiseptic solutions. However, the organisms are capable of survival on dry surfaces for long periods. Transfer of the organisms to a susceptible individual can be either by direct contact or by means of formites. (example : contaminated clothing or bed linens). Therefore medical personnel must use proper handwashing techniques to prevent transfer of staphylococci from themselves to patients or among patients (20).

2. *Staphylococcus aureus* : colonization and infections

The skin and nares of infants are colonized by *S. aureus* within a few days after birth, the carrier rate then drops, only to increase during childhood to the adult rate of approximately 30%. The organisms are most commonly found in the anterior nares and on skin and mucous membrane. The staphylococcal colonization presumbly involves an interaction between specific staphylococcal adhesins and cellular receptors. Endogenous infection is probably caused by the organisms in the nares (23).

S. aureus causes diseases by either production of toxin or direct invasion and destruction of tissue. The clinical manifestations of some staphylococcal diseases are almost due to toxin activity (example; staphylococcal scald skin syndrome, toxic shock syndrome, and staphylococcal food poisoning), whereas other diseases involve proliferation of the organisms with abscess formation and tissue destruction (Figure 3-3) (20).

The hallmark of invasion disease is abscess formation. Most often the abscesses are superficial (24), furuncle or boil, which begins as an infection of sebaceous glands on hair shafts. At full development, the center of the abscess shows liquefaction necrosis (pus) consisting of dead bacteria, phagocytes, and fluid;

surrounded by firm wall of fibrin, inflammatory cells and viable bacteria (23). In some cases the furuncle develop into burrowing lesions consisting of a number of interconnecting abscesses (carbuncle). Serious deep-seated disease usually is not seen in healthy people, but may occur in those debilitated by disease, malnutrition, extensive surgical procedures, and immunosuppression. Although *S. aureus* is probably the most common cause of bacterial infection in human, most infections are minor and superficial ; serious infections occur much more commonly in association with a predisposing condition include injury to normal skin (example ; traumatic wound , burns , and surgical incisions) , prior viral infections (example ; measles and influenza) , leukocyte defects , deficiency in humoral immunity , presence of foreign bodies (example ; sutures , pacemakers , and IV catheters) , altteration of normal flora through use of antimicrobial agents to which *S. aureus* is not susceptible , and presence of miscellaneous illnesses including diabetes mellitus , alcoholism , coronary artery disease and various malignant tumors (19). It is therefore not surprising that serious staphylococci disease is most often the result of hospital-acquired (nosocomial) infection (23).

No single component or product of *S. aureus* has been shown to be the primary determinant of either the initiator or the progression of invasive lesions. Therefore that a number of factors maybe involved. These include the intraphagocytic survival of few cells, inhibition of phagocytosis by capsules and protein A , and the toxic effects on cells and tissues of hemolysin, leukocidin and enzymatic products (24).

3. <u>Mechanisms of pathogenicity</u>

Proposed mechanisms of pathogenicity of staphylococci include encapsulation, toxin and enzyme production, colonization of specific host sites and slime production (Figure 3-4) (20). It should also be repeated that numerous host factors contribute to disease. Staphylococcal infections are usually associated with a breach in the normal host defense mechanisms ranging from injury to normal skin, to leukocyte defects

(congenital or associated with various conditions such as diabetes or malignancy), to the presence of an indwelling medical device.

1. Encapsulation

The certain strains of *S. aureus* producing capsule that inhibit phagocytosis of the phagocytes.

2. Toxins

S. aureus primarily produce toxins. They included hemolysins , leukocidin , enterotoxin , TSST-1 , and exfoliatin. Of these toxins, the last three appear to be most clearly associated with disease.

2.1 Hemolysins

Four different protein hemolysis of *S. aureus* are now recognized. All cause β-(clear) hemolysis, but they differ in red blood cell (RBC) species specificity and in their mechanism of action (Table 3-3) (25). A single strain may produce more than one. The hemolysins are antigenic proteins, activity is neutralized by specific antiserum. The tissue cells may also be damaged : some of hemolysins produce local necrosis and are lethal for experimental animals.

2.1.1 Alpha toxins (α-hemolysin)

Alpha toxin is the principal hemolysin of human strains of *S. aureus* that is genetically encoded on both the bacterial chromosome and a plasmid. It is most active against rabbit RBCs ; human RBCs are not susceptible , but human platelets and tissue culture cells are affected. Alpha toxin is known to damage smooth muscle as well as to kill skin cells (dermonecrotic) and is lethal when injected into mice or rabbits. This toxin is also toxic for human macrophages and platelets and causes degranulation of polymorphonuclear leukocytes through disruption of their lysosomes. Its reaction with erythrocytes results in a prelytic release of K^+ followed by complete lysis of the cell. The specific receptor on the erythrocyte membrane is a sialoglycoprotein. Alpha hemolysin is secreted as a water-soluble monomer of 34,000 daltons, which rearranges, on contact with a membrane. This cylinder not only

transverses the membrane but projects above the surface. There is a pore of 2 or 3 mm on the surface. This structure resembles the lytic C5b-9 (m) complex of complement.

2.1.2 Beta toxin (β-hemolysin)

Beta toxin is produced commonly by animal strains but by only 10% to 20% of human isolates. This toxin is a heat-labile protein that is toxic for a variety of cells, including erythrocytes, leukocytes, macrophages and fibroblasts. It is "hot-cold" hemolysin : its lytic effects are not fully developed unless mixtures with blood (or blood agar cultures) are placed at low temperature following inocubation at 37°C. Beta hemolysin is also called sphingomyelinase C of M_r 30,000 , that is activated by Mg^{2+} but not by Ca^{2+} ; it splits sphingomyelin into N-acylphingosine and phosphorylcholine. Sheep, human and guinea pig erythrocytes contain decreasing amounts of sphingomyelin and are decreasingly susceptible to beta hemolysin. The beta hemolysin is cytotoxic for a variety of tissue culture cells, and large doses are toxic for experimental animals. Beta toxin, together with alpha toxin, is believed to be responsible for the tissue destruction and abscess formation characteristic of staphylococcal diseases and the ability of *S. aureus* to proliferate in the presence of vigorous inflammatory response.

2.1.3. Gamma toxin (γ-hemolysin)

Gamma toxin consists of two basic protein subunits acting in concert. Rabbit, human, and sheep RBCs are susceptible, whereas 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.

2.1.4. Delta toxin (δ-hemolysin)

Delta toxin consists of aggregates of low molecular weight subunit of M_r 5,000 , and has a broad range of lytic and cytotoxic activity probably due to a nonspecific detergent-like action. Damage appears to results from the reaction of its hydrophobic amino acids with the phospholipids in the cell membrane. Delta toxin is produced by most human strains of *S. aureus*, and it acts on various cell types

including RBCs , leukocytes , cultured mammalian cells and bacterial protoplasts and is not species specific.

2.2 Panton-Valentine (P-V) leukocidin

Most *S. aureus* strains produce P-V leukocidin that acts only on human and rabbit polymorphonuclear cells and macrophages. This toxin consisted two components, F and S. S (electrophoretically slow moving) component first binds to ganglioside GM1 (the cholera toxin receptor) and activates an endogenous membrane-bound phospholipase A2. The products then bind F (fast moving) component, including a K^+-specific ion channel in the membrane and hence cytolysis. Both components are required for activity, and antibody to either component neutralizes toxicity. Bacteria producing leukocidin have increased resistance to phagocytosis.

