1. REVIEW OF LITERATURE
1.1 General characteristics of staphylococci

Staphylococci are spherical cells about 1 micrometer in diameter. They grow in clusters because staphylococci divide in two planes. Staphylococci are found in association with skin, skin glands, and mucous membranes of warm-blooded animals, although some species can be isolated from processed animal sources such as meat and diary products, or from environmental sources, such as soil, dust, air, and water. Members of the genus are usually facultative anaerobes, capable of generating energy by respiratory or fermentative pathways. Most species have relatively complex nutritional requirements, usually requiring several amino acids and B vitamins. They are tolerant to high concentrations of NaCl and temperature ranging from 10°C to 45°C. Staphylococci belong to the low G + C (30 to 38 mol percent) group of the gram-positive bacterial phylogenetic group (Wilkinson, 1997).

1.1.1 Taxonomy

The genus Staphylococcus represents gram-positive, catalase-positive cocci that historically belonged to the bacterial family Micrococcaceae (Witte, 2000). Phylogenetic classification indicated that over 50% of predicted proteins encoded by the *S. aureus* genome are closely related to those in *Bacillus subtilis* and *Bacillus halodurans*. They typically contain house-keeping genes, essential for bacteria to absorb nutrients from the environment, synthesize metabolic intermediates, and
multiply. They possess bacteriophages, pathogenicity islands, transposons, and insertion sequence elements distributed over the genomes (Kuroda, 2001).

1.1.2 Gram positive cell wall

**Figure.** Gram positive cell wall is composed of a thick peptidoglycan (murein) layer and ribitol teichoic acids. Lipoteichoic acid, which may protrude on the surface through the peptidoglycan layer, is linked to the cytoplasm. Cell wall ribitol teichoic acid is covalently linked to the peptidoglycan layer. The cell walls of most gram-positive bacterial species have an extensive meshwork of peptidoglycan layer that is typically 15-30 nm thick. Peptidoglycan polymers are cross-linked by pentaglycine bridges between L-lysine and the terminal D-alanine, building a huge macromolecular murein sacculus around each bacterium (Beveridge, 2000).
1.1.3 Basic structure of *S. aureus* genome

The staphylococcal genome consists of a single circular chromosome generally including prophages, transposons, insertion sequences, and other variable accessory elements plus irregularly present, autonomous plasmids. *Staphylococcus aureus* (*S. aureus*) genome is about 2.8 Megabase pairs long with approximately 2600 open reading frames. The genes for antibiotic resistance in *S. aureus* are located on plasmids, transposons, or on the chromosome (Kuroda, 2001). *S. aureus* NCTC 8325 has been the organism of choice for genetic and mapping studies of *S. aureus* chromosome. The parental strain is known to carry three proghages called ϕ11, ϕ12, and ϕ13. However, UV-cured (prophage minus) derivatives, such as 8325-4 (RN 450) or RN6390 have been used for most genetic studies (Iandolo, 2002).

**Figure.** Circular representation of *S. aureus* chromosome (N315) (Kuroda, 2001).
Whole genome sequences of two health-care-associated MRSA strains, N315 and Mu50, isolated in Japan in 1982 and 1997, respectively, and one community-acquired MRSA strain MW2 (MW standing for midwest USA) have been published (Kuroda, 2001; Baba, 2002). The three MRSA strains have closely related nucleotide sequences (99.7% identity between N315 and Mu50, 94.8% between MW2 and N315, and 94.7% between MW2 and Mu50). General features of the N315 and Mu50 genomes are described (Kuroda, 2001). They have a low G+C content (average 33%), and possess five rDNA operons. The N315 and Mu50 genomes contain 2595 and 2697 open reading frames, respectively. Most of the nucleotide differences between strains N315 and Mu50 are due to insertion of mobile genetic elements. Both strains possess several bacteriophages, pathogenicity islands, transposons, and insertion sequence (IS) elements distributed over the genomes. Additionally, 15 each strain has a distinct plasmid carrying different antibiotic resistance genes (Kuroda, 2001).

1.2 Pathogenesis of S. aureus infections

S. aureus expresses many potential virulence factors including:

- surface proteins that promote colonization of host tissues or endocytosis
- surface components that may inhibit phagocytic engulfment (capsule, protein A)
- membrane-damaging exotoxins that may lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin);
- superantigens that may lead to indirect damage of host tissues and toxic shock symptoms, such as staphylococcal enterotoxins (SE), toxic shock syndrome toxin (TSST), exfoliative toxins (EFT).

**Figure.** Potential virulence factors of *S. aureus.*

*S. aureus* has been recognized as serious pathogens for over a century, causing a wide range of diseases ranging from benign or more severe skin infection to often fatal forms of endocarditis or septic shock. The diseases spectrum of *S. aureus* includes superficial skin lesions such as boils, styes and furuncles, abscesses, and serious infections such as bacteremia, central nervous system infections, endocarditis, osteomyelitis, pneumonia, urinary track infections, and syndromes caused by exotoxins, including bullous impetigo, food poisoning, scaled skin syndrome, and toxic shock syndrome (Fred, 2000). Despite largescale efforts to halt their spread, particularly in hospitals, *S. aureus* remain the major
pathogens of community- and nosocomially-acquired bacteremia.

1.3 *Staphylococcus aureus* as a human pathogen

*Staphylococci* are ubiquitous in the environment. The most common cause of staphylococcal infections, *Staphylococcus aureus*, is a spherical bacterium that can cause a range of illnesses from minor skin infections and abscesses, to life-threatening diseases such as pneumonia, meningitis, endocarditis, toxic shock syndrome, and septicemia (Waldvogel, 2000). A Gram-positive coccus, it appears as grape-like clusters when viewed through a microscope and as large, round, golden-yellow colonies, often with β-hemolysis, when grown on blood agar plates. The golden appearance is the etymological root of the bacteria’s name: aureus means "gold" in Latin. *S. aureus* is catalase positive and thus able to convert hydrogen peroxide to water and oxygen, which makes the catalase test useful to distinguish staphylococci from enterococci and streptococci. *S. aureus* can be differentiated from most other staphylococci by the coagulase test. *S. aureus* is coagulase-positive, while most other staphylococcus species are coagulase-negative. *S. aureus* has about 2,600 genes and 2.8 million bp of DNA in its chromosome (Ryan *et al*., 2004). Humans are a natural reservoir for *S. aureus* and asymptomatic colonization is far more common than infection. As a commensal on human skin, colonization of the nasopharynx, perineum, or skin (especially if cutaneous disruption or damage is present) may occur shortly after birth and may recur anytime thereafter (Payne *et al*., 1965). Family members of a colonized infant may also become colonized. Transmission
occurs by direct contact with a colonized carrier. Carriage rates are reported to vary greatly. Notably, higher rates than in the general population have been documented in patients in intensive care units, patients with long-term indwelling intravascular catheters, and health-care workers. Young children tend to have higher colonization rates, possibly because of their frequent contact with respiratory secretions (Adcock et al., 1998). Colonization may be transient or persistent and can last for years. The finding of *S. aureus* under these circumstances does not always indicate infection and therefore does not always require treatment (indeed, treatment may be ineffective and re-colonization may occur). However, in infants *S. aureus* infection can cause a severe disease known as staphylococcal scalded skin syndrome. Deeply situated *S. aureus* infections can lead to bacteremia, staphylococcal endocarditis, and pneumonia, which may be rapidly fatal. Approximately 30% of healthy adults carry *Staphylococcus aureus* on their skin or in their anterior nares at any given time (Ryan et al., 2004). Worldwide, an estimated 2 billion people carry some form of *S. aureus*; of these, up to 53 million (2.7%) are thought to carry MRSA. In the United States, 95 million carry *S. aureus* in their noses; of these 2.5 million (2.6%) carry MRSA (Graham et al., 2006). A population review conducted in 3 communities in the US showed the annual incidence of community-associated MRSA during 2001–2002 to be 18–25.7/100,000; most isolates were associated with clinically relevant infections, and 23% of patients required hospitalization (Jernigan et al., 2007).
1.4 Colonization and Infection

