A COMPARATIVE EPIDEMIOLOGICAL ANALYSIS OF CLINICAL STRAINS OF *Staphylococcus aureus* in Nigeria and South Africa

By

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A dissertation submitted in fulfillment of the requirements for the degree of Doctor of Microbial Biotechnology in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, Faculty of Science and Agriculture, University of KwaZulu-Natal, South Africa

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Promoter: Professor Johnson Lin

DECLARATION

The Registrar (Academic)
UNIVERSITY OF KWAZULU-NATAL

I, Shittu Adebayo Osagie (Registration number: 200302022) hereby declare that this dissertation hereby submitted to the University of KwaZulu-Natal for the degree of Doctor of Microbial Biotechnology has not been previously submitted by me for a degree at this or any other University, that it is my own work in design and execution, and that all materials contained therein have been duly acknowledged.

Signature:

Date:

DEDICATION

This work is dedicated to the IMMORTAL, INVISIBLE, the only WISE GOD, and to my wife, Olaide Tiwalola and son, Victor Oluwadamilare Adekoyejo.

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ABSTRACT

Staphylococcus aureus is recognized as one of the main human pathogens, which has developed resistance to many classes of antibiotics. Information on antibiotic susceptibility pattern is of great importance for clinicians in the selection of empiric antimicrobial therapy. Moreover, data on phenotypic and genetic characterization of *S. aureus* isolates (especially MRSA) are crucial in monitoring and limiting the intra- and interhospital spread in the control of staphylococcal infections.

In this study, antibiotic susceptibility of *S. aureus* isolates obtained from clinical microbiology laboratories in Southwestern Nigeria (200 isolates) and KwaZulu-Natal (KZN) province, South Africa (227 isolates) was investigated. All the isolates from both countries were susceptible to teicoplanin, vancomycin and fusidic acid, while the prevalence of MRSA was 1.5% and 27% in Southwestern Nigeria and KZN province, South Africa, respectively. Resistance of methicillin-sensitive *S. aureus* (MSSA) to sulfonamides, tetracycline, and the number of multi-drug resistant MSSA was significantly higher in Nigeria compared with South Africa. Most of the MRSA isolates (87%) from KZN were resistant to at least four classes of antibiotics. Apart from vancomycin, teicoplanin and fusidic acid, quinipristin/dalfopristin, linezolid and fosfomycin are recommended for the treatment of MRSA infections in the two countries.

Characterization of MRSA from South Africa indicated the widespread dissemination of strains in pulsotypes a and b, suggesting that there appears to be a major clone circulating in health institutions in KZN, South Africa. Clonal relatedness between one MRSA from Ibadan, Nigeria and EMRSA-15 was identified indicating clonal dissemination of the pandemic clone. The characterization of low-level mupirocin

resistant *S. aureus* from South Africa revealed that a dominant clone exists in health institutions located in Durban. A 41.1kb plasmid mediating resistance to mupirocin was identified from a methicillin/mupirocin resistant *S. aureus* strain from South Africa. Base substitution at nucleotide position $671_{A\ to\ T}$ of the *ileS*-2 gene in the mupirocin-resistant strain from Nigeria indicated a change in the amino acid sequence from leucine to phenylalanine. These findings clearly indicate that urgent measures including strict antibiotic and infection control policies are needed in curtailing the spread and establishment of mupirocin and methicillin-resistant strains in both countries.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Staphylococcus aureus remains one of the important pathogenic microorganisms in many countries causing infection in hospitals and the community. One of the reasons for its persistence might be its great variability, occurring at different periods and places with diverse clonal types and antibiotic resistance patterns. Epidemiological investigations of *S. aureus* at the moment have observed four major trends. In many countries, infections caused by multiresistant strains (especially methicillin-resistant *S. aureus* - MRSA) are of great concern, whereas in other countries, the frequency of MRSA is low. The third trend is the emergence of MRSA in the community and finally the recent reports of vancomycin-intermediate and resistant *S. aureus* in hospitals.

