1.1. Staphylococci and staphylococal infections

1.1.1. Genome and virulence determinants of staphylococci

*Staphylococcus aureus* is one of the major causes of community-acquired and hospital-acquired infections. It produces numerous toxins including super-antigens that cause unique disease entities such as toxic-shock syndrome and staphylococcal scarlet fever, and has acquired resistance to practically all antibiotics. Whole genome sequences of two related *S. aureus* strains (N315 and Mu50) were determined by shot-gun random sequencing in year 2001 by Kuroda M. *et al.* (2001). N315 is a meticillin-resistant *S. aureus* (MRSA) strain isolated in 1982, and Mu50 is an MRSA strain with vancomycin resistance isolated in 1997 (Kuroda M. *et al.* 2001). The staphylococcus genome is composed of a complex mixture of genes many of which seem to have been acquired by lateral gene transfer. Most of the antibiotic resistance genes are carried either by plasmids or by mobile genetic elements including a unique resistance island. Three classes of new pathogenicity islands have been identified in the genome: a toxic-shock-syndrome toxin island family, exotoxin islands and enterotoxin islands. In the latter two pathogenicity islands, clusters of exotoxin and enterotoxin genes were found closely linked with other gene clusters encoding putative pathogenic factors. The remarkable ability of *S. aureus* to acquire useful genes from various organisms is through the observation of genome complexity and evidence of lateral gene transfer. Repeated duplication of genes encoding super-antigens is responsible for capability of *S. aureus* to infect humans and eliciting severe immune reactions (Kuroda M. *et al.* 2001). Whole genome sequences of MW2, a strain of community-acquired MRSA, were determined by shot-gun cloning and sequencing in year 2002 by Baba T. *et al.* (2002).
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MW2 strain carries a range of virulence and resistance genes that is distinct from those displayed on the chromosomes of extant *S. aureus* strains. Most genes are carried by specific allelic forms of genomic islands in the MW2 chromosome. The combination of allelic forms of genomic islands is the genetic basis that determines the pathogenicity of medically important phenotypes of *S. aureus*, including those of community-acquired MRSA strains (Baba T. *et al.* 2002). The genetic plasticity of *S. aureus* plays the important role in the evolution of many virulent and drug-resistant strains. Two disease-causing *S. aureus* strains were sequenced by Holden M.T. *et al.* (2004) (Figure 1.1). Two *S. aureus* strains were isolated from distinct clinical settings: a recent hospital-acquired representative of the epidemic methicillin-resistant *S. aureus* EMRSA-16 clone (MRSA252), a clinically important and globally prevalent lineage; and a representative of an invasive community acquired methicillin-susceptible *S. aureus* clone (MSSA476). The study was carried out to explore the mechanisms of evolution of clinically important *S. aureus* genomes and to identify regions affecting virulence and drug resistance. *S. aureus* produces a wide variety of exoproteins that contribute to its ability to colonize and cause disease in mammalian hosts. Nearly all strains secrete a group of enzymes and cytotoxins which includes four hemolysins (alpha, beta, gamma, and delta), nucleases, proteases, lipases, hyaluronidase, and collagenase. The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth. Some strains produce one or more additional exoproteins, which include toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins, exfoliative toxins and leukocidin. Each of these toxins is known to have potent effects on cells of the immune system, but many of them have other biological effects as well. Their primary function in vivo may be to inhibit host immune responses to *S. aureus*. TSST-1 and the staphylococcal enterotoxins are also known as pyrogenic toxin
super-antigens (PTSAgs). Two former names for TSST-1 were staphylococcal pyrogenic exotoxin C and staphylococcal enterotoxin F (Dinges, M.M. et al. 2000).

1.1.2. Staphylococcal infections

Among staphylococci, *S. aureus, S. epidermidis*, and *S. saprophyticus* have the greatest pathogenic potential and diversity. Disease causation by *S. aureus* is a very complex process and probably involves large numbers of factors, both cell associated and secreted (Dinges, M.M. et al. 2000). The spectrum of *S. aureus* infections includes toxic shock syndrome, food poisoning, meningitis as well as dermatological disorders ranging from minor infections and eczema to blisters and scalded skin syndrome. Pneumonia and bacteraemia account for the majority of MRSA serious clinical infections, but intra-abdominal infections, osteomyelitis, toxic shock syndrome, food poisoning, and deep tissue infections are also important clinical diseases (Haddadin A.S. et al. 2002). The most common life-threatening manifestation of *S. aureus* infection is bacteremia. *S. aureus* bacteremia is a common feature of a spectrum of conditions ranging from uncomplicated bacteremia to fixed endovascular infection, such as endocarditis. The mortality rate associated with *S. aureus* bacteremia remains significantly high in patients with complications (Tan T.Y. et al. 2001). *S. aureus* has a particular affinity for establishing infection in the endothelium. This bacterium binds specifically to the endothelial cell via interactions between adhesins and host receptors, which prompt the cell to initiate phagocytosis of the bacteria and thus protect the bacteria from host defense mechanisms (Lowy F.D. 1998). Meningitis caused by *S. aureus* is highly uncommon, accounting for only 1% to 9% of cases of bacterial meningitis (Jensen A.G. et al. 1993). Staphylococcal food poisoning, caused by enterotoxin-producing *S. aureus* is an important food-borne infection (Shimizu A. et al. 2000). Increased costs associated with MRSA infection, as well
as the importance of colonization pressure have been reported (Haddadin A.S. et al. 2002). Community-acquired methicillin-resistant *S. aureus* (CA-MRSA) infection among individuals without healthcare-associated (HCA) risk factors has now emerged as an epidemic that is responsible for rapidly progressive, fatal diseases including necrotizing pneumonia, severe sepsis and necrotizing fasciitis (Boyle-Vavra S. and Daum R.S. 2007).

Infections caused by *S. epidermidis*, include bacterial endocarditis prosthetic heart valve endocarditis, bacteraemia, surgical wound infections, intravascular catheters, postoperative endophthalmitis, conjunctivitis and keratitis. Several other coagulase negative staphylococci (CoNS) species have been implicated at low incidence in a variety of infections. Among CoNS species, *S. saprophyticus* is often regarded as a more important opportunistic pathogen than *S. epidermidis* in human urinary tract infections (UTIs), especially in young sexually active females. It was considered to be the second most common cause of acute cystitis or pyelonephritis in these patients. The major reservoir of staphylococci in hospitals are colonized/infected in-patients and colonized hospital workers, with carriers at risk for developing endogenous infection or transmitting infection to health care workers and patients, while transient hand carriage of the organism on the hands of health care workers account for the major mechanism for patient to patient transmission (Dar J.A. et al. 2006). Other staphylococcal species (*S. haemolyticus*, *S. hominis*, and *S. lugdunensis*) are usually found as contaminants of blood cultures but could also be associated with a variety of infections (Martineau F. et al. 2001).
**Figure 1.1.** Schematic circular diagrams of the methicillin-resistant *S. aureus* (MRSA) strain (MRSA252) and methicillin-sensitive *S. aureus* (MSSA) strain (MSSA476) chromosomes (Adapted from Holden M.T. et al. 2004).

Key to Outer Ring

- SCC element
- Integrated plasmid
- Prophage
- Transposon
- Genomic island unique to strain
- Genomic island shared with other *S. aureus* strains
1.2. **Staphylococci and antimicrobial resistance**

Antibiotics and their introduction into clinical medicine more than 60 years ago opened new door for medicine. Antibiotics have become major means of treating bacterial infections since their discovery. The excess usage of antibiotics leads to the evolution of resistance in bacteria at high frequency. Resistance to almost every antibiotic available in clinical practice has been observed and individual bacterial strain exhibit multi-resistance to number of antibiotics (Scaria J. *et al.* 2005).

1.2.1. **Historical development of antibiotics and emergence of resistance in *S. aureus***

Studying the history of development of both antibiotics and antibiotic resistance would help better understand the problems of antimicrobial resistance that exist throughout the world. The history of the antibiotics began with the discovery of first antibiotic penicillin, in 1929, based on the observation by Sir Alexander Fleming. He noticed inhibition of staphylococci on an agar plate contaminated by a *Penicillium* mold. Penicillin was the first natural antibiotic to be discovered. Penicillin was first isolated from *Penicillium notatum* by Alexander Fleming but he could not isolate and purify enough drugs for effective therapy (Fleming, A. 1929). Later in 1999, the new strain *Penicillium chrysogenum*, that was capable of producing higher yields of penicillin were discovered (Demain, A.L. and Elander, R. 1999). A series of different antibiotics were quickly discovered after penicillin was introduced to the market. From 1940 to 1960, penicillin was the dominant antibiotic which was regarded as the “magic bullet”. This name was given to penicillin due to it’s unbelievable ability of killing bacterial pathogens without harming the host that harbored them. This was a breakthrough for the treatment of infectious diseases.
With the discovery of penicillin there was no more major challenge for the medicine. The new challenge for medicine began when resistance to penicillin was reported in *S. aureus* in the United Kingdom (U.K.) in the year 1961. The drug of choice penicillin has never been effective against most Gram-negative pathogens (e.g. *Salmonella*, *Shigella*, *Bordetella pertussis*, *Yersinia pestis*, *Pseudomonas*) with the exception of *Neisseria gonorrhoeae*. Gram-negative bacteria are inherently resistant to penicillin because of their cell wall. The outer membrane layer of the cell wall in gram-negative bacteria prevents permeation of the penicillin molecule. Penicillin remains antibiotic of the choice for many types of gram-positive infections especially streptococcal infections. The discovery and introduction of streptomycin, chloramphenicol, and tetracycline, in late 1940s and early 1950s changed the era of antibiotic chemotherapy. These antibiotics were effective against a wide range of bacterial pathogens including Gram-positive and Gram-negative bacteria, intracellular parasites, and the tuberculosis bacillus.