Newborn infants are initially devoid of endogenous flora and thus are more prone to bacterial colonization with environmental organisms while in the neonatal intensive care unit (NICU). The 2 major modes of acquisition are the perinatal transfer of maternal vaginal flora to the infant (vertical transmission) and acquisition after birth from an environmental or human source (horizontal transmission). With the survival of an increasing number of premature infants, the average hospital stay of infants in the NICU has been longer; thus, the opportunity for acquiring micro-organisms while in the NICU environment is greater. Healthcare associated infections are defined as infections that manifest 48 hours after admission to the neonatal intensive care unit (Hemming et al., 1976). However, the incubation period of neonatal infections differs and some perinatally acquired infections are known to manifest after 48 hours of life, especially those with maternal predisposing factors for sepsis such as chorioamnionitis, premature rupture of membranes or maternal infections. In 1990 Fonseca et al., extensively reviewed the literature on healthcare associated *S. aureus*, with emphasis on the methodological soundness of studies, and classified them by applying standard epidemiological criteria of quality. They found that few studies concerning endemic rates of healthcare associated infections give an overall picture of the problem in the newborn population, a problem all the more acute since *S. aureus* and coagulase negative staphylococci (CNS) have now replaced gram-negative bacteria as the most common etiological agents. Skin infections, pneumonia and bacteremia were the
most common healthcare associated infections for infants. Risk factors included prolonged hospital stay, very low birth weight (<1500 g) and invasive procedures such as endotracheal intubations, hyper alimentation, or intravenous feeding. The question of the most significant risk factor for the neonatal acquisition of a healthcare associated infection was not satisfactorily answerable. However, the most reliable and reproducible studies with statistical analysis documented that mothers are not an important source of \textit{S. aureus} for their infants, that nursery personnel play a minor role whether as reservoir or source in outbreak situations; that other infants, once the pathogen has been introduced are themselves the reservoir for sustaining an outbreak, the primary localization being the nose or umbilicus; that the ultimate origin of new pathogenic strains is still uncertain but some evidence points to the health care personnel, that the environment is not the source of \textit{S. aureus} for the infants; that transmission is usually mechanical, on the hands of health care personnel; that colonization often precedes the development of disease, and finally that high rates of colonization may exist without the occurrence of an outbreak. They also found that prophylactic or non-specific topical use of antibiotics is not advisable and that monitoring the rate of overt infection is more efficacious than monitoring the rate of colonization. Investigations of \textit{Staphylococcus aureus} in the 1950s and 1960s in both longitudinal and epidemic situations provided the first solid data regarding the epidemiology of colonization and disease among newborn infants. Studies were designed to evaluate a variety of other factors which might influence colonization rates, including the use of central observation
nurseries, cohort nurseries, antibacterial agents on the infant's nares or umbilical cords, and the colonization rates of mothers and personnel. Many of these factors appeared to be interacting at different times (Lowy et al., 2004). Since many neonates are colonized within the first week of life, 20-30% of normal infants carry at least one strain of *S. aureus* in the anterior nares. The organisms may be transmitted from the nose to the skin, where colonization appears to be more transient. Repeated recovery of *S. aureus* from the skin would suggest repeated transfer rather than persistent skin colonization. However, persistent umbilical and perennial carriage occurs.

Transmission of *S. aureus* generally occurs by direct contact or by the spread of heavy particles over a distance of < 2m. Heavily colonized individual carriers are particularly effective disseminators. Autoinfection is thought to be common, and minor infections such as pustules, paronychia and styes may seed more disseminated infections. Consistent hand hygiene between patient contacts decreases the spread of staphylococci from patient to patient. Incidentally, older children and adults are more resistant than neonates to colonization (Behrman et al., 2004). Invasive disease may follow colonization. Antibiotic therapy with a drug to which *S. aureus* is resistant favors colonization and the development of infection. The antibiotics most commonly used empirically in the nursery are ampicillin and gentamicin. Third generation cephalosporins are restricted and are mainly used for treating infections that are the result of antibiotic-resistant organisms and for treating meningitis. Other factors that increase the likelihood of infection include
wounds, skin disease, ventriculoperitoneal shunts, intrathecal or intravenous catheterization, corticosteroid treatment, malnutrition, acidosis and azotemia. Viral infections of the respiratory tract may also predispose to secondary bacterial infection with staphylococci (Behrman et al., 2004).

In one study, rates of colonization by *Staphylococcus aureus* were determined for 9515 infants admitted to the nursery of a general hospital during a six-year period (Czarlinsky et al., 1979). The mean colonization rate was 14%, but there were no consistent phage types present or changes in the number of phage-typable staphylococci during this period. However, during one year of this study a certain phage type became endemic and accounted for 74% of colonization due to *S. aureus*. Anterior nares cultures of the mothers and nursing personnel, umbilical cord cultures of the infants at the time of admission, of the bath water following admission bathing, and of fomites within the nursery excluded these potential reservoirs as the cause of the high colonization rates. The primary reservoir of staphylococci was apparently the infants themselves and the postulated method of spread was hand carriage by healthcare workers and transmission of the organism from infant to infant. In another study, measuring umbilical colonization rates for *S. aureus* among the total infant population yielded a mean colonization rate of 18% over a six-year period. The data were analyzed by the use of simple analysis of variance. There was no significant change in the mean *S. aureus* colonization rate of umbilical cords following the discontinuation of hexachlorophene midway through the study period (Starkb et al., 1992).
1.5 Epidemiology

Many humans are regularly colonized by *S. aureus*, and asymptomatic colonization is far more common than infection (Chambers, 2001). Colonization of the nasopharynx, perineum, or skin, particularly if the cutaneous barrier has been disrupted or damaged, may occur shortly after birth and may recur anytime thereafter. Family members of a colonized infant may also become colonized. In some epidemics of MRSA, a relatively low level of nasal carriage (3%) has been found in hospital personnel, but more recent studies indicate a higher rate of MRSA nasal carriage in health care workers for endemic MRSA situations. The sharp increase in the prevalence of MRSA in many communities has led to consider outpatients as a potential source of contamination in an institution. Populations that are more susceptible to MRSA colonization include intravenous drug users, persons with dermatologic diseases, diabetes, and persons on kidney dialysis. Young children tend to have higher colonization rates, probably because of their frequent contact with respiratory secretions. Colonization may be transient or persistent and can last for years.

1.6 Nosocomial infections

*S. aureus* is a major pathogen of hospital-acquired infections. Colonization of healthy carriers represents a major source of nosocomial infections. A major concern of nosocomial *S. aureus* infections is the presence of endemic isolates exhibiting resistance to methicillin and frequently to several other antibiotics. The prevalence
of methicillin-resistant *S. aureus* (MRSA) has increased in many countries over the
world since 1980. The first strain of MRSA was isolated in 1961 (Jevons, 1961),
two years after the introduction of methicillin. MRSA mainly reside in
environments in which there is a constant antibiotic pressure such as hospitals.
Once endemically present, they are difficult to control and eradicate. A striking
property of MRSA is their tendency to accumulate additional unrelated resistance
determinants and incorporate them into their genome. Their adaptability and ready
response to antibiotic selection has led to the evolution of MRSA strains resistant to
almost all commonly used antibiotics. While vancomycin is still the antibiotic of
choice (and frequently of last resort) for treating MRSA infections, reports of
vancomycin-intermediate *S. aureus* (VISA) in Japan in 1997 (Hiramatsu, 1997) and
later in other countries caused widespread concern among physicians. In 2002, the
first vancomycin-resistant *S. aureus* (VRSA) isolates, exhibiting vancomycin MICs of
1024 and 32 μg/ml, respectively, were reported in Michigan and Pennsylvania
(CDC, 2002a; CDC, 2002b). Currently, there is no antibiotic class that is uniformly
effective against *S. aureus* (Robinson, 2004).

1.7 Methicillin-resistant *Staphylococcus aureus* (MRSA)

The global emergence of drug-resistant bacteria is a pressing public health
problem. Methicillin resistance in *S. aureus* is determined by the *mecA* gene, which is
a part of an additional DNA region, the staphylococcal cassette chromosome *mec*
(SCCmec) (Ibrahim et al., 2005). Mechanisms of MRSA dissemination are the spread
of individual MRSA clones and horizontal transfer of SCCmec among *S. aureus* and
other staphylococci (Ibrahem et al., 2005). Methicillin-resistant strains of *S. aureus* were first reported in 1961, and subsequently outbreaks of MRSA infections occurred worldwide forming a significant proportion of all *S. aureus* isolates in many countries (Diekema et al., 2001, Griffiths et al., 2004 and Moreillon et al., 2005). Widespread antibiotic use, presence of intravascular catheter, severe underlying disease, prolonged hospitalization, and poor adherence to infection control precautions have contributed to the rise in MRSA rates (Petti et al., 2002, Karchmer 2000 and Monnet et al., 2001). The incidence and prevalence of MRSA varies widely between countries, regions and even hospitals (McDonald et al., 2002, Tiemersma et al., 2004 and Boyce et al., 2005). In a survey from the SENTRY antimicrobial Surveillance Program, geographic variations of MRSA prevalence from all sites of infections were found, as follows: Western Pacific region 46%, Latin America 35%, United States 34%, Europe 26%, and Canada 6% (Diekema et al., 2001). In Europe, the proportion of MRSA rates ranged from ≤2% to 54%, and among Western Pacific countries from 24% to 70% (Diekema et al., 2001; Tiemersma et al., 2004). Finland is a country with a very low prevalence of methicillin resistance. For many years prevalence of MRSA was less than 1% among invasive infections. However, during 2004 increased incidence in the number of MRSA bacteremias was observed but its prevalence still remained below 3% (Lyytikäinen et al., 2005, Boyce et al., 2005 and Lyytikäinen et al., 2002). MRSA has been originally confined to nosocomial infection, with only rare community-associated cases. The prevalence of hospital-acquired MRSA isolates increased progressively in the United States from 2% in 1975 to 35%
in 1991 (Panlilio et al., 1992). The rate among bloodstream MRSA isolates during 1997-1999 was even higher, up to 45% (Diekema et al., 2001). MRSA has accounted for over 60% of all isolates in intensive care units (ICU) in the United States, especially among older patients (Kleven et al., 2006). However, a major change in the epidemiology of staphylococcal infections is the rapid emergence of community-acquired MRSA strains which frequently produce dangerous exotoxins (e.g., Panton-Valentine leukocidin) (Gillet et al., 2002; Naimi et al., 2003). According to a recent meta-analysis, (Salgado et al., 2003) the pooled prevalence rates of community-associated MRSA isolated from hospitalized patients were 30% to 37%. These infections usually involve the skin, and outbreaks have been described among prisoners or IDUs, and among patients without established risk factors such as young children, military recruits, or competitive sports participants (Fridkin et al., 2005, Kluytmans et al., 2006 and Maltezou et al., 2006). Generally, nosocomial MRSA isolates are multiresistant and clonal, whereas community-associated MRSA strains are pauciresistant and more polyclonal (Moreillon et al., 2005). Glycopeptide agents have been considered effective antibiotics against multidrug-resistant S. aureus. Therefore, reports of staphylococci with reduced susceptibility to these agents are alarming. The first clinical isolate of vancomycin-intermediate S. aureus was described in Japan in 1997 and since then these strains have continued to cause healthcare-associated infections worldwide. More recently, there have been single reports of vancomycin-resistant S. aureus infections which have not been observed in Finland. (Yoshiyama et al., 2001; Kok et al., 2005)
1.8 Methicillin-susceptible *Staphylococcus aureus* (MSSA)