Studies on the epidemiology of antibiotic-resistant *S. aureus* are important and necessary for many reasons. In order to have adequate information for treatment of staphylococcal infections, it is crucial to understand the trends in the antibiotic-resistance patterns of *S. aureus*. In addition, the occurrence and changes in types of *S. aureus*, clonal identities, and their geographic spread is essential for the establishment of adequate infection control programs in hospitals and in the community. The long term or epidemiologic question is whether the strain causing disease in one geographic area is related to those causing disease in other regions. There is presently a growing interest in tracking, identifying and understanding the diversity of major multi-drug resistant *S. aureus* clones, especially due to the increasing number of infections caused by methicillin-resistant

Staphylococcus aureus (MRSA). The tendency for specific strains of *S. aureus* to spread within a hospital and across geographic boundaries has also led to the evaluation and establishment of a number of techniques aimed at the strain-specific typing of clinical isolates.

The main objective of the study was to provide baseline data on the epidemiology of clinical isolates of *S. aureus* isolates in SouthWestern Nigeria and the KwaZulu-Natal province in South Africa. Data based on phenotypic and genetic characterization of isolates would be important in understanding and monitoring the geographic expansion of *S. aureus* clones in the global surveillance network, in different population. Although studies have been carried out in many countries on *S. aureus* (especially MRSA), there is paucity of data on the epidemiology of *S. aureus* (MSSA and MRSA) in Nigeria and South Africa using phenotypic and molecular techniques. In addition, there is no information on clonal identities and diversity of *S. aureus* in both countries. Furthermore, data does not exist on the emergence and dissemination of pandemic MRSA clones in the two countries.

This study is expected to provide baseline information for health personnel and policy makers, in the development of health intervention strategies. Furthermore, data from this study would be useful in the establishment of adequate infection control programmes, regulate national drug policies in the treatment of staphylococcal infections, and bring a better understanding on the epidemiology of *S. aureus* in both countries.

1.2 Literature review

1.2.1 *Staphylococcus aureus* infections

"Micrococcus, which when limited in its extent and activity, causes acute suppurative inflammation, produces, when more extensive and intense in its action on the human system, the most virulent forms of septicaemia and pyaemia" Ogston (1882).

Staphylococcus aureus has been known as a causative agent of infection since 1882, when Alexander Ogston identified its role in sepsis and abscess formation (Ogston, 1982). It has continued to be one of the commonest human pathogen in community and hospital acquired infections. *S. aureus* is an opportunistic bacterium, which is frequently part of the human microflora, causing disease when the immune system becomes compromised. Although *S. aureus* can be found in different parts of the body, the anterior nares are the primary ecological niche in humans. From here, staphylococci may spread to the skin (especially eczematous lesions), surgical wounds, foreign bodies (e.g. tracheostoma, external fixation devices), burns and the upper respiratory tract. Nasal carriage differs between individuals and it is one of the major risk factors for *S. aureus* infection (Kluytmans *et al.*, 1997). In the healthy populations, approximately 20% of the individuals carry *S. aureus* persistently, about 60% intermittently and about 20% do not carry this bacterium (Kluytmans *et al.*, 1997). Another important mode of transmission is via transiently colonised hands of health care workers who acquire the organism after close contact with colonised patients or contaminated equipment (Peacock *et al.*, 1980; Lammler *et al.*, 2001).

S. aureus can cause a wide range of infections (Figure 1.1). They include (i) superficial lesions such as wound infections (ii) systemic and life-threatening conditions such as endocarditis, osteomyelitis, pneumonia, brain abscesses, meningitis, and bacteremia; and (iii) toxinoses such as food poisoning, scalded skin syndrome and toxic shock syndrome (Tenover and Gaynes, 2000).