2.3 Exfoliatin (Epidermolytic toxin , ET)

Exfoliatin causes a variety of dermatologic lesions. This relatively heat-stable and acid-labile protein of M_r 24,000 is produced by approximately 5% of *S. aureus* strains , mostly of phage group II. Two distinct forms of exfoliative toxin (ETA and ETB) have been identified (Table 3-4) (20). ETA, produced by strains of various phage types, is chromosomal-coded, whereas, ETB , occurring primarily in strains of phage group II , is plasmid-coded. Many strains produce both types. The toxin acts by cleaving the stratum granulosum of the epidermis, probably by splitting desmosomes that link the cells of this layer.

2.4 Enterotoxins

Enterotoxins , which are exotoxins , cause food poisoning (intoxication) that is characterized by severe diarrhea and vomiting. Enterotoxins are excreted by about one third of all clinical isolates of staphylococci. This toxins are resistant to hydrolysis by gastric and jejunal enzymes and are stable to heating at 100°C for 30 minutes. The enterotoxins are antigenically distincted into 6 types ; A , B , C1 , C2 , D , and E. Enterotoxin A is most commonly associated with disease. Enterotoxin C and

D are associated with contaminated milk products, and enterotoxin B is associated with staphylocoeccal pseudomembranous enterocolitis.

Diarrhea is related to their ability to enhance fluid secretion in the small intestine. The staphylococcal enterotoxins can act as polyclonal T-cell mitogens apparently as a result of their ability to bind class II molecules on antigen-presenting cells.

2.5 Toxic Shock Syndrome Toxin-1 (TSST-1)

TSST-1, formerly called pyrogenicexotoxin C and enterotoxin E , is and exotoxin secreted during growth of some strains of *S. aureus* and can reproduce most of the clinical manifestations of toxic shock syndrome (TSS) in an experimental rabbit model. TSST-1 has not been found in staphylococcal isolates from all patients with TSS, however, most of these non-TSST-1 producing isolates are reported to produce enterotoxin B. The presence of TSST-1 in species other than *S. aureus* remains controvesal. However, it is now clear that coagulase-negative staphylococci and group A streptococci can cause TSS.

3. Enzymes

3.1 Coagulase

Culture filtrates of *S. aureus* clot the plasma of many animal species , as a result of production of the clotting factor , coagulase : the standard marker for *S. aureus*. All coagulase-producing staphylococci are , by definition , *S. aureus* and , as a result , coagulase production is considered the best laboratory evidence for the potential pathogenicity of a staphylococcus. Seven antigenically different extracellular coagulase have been identified from various staphylococci, but the only pathogenic role suggested for the enzyme is the coating of the organisms with fibrin to inhibit their phagocytosis.

Clotting requires interaction with a coagulase-reacting factor (CRF) in plasma, which is probably a derivertive of prothrombin : a coagulase-CRF complex converts fibrinogen to fibrin. Although the same fibrinopeptides are released as with

thrombin, the process differs from normal clotting in that the multiple accessory factors, including Ca^{2+}, are not required and the clot is more friable and does not retract.

In addition to extracellular coagulase , *S. aureus* also possesses a bound coagulase that causes the organisms to clump when mixed with plasma. This clumping factor can convert fibrinogen directly to fibrin and does not require the presence of CRF for activity.

3.2 Catalase

All staphylococci produce catalase , a protective enzyme that catalyzes the conversion of toxic hydrogen peroxide , which accumulates during bacterial metabolism or is released following phagocytosis , to water and oxygen.

3.3 Hyaluronidase

This enzyme hydrolyzes hyaluronic acids, the acidic mucopolysaccharides present in the acellular matrix of connective tissue. Hyaluronidase facilitates the spread of *S. aureus* in tissues. More than 90% of *S. aureus* strains produce this enzyme.

3.4 Fibrinolysin

This enzyme, also called staphylokinase, is produced by virtually all *S. aureus* strains and can dissolve fibrin clots. This enzyme link streptokinase and urokinase. It causes clot dissolution by activating conversion of the proenzyme plasminogen to the fibrinolytic enzyme plasmin. Staphylokinase is distinct from the fibrinolytic enzymes produced by streptococci.

3.5 Lipases

All *S. aureus* and more than 30% of coagulase-negative staphylococci produce several different lipases. As their name implies, these enzymes hydrolyze lipids, which is essential for the survival of staphylococci in the sebaceous areas of the body. It is believed that these enzymes are required for invasion of staphylococci into cutaneous and subcutaneous tissues and the formation of superficial skin infections

(example ; furuncles [boils] , carbuncles). The lipases are assayed by testing for the ability to produce opacity on egg yolk agar or to split Tween detergents.

3.6 Nuclease

Another marker for S. aureus is the presence of a thermostable nuclease. The role of this enzyme in pathogenesis is unknown. This enzyme has both endonuclease and exonuclease activity on both DNA and RNA producing 3' nucleotides.

3.7 Penicillinase

When penicillin was introduced, more than 90% of staphylococcal isolates were susceptible. However, resistance quickly developed and was primarily mediated by the production of penicillinase (β-lactamase). The widespread distribution of this enzyme is ensured by its presence on transmissible plasmids.

4. Adherence Mechanism

S. aureus demonstrate a selective adherence to nasal epithelial (mucosal) cells. The adherence of *S. aureus* is significantly greater for carriers of this species than for noncarriers.

5. Slime production

Slime is a viscous extracellular glycoconjugate produced by *S. aureus* and certain CNS. Several studies indicate that slime production plays an important role in disease caused by CNS. Of particular significance is the fact that it allows these organisms to adhere to the smooth surfaces such as catheters. Slime also appears to inhibit neutrophil chemotaxis and phagocytosis and to inhibit the antimicrobial action of the glycopeptide antimicrobial agents, vancomycin and teichoplanin. Slime also appears to have other effects on immune function. It has been suggested that factors in slime may act by interfering with the ability of T-helper cells to produce lymphokines that are necessary for the stimulation of other cells in the immune system.