The epidemiology of and risk factors for healthcare associated infections caused by methicillin-resistant *S. aureus* (MRSA) have been comparatively well-studied, less is known about methicillin-susceptible *S. aureus* (MSSA), especially in the NICU setting. Though MSSA is generally considered an endemic pathogen, there are reports of clusters or mini-epidemics of disease among hospitalized neonates, both in well-baby nurseries and in NICUs. The primary mode of transmission in neonatal nurseries is thought to be from the hands of staff, although *S. aureus* can also be carried in the nose and/or rectum of staff (Kluitmans et al., 1997). Overcrowding in neonatal nurseries has been shown to be a major factor in transmission of organisms among infants. There has been little study of mixed outbreaks of MRSA and MSSA (de Sousa et al., 2005) and infection control strategies to control these outbreaks. Because several infection control strategies are normally instituted simultaneously in order to halt the epidemic as soon as possible, the relative importance of specific measures is unclear. In one study, the number of infants who were known to be colonized in the umbilicus with *S. aureus* at the Darlington Maternity Hospital increased from 6.4% to 8.5% between 1981 and 1987. There was no evidence of any relationship between colonization and changes in either birth rate, new intakes of clinical staff or repairs and maintenance to the old building. However, infants were normally only cultured if they were thought to have a clinical infection, so colonization rates were probably underestimated (Starkb et al., 1992).
1.9 Risk factors for *Staphylococcus aureus* infections

1.9.1 *Staphylococcus aureus* colonization

Many healthy adults are persistently or intermittently colonized with *S. aureus* in their anterior nares. Approximately 20% of individuals are persistent nasal carriers, 30% are intermittent carriers, and 50% are non-carriers (Kluytmans et al., 1997; Wertheim et al., 2004). Nasal carriage of *S. aureus* is one of the most important risk factors for nosocomial and surgical site infections (Moreillon et al., 2005; Wertheim et al., 2004). Some subgroups such as IDUs, patients undergoing hemodialysis or peritoneal dialysis, and patients with diabetes, human immunodeficiency virus (HIV) or recurrent skin infections are at increased risk for skin and nasal colonization with *S. aureus* (Lipsky et al., 1987, Yu et al., 1986, Hoeger et al., 1992, Tuazon et al., 1974 and Miller et al., 2003). Studies among IDUs have shown that injection of contaminated drug and inhalational drug use may support nasal *S. aureus* colonization, probably by damaging the nasal mucosa (Bassetti et al., 2004; Lowy et al., 2002). In recently published studies, nearly 80% of nosocomial SAB were due to the same phage type of *S. aureus* strain isolated from the patients' anterior nares (Wertheim et al., 2004; Von et al., 2001). Decolonization with mupirocin has been shown to prevent staphylococcal disease in dialysis and surgical patients (Kluytmans et al., 1996; Boelaert et al., 1991). However, recent clinical trials in non-surgical and orthopedic patients uniformly failed to confirm these results (Kalmeijer et al., 2002; Wertheim et al., 2004). Comorbid conditions and predisposing factors Several factors have been suggested to increase the risk for invasive *S. aureus* infections. A
high proportion of patients with SAB have underlying diseases such as cardiovascular disease, chronic obstructive pulmonary disease, diabetes mellitus, malignancy, chronic renal failure, HIV infection, or hepatic cirrhosis (Kuikka et al., 1994, Laupland et al., 2003, Lautenschlager et al., 1993, Espersen et al., 1995, Jensen et al., 2003 and Mylotte et al., 1987). Only 3% to 5% of patients have had no underlying disease (Kuikka et al., 1994; Lautenschlager 1993).

Furthermore, recent surgery, prosthetic devices, or the presence of intravascular catheters are important risk factors (Petti et al., 2002). Older age (>60 years), male sex, alcohol abuse, hyponatremia, anemia, immunosuppressive treatment, injection drug use, preceding trauma, previous hospitalization, or prolonged treatment in ICU predispose to SAB (Lowy et al., 1998, Steinberg et al., 1996, Laupland et al., 2003, Fowler et al., 2005, Espersen et al., 1995, Jensen et al., 2003 and Gransden et al., 1984). Patients with chemotactic defects (e.g., Job's syndrome) and defects in phagocytosis are also at increased risk for staphylococcal disease (Moreillon et al., 2005). Historically, most cases of IE have occurred in association with community-acquired SAB (Lautenschlager et al., 1993; Nolan et al., 1976). Underlying cardiac diseases have remained one of the most important risk factors for endocarditis among patients with SAB (Espersen et al., 1986). Classic risk factors such as rheumatic heart disease are now being replaced by new ones, including IDUs, elderly patients with degenerative valve sclerosis, hemodialysis patients, and patients with an intravascular catheter or prosthetic valve (Fowler et al., 2005, Chang et al., 2003, Moreillon et al., 2005, Abraham et al., 2004 and Mylonakis et al., 2001).
addition, previous IE, mitral valve prolapse, unknown portal of entry, immunosuppression, and hospital-acquired bacteremia have been related to predisposing conditions for endocarditis (Mitchell et al., 2005, Cecchi et al., 2004, Cabell et al., 2002, Chang et al., 2003, Cabell et al., 2004 and Lesens et al., 2003) A variety of strategies have been used in attempt to identify patients who develop metastatic infections secondary to SAB. In recent studies, the most important risk factors for complicated SAB were community-acquisition, hemodialysis,( Fowler et al., 2005; Marr et al., 1998) one or more underlying diseases,( Lesens et al., 2003) persistent bacteremia or fever for more than 72 hours,( Fowler et al., 2003, Chang et al., 2003, Khatib et al., 2006 and Lesens et al., 2004) C-reactive protein (CRP) level >100 mg/L,( Lesens et al., 2003) the presence of a permanent foreign body, or a failure to remove an infected catheter (Fowler et al., 2005; Mermel et al., 2001). The impact of MRSA in complicated SAB has been evaluated in some studies. Patients with MRSA bacteremia did not have a higher rate of metastatic infections,(Soriano et al., 2000, Fowler et al., 2003, Romero-Vivas et al., 1995 and Harbarth et al., 1998) although in one study methicillin resistance was independently associated with an increased risk for metastatic infections in intravascular catheter-associated SAB (Fowler et al., 2005). The genetic properties of MRSA isolates may produce more virulent phenotypes in cases of catheter-associated bacteremia. Treatment of MRSA-infected patients with vancomycin has also been related to clinical failure and prolonged bacteremia (Small et al., 1990; Levine et al., 1991).
1.10 MRSA prevalence in hospitals

Since the mid to late 1990s, the prevalence of MRSA isolates in hospitals increased in Europe, the USA, and elsewhere. In one European study performed in 25 university hospitals, one-quarter of 3051 S. aureus isolates were MRSA, with a geographical bias towards higher rates in southern countries such as Italy (50.5%) and Portugal (54%), and lower rates in northern European countries, including the Netherlands (2%), Austria (9%) and Switzerland (2%) (Robinson, 2004; Enright, 2003). The National Nosocomial Infections Surveillance system (NNIS) reported a 40% increase in the rate of MRSA in 1999 compared to 1994-1998 data (NNIS system report, 2000). MRSA infections are associated with increased morbidity, mortality and length of hospital stay, and represent a major financial burden on healthcare services (Nathwani, 2003; Cosgrove, 2003). Epidemiological studies confirmed that MRSA infections are more costly to manage (screening, treatment and isolation) than other types of infection (Rubin, 1999). They also highlighted the importance of hospital length of stay as a key determinant of the total cost of an episode of infection. In summary, MRSA are now endemic in many hospitals, and represent one of the leading causes of nosocomial pneumonia and surgical site infections and the second leading cause of nosocomial bloodstream infections.

1.11 Molecular structure of the methicillin resistance gene

The structural gene for methicillin resistance, meca, encodes a novel penicillin binding protein (PBP)-2', which has reduced affinity for β-lactam
antibiotics (Hiramatsu, 1995; Chambers, 1988; Berger-Bachi, 2002). This gene is carried on a genetic element, staphylococcal chromosomal cassette (see figure), which is a 20-67-kb DNA element which precisely inserts into the \( S.\ aureus \) chromosome at the \( orfX \) locus. SCC\( mec \) is found in other staphylococcal species from which it is presumed to have been transferred (Ito, 2003). The staphylococcal cassette chromosome \( mec \) (SCC\( mec \)) is a special family of staphylococcal genomic islands (GI) characterized by its cassette chromosome recombinases A (CcrA) and B (CcrB) (Hiramatsu, 2001; Ito, 2003; Enright, 2003).