The new natural antibiotics were developed soon after the introduction of penicillin. These included chloramphenicol, erythromycin, streptomycin and tetracycline. The resistant *S. aureus* strains caused major problems in many hospitals. Multi-resistance to penicillin, tetracycline, and streptomycin were reported soon after. This situation was noticed by Sir Robert Williams who regarded the multi-resistance as the ‘Hallmark of dangerous strains’. He also noted that resistance was often clonal, associated with particular strains and particular phage types. The resistant strains often colonized the noses of hospital staff, which could be the source of transmission and spread of resistant strains. His observations have been repeatedly noticed again in the current situation of methicillin-resistant *S. aureus* (MRSA) (Livermore D.M. *et al.* 2001). In the early 1960s a new era of antibiotic development began. The new antibiotics were initially directed against *S. aureus*. 
The first cephalosporins, cephalothin and cephaloridine, were developed primarily for their stability to staphylococcal penicillinase. This was possible because of the discovery of how to replace the 6′ phenylacetyl group benzylpenicillin with other acyl substituents. This discovery provided the synthetic route for methicillin, nafcillin and the oxacillins. These compounds all have the bulky 6′ acyl groups that sterically hinder attack on the β-lactam ring, thus allowing activity to be retained against penicillinase-positive *S. aureus*. Staphylococci secrete their β-lactamase extracellularly thus protecting the entire bacterial population, therefore little or no subsequent selection of strains with more potent β-lactamase variants was observed. (Livermore D.M. and Williams J.D. 1996). In addition to the development of penicillinase-stable β-lactams, the early 1960s also saw the introduction of gentamicin, which had better anti-staphylococcal activity and less toxicity than earlier aminoglycosides. In the early 1960s, *S. aureus* strains resistant to penicillinase-resistant β-lactam drugs, such as methicillin, were reported in hospitals (Jevons, M.P. 1961). Methicillin-resistant *S. aureus* (MRSA) quickly spread around the world and are now a major nosocomial pathogen. Even though the new antibiotics are introduced into the market, microorganisms are capable of developing resistance very rapidly. The new strains that are resistant to almost all antibiotics are called Multi-Drug Resistant (MDR) strains. These strains were spread throughout the world quickly making the treatment of infectious diseases a challenging task for medicine.

1.2.2. Genetic basis and mechanisms of antimicrobial resistance

During the last decade the understanding and knowledge of the genetics and biochemistry of antimicrobial resistance has been increased progressively. The important factors that contribute towards the development of resistance include mutations in cellular
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genes or acquired genes that alter antimicrobial target sites or affect gene expression, the ability to exchange genes, and the acquisition and expression of new DNA by horizontal gene transfer. The ability of the bacteria to evolve these changes within themselves has lead to bacterial adaptation or resistance to various antibiotics.

The mechanisms by which the bacteria develop resistance are:

1) The production of an enzyme that inactivates the antimicrobial agent. The most common cause of resistance to β-lactam drugs is enzyme-mediated antibiotic degradation. β-lactamases are the commonest that have been identified that can hydrolyze β-lactam antibiotics. β-lactamases are produced by some Gram Positive Bacteria (GPB) and a number of GNB. β-lactamases produced by GPB are excreted extracellularly. Those produced by GNB are usually concentrated in periplasmic space and not excreted. Enzymes produced by GPB (predominantly by *S. aerues*) are distinct from those produced by GNB (Laura A *et al.* 1991).

2) The production of an alternative enzyme for the enzyme that is inhibited by antimicrobial agents. A major cause of trimethoprim resistance is the production of an altered (plasmid encoded) dihydrofolate reductase that lacks the capacity to bind trimethoprim (Laura A *et al.* 1991).

3) A mutation in the antimicrobial agent’s target. A mutation in the DNA gyrase of quinolone antibiotics is the most important mechanism for resistance to this important new group of antibiotics in both GPB and GNB, including some strains of methicillin resistant *S. aureus* (MRSA). Modification of antimicrobial agent’s target, which reduces binding of the antimicrobial agent is seen in methicillin resistance in *S. aureus* and is mediated through the production of an altered penicillin binding protein 2 (PBP2A or PBP2’), that
has much lower affinity for methicillin (and for most other β-lactam antibiotics) than for PBP2 (Laura A et al. 1991).

4) Reduced uptake of the antimicrobial agents. Aminoglycosides are hydrophilic molecules that require active transport to gain entry into bacteria. Diminished drug uptake is one of the mechanisms of resistance to aminoglycosides (Ahmad M.H. et al. 1980).

5) Active efflux of antimicrobial agent. Although macrolides resistance among coagulase negative staphylococci (CoNS) is usually due to presence of an *erm* C methylase, a significant number of strains are resistant as a result of active efflux of the drug (Eady A.E. et al. 1993).

6) Over production of the target of antimicrobial agents. Over production of the normal (chromosomally encoded) dihydrofolate reductase is an additional mechanism of trimethoprim resistance particularly in *E. coli* and *Klebsiella pneumoniae* (Burchall J.J. et al. 1982).

Bacteria often acquire the ability to use more than one of these resistance mechanisms through genetic mutation and transfer. Mutations can occur naturally within any species of the bacteria. An element in the environment which is not suitable to bacteria creates a selective pressure on it as a result of which the bacteria will undergo mutational changes that means the bacteria develops the ability to withstand the unpleasant environment and is able to survive better than the wild type one (non-mutated). Bacteria with the mutation will also have the advantage of the additional nutrients and the space left by the wild phenotype which has been eliminated from the environment. The greater the selective pressure exerted on bacteria, due to greater or more frequent exposure to the antibiotics the greater the potential advantage for development of beneficial mutations in the bacteria (Walsh C. 2000). This selective advantage caused by mutations will be passed
from parent bacteria to offspring. This is called vertical transmission. The gene mutations also can be transferred horizontally through plasmids or transposons, which are extra chromosomal genetic elements. Plasmids are circular double-stranded DNA that are located within the cell apart from the chromosomal DNA of the bacteria. In bacteria, the chromosomal DNA encodes for general characteristics of the cell as well as metabolic process and repair mechanisms. The plasmid DNA usually encodes for additional functions such as virulence and resistance (Kaye K.S. and kaye D. 2000). Transposons are small pieces of DNA capable of moving between chromosomal DNA and plasmids. Transposons are able to detach themselves from one part of DNA and attach themselves to another part. Transposons are able to carry resistance genes within themselves from one DNA to another (Walsh C, 2000). These mobile genetic elements are able to transfer resistance genes horizontally between the same species of the bacteria and also between different species within the genus (Kaye K.S. and kaye D. 2000 and Walsh, C. 2000). The genetic elements can move from one cell to another by three different processes called conjugation, transduction and transformation. During the conjugation process, the genetic material will be exchanged between bacteria through the conjugation tube. In the process called transduction, viruses can transfer small amounts of bacterial DNA from one bacterium to another. Some bacteria such as Pneumococci can go in to the phase known as competence phase. At this phase the bacteria can take up naked DNA from the environment in a process called transformation and incorporate the DNA into their own genome. This additional new piece of DNA can harbor the genes that encodes for antibiotic resistance, therefore the recipient strain will acquire a new resistant profile.
1.2.2.1. Methicillin resistance