Table 3-1 **Staphylococcus Species Found on Humans**

Species	Cause of human disease
S. aureus	**Common**
S. epidermidis	**Common**
S. saprophyticus	**Common**
S. haemolyticus	**Common**
S. lugdunensis	**Uncommon**
S. schleiferi	**Uncommon**
S. saccharolyticus	**Rare**
S. warneri	**Rare**
S. hominis	**Rare**
S. auricularis	**Rare**
S. xylosus	**Rare**
S. simulans	**Rare**
S. capitis	**Rare**
S. capitis* ssp. *ureolyticus	**Rare**
S. cohnii	**Rare**
***S. cohnii* ssp. *ureolyticum*.**	**Rare**

From : Murray *et al.*, Medical Microbiology 2nd edition , p166

Table 3-2 Cell structure and functions of Staphylococci

Structure	Function
Capsule	**-Inhibits opsonization and phagocytosis** **-Protects from C'-mediated leukocyte destruction**
Peptidoglycan	**-Osmotic stability** **-Stimulates production of endogenous pyrogen** **-Leukocyte chemoattractant** **-Inhibits phagocytosis and chemotaxis**
Protein A	**-Binds IgG1 , IgG2 , IgG4 Fc receptors** **-Inhibits opsonization and phagocytosis** **-Leukocyte chemoattractant** **-Anticomplementary**
Teichoic acid	**-Regulates cationic concentration at cell membrane** **-Receptor for bacteriophages** **-Attachment site for mucosal surface receptors**
Cytoplasmic membrane	**-Osmotic barrier** **-Regulates transport into and out of cell** **-Site of biosynthetic and respiratory enzymes**

From : Murray *et al.*, Medical Microbiology 2nd edition , p.167

Table 3-3 Classification of hemolysins by type of erythrocyte lysed

Type of Hemolysin	Red Blood Cell Lysed	Usual Source
alpha	Calf, rabbit, sheep	Human
beta	Human, ox, sheep (effective only as hot-cold lysis, i.e., 37°C for 1-2 hr followed by overnight in refrigerator)	Animal
gamma	Guinea pig, horse, human, ox, rabbit, rat, sheep	Human
delta	Guinea pig, horse, human, rabbit, rat, sheep	Human

From : Volk *et al.*, Essentials of Medical Microbiology 4th edition, p368

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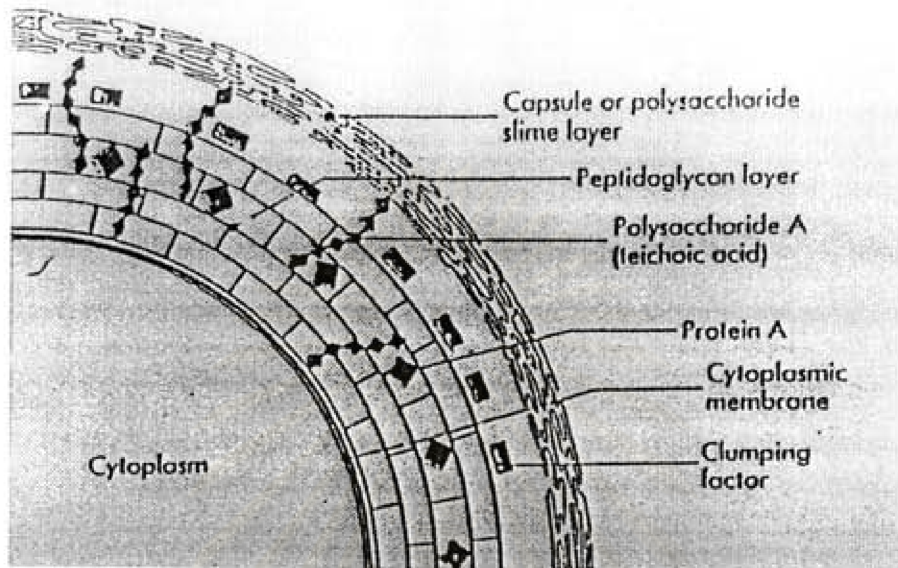
Table 3-4 **Characteristics of exfoliative toxins**

Properties	Exfoliative toxin A	Exfoliative toxin B
Size	24,000 daltons	24,000 daltons
Temperature tolerance	Stable (100°C , 20 min)	Labile (60°C , 30 min)
EDTA treatment	Inactivated	No effect
DNA	Chromosomal	Plasmid

From : Murray *et al.* , Medical Microbiology 2nd edition , p.169

Figure 3-1 Staphylococcal cell wall structure



From : Murray *et al.* , Medical Microbiology 2nd edition , p.167

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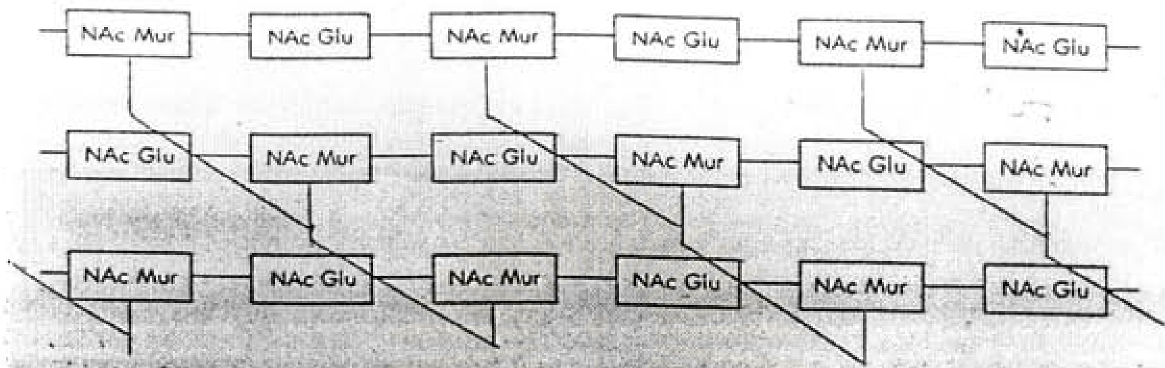

<u>Figure 3-2</u> The peptidoglycan layer consists of three integral parts. The glycan chains are built with 10 to 12 alternating N-acetylglucosamine (Nac Glu) and N-acetylmuramic acid (Nac Mur) subunits jointed with b-1,4 glycosidic bonds. Vertical tetrapeptide side chains (L-alanine , D-glutamine , L-lysine , D-alanine) are linked to the muramic acid subunits , and the side chains are in turn cross-linked with diagonal intrapeptide bridges. The glycan-chains in *S. aureus* are cross-linked with pentaglycine bridges attached to L-lysine in one tetrapeptide chain and D-alanine in an adjacent chain.