**Figure. Structures of four types of SCC\( mec \) genes.**

SCC\( mec \) is composed of two essential gene complexes, the \( ccr \) gene complex (orange) and the \( mec \) gene complex (gray). \( ccr \) gene complex is composed of \( ccrA \), \( ccrB \) genes which are responsible for the mobility of SCC\( mec \) and some \( orfs \).
surrounding them. *mec* gene complex is responsible for β-lactam resistance. Other areas (light gray) of the SCC*mec* are non-essential, and are divided into three regions. Type-IV SCC*mec* is mostly composed of essential gene complexes (Ito, 2001).

The *ccr* gene complex is composed of two site-specific recombinase genes, *ccrA* and *ccrB*, responsible for the mobility of SCC*mec*, and surrounding *orfs* of unknown function. The rest of the SCC*mec* is designated J regions (J stands for junkyard) that contain various genes or pseudo genes whose presence does not appear essential or useful for the bacterial cell. Figure illustrates the structure of the four SCC*mec* types and their subtypes. Three types of SCC*mec* element (type I, II, III) are found in hospital-acquired MRSA (HA-MRSA) strains and one type of SCC*mec* element (type IV) in community-acquired MRSA (CAMRSA). Type I SCC*mec*, a 34-kb element that was first described in 1960s MRSA isolates and does not contain any antibiotic resistance genes other than *mecA*. Type II SCC*mec*, a 53-kb element, carries the type-2 *ccr* gene complex, which was identified in 1982 and is ubiquitous in Japan, Korea, and the United States. MRSA N315 and Mu50 carry type-II SCC*mec*, containing integrated copy of plasmid pUB110 and transposon Tn554 in the J region. Type-III SCC*mec*, the largest element at 67 kb, was identified in 1985 and is prevalent in Germany, Austria, India, and other South Asia and Pacific areas, containing integrated copy of plasmid pT181, transposon Tn554, and pseudo Tn554 that encode resistance to tetracycline, erythromycin, and cadmium, respectively. Type IV SCC*mec* includes four subtypes, whose sizes vary from 20 to 24 kb, and is
much smaller than SCC\textit{mec} types I-III. SCC\textit{mec} type IVa is generally found in recently described (Okuma, 2002) community-acquired MRSA isolates (CA-MRSA). Types-IVa and - IVb SCC\textit{mec} do not harbor any antibiotic resistance genes except for \textit{mecA}. The community-acquired MRSA MW2 carry type-IVa SCC\textit{mec}.

1.12 Transmission of MRSA

Recent molecular studies on the genetic origin of methicillin resistance in \textit{S. aureus} have led to a greater understanding of the epidemiology of MRSA (Muto, 2003). \textit{De novo} development of MRSA results when a strain of MSSA acquires a large genomic element SSC\textit{mec}. Detailed genetic analysis of MRSA from diverse parts of the world suggests that the transfer of SSC\textit{mec} from a MRSA strain to a MSSA strain occurred rarely, and therefore the worldwide emergence of MRSA mostly resulted from dissemination of only a limited number of types rather than frequent \textit{de novo} introduction of new MRSA clones. These findings suggest that most patients acquired their MRSA infection or colonization by transmission from other colonized patients or health care workers. Transmission of MRSA within and between healthcare facilities has been well documented using molecular typing techniques, such as pulsed-field gel electrophoresis (PFGE). Outbreaks involving clonal spread within single facilities have been frequently reported. Geographic clustering of closely related genotypes within cities and geographic regions has been described, suggesting that spread beyond the boundaries of a single hospital may occur. Transmission of MRSA from one city to another, from country to
country, and even from continent to continent has been traced to the transfer of patients infected or colonized with MRSA.

1.13 Mechanisms of transmission

- Healthcare workers’ (HCWs) hands is the major source of cross-transmission: in several studies, MRSA or VRE have been isolated from the hands, gloves, or both of the HCWs involved in the care of infected or colonized patients.
- HCWs’ clothes: several investigators suggested that the contaminated HCWs’ clothing may result in the transmission of microbes from patient to patient;
- Contaminated equipment, environment, and air (in the case of staphylococcal pneumonia). In one study, 42% of nurses’ gloves became contaminated with MRSA when they touched surfaces in the room of a patient with MRSA even without touching the patient. Prolonged hospital stay, use of broad spectrum antibiotics, greater number and longer duration of antibiotic use, stay in an ICU or burn unit, surgical wounds, poor functional status and proximity to another patient with MRSA constitute risk factors for MRSA acquisition (Muto, 2003).

1.14 Control and preventive measures

- Active surveillance cultures are essential to identify the reservoir for spread of MRSA and VRE infections and implement infection control guidelines.
Many studies have shown that endemic and/or epidemic MRSA and VRE infections can be controlled by using surveillance cultures and contact precautions and has been proven to be effective (Pittet, 2000; Muto, 2003).

- Hand hygiene is an important measure for preventing MRSA transmission. Educating healthcare workers and patients on the importance of hand hygiene is very important for preventing MRSA transmission and has been proven to be effective (Pittet, 2000; Muto, 2003).

- Contact precautions include wearing gloves, gowns and masks (if expected contact with patient or environment), regular cleaning of equipments and items. Jernigan (Jernigan, 1996) found a 16-fold reduction in transmission when contact precautions were implemented.

- Antibiotic control may also decrease the risk of MRSA colonization. It has been shown that antibiotics in general and fluoroquinolone in particular must be used prudently in institutions where MRSA is endemic.

- Eradication of colonization in HCWs is necessary, in particular in outbreak situations.

- Information management: a hospital computer system can be used to store information regarding long-term isolation indicators for patients known to be colonized with MRSA or VRE. This was done at the University of Geneva Hospitals and resulted in a significant improvement in isolation of such patients on readmission (Pittet, 1996).
1.15 Principles and overview of current typing methods

Over the past two decades, a plethora of novel and often innovative typing methods has been developed. These range from methods that assess simple phenotypic traits to DNA sequencing. Previously, the comparison of phenotypic characters, which involves the comparison of apparent biological features of isolates, was often abandoned because of the problems with performance criteria already mentioned. Instead, methods involving the comparison of genomic DNA fragments were adopted. DNA molecules (or restriction fragments or amplified sections thereof) can be separated on the basis of their molecular size by gel electrophoresis. Such size comparisons assess differences in the length of DNA fragments obtained from DNA from different bacterial strains. Whether the fragments of DNA are natural (e.g., plasmids) or generated at random, by restriction enzymes or after amplification of the DNA using enzymatic DNA replication (PCR), does not matter; size differences, provided that they are accurately determined, can be excellent markers of strain differences. By definition, the genome of every bacterial isolate is unique. The mere fact that DNA polymerases make copying mistakes during replication suggests that no genome has a 100% identical counterpart (Yoshiyama et al., 2001). However, such mutations must be compatible with nature; they must be neutral or at least in line with existing structure–function relationships among the corresponding gene products. Hence, bacterial strains differ with respect to their complete genome sequence, and DNA sequencing methodologies can therefore be used to assess similarity of strains. A
challenge for the near future is to assess which DNA sequences are useful epidemiological markers, a task that is greatly assisted by whole genome sequencing (Kok et al., 2005, Scarselli et al., 2005 and Kuroda et al., 2004). Since far more detailed reviews exist concerning the technical aspects of typing methods [50, Norris et al., 1980] we will restrict ourselves to defining briefly the common aspects and quality characteristics of the methods, without any claim to completeness. The diversity and plethora of methods available to the scientific community are such that it is impossible to be comprehensive in the subsequent sections. Strategic literature references will be included to facilitate and stimulate further reading. Important overviews of typing methods can also be found in several general textbooks on the practical and theoretical aspects of bacterial typing.

1.15.1 Phenotypic typing methods

Phenotyping may involve colony morphology, colour, odour and other macroscopic features, but most typing methods rely on traits that require specialised technology in order to be documented. For example, they may assess, qualitatively and quantitatively, the ability of isolates to grow in the presence of specific substances (be they metabolites, drugs, bacterial toxins or bacteriophages) and their expression of specific molecules (be they surface antigens or allelic variants of housekeeping enzymes). All methods require strict standardisation of experimental conditions, since phenotypes are generally quite susceptible to changes in environmental conditions. In a simple statement: phenotyping results in
the grouping of organisms according to their similarity in characters resulting from the expression of their genotypes.