The first methicillin-resistant *S. aureus* (MRSA) were discovered in the year 1961, when methicillin was introduced into the market (Jevons M.P. 1961). In past decades methicillin-resistant *S. aureus* (MRSA) has been reported as an important pathogen with increasing prevalence rates throughout the world. MRSA isolates have been reported as both nosocomial and community-acquired pathogens (Maltezou H. and Giamarellou H. 2006). Originally *S. aureus* contains three penicillin-binding proteins, PBPs 1, 2, and 3, to catalyse cross-linking of peptidoglycan. The MRSA isolates have an additional component, PBP 2’ or 2a, which has low affinity for β-lactams (Georgopapadakou N.H. and Liu, F.Y. 1980 and Reynolds P.E. 1988). MRSA isolates are resistant to all β-lactams. The structural gene for methicillin resistance, *mecA*, encodes a novel penicillin binding protein PBP 2’, which has much lower affinity for β-lactam antibiotics. It is absent in methicillin susceptible strains (Hiramatsu K. *et al*. 1996). This gene is carried on a genetic element, staphylococcal chromosomal cassette (SCC)mec, which inserts precisely into the *S. aureus* chromosome at *orfX* (Hiramatsu K. *et al*. 2001). SCCmec is a novel genetic element containing two recombinase genes (*ccrA* and *ccrB*) and *mecA* and its regulatory genes (Ito T. *et al*. 2001). The SCCmec types I-V (21–67 kb) vary in their overall genetic composition, type of recombinase genes (*ccrAB* and *ccrC*) and class A, B, C, or D mec classes. Several *mec* and *ccr* allotypes have been found among SCCmec elements and they have been classified as Type I SCCmec, carrying class B *mec* and type 1 *ccr* ; Type II SCCmec, with class A *mec* and type 2 *ccr* ; Type III SCCmec, with class A *mec* and type 3 *ccr* ; Type IV SCCmec, with class B *mec* and type 2 *ccr* ; and Type V SCCmec, with class C *mec* and type 5 *ccr* (Chongtrakool P. *et al*. 2006) (Figure 1.2).
**Figure 1.2.** Structural comparison of SCC*mec* elements (Adapted from Chongtrakool P. *et al.* 2006).
SCCmec is found in other staphylococcal species from which it is presumed to have been transferred; however, the original donor of mecA to staphylococci is unknown, as the element has not yet been identified outside this genus. S. sciuri has an intrinsic PBP that shares 87.8% amino acid homology with PBP2’, and it has been suggested that this may be a precursor to its homologue in S. aureus (Wu S. et al. 1996). mec DNA has approximately 30 to 50 kb additional chromosomal DNA which is present only in methicillin resistant strains (Hiramatsu K. et al. 1996). mecDNA contains mecA, the structural gene, mecI and mecR1, regulatory element controlling mecA transcription, and 20 to 45 kb of mec associated DNA. mecA is highly conserved among staphylococci species. Expression of mecA is either constitutive or inducible by some β-lactam antibiotics, but not by metihicillin or oxacillin, or heterogeneous, with only a few cells in a population expressing the gene. mecA expression may be regulation dependent. Presence of the mecA gene in staphylococcal isolates is considered synonymous with oxacillin resistance. Low level resistance is generally the result of β-lactamase over production, increased levels of intrinsic PBPs or reduction of their binding affinity. High level resistance is always dependent on the expressions of PBP2’. Regulation is complex: mecI encodes a repressor for mecA, and expression of methicillin resistance thus demands that this gene is inactivated by mutation or deletion; transcription of mecA is further modulated by interactions involving the chromosomal fem genes, which encode peptidoglycan-modifying enzymes. These multiple interactions explain the variable expression of methicillin resistance among mecA-positive lineages. Some MRSA have homogeneous resistance to β-lactams, with all the cells in a population expressing resistance; others have heterogeneous resistance, with resistance only expressed by a small minority of the cell population unless salt is added or the incubation temperature is lowered (Chambers H.F. 1997 and Fluit A.D.C. 2001).
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The heterogeneous nature of methicillin resistance is an inherent limitation to the accuracy of susceptibility testing. The commonly used methods for detection of methicillin resistance rely on modified culture conditions to enhance the expression of resistance. The oxacillin disk diffusion method is the least reliable method for detection of methicillin resistance. Recent investigations suggest that disk diffusion using cefoxitin is superior to most previously recommended phenotypic methods, including oxacillin disk diffusion and oxacillin screen agar testing (Skov R. et al. 2006). In 2005, the Clinical and Laboratory Standards Institute (CLSI) published guidelines for cefoxitin disk usage (CLSI, 2005). The detection of meca gene by PCR as a rapid method of identification of MRSA has been well established (Martineau F. et al. 2000).

1.2.2.2. Aminoglycoside resistance

The main mechanism of aminoglycoside resistance in staphylococci is drug inactivation by cellular aminoglycoside-modifying enzymes. Several distinct gene loci encoding such modifying enzymes have been characterized in staphylococci. Plasmid mediated aminoglycoside modifying enzymes of all three classes (aminoglycoside phosphotransferases, acetyltransferases, and nucleotidyldtransferases) have been found in staphylococci (Shaw K.J. et al. 1993). Resistance to gentamicin and concomitant resistance to tobramycin and kanamycin in staphylococci is mediated by a functional enzyme displaying AAC (6\(^{\prime}\)) and APH (2\(^{\prime}\)) activity. The aacA-aphD gene encodes this bi-functional enzyme and is located on the composite transposon Tn4001. Tn4001 like element is widely distributed in both S. aureus and CoNS. In early 1980s the endemic MRSA strains carrying multiple resistance determinants were reported to cause worldwide nosocomial infections (Hryniewicz W. 1999). These multi-resistance phenotypic patterns were observed in the
majority of MRSA strains until the mid-1990s. Hence most of the tests that were developed focused on the detection of only \textit{mecA} gene which gives a proper clue for choosing glycopeptides as alternative therapeutic agents (Strommenger B. \textit{et al.} 2003, Kearns A.M. \textit{et al.} 1999 and Schmitz F.J. \textit{et al.} 1997). However during the past 8 years MRSA clones having less broad resistance patterns have been reported especially in Europe where they have emerged as epidemic strains (Witte W. \textit{et al.} 2001). The detection of \textit{mecA} gene alone is no more informative when deciding alternative therapy. Therefore the genotypic tests for the identification of MRSA need to include the other relevant antibiotic resistant genes as well, since the resistance to these older antibiotics such as aminoglycoside have become clinically relevant again (Strommenger B. \textit{et al.} 2003). A number of conventional gel-based PCR methods in the multiplex format have been published. (Ardic N. \textit{et al.} 2006 and Strommenger B. \textit{et al.} 2003)

1.2.2.3. Erythromycin resistance

Resistance to erythromycin in staphylococci is usually associated with resistance to other macrolides, to the lincosamides, and to typeB streptogramin (MLS). The most important mechanism conferring high-level resistance is structural changes in ribosomal RNA (rRNA) that prevent macrolide binding. Different bacterial species are able to synthesise an enzyme that methylates rRNA encoded by a series of structurally related erythromycin-resistant (\textit{erm}) methylase genes (Pechère J.C. 2001). Production of methylase results in the N6-dimethylation of an adenine residue at position 2058 of 23S rRNA. The conformational changes that occur in the P site of rRNA 23 prevent macrolide binding; therefore the inhibitory effect of the macrolide on protein synthesis is overcome. The erythromycin genes that have been identified in staphylococci are \textit{ermA}, \textit{ermB}, \textit{ermC} and
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*ermM*, with *ermA* being the predominant gene (Sekiguchi, J. *et al.* 2003). Another mechanism of inducible resistance to erythromycin is conferred by the gene *msrA*, which encodes an ATP-dependent efflux pump (Westh H. *et al.* 1995). Investigation of resistance to the older antibiotics such as macrolides has become clinically important again (Strommenger B. *et al.* 2003 and Klein N.C. 2001). The conventional gel-based PCR methods in the multiplex format which can detect erythromycin resistance genes have been published (Ardic N. *et al.* 2006 and Strommenger B. *et al.* 2003).