From : Murray *et al.* , Medical Microbiology 2nd edition , 6.168

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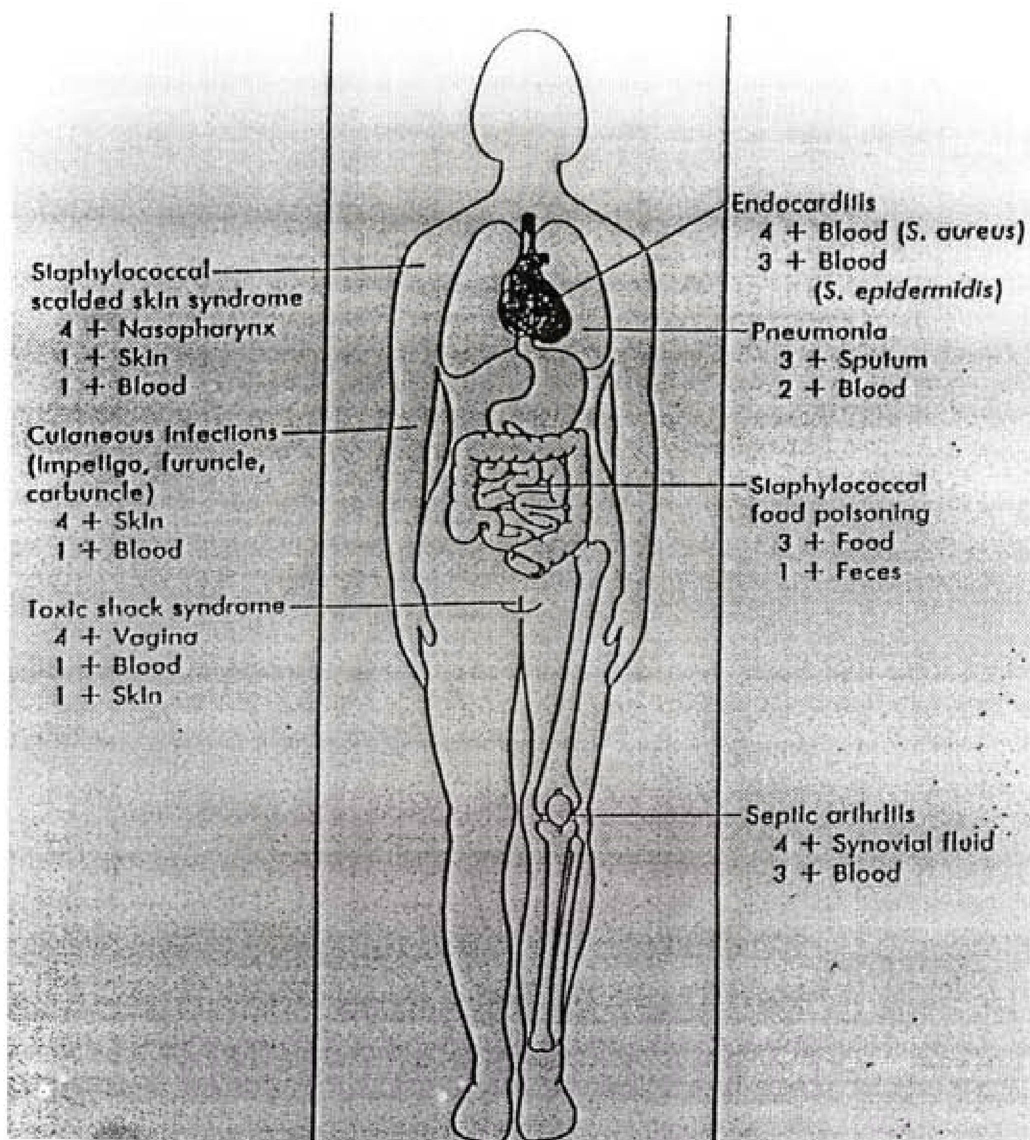
Figure 3-3 **Staphylococcal disease. Isolation of staphylococci from sites of infection.** ***1+***, **<10% positive cultures ;** ***2+***, **10% to 50% positive cultures ;** ***3+***, **50% to 90% positive cultures ;** ***4+***, **>90% positive cultures.**



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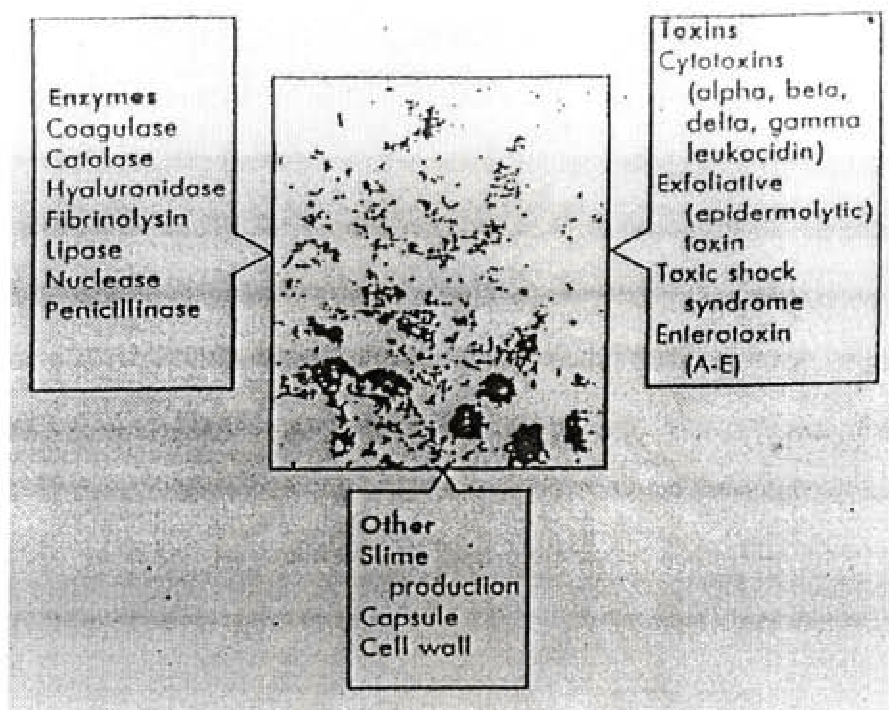
From : Murray *et al.*, Medical Microbiology 2nd edition , p.172

Figure 3-4 **Staphylococcal virulence factors.**



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4. Methicillin-resistant *Staphylococcus aureus* (MRSA)

4.1 Historical prespective

Benzylpennicillin (pennicillin G) was introduced to solved the staphylococcal infection problem in the early 1940s, but the continuously using of this drug caused the selection of resistant strains, which produced penicillinase (β-lactamase). By 1948, the prevalance of resistant strains had seriously reduced the value of benzylpenicillin. (2,27) By the end of 1950s, *S. aureus* became resistance to all available systemic antibiotics , including erythromycin , streptomycin , and tetracycline , and its virulence remained undiminished. The nosocomial staphylococus of phage type 80/81 became untreatable with antibiotics. The problem seems to be solving in 1960, with the advent of β-lactamase-resistant semisynthetic penicillin such as methicillin (28), oxacillin , nafcillin , dicloxacillin ,and floxacillin. Cephalosporin, cephaloridine and cefazolin were introduced a few years later, thus adding to the β-lactam antibiotics available to treat staphylococcal infections. Although modern nosocomially acquired strains of staphylococcal infections remain not only common but also life-threatening throughout the world. (29)

Strains of methicillin-resistant *S. aureus* (MRSA) were detected in 1961, shortly after methicillin came into clinical use. (30-32) However, such strains (classic methicillin-resistant *S. aueus*) which occurred sporadically were resistant only to β-lactam antibiotics, and never caused any major problem. 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.

The circumstances surrounding isolation of these three strains are noteworthy because they typify 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 multiply 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. (26) These original strains to that resistance to methicillin were heterogenous. Only rare cells in the population expresses the resistance : 1 cell in 10^8 cell 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. (26) Unlike susceptible strains that had been selected for resistance to methicillin in the laboratory, which grew poorly and were avirulent , these naturally resistant strains showed normal growth and virulence. (34)

In 1963, the first major nosocomial epidemic of methicillin-resistant strains of *S. aureus* was described. (33) The strain was firstly isolated from infant who had been treated with penicillin. Then these strains were isolated from one nurse and 37 children in eight wards. A child that treated with methicillin and streptomycin for a wound infection became infected with this strain and died. Unlike previous three strains, this strain displayed more uniform growth in the presence of methicillin and was cross-resistant to cephalosporin , oxacillin , and cloxacillin.