1.15.1.1 Biotyping

This method assesses biochemical characteristics that are known to vary within a given species. Typeability is usually excellent. Discriminatory power is variable and to optimize it, a large number of well-selected characteristics, e.g., metabolic reactions need to be included in the test scheme. Stability is dependent on the species and characteristic under consideration. The methods are usually technically easy and inexpensive, the data generated are simple to score and interpret, and all tests can be performed, even in the smallest of laboratories, on large numbers of isolates. If reproducibility is demonstrated, it can be used as a library typing method (Bouvet and Grimont, 1987; Kuhn et al., 1991). For instance, commercial systems facilitating the measurement of large panels of 'biotype characteristics' have been developed. These systems use versatile redox technologies, enabling the quantification of various biochemical reactions by colour readings (Bochner, 1989, 1993, Pitulle, 1999 and Carnahan et al., 1989). The main power of the system lies in its ability to distinguish among strains within a species (Di Giovanni et al., 1999; Bochner et al., 2001). Phenotype reaction arrays are available and are useful tools in addition to DNA and proteomic technologies. The reproducibility of biotyping is organism and character-dependent. It is rarely 100%.
1.15.1.2 Antimicrobial susceptibility testing (antibiogram-based typing)

The antibiogram can be performed either by drug diffusion in solid growth media or drug dilution in liquid media using a variety of measurement systems. Most clinical microbiology laboratories perform some sort of antibiogram typing, since its results are commonly used to guide chemotherapy. Therefore, this method has immediate clinical consequences also. Antibiogram-based typing can, with appropriate selection of drugs, be applied to most species. Discrimination is dependent on the diversity, stability and relative prevalence of the detectable acquired resistance mechanisms in study isolates. It is also dependent on the number of antimicrobials (including antibiotics no longer in use, such as neomycin, which are adequate for revealing specific resistance mechanisms). Testing for resistance to heavy metals (resistotyping), as well as to disinfectants and antiseptics, can provide useful typing information. The utility of this method can vary according to the stability of resistance patterns, which can be insufficient for use as a clonal marker. Some resistance determinants are plasmid-borne and can be readily lost in the absence of selective conditions; in addition, resistance expression can be under the control of complex regulatory systems. Susceptibility profiles expressed as diameters of inhibition zones combined with cluster analysis can provide useful typing data as an adjunct to data generated by other methods (Sloos et al., 1998; Dijkshoorn et al., 1996). There exist large, international databases built around antibiograms, including data on the geographical origin and clinical nature of the isolates. Although these are primarily used to estimate incidences of resistance, they
may, of course, also be consulted for epidemiological queries concerning the spread of specific resistance markers (Fluit \textit{et al.}, 2006; Bronzwaer \textit{et al.}, 2002). It is of note that similar resistance patterns may be due to convergent evolution (as is the case with many extended-spectrum \( \beta \)-lactamase-producing microorganisms, for instance), which is a strongly confounding phenomenon.

\subsection{1.15.1.3 Serotyping}

This is traditionally the most important phenotypic method that has been developed from the early days of microbiology. It has led to comprehensive systems for typing of, for example, Salmonella and \textit{E. coli} isolates. Most typing sera react with surface antigens. These systems are still widely used in healthcare-associated or food-associated microbiology laboratories. High-throughput procedures using defined sets of polyclonal or monoclonal antibodies have been made available (Frasch, 1994). Typeability and discrimination, complicated by cross-reactions, are variable. With adequate quality control of both reagent and method, serotyping can be a reproducible, library typing method of wide applicability. Standardisation of preparation and testing conditions is important. Discrimination can sometimes be improved by combining serotyping with SDS-PAGE, resulting in ‘western’ (immuno) blotting (Vauterin \textit{et al.}, 1993) Some serotyping schemes (e.g., the one for \textit{E. coli} or \( M \)-protein typing of \textit{Streptococcus pyogenes} (Stanley \textit{et al.}, 1995)) are now being replaced by their genotypic equivalents, where variability is assessed at the level of genes encoding for the antigens (Nowakowska \textit{et al.}, 2006; Gimenez, 1976).
Similarly, restriction analysis of the amplified O-antigen gene cluster ('molecular serotyping') has proven to be an interesting alternative for classic serotyping of *E. coli* and *Shigella* isolates (Coimbra *et al.*, 1999, 2000). Genetic instability per se, horizontal gene transfer and convergence due to natural or vaccine-driven herd immunity intrinsically limit the power of serotyping methods.

1.15.1.4 Phage and bacteriocin typing

To assess the lytic patterns of test isolates those have been exposed to a defined set of bacteriophages, or bactericidal toxins (bacteriocins). These traditional typing methods are restricted to a limited numbers of species for which such agents have been identified in numbers large enough to provide a useful degree of discrimination. In addition, when new bacterial clones are discovered, additional phages may need to be included in the typing scheme. Types can change over the longer term, and this in itself can be a useful characteristic in endemic situations. Discrimination is therefore variable, typeability often partial, and reproducibility poor. The production and continuous quality control of phages is important, requiring extensive expertise and time-consuming efforts. However, large numbers of isolates can be processed readily, which is not the case with most current DNA fragment-based typing methods. Interpretation of results is not easy and requires training and experience (Weller, 2000; Godovannyi *et al.*, 1974). Nowadays, acquisition or loss of phages, which may play a role in virulence, can be traced by molecular typing, providing a modern extension of the role of phage typing (Goerke
et al., 2004). Phage typing has long been an important tool with which to study the epidemiology of S. aureus for example, but today it has lost its position as a reference typing method.

1.15.1.5 SDS-PAGE of cellular and extracellular components

This can give rise to highly discriminatory typing methods, with applications in taxonomy also [16, Dijkshoorn et al., 1987, Holmes et al., 1991, Van Alphen et al., 1988 and Pot et al., 1993]. In the 1980s, these methods were applied to a variety of organisms, but since the 1990s they have been largely superseded by DNA-based methods. Interestingly, the need for comparative analysis of the complex banding patterns obtained by protein SDS-PAGE was the trigger for the development of dedicated computer software that is now successfully applied to DNA fragment analysis. By protein SDS-PAGE, cell envelope fractions obtained by sonication and stepwise centrifugation, or whole cells, are solubilised in buffer with the denaturing agent SDS and separated under denaturing conditions by PAGE. After staining, the gels are digitised and the images subjected to cluster analysis. If growth conditions, sample preparation and electrophoresis are rigorously standardised, the profiles are reproducible and suited for databases for longitudinal analysis. Protein SDS-PAGE is rather laborious and requires experience; the advantage is that reagents and equipment are relatively inexpensive. The step from protein SDS-PAGE to lipopolysaccharide (LPS) gel electrophoresis is relatively small, since the samples prepared for protein analysis can be treated with proteinase K, after which they can
be used for electrophoretic separation of LPS molecules, followed by silver staining to visualise them. 'Ladder-type' LPS gel electrophoresis can be strain-specific and has been used for comparative typing, but the method is not widely used because it is laborious (Aucken et al., 1993, Pantophlet et al., 1998 and Garin et al., 1990).

1.15.1.6 Multilocus enzyme electrophoresis (MLEE)

This method identifies electrophoretic variants of a set of housekeeping enzymes, encoded by different alleles of the same gene, thus giving rise to small but detectable variations in protein size and charge (Selander et al., 1986). MLEE has been used as a reference method for defining the phylogenetic structure of clonal lineages in bacterial populations. Although it is neither a rapid nor a widely applied system, it has been very important in shaping the bacterial population biology landscape. Its molecular progeny, MLST (see below), is much more practical and, hence, more widely used nowadays. Mass spectrometry (MS) is a technique originally developed for the identification of (primarily organic) molecules of a low molecular weight in complex mixtures (Duffield, 1974). Nowadays, the technology can also be used to characterise mixtures of complex biological macromolecules, through their specific degradation products. Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) MS facilitates the generation of molecular fingerprints for entire organisms (Dworzanski et al., 2005, Jackson et al., 2005 and Lechner et al., 2005). The method uses intense laser light to evaporate the biological material, which is subsequently subjected to a strong electrical field. Small ions
move at high speed and reach a detector before the larger ones. The signals generated are recorded and give rise to complex spectra, characteristic for the molecular content of a bacterial cell. When these spectra are compared using appropriate computer software, bacterial types can be distinguished. MALDI-TOF MS is also suited for the analysis of less complex mixtures of, for instance, DNA molecules (Kirschner et al., 2001, Lefmann et al., 2004, Stanssens et al., 2004 and Honisch et al., 2004). (Other spectroscopic methods, based on alternative biophysical strategies, can be used as well. Infrared (IR) or Raman spectroscopy are two such methods that can be used for isolate comparison (Goodacre et al., 1998, Maquelin et al., 2006 and Seltmann et al., 1994). Both use focused illumination of bacterial biomass and record the emission spectra generated. The complexity of the spectrum reflects molecular complexity and, although not every peak in the spectrum can be assigned to a submolecular particle, the composite patterns can allow comparisons to be performed and types to be assigned. Other spectroscopic and chromatographic methods have been commercialized successfully and can provide useful platforms for certain formats of bacterial typing. Gas-liquid chromatography (GLC; the widely used MIDI system) and Fourier-transform (FT)-IR spectrometry/FT-IR microscopy are merely two examples (Kirschner et al., 2001; Naumann et al., 1991).

1.15.2 Genotypic typing methods

Genotypic typing methods assess variation in the genomes of bacterial isolates with respect to composition (e.g., presence or absence of plasmids), overall
structure (e.g., restriction endonuclease profiles, number and positions of repetitive elements), or precise nucleotide sequence (of one or more genes or intergenic regions). Basic genetic analysis of the molecular event(s) (acquisition, multiplication, mutation, deletion, insertion) associated with pattern variation is the preferred approach to measuring inter-strain relatedness, but is neither always required nor generally feasible [Hall, 1994]. A wide variety of genotypic methods has been presented, of which the most widely used will be discussed below in a 'rational-historical' order. The increasing availability of bacterial genome sequences has had, and is still exerting, a great impact on the evolution of these methods, by facilitating the choice of successful typing targets. Hybridisation-mediated methods.