**1.2.3. Epidemiology of methicillin-resistant S. aureus**

Since 1961, methicillin-resistant *S. aureus* (MRSA) have spread throughout hospitals and other healthcare facilities worldwide. MRSA is now the most commonly isolated antimicrobial-resistant pathogen in many countries. The widespread emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), especially in various types of nosocomial infections, is a serious clinical problem worldwide. The incidence of methicillin resistance among nosocomial isolates of *S. aureus* is higher than 70% in some Asian countries such as Taiwan, China, and Korea (Ko K.S. *et al.* 2005). Although some Asian countries showed the highest prevalence of MRSA in the world, very limited data are available with regard to the evolution and population genetics of MRSA isolates in this region. One of the specific features of the rapid emergence of MRSA in many parts of the world is the dissemination of specific clones (Ko K.S. *et al.* 2005). In 1980s, widespread occurrence of MRSA resulted in empiric therapy using vancomycin in many health care institutions. Vancomycin use in United States (U.S.A.) also increased during this period because of the growing numbers of infections with *Clostridium difficile* and coagulase negative staphylococci (CoNS) in health care institutions. As a consequence, selective
pressure was established that eventually lead to the emergence of strains of *S. aureus* and other species of staphylococci with decreased susceptibility to vancomycin and other glycopeptides. In 1997, in Japan, vancomycin-resistant *S. aureus* (VRSA) was first isolated (Hiramatsu, K. et al. 1997). Shortly after VRSA isolates in the U.S.A. and other countries were also reported (Srinivasan A. *et al.* 2002). The introduction of VRSA in medical settings lead to the presumption of that vancomycin resistance had come from vancomycin-resistant *enterococci* (VRE), especially after it was reported that the vancomycin resistance gene *vanA* could be transferred from *E. faecalis* to *S. aureus* in vitro (Noble W.C. *et al.* 1992). There has been some disagreement over the designation VRSA, as many of the strains that have been isolated do not meet the Clinical and Laboratory Standards Institute (CLSI) standards for resistance. Therefore, the term, vancomycin-intermediate *S. aureus* (VISA), has come into use. The Clinical and Laboratory Standards Institute (CLSI) recommended the criteria for vancomycin susceptibility and resistance of *Staphylococcus aureus* in 2006. The earlier criteria had established that *S. aureus* with minimum inhibitory concentrations (MICs) of vancomycin of \( < \text{ or } \leq 4 \text{ microg/ml, 8 to 16 microg/ml, and } > \text{ or } \geq 32 \text{ microg/ml} \) were vancomycin-susceptible, -intermediate-resistant and -resistant, respectively. The revised recommendation states that bacteria showing vancomycin MICs of \( < \text{ or } \leq 2 \text{ microg/ml, 4 to 8 microg/ml, and } > \text{ or } \geq 16 \text{ microg/ml} \) are -susceptible, -intermediate-resistant, and -resistant, respectively (Hanaki H. *et al.* 2007). Strains of vancomycin-intermediate *S. aureus* (VISA) with vancomycin MIC of 8 µg/ml have been reported from Japan, United States (U.S.A.), France, United Kingdom (U.K.) and Germany. Most of these isolates appear to have developed from pre-existing MRSA infections. Until recently vancomycin resistance among gram-positive bacteria had been thought to be uncommon but the confirmed reports of vancomycin resistance in *Enterococcus* spp; *S.*
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*S. aureus* and CoNS have been reported from various parts of the world. Widespread use of vancomycin to treat infections caused by MRSA and other gram-positive cocci has led to the emergence of vancomycin resistance (Tiwari H.K. and Sen M.R. 2006). MRSA in the hospital setting were established as the strictly nosocomial pathogen. Originally it was hardly ever isolated from the community. This observation was due to the much slower growth rates of earlier MRSA isolates (probably a result of the fitness costs of SCCmecl to III) compared to methicillin-susceptible *S. aureus* (MSSA) isolates. The initial reports of sporadic cases of community-associated MRSA (CA-MRSA) infections in patients without the usual risk factors for nosocomial MRSA acquisition did not generate much attention. However, the deaths of 4 children in North Dakota and Minnesota from severe CA-MRSA infections stated the potential importance of treatment of these organisms (Wijaya L. *et al.* 2006). In Michigan, U.S.A. in 1981, the first sporadic cases of MRSA infections in patients without the usual risk factors for nosocomial MRSA acquisition were reported. This was followed in the late 1980s in West Australia and Chicago. The term “community-associated MRSA” (CA-MRSA) was coined for this phenomenon, which has since become global in scope (Hsu L.Y. *et al.* 2006). A recent report states that with the emergence of CA-MRSA strains, MRSA should no longer be regarded as a strictly nosocomial pathogen (Maltezou H.C. and Giamarellou H. 2006). The terms *community-acquired* and *community-associated* have been used to classify CA-MRSA. Currently, the Centers for Disease Control and Prevention (CDC) prefer the term *community-associated* because of the difficulty of establishing the origins of MRSA strains in the community. Most MRSA outbreaks among athletic teams have been linked to community-associated strains. These strains are distinct from other MRSA strains with regard to molecular characteristics (type of strain), clinical spectrum (type and location of infection), epidemiology (location of outbreaks), and
resistance pattern (susceptibility to antibiotics) (Beam J.W. and Buckley B. 2006). CA-MRSA has been spreading in epidemic proportions in parts of the U.S.A. where conditions of overcrowding and poor sanitation prevail. Recently, MRSA has also emerged in the community setting in Asian countries (Ko K.S. et al. 2005). The concept that CA-MRSA initiated from nosocomial isolates that had somehow managed to spread in the community was pointed out by a series of comparison studies demonstrating differing clinical presentations and antimicrobial susceptibilities, SCCmec types and genetic heritage. Five major structural types of SCCmec have been described to date. Three types are typically found among hospital-acquired MRSA (HA-MRSA) isolates. Type I (34 kb) was identified in a 1960s isolate (strain NCTC10442); type II (53 kb) was identified in a 1982 isolate (strain N315) which is ubiquitous in Japan, Korea, and the United States; and type III (67 kb) was identified in a 1985 isolate (strain 85/2082) which is prevalent in Germany, Austria, India, and other South Asian and Pacific areas. Type IV (20 to 24 kb) is generally carried by CA-MRSA isolates, and at least four subtypes have been reported. In 2004, type V was reported in an isolate of CA-MRSA, in which the only difference was the presence of a restriction-modification system composed of the ccrC gene and the surrounding open reading frames (Yang J.A. et al. 2006). It is believed that these elements were horizontally transferred into methicillin-susceptible *Staphylococcus aureus* (MSSA) at different time points by distantly-related bacterial species. The majority of CA-MRSA isolates possessed geographically-related genetic backgrounds, SCCmec IV and panton-valentine leukocidin (PVL) genes, although this is not necessarily true of CA-MRSA described from all countries.

Epidemic clones from five major lineages of MRSA have spread worldwide and are responsible for practically all healthcare-associated MRSA infections globally. The
predominant concern with CA-MRSA is neither the clinical diseases nor the epidemiological problems caused by these organisms; rather, it is the fact that this phenomenon probably represents yet another evolutionary step forward for \textit{S. aureus}. It is conceivable that the development of methicillin resistance will mirror that of penicillin resistance, which had initially also been restricted to nosocomial \textit{S. aureus} strains, but is now so widespread that few doctors would contemplate using penicillin for the empiric treatment of staphylococcal infections. Salgado C.D. \textit{et al.} (2003) presented a number of studies that assessed certain risk factors for MRSA acquisition; these risk factors included recent hospitalization, outpatient visit, nursing home admission, antibiotic exposure, chronic illness, injection drug use, and close contact with a person with risk factor(s). The most common risk factors assessed were recent hospitalization and chronic illness requiring health care visits.

\textbf{Prevention and effective control of CA-MRSA}

In the community, including athletic training settings, the Centre for Disease Control and Prevention (CDC, 2003) recommend the following measures to control CA-MRSA: These are (1) increased recognition of MRSA infection through prospective surveillance, education of athletes on signs/symptoms and reporting procedures, and coordination of referral services (2) conduct appropriate treatment of MRSA infections by obtaining cultures, draining abscesses when necessary, and using antimicrobial medications concordant with susceptibility patterns (3) care for and containment of wounds through education of athletes; use of clean, dry dressings to cover infected wounds; and hand washing after contact with wounds (4) promote enhanced personal hygiene by encouraging hand washing and bathing/showering among all athletes and staff, using antimicrobial
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soaps (liquid if possible) and alcohol based hand gels and limiting sharing of personal items such as razors and towels (5) exclude athletes from routine activities if proper hand and personal hygiene and wound coverage cannot be assured, and exclude athletes from whirlpools and common-use water facilities if open wounds are present and (6) maintain a clean environment by performing cleansing duties consistent with manufacturers’ recommendations and target cleaning of areas and equipment where known MRSA-infected individuals had recent contact.

Treatment

The treatment of serious MRSA infections presents a great challenge to clinicians, particularly bacteremias and infective endocarditis, for which bactericidal therapy is essential to maximize successful clinical outcomes. Vancomycin has been the preferred antimicrobial agent to treat such MRSA infections. However, the clinical efficacy of this glycopeptide has become more limited (Sader H.S. et al. 2007). Widespread use of vancomycin to treat infections caused by MRSA and other gram-positive cocci has led to the emergence of vancomycin resistance. Linezolid and quinupristin-dalfopristin represent alternative treatment options for serious MRSA infections; however, these compounds also possess important limitations. Quinupristin-dalfopristin, a streptogramin combination, requires a central venous access to be administrated and has been linked to some adverse events such as arthralgia and myalgia (Sader H.S. et al. 2007). Concerns with linezolid, an oxazolidinone, include possible hematologic toxicity of long-term treatment and the fact that it is a bacteriostatic agent against staphylococci is then not indicative for the treatment endocarditis and serious infections in immuno-suppressed patients. The current approach towards a suspected S. aureus infection from the community involves the use of beta-
lactam antibiotics along with removal of foci of infection, e.g., abscess drainage. Severe community-acquired infections such as pneumonia or septicaemia are treated with broad-spectrum intravenous beta-lactam antibiotics in the majority of cases. While uncomplicated cutaneous abscesses caused by CA-MRSA are often cured by incision and drainage alone, the delay in initiating appropriate antibiotic therapy for severe MRSA infections might prove costly (Lee M.C. et al. 2004). In regions where CA-MRSA incidence is high, empiric antimicrobial therapy guidelines have been changed to reflect this. Intravenous vancomycin is now given empirically for selected septicaemic patients in Northern Territory, Australia (Murray R.J. et al. 2004) and oral clindamycin and trimethoprim/sulfamethoxazole are now first-line drugs for the treatment of cutaneous infections in parts of the USA (Buescher E. S. 2005). However, although CA-MRSA infections – in contrast to the majority of HA-MRSA – are generally susceptible to most non-beta-lactam antibiotics (Vandenesch F. et al. 2003), resistance to various older non-beta-lactam antibiotics may be relatively easily acquired. Widespread use of clindamycin had resulted in a small but significant rise in resistance within 2 years in northeast U.S.A. (Braun L. et al. 2005). The recent and impending release of new antibiotics targeting gram-positive organisms such as linezolid, daptomycin, tigecycline and newer glycopeptides appears to blunt the impact of CA-MRSA (Sader H.S. et al. 2007).