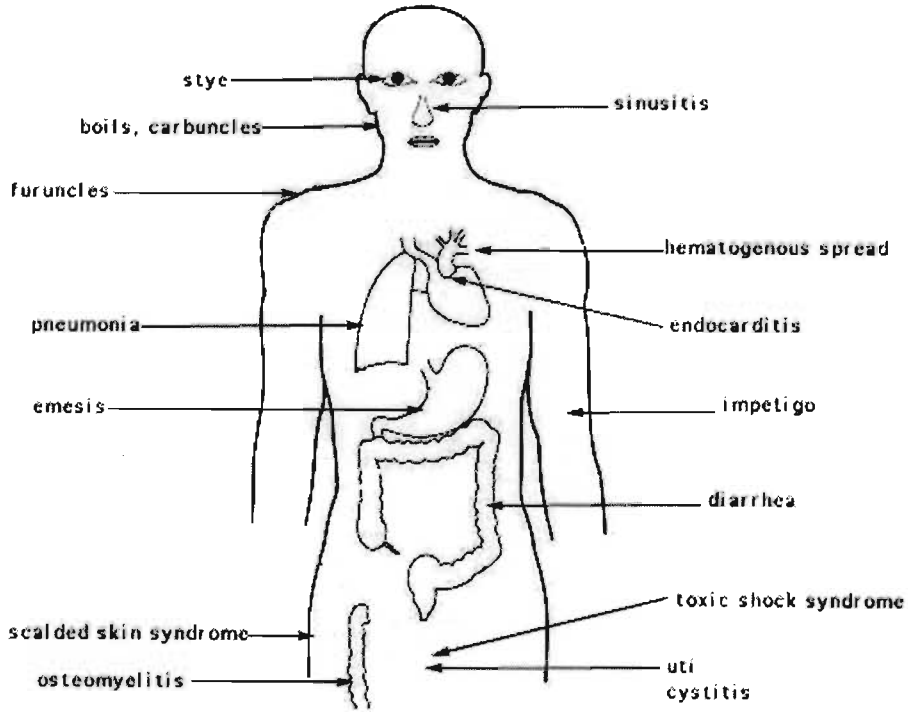

Figure 1.1: Sites of infection and diseases caused by *Staphylococcus aureus*

Reference: http://textbookofbacteriology.net/staph.html

1.2.2 Emergence of antibiotic resistance – a silent revolution

Major discoveries in antibiotics were made in succession from the 1950s through the 1970s, an era that has come to be known as the "Golden Age of Antibiotics". These accomplishments created a sense of euphoria in the medical community, as it perceived that bacterial infections were curable. Victory over bacteria was declared and financial resources were redirected toward more pressing scientific questions. By the late 1980s to the early 1990s, a decline in research for discovery and development of new types of antibiotics in many pharmaceutical companies was observed, a trend that was shadowed by federal agencies such as the National Institutes of Health, which showed more inclination to support studies of non-microbial systems during that period (Golemi-Kotra *et al.*, 2003).

Antimicrobial agents are a critical element of the therapeutic armamentarium of modern medicine. Antimicrobial drug consumption costs more than seven billion dollars annually in the United States, of which four billion dollars are used for treatment of hospital-acquired infections due to antibiotic resistant bacteria (Guven and Uzun, 2003). Meanwhile, liberal use of antibiotics in the clinics, in agriculture, in aquaculture, and in animal husbandry was facilitating a quiet revolution in microbial populations. These events resulted in antibiotic-resistant organisms that were perfectly treatable a decade or two earlier. These organisms were included among those defined as re-emerging infectious agents (IOM, 1992; Heymann and Rodier, 2001). While this trend was progressing steadily over the past decades, previously unknown infectious agents were also being discovered (WHO, 1996; Desselberger, 2000). A decline in the effectiveness of existing antimicrobial agents began to emerge, and thus infections became more

difficult and expensive to treat and epidemics became harder to control (Moellering, 1998a; Mah and Memish, 2000). These events contributed to high morbidity and mortality of previously treatable infectious diseases such as tuberculosis, malaria, acute respiratory diseases and diarrhoea (Cohen, 1994; Sack *et al.*, 1997; Espinal, 2000; WHO, 2000a; 2000b).