1.15.2.1 Direct (and reverse) hybridization

Direct hybridization testing of bacterial genomic DNA (without restriction enzyme treatment) is feasible. In all methods, the immobilised DNA to be investigated is probed with DNA molecules that are selective; some templates are recognised, and others are not. The technologies employed vary widely, but the core technology was developed by Southern and colleagues (Southern, 1975) (hence 'Southern hybridisation'). As a recent example, 'binary' typing has been developed for S. aureus through the isolation of DNA probes that are specific for some S. aureus strains (Van Leeuwen et al., 1999, 2001). The method proved to be reproducible and easy to perform (Van Leeuwen et al., 1998). Similar systems have been developed for other bacterial species (Lemee et al., 2005; Shepard et al., 2005).
Direct hybridization tests can also be used to define the nature of mobile elements involved in methicillin resistance or to identify determinants of glycopeptide resistance in *S. aureus*. The same methodology can be used for typing of DNA amplified by PCR. For instance, *Mycobacterium tuberculosis* ‘spoligotyping’ includes amplification of a locus harbouring tandem repeats with some internal sequence variation. These variants are then identified by hybridisation using repeat-specific DNA probes (Glynn *et al.*, 2002; Wootton *et al.*, 2005).

1.15.2.3 Ribotyping

This is a classic variant of a Southern hybridisation-mediated assay (Grimont *et al.*, 1986) that estimates the number of ribosomal gene loci and their position in the chromosome. It is reproducible and applicable to (fast-growing) bacteria, but has a discriminatory power that is usually lower than that of, for example, PFGE (Blanc *et al.*, 1994). Fully automated robots for ribotyping have been made available, reducing hands-on time, albeit at a significant price (Bruce *et al.*, 1996; Arvik *et al.*, 2005). The automated method has been compared with a variety of other genotyping methods (Ito *et al.*, 2003, Botes *et al.*, 2003, Grif *et al.*, 2003, Brisse *et al.*, 2000, Verduin *et al.*, 2000, Brisse *et al.*, 2002., Pfaller *et al.*, 2005 and Dalsgaard *et al.*, 1999) and, although it was demonstrated to be useful for various bacterial species, it did not always stand out as a superior method (Skinner *et al.*, 2000) since its discriminatory power is relatively limited. Nevertheless, it is robust, and profiles can be compared among laboratories and be used for the generation of databases;
hence, it was adopted for some pathogens important in food microbiology (Arvik et al., 2005) Reproducibility has been documented experimentally during clinical microbiological usage (Pfaller et al., 2005; Tettelin et al., 2005)

1.15.2.4 Genome analysis by array hybridization

Array systems currently represent state-of-the-art hybridisation-mediated testing. This method capitalizes on the technological possibility of immobilizing up to several hundred thousands of DNA probes per square centimetre of a solid matrix. For most of the clinically relevant microorganisms, whole genome arrays have been developed, based on the available whole genome sequences, and covering all of the genes identified. Probes may be PCR products of defined length, but synthetic oligonucleotides are more frequently used. These platforms facilitate bacterial typing in unprecedented detail. As the method is not yet suited for day-to-day clinical application, careful consideration of target genes is necessary in order to achieve optimal epidemiological concordance. Currently, costs and accessibility also remain problematic. A recent comparison of multiple genomes of strains of the same species has shown that considerable gene variation exists within a species, and the term 'pan genome' was coined to denote the cumulative genome deduced from the individual genome sequences (Eisgruber et al., 1995). Hence, it is emphasised that analyses based on single-strain genomes of a given species are not likely to be sufficient to make generalisations about the species as a whole.
1.15.3 Fragment-based methods

1.15.3.1 Plasmid typing

This assesses the number size and/or restriction endonuclease digestion profiles, after agarose gel electrophoresis, of these bacterial extrachromosomal genetic elements. It has been used for typing of many bacterial species [9]. Typeability and discrimination are variable, depending on the bacterial species [9]. However, the lack of stability of plasmid content rendered it unsuitable for use as a reliable clonal marker in some studies (Pang et al., 2005). It is best combined with other genomic typing methods, to distinguish, for example, between spread of a resistant clone and that of a resistance plasmid [23]. Plasmid typing is still used frequently in combination with testing of antimicrobial susceptibility in modern clinical microbiology laboratories (Wei et al., 2005; Crawford et al., 2003) to assess whether an antibiotic resistance gene is plasmid-borne and can be transferred.

1.15.3.2 Among restriction fragment length polymorphism (RFLP) methods

This was the first to be widely used. The chromosome is digested by frequently cutting restriction enzymes into several hundreds of small fragments, which are separated by horizontal gel electrophoresis into complex patterns [10]. It is rapid and, under standardised conditions, very reproducible and discriminatory. However, the complex patterns produced complicate interpretation and hinder data exchange among laboratories. In order to simplify the interpretation of REA results, Southern blot and hybridization steps were added. A variant, which is very
important historically, is ribotyping (mentioned above), a method that couples genome digestion by a ‘frequent-cutting’ restriction endonuclease with a 4-bp recognition sequence, and hybridization with a probe complementary to rDNA. Some of the hybridisation probes used are restricted to a single species; the most illustrious and popular example is IS6110 typing of M. tuberculosis. This method has been the agreed standard among tuberculosis reference laboratories worldwide over the past 15 years. It has been applied during hundreds of studies, and its output has been shown to be communicable among institutions and over the years, as thousands of profiles generated in different laboratories have been integrated in a central database (Dale, 1995 and Wagner et al., 1994). A new electrophoresis technique, PFGE, made it possible to separate large DNA fragments in agarose gels by periodic alternation of the angle of the electric field’s direction. These DNA ‘macrorestriction’ fragments are generated with restriction endonucleases with six or more base pair recognition sites (‘rare cutters’), usually yielding fewer than 30 large fragments, normally ranging in size between 20 and 600 kbp. PFGE was originally used for electrophoretic separation of the chromosomes of lower eukaryotes, and has enabled epidemiological studies of yeasts and fungi (Beadle et al., 2003; Barney et al., 2001). Only in the case of excessive endogenous endonuclease or DNA methylation activities has PFGE been problematic (Bens et al., 2006). However, even these technical problems can be overcome by the use of chemical endonuclease inhibitors and alternative restriction endonucleases. PFGE has remarkable discriminatory power and reproducibility, and has therefore become a
widely applicable method for comparative typing of almost all bacterial species (Coenye et al., 2002 and De Zoysa et al., 1999). With careful standardisation, acceptable levels of interlaboratory reproducibility can be achieved, which have allowed the creation and maintenance of international databases, with the PulseNet effort representing an important achievement (Tenover et al., 2006, Cardinali et al., 2002, Van et al., 1998, Samore et al., 1997, Foissaud et al., 1999, Walker et al., 1999, Zinn et al., 2004 and Murchan et al., 2003). However, 2–4 days are required to obtain results and relatively expensive PFGE equipment is required. Gels need to be analysed closely and carefully, even after digitalisation and computerised processing (Chung et al., 2000). To confirm the outcome of the mathematical analysis and to verify, establish or refute finer discrimination, quality control is essential.

PCR fingerprinting relies on the amplification of genomic fragments flanked by one or two oligonucleotide sequences used as primers. These primers should preferably be cognate to the species being typed. Cognate primers allow for relatively high annealing temperatures, thus contributing to high reproducibility, in contrast to non-cognate primers such as the very widely used ‘arbitrary’, randomsequence primers that range between six and ten nucleotides in length. Primer pairs are often designed to be directed outwards from repetitive elements, to amplify short spacer sequences lying between these elements. It is a quasiuniversal typing method, exhibiting an easily adjustable level of discrimination (Deplano et al., 2000). Its major advantages include flexibility, technical simplicity, wide availability of equipment and reagents, and rapid, same-day turnover. However, interpretation of
band differences, of necessity, remains biologically unfounded (it can never be known, for example, if other unobserved ‘spacer sequences’ existed that were longer than what could be amplified by the DNA polymerase used) and, as suggested, this method can rarely be considered a ‘library’ method. PCR ‘fingerprinting’ data, in general, are considered to be non-exchangeable among laboratories (Coenye et al., 2002, Caetano-Anolles et al., 1996 and Al-Thawadi et al., 2003), although commercial tests claim the contrary (Krawczyk et al., 2003). In order to increase the resolution of PCR fingerprinting, an RFLP step is sometimes added. One example of a PCR-RFLP method is amplified ribosomal DNA restriction analysis (ARDRA), which has been used successfully for species identification of various organisms, including acinetobacters (Healy et al., 2005), while there are numerous examples of this methodology for typing of other bacterial species. Essentially, PCR-RFLP monitors for a variety of mutations that can occur in restriction sites and, as such, is a variant method for detection of single nucleotide polymorphisms (SNPs) (see later in ‘Sequence-based methods’).