In routine practices, the knowledge of biochemical mechanisms involved in development of antimicrobial resistance have been the basic guide for improving antimicrobial susceptibility testing and therapeutic interpretation of resistance phenotypes (Courvalin P. 1996 and Livermore D.M. et al. 2001). Interpretive reading of anti-biogram data takes advantage of an observed phenotype in combination with an understanding of the underlying resistance mechanism. The interpretation predicts the resistance mechanisms
from the actual phenotype and acts as a basis for decision making in antimicrobial therapy. For example, the detection of methicillin resistance in staphylococci allows reporting of resistance to other β-lactams that have not been tested because the resistance mechanism predicts treatment failure. Qualified interpretation requires accurate bacterial speciation and careful selection of indicator drugs that are best able to discern certain resistance mechanisms. This notion is illustrated by the use of oxacillin to screen for reduced susceptibility to penicillin in *Pneumococci*. Finally, the concept of detection and characterization of antimicrobial resistance at the genetic level has evolved as a direct consequence of our increased understanding of antimicrobial resistance at the molecular level (Courvalin, P. 1991). Our increasing knowledge and understanding of basic mechanisms involved for each class of existing antibiotics and newly developed one are fundamental basis for development of the rapid and reliable molecular technique. Rapid and accurate identification of MRSA in clinical samples is of considerable importance for the institution of early correct therapy and to reduce the work load associated with MRSA control and surveillance. Numerous molecular methods have therefore been developed to confirm phenotypically suspected MRSA and to reduce the detection time of MRSA in clinical samples, including blood culture. Considerable progress has recently been made in the characterization of the genetic support of the *mecA* gene. Several molecular markers have been identified that may be useful for epidemiological studies as well as diagnostic purposes. (Francois P. *et al.* 2004, Hiramatsu K. *et al.* 2001, Ito T. *et al.* 2003 and Oliveira D.C. and de-Lencastre, H. 2002).
1.3. Laboratory diagnosis and molecular study of antimicrobial susceptibility/resistance

1.3.1. Overview

One of the important functions of a diagnostic microbiology laboratory is determination of antimicrobial susceptibility pattern of bacterial pathogens. The antimicrobial susceptibility testing results will have major impact on treatment of the patients. For treatment of infectious diseases empiric treatment schemes will be drawn based on accumulated susceptibility testing data gathered at local, regional or national level. The testing is required for choosing proper and correct therapeutic options. It also helps to monitor the spread of resistant organisms or resistance genes throughout the hospital and community. A critical strategy to minimize spread of antimicrobial-resistant bacteria in health care institutions is detection of colonized patients and health care workers to initiate efficient infection control measures. The global emergence and spread of antimicrobial resistance poses a major risk for human health due to the impact on morbidity, mortality, and health care costs (Sundsfjord A. et al. 2004). For performing conventional antimicrobial susceptibility testing the bacteria in pure culture is needed. These tests are relatively simple to perform. It may routinely take at least 24-48 hrs to obtain an antimicrobial susceptibility profiles. Interpretation of susceptibility is based on break points of different values determined by the pharmacokinetic properties of the drug, inherent characteristics of the organism and the sites of infections. These values are constantly changing therefore the simple phenotypic tests have their limitations. The susceptibility testing are highly dependent on experimental conditions and often more than one method would need to be performed to obtain an accurate susceptibility profile.
To increase the rapidity and accuracy of susceptibility testing, the application of alternative identification methods that can be more reliable has recently been advised. The development of rapid genotypic assays is an attractive approach for determining resistance profile targeting specific genes. The nucleic acid – based detection systems that can offer rapid and sensitive methods to detect the presence of resistance genes are becoming more accessible to clinical microbiology laboratories. Several commercial systems for rapid identification and determination of antimicrobial resistance have been developed as an alternative to the classical identification and detection protocols. The advantages of genotypic methods for the detection of antibiotic resistance include: (i) detecting the presence or absence of a particular resistant gene (ii) being independent of categories involved in phenotypic expressions such as susceptibility, intermediate susceptibility and resistance for which breakpoints may vary between countries (iii) determination of low-level resistance (iv) can be performed directly on clinical specimens thus reducing the detection time and (v) early interpretation leading to earlier therapeutic predictions (Sundsfjord et al. 2004). On the other hand, the genotypic methods do have their limitations. These are (i) genotypic methods detects resistance determinants whereas decision making in antimicrobial therapy is preferably based on detection of susceptibility (ii) some silent genes can cause false positive results and (iii) the presence of mutations in primer binding sites can affects PCR amplification, generating false-negative results. (Sundsfjord A. et al. 2004).

To complement the conventional phenotypic tests genetic assays can be included in routine diagnostic laboratory settings. Confirmatory tests for the detection of specific resistance mechanisms in parallel to phenotypic assays. For example, methicillin resistance in staphylococci may exhibit heterogeneous low-level expression pattern that cannot be
detected by culture-based methods. Differentiation between MRSA and borderline
oxacillin-resistant *S. aureus* (BORSA) strains is very difficult using phenotypic tests (Louie
L. *et al.* 2000). They can be used for rapid detection of resistance determinants in clinical
specimens can thus assist early intervention in infection control strategies. Finally genetic
assays can be utilized for molecular epidemiological purposes to analyze the spread of
specific resistant pathogens and/or resistance determinants (Kirkland K.B. *et al.* 1999).

The application of genetic assays for detection of antimicrobial resistance is
dependent upon total cost per test and user-friendly format of the particular test for training
the lab technicians. The validation and quality assurance in the performance of the
genotypic tests at diagnostic microbiology laboratory are crucial. The in-house developed
PCR methods need to be validated before they can be used for clinical diagnostic purposes.
This also applies to published methods as they may not have undergone rigorous testing.
Commercial kits that have been properly validated can help to overcome some of the
problems associated with “in house” tests but this depends on costs associated with them
(Sundsfjord A. *et al.* 2004). The recent developments in multiplex and real-time PCR
assays have fulfilled the clinical acceptance of genetic tests in laboratories. This would
certainly lead to increased applications of genotypic tests at diagnostic microbiology
laboratories. (Espy M.J. *et al.* 2006). Genetic methods for the detection of antimicrobial
resistance genes and their expression take advantage of the development of nucleic acid
amplification and hybridization techniques. The available genetic information in large
databases is used to design oligonucleotide primers complementary to the target of interest
and labeled single-stranded nucleic acid probes for amplification of the particular gene. The
available information on conserved and variable regions within the antimicrobial resistance
gene is of fundamental importance for the analytic sensitivity and specificity of the
amplification methods. The nucleic acid primers and probes may be specific for a defined gene or single nucleotide polymorphism or universal for a group of related resistance determinants. Alignment of multiple nucleotide sequences from the target of interest that are available from public databases can be used for primer or probe selection. Several commercial program as well as free software on the internet is available for DNA sequence analysis that can be used for designing amplification primers. Selected primers and probes should be carefully checked to exclude potential cross reacting sequences (Sundsfjord A. et al. 2004).