The emergence of antimicrobial resistance is a complex problem driven by numerous interconnected factors, many of which are linked to the use of antimicrobials in animals, plants and man (DuPont & Steele, 1987; Schwarz *et al.*, 2001; Tan *et al.*, 2002; Vidaver, 2002). Antimicrobial use has been credited as the single most important factor responsible for increased antimicrobial resistance (Mitema *et al.*, 2001; Rubin and Samore, 2002; McGarock, 2002). Antimicrobial use is influenced by a number of factors including knowledge, expectations, and interactions of prescribers and patients, economic incentives, characteristics of the health systems, the regulatory environment and availability of resources. There are factors that are inherent within the organisms themselves, which are enhanced by other environmental features (Allen *et al.*, 1999; Tumbarello *et al.*, 2002; Harris *et al.*, 2002; Zaidi *et al.*, 2003).

Hospitals are critical in the development of resistance. In addition to the heavy burden of infections acquired in the community setting, lack or underutilization of diagnostic tests, non-compliance with basic infection control measures, as well as a false feeling of security once the patient is placed on antibiotics, are the common reasons for irrational use in hospitals. Travel and migration also increase the potential for spread of plasmid-mediated, multi-resistant bacteria throughout the world (Guven and Uzun, 2003). Overall, these trends could have a substantial impact on health care costs. Each year

about two million patients acquire nosocomial infections in the United States, and about 60% of these infections involve antibiotic resistant bacteria (Lowy, 1998). In a study conducted in a university-based tertiary-care medical center, the average hospital bill increases by about $9,000 if a hospital patient becomes infected with a methicillin-susceptible *S. aureus* in primary blood stream infections (Abramson and Sexton, 1999). Moreover, if the strain is methicillin-resistant as well, the cost triples to $27,000, which add up to substantially increased expenditures in local hospitals. A study by Rubin *et al.* (1999) in New York City indicated that the cost for treating resistant *S. aureus* exceeded $400 million in 1999. Palumbi (2000) also reported that the hundreds of thousands of penicillin-resistant and methicillin-resistant infections in the United States add to the national health care bill by an estimated $30 billion.

1.2.2.1 History of penicillin and epidemiology of penicillin resistance in *S. aureus*

The rapid progression from a uniformly antibiotic-sensitive bacterium to a uniformly antibiotic-resistant species is well demonstrated in the case of *S. aureus*. Before the antibiotic era, *S. aureus* diseases had high mortality rates. In 1941, the mortality rate of *S. aureus* at the Boston City hospital was reported to be 82%, and over 70% developed metastatic infections (Skinner and Keefer, 1941). In 1940, a policeman was admitted to the John Radcliffe Infirmary at Oxford with an aggressive cellulitis spreading from a lesion on the corner of his mouth (Jevons, 1961). *S. aureus* was isolated together with *Streptococcus pyogenes*. Because the *S. aureus* strain was already suphonamide resistant, the first experimental preparation of penicillin was used as treatment. The patient began to respond, but penicillin could not be produced rapidly

enough and he died. This experience nevertheless revealed the potential of penicillin, and provided the impetus for further development. In the early 1940s, when penicillin was introduced into therapy, all strains of staphylococci were highly sensitive to this antibiotic. The first successes of penicillin therapy were related to the cure of formerly untreatable staphylococcal diseases (Abraham *et al.*, 1941). When Kirby's description of penicillinase-producing strains of *S. aureus* was published in 1944 (Kirby, 1944), resistance was infrequently encountered, with only a handful of strains available for study.