1.15.3.3 Amplified fragment length polymorphism (AFLP) analysis.

AFLP is the patented name of a method designed to selectively amplify subsets of genomic fragments generated with one or two restriction enzymes, usually a ‘rare’ and a ‘frequent cutter’ (Vaneechoutte et al., 1998; Vos et al., 1995). After ligation of adapters to the restriction fragments, selective amplification is achieved by the use of primers that consist of the adapter-derived core sequence,
including the 3' part of the restriction half-site, and an extension of one or more selective bases. Elongation will only take place if a nucleotide complementary to the selective base in the primer sequence is present in the fragment. Products can be separated in agarose gels (Janssen et al., 1996, Valsangiacomo et al., 1995 and Nemec et al., 2001), but usually one primer is labelled and fragment separation is obtained using an automatic DNA sequencing instrument with automated data capture. The digitised and complex DNA fingerprints are generally highly reproducible and have been used very successfully for the high-throughput molecular typing of large numbers of bacterial isolates (Wroblewska et al., 2004; Melles et al., 2004). Essentially, nearly whole genome coverage can be attained. For some bacterial species, databases have been developed and inter-centre reproducibility assessed (Van den Braak et al., 2004, Fry et al., 2005, Horvath et al., 2004 and Fry et al., 2002). Recent and, as yet, unpublished studies have revealed that AFLP may also suffer from the absence of inter-centre reproducibility, especially when different electrophoresis platforms are being employed (Van den Braak et al., 2004, Fry et al., 2005 and Hong et al., 2005). For Acinetobacter spp., the method is useful to identify species (Nemec et al., 2001), clones within Acinetobacter baumannii (Wroblewska et al., 2004) and epidemic strains (Wroblewska et al., 2004). The profiles generated with labelled primers and automated sequencing equipment is highly complex, and dedicated software for cluster analysis is therefore mandatory.
1.15.3.4 Multilocus variable number tandem repeat (VNTR) analysis (MLVA)

These are also a PCR-based typing method that capitalizes on the inherent variability encountered in many regions of repetitive DNA. Repetitive DNA is often incorrectly copied in bacterial species, through slipped strand mispairing (SSM) (Van Belkum et al., 1998, Hammerschmidt et al., 1996 and Lindstedt et al., 2005), thus resulting in shortening or lengthening of the repeat region due to deletion or insertion of repeat units, respectively (Hammerschmidt et al., 1996). This type of DNA variation can be simply assessed by performing repeat-spanning PCRs and determining the length of the PCR product. In the case of large repeat units, the analysis system can be simple (e.g., agarose gel electrophoresis). However, for shorter repeats, more complex electrophoresis or MS methods are required. For each repeat locus, a digit can be assigned, representing the number of repeats implied (by electrophoresis) or demonstrated (by sequencing). When assessing the length of the product, normalization of the migration distances is required to guarantee accurate length measurement. When several repeat loci are analyzed per isolate, several such digits are obtained, resulting in a multi-digit, specific strain code (Hammerschmidt et al., 1996). Dedicated MLVA systems have been developed for a variety of species (Lindstedt et al., 2005, Onteniente et al., 2003, Francois et al., 2005, Malachowa et al., 2005, Titze Almeida de et al., 2004, Johansson et al., 2006, Noller et al., 2006, Arricou-Bouvery et al., 2006, Sawires et al., 2005 and Stratilo et al., 2006). When compared with other genotyping methods, MLVA has, in general, performed well (Francois et al., 2005, Kremer et al., 2005 and Schouls et al., 2006). However, few multicentre
studies have been undertaken. Given its technical simplicity, MLVA may have a successful future. The major drawback is that the evolution of repetitive DNA may be too rapid, compromising epidemiological concordance. When the mutation frequency in a locus is known and the frequency of certain alleles in a population is documented, it is possible to calculate whether two isolates are identical on the basis of chance. This is not feasible with other fragment-based methods. Also, as for all of the methods that rely on the estimation of molecular size based on standard curves, the accurate sizing of fragments, even using fluorescent detection systems, is not a simple task, as it is mobility dependent on sequence composition as well as length.

1.15.3.5 Sequence-based methods Single-locus sequence typing (SLST)

SLST is an umbrella term for a variety of methods, in which sequencing of a single genetic locus has been shown to provide valuable typing results. Analysing a single locus means that the amount of DNA to be sequenced is limited, but it is imperative to select gene sequences that are (highly) variable. The best example of an established, epidemiologically significant SLST scheme is that of emm typing for S. pyogenes, which is the 'genotypic descendant' of M-serotyping, and relies on DNA sequencing of only 150 nucleotides, coding for the N-terminal end of an isolate's M protein (Facklam et al., 1999). An international database, incorporating a query module, ensures the continuing enrichment of the type repertoire, and already includes over twice as many types as those acquired through the previous use of antisera (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). Another
more recent example is that of the S. aureus protein A gene, spa, whose repeats are variable in number and individual sequence (Facklam et al., 1999). This feature formed the basis for the currently used sequencing system, which has been further elaborated upon and validated (Frenay et al., 1996; Shopsin et al., 1999). The development of dedicated software and the possibility of determining sequences rapidly have now led to an automated system, which is 100% reproducible among different centres (Koreen et al., 2004, Harmsen et al., 2003 and Mellmann et al., 2006). In the case of typing studies performed on the basis of DNA sequences in hypermutable regions, it should be noted that generation of variation may exceed the speed of spread; mutants may arise during an outbreak and thus falsely suggest that the outbreak has multiple sources rather than one.

1.15.3.6 Multi locus sequence typing (MLST)

MLST is the genotypic descendant of MLEE and assesses DNA sequence variation among the alleles (usually five to ten) of housekeeping genes [71]. It has been widely accepted and constitutes one of the major ‘typing successes’ of the past decade. It is very important to note that the ‘wet lab’ developments were paralleled by very important efforts to standardise the interpretative, free software (e.g., eBURST) and to make data freely available via the internet (Aires de Sousa et al., 2006, Feil et al., 2004, Enright et al., 2002 and Serrano et al., 2005 ). The implications for population genetics and dynamics may be more significant than those for
bacterial epidemiology, since polymorphism in the slowly evolving genes, which are its targets, may not be high enough for useful epidemiological comparisons.

1.16 Mechanisms of action of antimicrobials

Currently available antimicrobial agents are targeting key components of cell wall synthesis, protein synthesis, RNA synthesis, DNA synthesis, or intermediary metabolism. Bacterial cell wall synthesis has been the target which is most extensively exploited for antimicrobial development. The components of the cell wall synthesis machinery are appealing antimicrobial targets because of the absence of counterparts in human, thereby providing intrinsic target selectivity. β-Lactams as transpeptidase inhibitors thus block the conversion of immature to mature peptidoglycan (Hooper, 2001a). Vancomycin is a glycopeptide that is known to bind tightly to the terminal D-ALA-D-ALA of the peptide side chain of the immature peptidoglycan. Thus, vancomycin produces a block at the same step as β-lactams by producing steric hindrance to transpeptidase action, thereby preventing conversion of immature to mature peptidoglycan.

Staphylococcus aureus is one of the most common causes of nosocomial infections, especially pneumonia, surgical site infections, and bloodstream infections. This bacterium has the ability to rapidly acquire antimicrobial resistance. Most S. aureus strains (>90%) are resistant to penicillin, and since the 1980s, methicillin-resistant S. aureus (MRSA) strains have become endemic in hospitals worldwide. One decade later during the 1990s, S. aureus isolates with diminished
susceptibility to vancomycin (vancomycin-intermediate S. aureus [VISA]) were reported (Bozdogan et al., 2004; Avison et al., 2002; Bhateja et al., 2005). Glycopeptides, such as vancomycin, are frequently the antibiotics of choice for treatment of infections caused by the now common methicillin-resistant Staphylococcus aureus (MRSA). Incidences of vancomycin resistance in S. aureus (VRSA) have been increasing worldwide for the last 5 years. (Avison et al., 2002) The first clinical VRSA, Mu50, was isolated in Japan in 1997. Glycopeptides, such as vancomycin, are often the therapeutic drugs of choice for serious MRSA infections. However, failures of vancomycin therapy against S. aureus, due to the emergence of strains that are significantly less susceptible to vancomycin [vancomycin-resistant S. aureus (VRSA)], are now well established (Avison et al., 2002). Recent reports of three S. aureus clinical strains with the vanA gene open a new era in staphylococcal antibacterial resistance. This latter development limits further potentially therapeutic options against these strains (Bozdogan et al., 2004).

A thickened cell wall is responsible for the vancomycin resistance of VRSA strain Mu50, however, the mechanism of vancomycin resistance in other VRSA strains remained unclear (Cui et al., 2003). Thickening of the cell wall is a common phenotype of clinical VRSA strains and may be a phenotypic determinant for Vancomycin resistance in S. aureus. (Cui et al., 2003). VRSA strain Mu50 produces excessive amounts of peptidoglycan to make the thickened cell wall which become thinner with the loss of vancomycin resistance during drug-free passages and again became thick in the resistant mutant strains (Cui et al., 2003). The differences in the
cell wall thickness between VRSA and passage-derived strains, vancomycin-resistant mutant strains and passage-derived strains, and VRSA and control strains were all statistically significant (P < 0.001) (Cui et al., 2003). Vancomycin binds to the stem peptide of the membrane-anchored murein monomer (lipid II) at its Lys-d-Ala-d-Ala residue and inhibits the transglycosylation and transpeptidation reactions, preventing incorporation of the precursors into the bacterial cell wall (Périchon et al., 2004). The amount of glutamine-non-amidated muropeptide subunits (i.e. those containing d-glutamate rather than d-glutamine) in the Mu50 cell wall increases, and non-amidated muropeptides are poorer substrates for transpeptidases than amidated ones, although this is yet to be confirmed. Cell wall thickness and cross-linking result in vancomycin resistance is that the modified cell wall binds more vancomycin, due to the increased amount of terminal d-alanyl-d-alanine dipeptide (Avison et al., 2002). And thus the thickened cell wall not only traps a greater number of vancomycin molecules but also significantly reduces the time that vancomycin completely inhibits peptidoglycan synthesis (Cui et al., 2003).