In the late 1970s, there were resistant strains appeared in Australia. These strains have received considerable attention, because the entire characteristics of these organisms were different from those of the MRSA strains detected in 1961. They are resistant to other antibiotics in addition to beta-lactam compounds. They have also spread around the world while retaining their virulence. These strains now pose a serious problem to patients as well as their care gives. (2)

The rapidly spread of the organisms throughout the world have created therapeutic problems for physicians , management difficulties for nurses , confusion for infection control practitioners , and resource-allocation uncertainties for hospital administrations. The outbreaks of MRSA have been reported from medical centers all over the world. (6-8,32,35-37)

4.2 Properties of methicillin resistance

4.2.1 Heterogenous resistance : The major properties of methicillin resistance is heterogenous. (26) Only rare cells (1 in 10^4 to 10^8) express the resistance characteristics and grow in the presence of high concentration of drug (eg. 1 to 5 μg of methicillin per ml). Thus , the heterogenous strains are composed of two population of cells : highly resistant cells and relatively susceptible cells.

4.2.2 Homogenous resistance : The minority of the strains are homogenous. The cells are uniformly express resistance in high concentration of drug. Thus, homogenous strains are composed of a single population of cells that tend to be highly resistant.

In 1986, Hartman and Thomasz have classified resistant strains into heterogenous and homogenous groups based on efficiency of plating that defined as a number of 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 30°C after 72 to 90 h of incubation. For heterogenous strain, $<$ 1% of colony forming unit grow; for homogenous strain, 1% or more do so. The alteration of conditions may influence the pattern of resistance expressed by strains.

4.3 Mode of transmission

There are two major mechanisms for introduction of MRSA into an institution : the most common is the admission of infected or colonized patients who serves as a reservoir (32,41-43) ; less frequent is the arrival of a colonized or infected health care worker who disseminates the organism directly. (44) Chronically colonized healthcare workers can disseminate the organism directly, but such occurrence is less common. (41-42) MRSA could be community organism according to the study by Moreno *et al.* (1995) (12) who demonstrated that MRSA might be present on admission to the hospital in patients. They found that the total number of MRSA isolates increased during 21-month period that correlated with the increase in the

number of community cases. Thus, the patients in such study could possibly receive MRSA from community.

Several studies suggested that the principal mode of transmission within an institution was from patient to patient via the transiently colonized hands of hospital personnel who acquired the organism after direct patient contact or after handling contaminated material. (12,32,41)

Transmission via the inanimate environment maybe important for special population such as those in burn or intensive care units where extensive environmental MRSA contamination occur. (45) MRSA have been recovered from many sites including floors, hydrotherapy tubs, linens, air vents, medical equipment, hospital furnishing, patient charts, tourniquets, and topical medication. However, environmental contamination may be common but not contribute to transmission in the long-term care setting. (41)

4.4 Controlling spread of MRSA

The ideal goal for any institution would be the eradication of MRSA from all patients and the inanimate environment. The environment in patient's room should be clean. There should be only essential items in patient's room. Medical personnel (physicians, nurses, ward attendants) should wear protective barrier while contact the patients. Gloves, masks and gowns must be required, especially in case of MRSA pneumonia or avoidance of cough or body contact with patients. The patients should be covered with clean gowns and all wound are covered. The primarily way of effectively preventing the spread of *S. aureus* is hand washing after each patient contact (38), and use disposable gloves during all contacts. (39)

The control of MRSA epidemic include:

1) surveillance culture to identify unrecognized colonized patients
2) surveillance culture of colonized staff members
3) isolation of all colonized patients
4) treatment infected patients with active antimicrobial agents

5) treatment all colonized staffs and patients with antistaphylococcus antiseptic solution. (2)

4.5 <u>Treatment of MRSA infecton</u>

Treatment of MRSA infection include the use of appropriate antibiotics, isolation of infected patients, the protection of high-risk patients from exposure of MRSA, and identification and treatment of carriers. The efficiency of infection treatment depend on the use of effective antimicrobial agent for the time that sufficient to completely eradicate MRSA. Intravenous vancomycin is the antibiotic of choice for treatment MRSA infections. (40-41) The occasional failure of patients with severe MRSA infection to respond to vancomycin therapy and the side effect associated with vancomycin have led to an ongoing search for alternative agent. Rifampin and trimethoprim-sulfamethoxazole are another drug-of --choice to treat MRSA infection.

For nasal colonization, the use of topical antimicrobial substances, such as bacitracin, tetracycline or chlorhexidine results in the suppression of nasal colonization, as opposed to its eradiation. (2)

4.6 <u>Mechanisms of Methicillin Resistance in *Staphylococcus aureus*</u>

Methicillin is a beta-lactam antibiotic, which is not hydrolyzed by small quantities of staphylococcal penicillinase. However, producers of a high amount of penicillinase tend to resist. The effect of methicillin though partial hydrolysis of antibiotic, making the susceptibity of the strain to methicillin borderline between susceptible and resistant. (46-47) Another mechanism of methicillin resistance involves the alterations of penicillin-binding proteins (PBPs), the peptidoglycan synthesis enzymes which are targets of beta-lactam antibiotics. Methicillin sensitive *S. aureus* (MSSA) has four PBPs, PBP1 to 4, and MRSA has an additional PBP, PBP2a , which is recognized as a major cause of its high resistance to beta-lactam antibiotics. (48-49) A decrease in the penicillin-binding affinities of the PBPs, an increase in the production of PBPs, or a combination of both are considered to account for some cases of raised resistance to beta-lactam antibiotic. MICs of methicillin to those producing

an excessive amount of penicillinase increase only marginally (MIC = 2-4 μg/ml) compared to that of susceptible *S. aureus* strain (MIC < 2), while an *S. aureus* clinical strain with an MIC of 12.5 μg/ml , and with increased production of PBP2 has also been found. (50) Thus, the most significant and most prevalent mechanism of methicillin resistance is associated with the acquisition of the additional PBP, designated PBP2a (or PBP2') , by staphylococci. (42;47-49)

PBPs are enzymes that catalyze the cross-linking reaction between peptidoglycan polymers, which is one of the final steps in bacterial cell wall assembly. Thus, beta-lactam antibiotics are potent inhibitors of cell wall synthesis. Susceptible strains of *S. aureus* produces four or five PBPs that are distinguished by molecular weight : 85,000 (PBP1) , 80,000 (PBP2) , 75,000 (PBP3) , 70,000 (PBP3') , and 45,000 (PBP4). (26)

The specific functions of staphylococcal PBPs as transpeptidases, endopeptidases , and carboxypeptidase have not been defined completely. Some PBPs essential for cell growth and survival, and others are not. The essential PBPs are deduced from experiments in which conditional lethal mutants lacking PBPs are constructed and from experiments in which binding of beta-lactam antibiotics to PBPs is correlated with their inhibitory of lethal concentrations *in vitro*. PBP4, which probably serves as a transpeptidase *in vivo*, is not essential. (26) Wada *et al*. (1998) (48) reported that PBP1 of *S. aureus* probably contributes to the synthesis of peptidoglycan septum. Further investigation by immuno and/or fluorescence staining of PBP1 to show its localization in dividing cells of S. aureus will provide more information about the function of PBP1. Both PBP2 and PBP3 are essential. PBP3 have been hypothesized to be the primary transpeptidase for the incorporation of newly synthesized peptidoglycan. (53)