Penicillinase-producing strains were first isolated from hospitalized patients and by 1946; about 6% of *S. aureus* produced penicillinase (β-lactamase), and were resistant to penicillin (Barber and Rozwadowska-Dowzenko, 1948). Studies in London then showed an increase in the proportion of penicillinase-producing isolates during the late 1940s (Barber and Rozwadowska-Dowzenko, 1948). The reasons why so many isolates had penicillinase as early as 1946 are unclear, but reasons for the subsequent rise were linked to heavy and inappropriate antibiotic use. Penicillin powder was dusted on infected wounds and penicillin-containing snuff was available to treat respiratory infections (Livermore, 2000). Colonization of hospital staff by penicillin-resistant strains and their role in transmission were also notable features of these early reports. By 1948, over 50% of hospital *S. aureus* were penicillin-resistant (Barber and Rozwadowska-Dowzenko, 1948). During the mid 1950s, the number of *S. aureus* clinical isolates showing high-level resistance to penicillin increased rapidly, to such an extent that penicillin ceased to be a useful therapeutic agent against staphylococcal infections. This proportion has subsequently grown to 80-90% (Chen *et al.*, 1993; Henwood *et al.*, 2000).

Although penicillinase-producing strains were universally present in hospitals in the early 1950s, community isolates of *S. aureus* were considered to be largely penicillin susceptible. Penicillin continued to be recommended as an effective anti-staphylococcal agent as late as the early 1970s (Weinstein, 1975). Spread to household contacts of patients with hospital-acquired penicillin-resistant strains was recognized as one notable exception, but the capacity for community transmission was largely ignored (Gerberding and Chambers, 2001). Furthermore, there was no systematic surveillance for antibiotic resistance among *S. aureus* isolates circulating within communities. *S. aureus* acquired the penicillinase-based resistance mechanism from an unknown "extra species" source and penicillin resistance spread across the entire species with the "plasmid epidemic" (Tomasz, 2003). The effects of this "plasmid epidemic" which were first seen on hospital isolates of *S. aureus* afterwards found its way into community isolates (Jessen *et al.*, 1969).

A comprehensive description and accurate assessment on the epidemiology of drug-resistant *S. aureus* strains was described by Jessen *et al.* (1969). Examination of more than 2,000 blood culture isolates of *S. aureus* received at the Statens Seruminstitut in Copenhagen from 1957 to 1966 for which detailed information on the origin of infection (hospital or community) was available confirmed a high prevalence of penicillin resistance (85% to 90%) for hospital isolates of *S. aureus*. Somewhat unexpected was that penicillinase-producing strains were almost as common in the community, with 65% to 70% of isolates resistant to penicillin. The community-acquired isolates were resistant only to penicillin, whereas nosocomial strains typically were resistant to multiple antibiotics. By the 1970s, it was apparent that the high prevalence of penicillin resistance

among community isolates was not limited to Denmark. A prevalence of 70-80% of penicillinase-producing strains was observed regardless of location in inner cities, surburbs and rural areas within and outside the United States (Ross *et al.*, 1974; Hughes *et al.*, 1976; Hahn and Baker, 1980). Furthermore, a population-based study conducted in 1972 revealed that 47% of healthy school-aged children under 10 years of age were carriers of *S. aureus* and that 68% of colonizing strains were penicillin-resistant (Ross *et al.*, 1974). An evaluation of resistance pattern of 551,563 *S. aureus* community and hospital isolates from 1961 to 1990 by Faber and Rosdahl (1993) also confirmed this trend.

The final stage of this remarkable and sweeping genetic change, is documented in a study conducted in Portugal (Sá-Leão *et al.*, 2001). Screening the *S. aureus* nasal flora recovered from 1,000 young and healthy volunteers who had never received antibiotics showed that 97% of the *S. aureus* colonizing these individuals produced penicillinase and was resistant to penicillin (Sá-Leão *et al.*, 2001). It became clear that the extra-species drug-resistance gene penicillinase had become a domesticated genetic component of *S. aureus* without causing any survival deficit to the cells.