1.3.2. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) has been the most commonly need nucleic acid amplification technique for detecting of antimicrobial resistant genes. (Arthur M. et al. 1990). Polymerase chain reaction is a “target amplification” technique. First the target sequence of DNA is identified, and then it will be amplified to the level that it can be detected. PCR is performed in “hot block” machine which is known as a thermal cycler. Basic components of a PCR reaction include single stranded oligonucleotide “primers”, deoxynucleotide triphosphate “dNTPs”, Tag DNA polymerase and the template/extracted nucleic acid. All these components will be placed together in a small vial which in turn is placed in the thermal cycler unit. As denaturation occurs oligonucleotide primes that flank the nucleic acid region of interest anneal to the denatured, unamplified DNA. The primers are extended by heat-stable DNA polymerase (Taq polymerase) that synthesizes complementary strands of the denatured DNA (cycle 1). The resulting dsDNA molecules are then denatured by heat again, the primers anneal to these ssDNA molecules, and Taq polymerase, in turn, synthesizes complementary strands (cycle 2). This cycle is repeated
many times, and with each cycle, the numbers of copies of the DNA sequence located between the opposing primers is doubled. This result in at least $10^5$ fold increase in copy numbers of the DNA sequence. PCR amplification of DNA involves 3 repetitive cycles which are; denaturation, annealing and primer extension. Each 3 repetitive cycle during the PCR amplification will be carried out at different temperature and time durations depend on the individual experiment. In general DNA denaturation occurs at $92^\circ$ C to $96^\circ$ C and the time required and suitable temperature at this step depends on high G+C contents. The temperature at the annealing step varies from $37^\circ$ C to $70^\circ$ C depending on base composition of the oligonucleotide primers. At the extension step temperatures range between $70^\circ$ C to $74^\circ$ C while the time required at this step depends on the length of PCR product. A typical PCR protocol for amplification of a target sequence consists of 30 to 50 thermal cycles, with a doubling of the number of target sequences occurring with each cycle. Detection of the PCR end-products may be accomplished by electrophoretically separating the components of the final amplified sample in agarose gel, followed by staining of the gel using ethidium bromide. The PCR assays with only single primer pairs are simple to perform, however these assays are vulnerable to inhibition. The addition of a second primer set as internal control is favorable (Fluit A.D.C. et al. 2001). Therefore multiplex PCRs were soon developed as the improved version of the PCR that can give simultaneous information by amplifying a number of genes together in the single assay. Multiplex PCR was invented first time when multiple loci in the human dystrophin gene (Chamberlain J.S. et al. 1988) were amplified simultaneously. Multiplex PCR is an amplification reaction in which two or more sets of primer pairs specific for different targets are introduced in the same amplification mixture. Thus, more than one unique target DNA sequence in a specimen can be amplified at the same time. Primers used in multiplex
reactions must be carefully designed to have a similar annealing temperature, which often requires extensive empirical testing. This coamplification of multiple targets can be used for various purposes. For diagnostic uses, multiplex PCR can be set up to detect internal controls or to detect multiple pathogens from a single specimen. Quantitative PCR, a variation of multiplex PCR, can be used to quantify the amount of target DNA or RNA in a specimen (Tang Y-W. et al. 1997). The laborious post-PCR work and problems with carry-over contamination have been largely removed by the advent of real-time PCR defined as the ability to monitor the amplified product during amplification. Real-time PCR techniques have permitted the improvements in routine diagnostic microbiology laboratory. The ability to monitor the accumulating amplicon in real time is based on labeled primers, oligonucleotide probes and/or fluorescing amplicons producing a detectable quantitative signal related to the amount and specificity of the amplicon (Sundsfjord A. et al. 2004). The most important application of Polymerase Chain Reaction (PCR) in microbiology and infectious diseases are typing methods based on the microbial genotype or DNA sequence. Using PCR enables amplification of specific genetic loci and differences indicate strain variation and/or antimicrobial resistance. The best example is PCR-based locus-specific Restriction Fragment Length Polymorphism (RFLP). The specific locus to be examined is amplified with gene-specific primers and subjected to RFLP analysis. The DNA fragments are separated on an agarose or small polyacrylamide gel, and the digestion patterns are visualized following ethidium bromide staining. Locus-specific RFLP has been used in epidemiological studies of hepatitis C virus (HCV) by Davidson et al. (1995). Application of the PCR in molecular typing also has been applied in random amplified polymorphic DNA (RAPD) assay which is referred to as arbitrary primed PCR. This was first described by Williams J.G. et al. (1990) and Welsh J. and McClelland M. (1990). RAPD assays are
based on the use of short random sequence primers, 9 to 10 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of the bacterial genome. If two RAPD primers anneal within a few kilobases of each other in the proper orientation, a PCR product with a molecular length corresponding to the distance between the two primers results. The number and location of these random primer sites vary for different strains of a bacterial species. Thus, following separation of the amplification products by agarose gel electrophoresis, a pattern of bands which, in theory, is characteristic of the particular bacterial strain results. A number of studies have reported success in using RAPD assays to distinguish bacterial strains among diverse species, for example in \textit{A. baumannii} (Reboli A.C. \textit{et al.} 1994) and \textit{S. aureus} (Saulnier, P. \textit{et al.} 1993).

1.3.3. Real-Time PCR

Real-time PCR assay is the PCR amplification reaction whereby, specific fluorescent oligonucleotide probe allow for real-time monitoring of polymerase chain reaction. First demonstration of real-time PCR used the non-specific reporter ethidium-bromide (Higuchi R. \textit{et al.} 1993). It is the exponential phase of amplification that provides the most useful and reproducible data. There is a quantitative relationship between the amount of starting target DNA and the amount of amplification product during the exponential phase of a cycling program. This is the very basis for real-time amplification. The advent of DNA intercalating dyes and probe specific chemistries, the study of the amplification process has improved by quantum leaps as a result of real-time detection. The real-time instruments are comprised of a fluoro-meter and a thermal cycler for the detection of fluorescence during the cycling process. A computer that communicates with the real-time machine collects fluorescence data which is displayed in a graphical format. The
simple method for nucleic acid detection with real-time PCR uses SYBR Green to detect the accumulation of any double-stranded DNA product. SYBR Green detection is sensitive but is not specific. In SYBR Green reaction the melting curve analysis will be performed to determine the melting temperature, $T_m$, which permits detection of different amplification products based upon the percentage of G+C content and length of the amplification product. This platform is similar to agarose gel electrophoresis where the separation is based primarily on length. The SYBR Green assays are often used for screening assays where further analysis of specimens is performed to confirm the results. The more improved version of the assay whereby both sensitive and specific detection is possible with real-time PCR using fluorescent probe technology probes (Espy M.J. et al. 2006). Three types of fluorescent probe are most frequently used in real-time PCR testing platforms in clinical microbiology: 5´nuclease (TaqMan probes), molecular beacons, and FRET hybridization probes (Figure1.2. A-C). A TaqMan probe is a short oligonucleotide (DNA) that contains a 5´fluorescent dye and 3´quenching dye (Figure1.3.A). In a fluorescent Taqman assay, the probe is labeled at the 5´ end with a fluorescent reporter molecule, usually a tetramethyl rhodamine derivative, which acts as a quencher for the reporter (Heid C.A. et al. 1996). During real-time PCR, the fluorogenic probe and the PCR amplimers first hybridize to their DNA targets with fluorogenic still intact, the emission of the reporter dye is quenched, but during the PCR extension phase the probe is cleaved by the 5´-exonuclease activity of the Taq DNA polymerase. This cleavage interrupts the fluorescence resonance energy transfer and permits the reporter dye to fluoresce, with the level of fluorescence produced being in proportion to the level of PCR product accumulation. (Oliver G. et al. 2001). Molecular beacons probes have a fluorescent dye on the 5´ end and a quencher dye on the 3´ end of the oligonucleotide probe (Figure 1.3.B). A
region at each end of the molecular beacon probe is designed to be complementary to itself, so at low temperatures, the ends anneal, creating a hairpin structure. At high temperatures, both the PCR amplification product and probe is single stranded. As the temperature of the PCR is lowered, the central region of the molecular beacon probe binds to the PCR product and forces the separation of the fluorescent reporter dye from the quenching dye. The effects of the quencher dye are obviated and a light signal from the reporter dye can be detected. If no PCR amplification product is available for binding, the probe re-anneals to itself, forcing the reporter dye and quencher dye together, and preventing fluorescent signal. Typically, a single molecular beacon is used for detection of a PCR amplification product and multiple beacon probes with different reporter dyes are used for single nucleotide polymorphism detection. FRET hybridization probes are two DNA probes designed to anneal next to each other in a head-to-tail configuration on the PCR product (Figure 1.3.C). The upstream probe has a fluorescent dye on the 3´end and the downstream probe has an acceptor dye on the 5´ end. If both probes anneal to the target PCR product, fluorescence from the 3´ dye is absorbed by the adjacent acceptor dye on the 5´ end of the second probe. The second dye is excited and emits light at a third wavelength and this third wavelength is detected. A design detail of FRET hybridization probes is that the 3´ end of the second (downstream) probe is phosphorylated to prevent it from being used as a primer by Taq during PCR amplification. The two probes encompass a region of 40 to 50 DNA base pairs, providing exquisite specificity. FRET hybridization probe technology permits melting curve analysis of the amplification product (Uhl J.R. and Cockerill F.R.I. 2004).
Figure 1.3 (A-C) Real-time probe chemistry (Adapted from Espy M.J. et al. 2006).

A) 5' Nuclease probe

B) Molecular beacons

C) FRET probes
The development of real-time PCR assay includes the following steps (i) target nucleic acids selections (ii) design of specific primers; the primers amplify one product that can provide the best assay sensitivity. PCR primers should be chosen with low potency to form secondary structures, including self and cross hybridization with other oligonucleotides in the PCR. This can be more problematic as more oligonucleotides are added to the reaction i.e multiplex format. A search for the primer sequence in a DNA database such as the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/) may reveal cross-reactivity. However, since the databases currently available represent only a small portion of the nucleic acid sequences for microorganisms the specimens and related microorganisms must also be tested to confirm the lack of cross-reactivity. The target nucleic acid sequence should also be conserved in the organism to be identified or quantitated. The more conserved site should be chosen if the sequence data of the intended target area shows a significant frequency of polymorphisms. (iii) optimization of real-time PCR assay; real-time PCR optimization experiments can be performed within hours hence making it more rapid and convenient than the conventional PCR optimization steps. In real-time PCR assay few components need to be optimized in order to achieve optimal and best results. These factors include magnesium concentration, which allows the polymerase enzyme to function at an optimal level; primer and probe concentrations, which affect the sensitivity and specificity of the assay respectively; and the use of additives such as di-methyl sulfoxide, which can aid in the de-naturation of nucleic acids with high G+C contents. The type and quality of polymerase enzyme utilized can also play a significant role, polymerases which permit hot-start PCR are preferable options. These enzymes do not function until a critical maximum temperature is reached, which reduces the generation of nonspecific sequence fragments.
Real-time PCR assays have significantly improved the application of PCR for these purposes.