PBP2a has a low binding affinity for beta-lactam antibiotic. PBP2a is expressed in addition to the normal complement of resident PBPs and remains the sole unsaturated PBP in the presence of beta-lactam antibiotics, alone capable to maintain

cell wall integrity. (26,51) Hartman *et al.* (1986) (52) interpreted the presence of PBP2a in the heterogenous and thermosensitive heterogenous cultures as an indication that these strains not only carry the structural gene of PBP2a, but they also transcribe and translate this protein. PBP2a is also produced in homogenous strains of MRSA and MSSA. (54)

PBP2a is highly conserved. Limited proteolysis of PBP2a from unrelated strains of *S. aureus* and coagulase-negative staphylococci, whether homogenous or heterogenous, generates remarkably similar peptide fragments. In contrast to other staphylococcal PBPs, which generally bind beta-lactam antibiotic at low concentrations, PBP2a binds beta-lactam antibiotic at high concentrations. Presumably PBP2a can substitute for essential PBPs when these have been saturated by drug and can perform the function necessary for cell wall assembly. (26)

In some strains, PBP2a is inducible by beta-lactam antibiotics and its production differs according to growth conditions. (55) PBP2a encoded by the chromosomally located gene *mecA*. This *mecA* gene and $\geq$ 30 kb of flanking DNA are unique to MRSA ; and there are no allelic equivalents in methicillin susceptible strains. (47-48,51) The *mecA* gene was cloned by Matsuhashi *et al.* by selecting a tobramycin-resistant *Escherichia coli* clone having a genomic fragment of MRSA in which sequences encoding methicillin and tobramycin resistance were linked. Introduction of the *mecA* gene confers methicillin resistance on MSSA strains, and transposon mutagenesis of the gene renders highly MRSA strains susceptible to methicillin. (57) Therefore, the principal role of the *mecA* gene in the expression of methicillin-resistance has been well established. Nucleotide sequencing study has revealed that the gene is composed of two regions having homology in two district gene ; the 5' region of the gene has homologous to the penicillinase gene (*blaZ*) of *S. aureus* , and the rest of the gene has homology to *E. coli* PBPs2 and 3. (45,60) Based on this, Song *et al.* reported that *mecA* gene emerged in the past by homologous recombination event between PBP and beta-lactamase gene in an as yet unknown organism. (58) This gene

is found in practically all MRSA (MIC $\geq$ 16 μg/ml at 30-35 °C or if oxacillin MIC $\geq$ 4 μg/ml (60), and is absent in MSSA clinical strains. Thus, the presence of *mecA* gene is the hallmark for the identification of MRSA in clinical laboratories, and this gene is also known to be widely distributed in the species of genus *Staphylococcus* including MRSA and methicillin-resistant coagulase-negative staphylococci (MRC-NS), but it has not been found in any other genus of bacteria.

Inducibility and differences in amounts of PBP2a produced might appear to account for heterogenous expression of resistance, but there are no correlation exists between inducibility or amount of PBP2a and the pattern of resistance. PBP2a production can be constitutive and the strain can be heterogenous. The cells can be induced to produce large amount of PBP2a and yet promptly lyse upon exposure low concentration of antibiotics. (26)

Homogenous and heterogenous strains are similar in binding affinity of beta-lactam antibiotic to PBP2a, and this ability is not altered by conditions, such as addition of NaCl to the medium, that enhance expression of resistance. (59)

4.7 Epidemiological studies of MRSA infection

Numerous reports of clinical epidemiology of MRSA have made this organism to be a focus of epidemiological studies. MRSA becomes common nosocomial isolate that can be transmitted from person to person, and can be colonized in any sites of body as carrier state. In the clinical role, MRSA is the major pathogen, producing pneumonia, empyema, osteomyelitis, lung abscess, enterocolitis, bacteria with sepsis, and wound infection. (61) The high mortality rates for patients with MRSA infections have been reported from several centers. (45,62)

Between the year 1984 to 1985, the epidemic methicillin-resistant *S. aureus* (EMRSA) in England caused an increase in the infection and the number of colonized patients when compared to those occurred between the year 1969 to 1983, and this strain was rapidly spread. A change in infection control procedures was usually required to control the EMRSA. The other MRSA (OMRSA) were still

encounter between 1984 and 1986, but did not spread or required change in infection control procedures. The EMRSA is rarely spread between ward, unless the patients have been transferred. Only one possible way of interward spread occurred when a staff member was infected with EMRSA that implicated 'transient' MRSA carriage in nursing staff, following close contact to the patient. Agency staff working on a number of wards on the same day may thus spread the organisms from ward to ward within the hospital. (63)

EMRSA have a special ability to colonized patients and staff. (63) In 1989, Cookson *et al.* proposed that the transient or short term carriage in nurses probably resulted in transfer of the EMRSA among patients and staff decontamination should be considered following a period of cohort nursing of EMRSA patients, especially if staff members are shortly to nurse unaffected patients. (64)

Patients who have high risk of MRSA infection are those with cutaneous wound with antibiotic administration, lower respiratory infection, and those who are immunocompromised hosts. These patients are the potential source of MRSA transmission and can directly contact to another and cause airborne transmission. (11)

The outbreaks of MRSA cause important focus at critical care unit, especially, burn unit. The colonized or infected patient is a reservoir and the medical personnel' hands were vehicles of MRSA. And the spread can be occurring by direct contact. (11,18)

Burn units as a source of MRSA infection. The burn wound is particularly susceptible to bacterial colonization and infection due to the physical disruption of the normal skin barrier and the accompanying reduction of cell mediated immunity. Experiment in mice suggest that MRSA appears to be less virulent in the normal subject but equally virulent in the immunocompromised. *S. aureus* can survive intracellularly in polymorphonuclear leukocytes (PMNs) , the function of which is known to be decreased in burn patients. (65)

MRSA may be introduced into burn units when burn patients are transferred from one hospital to another (45) or maybe transmitted from nonburn patients to burn patients within the same hospital. (15) Spread of MRSA within burn units may occur through airborne and direct contact transmission by transiently colonized personnel.

Boyce *et al.* (15) suggested that the occurrence of MRSA infections may lead to increase transmission of the organisms from non burn patients to burn patients and the control of the infection in this unit may reduce the spread of the organism to patient without burns. Once MRSA infection has become established in burn unit, this ward often becomes a persisting reservoir of the organism within institution.

In 1983, Rutala *et al.* studied the environment in burn unit during the MRSA outbreak. They found that there are 16%, 31%, and 40% of MRSA contaminated in air, elevated surfaces and floor surfaces, respectively. The infected patient rooms were higher in environmental contamination level than the adjacent work area, and there was ample opportunity for contamination of personnel through the inanimate environment in this unit. (13)

Lacey *et al.* (1986) (66) studied the properties of MRSA in burn unit. They found that multi-resistant clone of *S. aureus* has colonized in the burn unit. It possess many features typical of other recent isolates of MRSA; sensitivity to unsaturated fatty acids, survival in serum and plasma or desiccation. Containment of the strain within the unit may result from deficiency in protein A and/or clumping factor.