In summary, penicillin-resistant *S. aureus* was reported shortly after penicillin was introduced; within less than a decade, more than 25% of hospital strains were resistant, and within two decades, more than 75% were resistant (Gould and Cruikshank 1957; Harris and Wise, 1969). This rapid increase in the prevalence in hospitals was followed a decade or so later by a similar rate of increase in the community. By the early 1980s, roughly four decades after penicillin was widely available for civilian use, the prevalence of penicillin resistance in health care and community settings was virtually

identical and exceeded 85%, a situation which persists today (Gerberding and Chambers, 2001; Tomasz, 2003).

Some natural antibiotics were developed soon after the introduction of penicillin (Figure 1.2). These included chloramphenicol, erythromycin, streptomycin and tetracycline. However, the immense genetic repertoire of this bacterium for adapting to rapidly changing and uniformly hostile environments was repeatedly shown by the emergence of *S. aureus* strains that acquired resistance mechanisms to antimicrobial agents shortly after the introduction of these drugs into clinical practice (Oliveira *et al.*, 2002). Records of the Danish Health Board registered the years of introduction of various antimicrobials into clinical practice, beginning with penicillin in 1944-1946, streptomycin in 1948, tetracycline in 1950, and erythromycin in 1953 (Jessen *et al.*, 1969). The same records indicate that *S. aureus* bloodstream isolates resistant to penicillin, streptomycin, tetracycline and erythromycin were recovered as early as 1957, and resistance to these antibiotics was often mediated by plasmids and transposons (Lacey, 1984). As the 1950s ended, resistant *S. aureus* posed major problems in many hospitals. International concerns centred upon the phage type 80/81 strain (resistant to penicillin), which caused rapid and devastating skin and wound infections while less devastating strains were multi-resistant to penicillin, tetracycline and streptomycin (Rountree and Freeman, 1955; Williams, 1959).

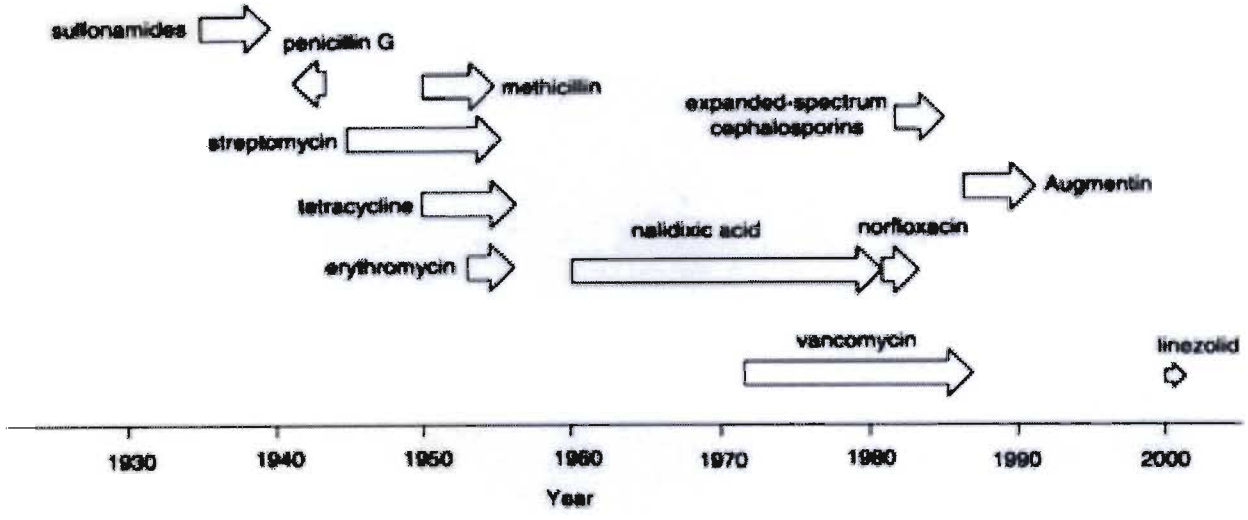

Figure 1.2: Clinical use of antibiotics. The beginning of each arrow indicates the time when the given antibiotic was introduced for clinical use and the tip of the arrow indicates when resistance emerged. The arrow is in reverse for penicillin G, since the first case of resistance to penicillin was reported two years prior to the first large-scale clinical use of penicillin in 1942. (Abraham and Chain, 1940). Reference: Wong and Pompliano (1998); Golemi-Kotra *et al.* (2003).