1.4. Genetic relationship and molecular study of methicillin-sensitive and methicillin-resistant S. aureus strains

Emerging antibiotic resistance can be the product of close evolutionary relationships between bacterial species and the administration of antimicrobial agents. Isolates collected before the use of antibiotics harbour no resistance genes (O’brien T.F. 2002). Many strains of staphylococcus show not only resistance but also multi-drug resistance. Genomic similarities allow for highly facilitated gene transfer. The close evolutionary relationship between staphylococci species leads to increased ability to transfer genes. A study conducted by Holden M.T.G. et al. (2004) showed that a genomic stretch in several strains of methicillin-resistant S. aureus was absent in methicillin-susceptible ones. Staphylococci are gram-positive and are coated with a very thick layer of peptidoglycan. β-lactam antibiotics, such as penicillin and cephalosporin-based medicines, target this type of bacteria by interfering with synthesis of the cell wall. Their molecular structures contain beta-lactam rings that interfere with the enzymes responsible for cell wall construction (Robinson G.N. 1998). Strains of staphylococcus exhibiting resistance to β-lactam antibiotics have a common resistance gene. This gene, denoted as meca, is involved in the normal process of cell wall synthesis and does not contribute to resistance in the wild. However, over-expression of the gene is shown to increase antibiotic resistance. Isolates of resistant strains acquired in hospitals have much larger regions of this gene and tend to show more resistance to the antibiotics (Samore M.H. et al. 2005). The cellular mechanism by which S. aureus is capable of withstanding the inhibitory effects of all β-lactam antibiotics, including the semisynthetic ones such as methicillin, is based on acquisition of
the *mecA* gene. The resistance mechanism by which the *mecA* can be transferred has been studied extensively not only because of the importance of resistant strains in the clinical setting but also because of many important unanswered questions regarding the mechanism itself. The mechanism(s) responsible for *mecA* transfer is not known, but evidence supports horizontal transfer of *mec* DNA between staphylococcal species and of the *mecA* gene between different gram-positive genera. The assumption is that the *ccr* and *mec* genes were brought together in coagulase-negative staphylococci (CoNS) from an unknown source, where deletion in the *mec* regulatory genes occurred, before the genes were transferred into *S. aureus* to generate MRSA (Jansen W.T.M. *et al.* 2006). Studies with human staphylococcal strains indicate that *Staphylococcus epidermidis* is a reservoir of antibiotic resistance genes that can be transferred to *S. aureus* under in vitro and in vivo conditions. The transfer of *mecA* from *S. epidermidis* to *S. aureus* was shown to occur in vivo, suggesting that *mecA* may transfer more frequently to MSSA (Wielders, C.L. *et al.* 2001). A high degree of diversity has been observed among methicillin-sensitive *S. aureus* (MSSA) strains. However, Schlichting C. *et al.* (1993) noticed that epidemiologically unrelated MSSA strains with similar PFGE patterns were found in different geographical regions, suggesting that some genotypes are ubiquitous. Multi-locus enzyme electrophoresis demonstrated the presence of clones in natural populations of *S. aureus* and, in particular, the presence of a clone responsible for most cases of toxic shock syndrome. In contrast to MSSA, several studies showed that MRSA strains were represented by a limited number of genotypes. There has been considerable speculation about the origin and evolution of the MRSA strains. According to Fitzgerald J.R. *et al.* (2001) the *mec* gene has been horizontally transferred into distinct *S. aureus* chromosomal backgrounds, demonstrating that methicillin-resistant strains have evolved several times independently.
The PCR-based method was also used in combination with the selective antibiotic screening method to study the direction and mechanism of resistance transfer between poultry and human staphylococcal isolates (khan, S.A. et al. 2000). The DNA microarray technology has helped in detecting meca in at least five divergent lineages, emphasizing that horizontal meca transfer has played a fundamental role in the evolution of MRSA (Fitzgerald, J.R. et al. 2001). Knowledge of the underlying genetic structure is important in understanding the epidemiological relationship between the strains as well. The molecular technique can help to study underlying genetic structure and relationship between sensitive and resistant strains. Applications of these techniques have revealed lots of information about genetic relationship between resistant and sensitive strains. Some of the molecular approaches that can help to study genetic relationship and molecular analysis of MRSA and MSSA strains include typing by pulsed-field gel electrophoresis (PFGE) (Kirikae T. et al. 2004) and multi-locus sequence typing (MLST) (Ko K.S. et al. 2005).

Molecular typing plays an important role in epidemiological studies of nosocomial infection, such as methicillin-resistant Staphylococcus aureus (MRSA) infection. Pulsed-field gel electrophoresis and multi-locus sequence typing are considered the most discriminatory and reliable methods of typing, but they are technically complex, time consuming, and expensive. PCR-RFLP has been reported as a preliminary and simple screening method for molecular typing of S. aureus isolates. In RFLP analysis, the amplified DNA is digested (fragmented) with restriction enzymes. These endonucleases will only cleave DNA molecules at specific sites, i.e., unique short sequences of nucleic acid. Therefore, if the sequence of the target DNA is known, RFLP analysis can be used to confirm the target DNA in its amplified form (Olive, D.M. and Bean P. 1999). Coagulase serotyping is widely used in some Asian countries (Japan) in addition to conventional and
genetic methods for distinguishing *S. aureus* strains. Coagulase production is the principle criterion used by the clinical microbiology laboratory for identification of *S. aureus* isolates. Numerous allelic forms of *S. aureus* coagulase exist and each isolate may produce one or more of different variants of this enzyme. The 3’ coding region of the coagulase gene contains a series of repeats that are well conserved but these repeats differ by the presence or absence of *AluI* and *CfoI* restriction sites. Therefore the *AluI* and *CfoI* restriction enzyme digestion of amplified PCR products produces fragments with different length sizes. The *S. aureus* isolate can be discriminated by Restriction Fragment Length Polymorphism. A typing procedure for *S. aureus* was developed by Hookey J.V. et al. (1998) based on improved PCR amplification of coagulase gene and restriction fragment length polymorphism (RFLP) analysis of the products.

Acquired resistance to antibiotics occurs either by mutations (point mutations, deletions, inversions etc. in bacterial genome) or by horizontal transfer of resistance genes located on various types of mobile DNA elements. The escape of resistance genes to mobile DNA fragments (plasmids) is enabling the process of transfer of antimicrobial resistance not only between bacteria of the same population, but between bacterial genera. In bacterial populations (plasmid bearing or not), the generation of antibiotic resistance depends on the rate of emergence of resistant mutants, i.e., on the bacterial mutation rate. Mutation frequencies to resistance can vary dramatically depending on the mechanism of resistance and whether or not organism exhibits a mutator phenotype. The resistance gene sequences are integrated by recombination into several classes of naturally occurring gene expression cassettes and disseminated within the microbial population by horizontal gene transfer mechanisms. Three mechanisms of gene transfer in bacteria have been identified: transformation, involving the uptake and incorporation of naked DNA; conjugation, a cell
contact-dependent DNA transfer mechanism found to occur in most bacterial genera; and transduction, whereby host DNA is encapsidated into a bacteriophage which acts as the vector for its injection into a recipient cell. Genes encoding for the resistance to antibiotics are often carried by large self-transmissible plasmids, or by smaller plasmids that can be mobilized by self-transmissible plasmids. They are frequently part of transposons or conjugative transposons. Self-replicating plasmids, prophages, transposons, integrons and resistance islands all represent DNA elements that frequently carry resistance genes into sensitive organisms. These elements add DNA to the microbe and utilize site specific recombinases-integrases for their integration into the genome. The majority of reports of bacterial gene transfer in the environment concern conjugation. Many plasmids and conjugative transposons are of very wide host range. Such transfer systems may have wide evolutionary consequences and have been implicated in the horizontal transfer of antibiotic resistance and xenobiotic degradation genes (Dzidic, S. and Bedecovic, V. 2003). Significant advances in field of molecular genetics play important role in our understanding of how methicillin resistance is acquired by S. aureus. Integration of a staphylococcal cassette chromosome mec (SCCmec) element into the chromosome converts drug-sensitive S. aureus into hospital pathogen methicillin-resistant S. aureus (MRSA), which is resistant to practically all β-lactam antibiotics. SCCmec is a novel class of mobile genetic element that is composed of the mec gene complex encoding methicillin resistance and the ccr gene complex that encodes recombinases responsible for its mobility. These elements also carry various resistance genes for non-beta-lactam antibiotics. After acquiring a SCCmec element, MRSA undergoes several mutational events and evolves into the most difficult-to-treat pathogen in hospitals, against which all extant antibiotics including vancomycin are ineffective (Hiramatsu K. et al. 2001).
1.5. **Microarray-based study to simultaneously and globally examine the transcriptional response at the genomic level**