The fine structure of the Mu50 cell wall is similar to that of an MRSA strain such as N315, except that the Mu50 peptidoglycan chains show significantly less cross-linking, and an increased content of pentapeptide chains (Avison et al., 2002). However, the resistance in enterococci (VRE) is due to synthesis of VanA-type glycopeptide, modified precursors, ending in d-Ala-d-lactate (d-Ala-d-Lac) in place of d-Ala-d-Ala that result in 1,000-fold-lower affinity for glycopeptides (Périchon et al., 2004).
1.17 Development of resistance in the hospital ecological environment

Isolates of vancomycin resistant S. aureus have emerged in many parts of the world. These isolates appear to achieve clinically relevant levels of resistance to vancomycin that leads to treatment failure (Bhateja et al., 2005). At present, the proportion of MRSA with reduced susceptibility to vancomycin is well known. Only 21 strains have so far been reported in literature, the first VRSA and hVRSA reported from Japan were MU 50 and MU 3, respectively. (Bhateja et al., 2005). Thus, the passage-derived strains with decreased MICs of vancomycin are best explained by the mechanism of heterogeneous resistance. The hetero-VRSA is the precedent strain of VRSA. Emergence of VRSA would be the result of vancomycin selection exerted upon a hetero-VRSA strain in the hospital, and the strain would return to hetero-VRSA status when vancomycin is not used for a while and its selective pressure lifted (Cui et al., 2003). Beta-lactam antibiotics are suspected to play a role in the dissemination of hetero-VRSA. In Japan, hetero-VRSA strains were found quite frequently in clinical isolates in the late 1980s before the introduction of vancomycin (vancomycin was introduced in 1991, and teicoplanin was introduced in 1998). This indicates that the conversion of VSSA to hetero-VRSA can be achieved in association with hetero- to homoconversion of methicillin resistance caused by beta-lactam selection (Cui et al., 2003). This suggests that the use of beta-lactam antibiotics for MRSA infection is a risk factor for the emergence of hetero-VRSA, although the precise genetic mechanism remains to be clarified. (Cui et al., 2003).
The unusual feature of cell wall physiology of the vancomycin stressed S. aureus makes the culprit to develop resistance easily and faster. This may challenge the clinical microbiologist more than any other antibiotics used and practiced previously.

1.18 Genetic analysis for Vancomycin resistance

Complex mechanisms producing changes in cell wall content and composition generate the VRSA phenotype, but the genetic basis of these changes has not yet been determined (Avison et al., 2002). In 2002, the first two clinical isolates of vancomycin-resistant Staphylococcus aureus (VRSA) possessing acquired vanA operon containing Tn1546 were recovered in Michigan and Pennsylvania (Clark et al., 2005) on conjugative plasmids with a broad host range in Gram-positive bacteria. (Bozdogan et al., 2003). The first two VRSA isolates arose from independent genetic events (Clark et al., 2005). The gene clusters are not stable genetic elements and serial passage of the strain in the absence of vancomycin led to loss of the vanA cluster and, consequently to restoration of vancomycin susceptibility (Bozdogan et al., 2003). VanA-type resistance is characterized by high-level inducible resistance to both vancomycin and teicoplanin and is mediated by Tn1546 or closely related elements. Tn1546, which belongs to the Tn3 family of transposons, is composed of seven genes, two of which encode a transposase and a resolvase responsible for the movements of the elements of the vanA operon, and is delineated by imperfect inverted repeats. The vanH, vanA, and vanX genes code for
proteins that are necessary for the expression of resistance. VanH is a dehydrogenase that converts pyruvate to d-Lac, VanA a ligase that uses d-Lac and a d-Ala residue to synthesize the depsipeptide d-Ala-d-Lac, which is incorporated into the peptidoglycan precursors, and VanX is a d,d-dipeptidase that hydrolyzes the dipeptide d-Ala-d-Ala formed by the endogenous Ddl chromosomal d-Ala:D-Ala ligase, thus reducing the level of normal peptidoglycan precursors ending in d-Ala-d-Ala. The VanY d,d-carboxypeptidase, not essential for resistance, cleaves the d-Ala C-terminal residue of pentapeptide precursors synthesized by using d-Ala-d-Ala dipeptides that have escaped VanX hydrolysis. The vanZ gene is implicated in teicoplanin resistance by an unknown mechanism. Expression of the vanA operon is regulated by two genes, vanR and vanS, located upstream from vanH that form a two-component regulatory system (Périchon et al., 2004). It is difficult to rationalize a role for these mutations in the VRSA phenotype. It is well documented that Mu50 grows more slowly than N315, which may be explained by the loss of such important functions. It has been hypothesized that the basis of the VRSA phenotype in Mu50 is an increased level of peptidoglycan biosynthesis, resulting in a considerably thicker cell wall. In addition to this, there is a reduced level of glutamate amidation in the cell wall, which may be responsible for reduced cross-linking and the reduced ability of d-d-carboxypeptidases to act on the peptidoglycan network, conserving d-alanyl-d-alanine terminal dipeptides, which bind and sequester vancomycin (Avison et al., 2002). Our analysis has identified loss-of-function mutations in genes encoding enzymes responsible, in part, for the
committed stage of peptidoglycan biosynthesis and for the manufacture of intermediary metabolites that are precursors of glutamine, and hence glucosamine. These findings will inform future targeted study of the biochemistry of vancomycin resistance in S. aureus (Avison et al., 2002). To facilitate the genetic investigation, entire genome sequences of the archetypal VRSA (Mu50), and vancomycin-susceptible MRSA strains N315, EMRSA 16 and COL were compared. The in silico analysis revealed several loss-of-function mutations in Mu50, affecting important cell wall biosynthesis and intermediary metabolism genes, not previously reported. The new findings provide further evidence for the hypothesis that vancomycin resistance in Mu50 is due to fundamental changes, important to metabolic pathways that impinge on peptidoglycan biosynthesis (Avison et al., 2002). The complete sequence flat-files of the genomes of Mu50 and N315 were using a specially written macro that eliminates all non-amino acid sequence information to produce a ‘proteome’ for each genome for all predicted open reading frames (ORFs) joined end to end. 164 individual differences between the proteomes of Mu50 and N315 were found. Of these, 114 are minor, i.e. single amino acid substitutions, and their effects cannot be predicted, though it cannot be assumed that they are neutral. Accordingly, each product would have to be investigated individually. DNA sequences for the remaining 50 Mu50 ORFs showing more marked differences from those in N315 were obtained. In 13 instances, the sequence in N315 was different from those in the other comparators (genome sequence data from MRSA COL), so these changes could not be linked to the VRSA phenotype. Where the Mu50
genomic copy was found to differ from those in the three MRSA strains (37 instances), many of the changes were located in 'hypothetical' ORFs, and genes clearly unrelated to vancomycin resistance (e.g. endotoxin genes and antigenic determinants, etc.). When these are eliminated, a total of 17 loss-of-function mutations specific to the Mu50 genome in genes encoding characterized functions were identified. A link between five of these disruptions and the biochemical differences that have been previously noted between Mu50 and MRSA strains is compelling (Avison et al., 2002). The authors explained the complementary genes and functions for those ones that seemed to be critical to cell wall physiology related to vancomycin stress, e.g., (a) murZ for murA that encodes UDP-GlcNAc-enolpyruvyl transferase, mrp for fmtB homologue, and (b) 2-ketoglutarate oxidoreductase and succinyl-CoA synthase for 2-ketoglutarate dehydrogenase (Kdh, odhA) and succinate dehydrogenase (Sdh, sdhB) respectively (Avison et al., 2002). Comparison of the PFGE patterns of the parent and passage-derived strains demonstrated that 13 of the 16 pairs shared identical banding patterns (Cui L et al., 2003). Avison et al. (2002) pointed out that Glutamine synthetase (GS) and glucosamine synthase activity in Mu50 is higher than in N315 suggesting that Mu50 is geared to direct more carbon into synthesis of GlcNAc. One side-effect of hyperproduction of GlcNAc would be a shrinking of the l-glutamine pool, because it would be used faster to produce glucosamine. This, in turn, would mean that the amount of l-glutamine available for amidation of d-glutamate in cell wall precursors would be reduced. The observed disruption of the genes odhA and sdhB, if
confirmed in other VRSA strains, does fit with the hypothesis that perturbation in this branch of intermediary metabolism is important in the VRSA phenotype of Mu50 (Avison et al., 2002). The analysis is necessarily limited in a number of ways. There may well be other DNA sequence differences in Mu50 that do not cause coding changes but do influence susceptibility to vancomycin, such as mutations in promoter regions or transcription regulatory sites that affect the expression of relevant genes. The analysis of global gene expression in VRSA strains is in progress, although limited analysis of Mu50 has yielded some interesting results. A major limitation is that, of necessity, the genetic analysis only relates to one VRSA strain, Mu50. An investigation to determine whether mutations similar to those found in Mu50 are also present in other clinical VRSA strains is underway (Avison et al., 2002).