In 1989, the Swedish researchers described two outbreaks of MRSA between two burn units. They found that the MRSA epidemics in the units seem to start with infected patient or infected staff. Carrier among the staff was not shown to be significance in this study. The spread of MRSA from the unit to other parts of the hospital was prevented by early identification of colonized patients and by restricted patient and staff movement. (67)

Farrington *et al.* (1990) (14) studied the MRSA outbreak in burn unit at new Hong Kong hospital. They found that the MRSA were spread by a combination of the airborne, transient hand-borne and environmental-contaminant. The burn patients became a source of hospital outbreak, and the most outbreaks were often occurred in the burn unit, which lack private room for patients. Additional, the heavy environmental contaminant by MRSA is the important source of prolonged staff carriage.

Phillips *et al.* (1992) (68) studied MRSA in burn and trauma unit, and they concluded that hand-washing and the wearing gloves, gowns, and masks can limit cross-contamination among patients and among patients and staff members. It has be shown that patients can be harboring MRSA at the time of admission and the multiple antibiotic resistance can be developed among organisms that reside in the patients through plasmid-mediated transfer of resistant gene. Excessive use of the synthetic penicillins and second- and third-generation cephalosporins has selected for survival of these organisms.

In Burn center of Oman, the proportion of patients developing MRSA infection rose from 48% in 1995 to 52.7% in 1996. Vancomycin was the antibiotic to which most cultures were susceptible, but partial resistance was reported due to the very low susceptibility observed in 1.04% of isolates in 1995 and 1.1% of isolates in 1996. However, prudent use of antibiotics , particularly vancomycin should be a major prevention strategy against emergence of vancomycin-resistant *S. aureus* (VRSA)

In Thailand , Danchaivijitr *et al.* (1995) (10) studied the outbreak of MRSA in burn unit at Siriraj hospital. The incidence of MRSA in burn patient during their study was as high as 14 in 29 patients (48.3%), and these MRSA first appeared in these patients during the first and second week of admission. The epidemic of MRSA infections persisted despite all control measures resulting in temporary closure of the ward.

MRSA is not only epidemic in burn unit, but also in other ward ; such as surgical intensive care unit (45,71-72) , long term care facility (70) , trauma center (73). In long term care facility, Muder *et al.* (1991) (70) found that 32 patients of 197 patients were persistent carriers of MRSA, and 25% of MRSA carrier had an episode of staphylococcal infection. They concluded that the colonization of MRSA in the anterior nares predicted the development of staphylococcal infection in this unit and most infections arise from endogenous carried strains. Thus, the MRSA colonization indicated a significantly greater risk for infection than MSSA colonization.

4.8 Epidemiological typing of MRSA

Typing of MRSA isolates can be used to identify the potential sources of different MRSA strains, to evaluate the role of human carriers , and to differentiate epidemic from endemic strains. (41) Reducing the number of MRSA infections by detecting and eradicating the sources of the organisms or by interrupting their path of transmission to the patients is an important goal and requires the ability to distinguish individual strains. (74) Improved typing systems are important for implementing appropriate infection control measures and for the clinical management of MRSA infections, particularly in evaluating the efficacy of therapy for infected or colonized patients. The ideal typing system should be rapid , inexpensive , technically simple , and readily available. (75)

Each typing system should have five criteria : typeability , reproducibility, discriminatory power , ease of interpretation , and ease of use.

1) Typeability refers to the ability of the test to provide an unambiguous result for each isolate examined; nontypable isolates are those that produce a null or ambiguous result.

2) Reproducibility refers to the ability of a technique to produce the same result when a strain is tested repeatedly.

3) Discriminatory power defines the ability of the test to discriminate between unrelated isolates. This discrimination is important because some typing

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systems tend to group organisms into a few broad groups, while others divide collections of isolates into many small clusters, often subdividing groups of isolates that are tightly linked by epidemiological data

4) Ease of interpretation and use are also key issues for many techniques. The greater the expertise that is required to discern differences between strains, the less likely the techniques is to be readily accepted by clinical microbiologists who generally lack such expertise. (76)

However, the choice of the right typing method is often difficult because of inherent theoretical and technical limitation. (78) Epidemiological typing systems for differentiating among strains of MRSA are phenotyping and genotyping.

4.8.1. Phenotyping

Phenotypic characteristics, such as antibiogram and phage typing, have been widely used in epidemiological studies of MRSA. However, because these methods detect phenotypic variation that is difficult to relate to allelic variation at specific gene loci, they have not provided the information on frequencies of alleles. (77) Isolates of the same genotypes can exhibit either positive or negative phenotype. Details of each main typing methods including advantages and disadvantages are described briefly in the following section.

a. Antimicrobial susceptibility patterns

In epidemiological investigations, antibiogram typing is a simplest and traditional typing method used to distinguish between individual strains. It is readily available, easy to perform, and relatively inexpensive. However, one drawback of the method is that markers of antibiotics resistance are often carried by labile or movable genetic elements (e.g., plasmids or transposons) whose selection of expression may depend on environmental condition. They may be gained or lost over time. (18,79) Antibiogram typing worked reasonably well , but only when zone diameters and not the categorical interpretations of susceptible , intermediate , or resistant were used as strain markers. (76) Thus, antibiogram typing is considered to

have poor discriminatory power and is used by microbiologists only in the first instance for rapid screening of the similarities between different clinical isolates

Blanc et al. (1994) (79) reported that the good results of antibiogram typing in their study were probably due to these following reason : i) the usual qualitative antibiogram analysis was refined by adding quantitative measurement of inhibition zone around antibiotic disks , ii) the five antibiotics used (i.e. erythromycin , clindamycin , co-trimoxazole , gentamicin , and ciprofloxacin) had been selected because they were mostly chromosomal markers which were unlinked and varied among the MRSA strains isolates in our hospital ; and iii) the analysis was restricted to isolates of a well-defined epidemiological setting. Although some genetically unrelated strains show great similarity by antibiogram typing, this appeared to be relatively infrequent and may be offset by the speed, availability , and the relatively high discriminatory power of this technique. The method could easily be adapted to the epidemiological setting of the other hospitals by changing or adding antibiotics to increase the technique's potential for discrimination (73) and redefining the cut off value to fit the particular hospital and laboratory setting.

Although antibiogram is valuable, especially in routine laboratories , as a first-line screening method to determine strain relatedness. It may allow quick and early recognition of a previously defined epidemic strain in a particular hospital setting. (83)

b. Bacteriophage typing

This method has been used widely for typing *S. aureus* for many years, however, its limitation are clearly recognized, a substantial number of *S. aureus* isolates can not be typed by phages. (76,80-81) This technique also requires maintenance of a large number of phage stocks and propagating strains, laborious, and it characterized isolates on the basis of a phenotypic marker that has poor reproducibility. (16,82) Whatever bacteriophage typing is not a cost-effective method

of typing *S. aureus* for most clinical laboratories, particularly since other available methods can be used to type a broader range of other microorganisms. (76)

c. Capsular typing

Capsular typing has only a very low power of discriminating between *S. aureus* strains. This method is not detected individual strains or clonal population. (78)

d. Electrophoretic protein typing and immunoblots

The proteins and other bacterial products expressed by MRSA can be analyzed using the procedure based on sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The materials in the gel can be detected directly by staining or indirectly by preparation of immunoblots. Additionally, the protein can be also labeled by radioisotope and detected by autoradiography. All strains can be typeable by these approaches, which typically identify variation among independent isolates and provide good discriminatory potential. However, due to the complexity of the patterns resolved, comparison among multiple strains can be difficult and significance of small differences is uncertain.