β-Lactam antibiotics are enzyme inhibitors and their mechanism of action involves inhibition of bacterial transpeptidases (penicillin-binding proteins, PBPs) that catalyze cell wall assembly. In contrast, glycopeptide antibiotics like vancomycin bind to the C-terminal of the cell wall precursor pentapeptide (Lipid II) and prevent it from being utilized for cell wall synthesis. The mechanisms of β-lactam and glycopeptide resistance match the mode of action of these antibiotics: β-lactam resistant bacteria produce a surrogate transpeptidase with low affinity for β-lactams. The mechanism of *mecA*-based β-lactam resistance involves the production of a new cell wall biosynthetic enzyme, the penicillin-binding protein 2’ (PBP2’) (Severin A. *et al.* 2004). *S. aureus* is known to produce a cell wall of unique muropeptide composition when grown in the presence of β-lactam antibiotics. This cell wall is composed primarily of monomeric, dimeric, and trimeric muropeptides. It was proposed that this abnormal peptidoglycan is the product of PBP2’, the protein encoded by the resistance gene *mecA*. In *S. aureus* exposed to β-lactam antibiotics the four native PBPs become inactivated and their transpeptidase function is taken over by PBP2’, which has very low affinity for most members of this family of antimicrobial agents (Severin A. *et al.* 2005). Microarray studies offer a unique opportunity to evaluate gene expression changes associated with resistance phenotype and to compare changes in resistant *S. aureus* isolates growing at different environmental conditions in presence of antibiotics. Detailed study of global gene regulation in isolates from different clinically susceptible and resistant *S. aureus* strains may bring key information for a deeper understanding of the molecular basis
of the development of the mechanism of the resistance to the cell wall active antibiotics in this highly important nosocomial and community-acquired pathogen.

**Microarray technology**

Genomics studies the genome of organisms as a whole. It allows studying the wide picture of gene characteristics which is based on high-throughput techniques. The most recent and advance high throughput techniques are arrays, which are an orderly arrangement of a great number of genes that can be used for large-scale studies. The development of the first arrays to study a great number of genes at a time started about 15 years ago and since then the technique has widely been expanded. Microarrays (or microchips) are a recently developed, powerful genomic technology that are widely used to monitor gene expression under different cell growth conditions, detect specific mutations in DNA sequences and characterize microorganisms in environmental samples. Microarray is based on standard molecular biology and it is improved version of traditional filter and blotting techniques with a principal advantage of being higher throughput. Microarrays use high-density microscopic array elements, planar glass substrates, low reaction volumes, multi-color fluorescent labelling, high binding specificity, high-speed instrumentation for manufacture and detection, and sophisticated software for data analysis and modelling. The array elements react specifically with labeled mixtures, producing signals that reveal the identity and concentration of each labeled species in solution. These specifications provide biological assays the exploration of any organism on a genomic scale.
Microarray technology can be used for three main applications:

1. **Gene expression profiling** – mRNA extracted from a biological sample is applied to the microarray. The result reveals the level of expression of tens of thousands of genes in that sample. This result is known as a gene expression “profile” or “signature”. The first pathogen array to examine gene expression profiles investigated the human cytomegalovirus (HCMV) genome (Chambers J. et al. 1999 and Whitney A.R. et al. 2003).

2. **Genotyping** – DNA, extracted from a biological sample, is amplified by a polymerase chain reaction and applied to the microarray. The genotype for hundreds or thousands of genetic markers across the genome can be determined in a single experiment. This approach has considerable potential in disease risk assessment, both in research and clinical practice. DNA microarrays have been used for determining genotype variations in several viruses. (Kozal M.J. et al. 1996, Livache T. et al. 1998, Bean P. and Wilson J. 2000 and Li J.P. et al. 2001). Wang D. et al. (2002) have described a long oligonucleotide microarray capable of detecting over 140 respiratory viruses.

3. **DNA sequencing** – DNA extracted from a biological sample is amplified and applied to specific “sequencing” microarrays. Thousands of base pairs of DNA can be screened on a single microarray for polymorphisms in specific genes whose sequence is already known. This greatly increases the scope for precise molecular diagnosis of a single gene and genetically complex diseases. Read T.D. et al. (2002) have used pathogen microarrays to determine the origin and mode of anthrax infection in an outbreak.
DNA microarray technology evolved from the use of solid substrates in performing southern blot experiments and the multiplexing of this technology has found greater utility to date than corresponding protein chips (Epstein J.R. et al. 2002). DNA microarrays generally contain hundreds or thousands of gene probes which are simultaneously exposed to a target sample. The gene probes are generally selected from cDNA fragments derived by polymerase chain reaction (PCR) or from synthetic oligonucleotides which can either be synthesized and robotically spotted onto a solid support or synthesized in situ using standard photolithographic techniques. Global profiling of gene expression is one attractive approach for assessing the gene function. Because a gene is usually transcribed only when and where its function is required, determining the locations and conditions under which a gene is expressed allows inferences about its function. The transcriptional profiling is the most widely used application at present. RNA profiling represents an advanced microarray applications. A microarray – or “GeneChip” – measures the expression level of a gene by determining the amount of messenger RNA that is present (mRNA abundance). Production of arrays begins with the selection of the probes to be printed on an array. These are often chosen directly from gene databases (e.g., GenBank, http://www.ncbi.nlm.nih.gov). The difference between the various array methods includes: nature and length of the probes (cDNA or oligonucleotides), immobilisation technology used to attach them to membranes or slides and labelling technologies for the targets. Two main methods are used to generate microarrays. In the first one cDNA array technology representing the complementary DNA (cDNA) code of specific genes, are spotted onto a glass slide. In the second one oligonucleotides consisting of nucleic acids are synthesised on to a silica slide by a process known as photolithography. The target, ie, purified mRNA from the biological sample (e.g., blood or tissue) is labelled by fluorescence or radioactivity and then hybridised for several
hours to the microarray. mRNA consists of a sequence built up of 4 different oligonucleotides (thymidine, guanine, cytosine, and uracil), which is specific for each gene. Such a specific mRNA sequence is able to bind to a single complementary sequence of oligonucleotides only. This specificity of binding is used in microarrays. If the sequence of oligonucleotides matches with the sequence of oligonucleotides on a specific spot of the microarray, hybridisation occurs. On each of the up to 450,000 spots, different binding intensities occur depending on the concentration of the different genes in the biological sample tested. Thus, the concentration of mRNA (gene expression) can be measured quantitatively. The analysis of microarray data is complex and involves several steps. After hybridisation, microarrays are scanned and images representing the intensity of the fluorescence signal are generated. After image processing, it is necessary to normalise the fluorescence intensities. The normalisation is done for each microarray. Typically, the signal for each gene is divided by the median gene signal. This process is called per chip normalisation. Often, a second normalisation, called per gene normalisation, is applied, where the signals for a specific gene throughout the different microarrays are divided by the median gene signal. Such normalised signal intensities of different microarrays, representing different conditions, can be compared. Genes, for which the mRNA is over-represented, or under-represented, are called up-regulated, or down-regulated, respectively. Most published studies have used a post-normalisation cut off of two-fold increase or decrease in measured level to define differential expression. Although it is generally accepted that one cannot rely on a single gene chip experiment, there is no clear consensus about the number of experimental replicates that are needed for robust results. Indeed, the answer would differ for gene to gene, as each gene has a different gene expression variability and expression range. Clearly, the trend is “the more the better”. Apart from
identifying genes by the arbitrary two-fold increase or decrease, the expression variability of the genes can be compared using classical statistical significance testing. Before interpreting the data, p-values need to be corrected with specific algorithms due to multiple comparisons. However, the true power of microarray analysis is to identify common patterns of gene expression associated with a specific experimental condition. There exists a large group of statistical methods for pattern recognition, such as principal component analysis, cluster analysis, and intelligent map.
1.6. Objectives

The widespread emergence of methicillin-resistant *S. aureus* (MRSA), especially in various types of nosocomial infections, is a serious clinical problem worldwide. The incidence of methicillin resistance among nosocomial isolates of *S. aureus* have increased in some Asian countries such as Taiwan, China, and Korea. This study aims to utilize molecular technique to study important antibiotic resistant determinants and characterize *S. aureus* isolates from Malaysia in relation to antibiotic resistance.

The objectives are:

1) To determine susceptibility of the clinical isolates to various antibiotics
2) To develop multiplex PCR and multiplex real-time PCR assays for simultaneous identification and detection of antibiotic resistant genes
3) To type *S. aureus* strains from University Malaya Medical Center (UMMC) by the simple method of RFLP
4) To determine the ability of antibiotic resistant gene transfer in clinical isolates
5) To study gene expression profile of the *S. aureus* isolates on exposure to antibiotics