The various electrophoretic implementations may provide different result due to differences in the method for extracting the material from the organisms, the methods detecting proteins or the choice of antibody for developing immunoblots.

e. Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MLEE) has shown to be the powerful tool for population genetic studies of various bacteria, including *S. aureus*. (84) Isolates are characterized by the relative electrophoretic mobilities of a large number of water-soluble cellular enzymes. (77) The enzyme binding patterns generated by starch gel electrophoresis during MLEE were easy to interpret, but the overall analysis required application of sophisticated algorithms and computer

software not readily available in most laboratories. This makes this technique inaccessible to most clinical laboratories. (76)

4.8.2 Genotyping (DNA-based study)

DNA-based typing of bacterial strains is based on the principle that epidemiologically related bacterial isolates have genetic feature that distinguishes them from other epidemiologically unrelated strains. Because MRSA strains probably originate from a single clone or at least a few strain types , the techniques used to type MRSA must be particularly discriminatory. Suitable typing procedures should combine typeability , good discriminatory potential , easily interpretable results and practicality. (85)

Molecular typing systems can identify different strains within a species, generating data useful for taxonomic or epidemiological purposes. A frequently observed fault of typing systems is their lack of reproducibility: most typing systems do not provide a definitive strain identification, which is usually due to the variability of the technique and the lack of large databases containing fragment patterns from a wide variety of organisms to which unknowns can be compared. (86)

a. Plasmid analysis

Plasmid analysis was the first DNA-based method to be applied to *S. aureus* and it has been used in a number of outbreak investigations (16,81,87-90) This techniques have been reported to be superior to phage typing of MRSA. (88) This technique offers only moderate reproducibility (76,82) and has limitations that some strains, which unrelated to the outbreak may have the epidemic profiles. The potential usefulness of plasmid digestion profiles to bacterial epidemiology depends upon the degree to which four requirements are satisfied.

i) The bacterial strains under study must contain plasmids

ii) Plasmids must be sufficiently diverse so that independent isolates, not recently derived from a common progenitor , are likely to carry distinctly different plasmid DNA.

iii) Differences between plasmids must be distinguishable by the fragments that appear after restriction endonuclease digestion.

iv) Methods for plasmid profiling must be rapid, reproducible, inexpensive, and excessible to clinical laboratories. (89)

This technique also has several drawback as following (91) : 1) not all pathogenic strains have plasmids , 2) plasmids are exchange and lost readily (76,81,84,88) , 3) plasmids are subject to rearrangements , and 4) some specific plasmid isolation protocols may limit the size of recoverable plasmids and thereby preclude the isolation of large R-factors.

b. Restriction enzyme analysis of chromosomal DNA

In the conventional restriction enzyme analysis, chromosomal DNA is digested with restriction enzymes which have frequent recognition site , and gains a large numbers of relative small restriction fragments. Then analyze the digested profiles by constant field agarose gel electrophoresis, which separates the restriction fragment by size. The different profiles of the bacterial isolates from the same species can occur because of the nucleotide sequence variations that alters the distribution of the restriction sites along the chromosome. The results show a large number of fragments that are close together on an agarose gel and must be difficult to analyze. (16,91)

c. Southern blot analysis of chromosomal DNA

The DNA restriction fragments separated by electrophoresis can be transferred from agarose gel onto nitrocellulose membrane which the DNA binds tightly. This membrane can be hybridized with specific DNA probe to identify the restriction fragments carrying sequences complementary to the specific probe. The variation in the number and size of such fragments are referred to as restricted fragment length polymorphisms (RFLPs) and reflected variations in the nucleotide sequences within or flanking the loci of interest. All strains carrying these sequences of interest can be typed by this technique.

Ribotyping is based on Southern Blot analysis of the RFLPs associated with the ribosomal RNA operons, which carry by all bacteria. Therefore all strains are typeable. The random DNA mutations alter the distribution of restriction sites within and adjacent to the loci being probed. Such changes are relatively infrequent within the bacterial rDNA operons and other coding sequences, but are most common in noncoding flanking regions The most appropriate criterion for interpreting ribotype banding patterns remains unresolved. (76)

d. Polymerase Chain Reaction (PCR)

PCR becomes more widespread in clinical laboratories (75,83-85,92-94). This technique is highly sensitive to detect outbreak-related strains, however, it failed to exclude the nonrelated isolates from the cluster.(76) The PCR assay appears to be more reliable than routine susceptibility testing and as accurate as DNA probe hybridization. The PCR assay is simpler, more rapid test for detection of the MRSA-PBP gene than Southern Blot hybridization. (92) PCR assay could be particularly useful in outbreaks where rapid control of MRSA is required and in countries where high incidence of clonal MRSA strains are observed in hospitals. But their sensitivity is not enough. (94) Even PCR-based typing methods are clearly faster and easier to perform, but this technique has less discriminatory power than Pulsed-field Gel Electrophoresis. (85)

e. Pulsed-Field Gel Electrophoresis (PFGE)

In recent years, there has been much interest in chromosomal genotyping of MRSA. The method is attractive because of its potential to type all organisms. However the difficulties in interpretation of the results of digestion with restriction enzyme which cut at frequent sites led to examination of alternative approach. One of these has been to prepare chromosomal DNA in agarose blocks, which minimise, shearing, and then to digest it with rare cutters. The large fragments are pulsed electrically into the agarose. (95)

The development of PFGE enabled large fragments of DNA (> 9000 kb) to be separated. Variations of this technique include contour-clamp homogenous electric field (CHEF) and field inversion gel electrophoresis (FIGE). (95) PFGE is multidirectional, continually changing the location of the positive charge. The DNA molecules respond by continuously reorienting their directions of migration through the agarose gel. In addition, electrical pulses of different duration favor the reorientation of different sized DNA molecules. (96)

PFGE demonstrated the best discrimination, although this method is the most time consuming when compares to the other typing method, taking excess of 48 h to obtain result. Several publication have already confirmed the value of PFGE in *S. aureus* typing , reproducibility and discrimination between unrelated clones (16-18,39,74,76,78,82,85-86,88,97-102)

PFGE has been proposed as a good typing technique for epidemiological studies of most species. Profiles types generated by this technique appear stable and reproducible. Chromosomal stability makes this technique suitable for the long-term follow-up of epidemic strains of MRSA. (74)

Two main advantages of using PFGE are : i) it had universal *S. aureus* strain typing ability and ii) it was better than other typing techniques at distinguishing epidemiologically related strains from unrelated strains. Because of these advantages, at present the Center of Disease Control and Prevention (CDC) uses PFGE in epidemiologic investigation of *S. aureus*. (82)

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