1.2.2.2 Mechanisms of penicillin resistance

The report by Kirby (1944) demonstrated that penicillin was inactivated by penicillin-resistant strains of *S. aureus*. Bondi and Dietz (1945) subsequently identified the specific role of penicillinase. The mechanism of penicillin resistance involved the acquisition of a plasmid-borne penicillinase capable of degrading the antibiotic before it reaches its cellular targets. The gene for β-lactamase is part of a transposable element located on a large plasmid, often with additional antimicrobial resistance genes (e.g. gentamycin and erythromycin).

Staphylococcal resistance to penicillin is mediated by *blaZ*, the gene that encoded β-lactamase production (Figure 1.3). This predominantly extracellular enzyme, synthesized when staphylococci are exposed to β-lactam antibiotics, hydrolyses the β-lactam ring, rendering the β-lactam inactive. *BlaZ* is under the control of two adjacent

regulatory genes, the antirepressor *blaRI* and the repressor *blaI* (Kernodle, 2000). Studies have also demonstrated that the signaling pathway responsible for β-lactamase synthesis requires sequential cleavage of the regulatory proteins BlaRI and BlaI. Following exposure to β-lactams, BlaRI, a transmembrane sensor-transducer, cleaves itself (Gregory *et al.*, 1997; Zhang *et al.*, 2001). Zhang and his co-workers hypothesize that the cleaved protein functions as a protease that cleaves repressor BlaI, directly or indirectly (an additional protein, BlaR2, may be involved in this pathway) and allows *blaZ* to synthesize the extracellular enzyme.

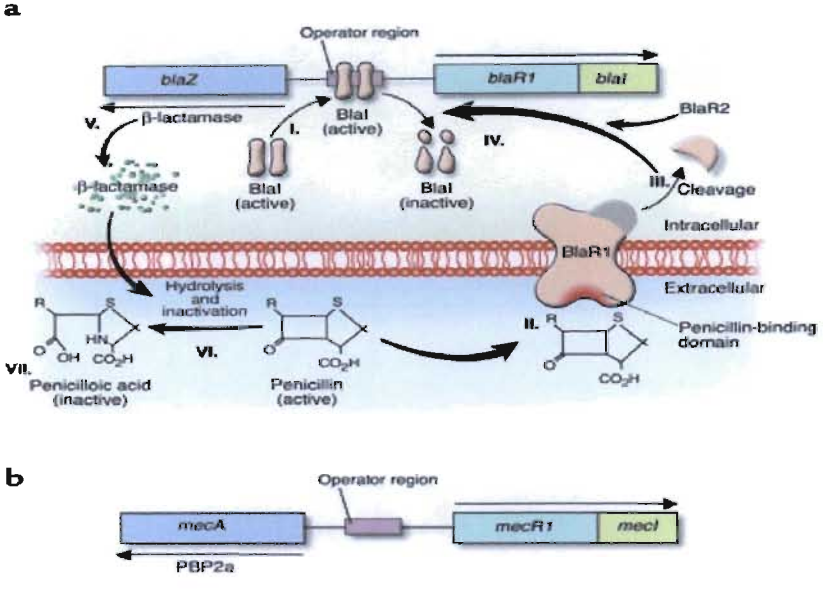

Figure 1.3: Mechanisms for penicillin and methicillin resistance. (**a**) Induction of staphylococcal ß-lactamase synthesis in the presence of the ß-lactam antibiotic penicillin. I. The DNA-binding protein BlaI binds to the operator region, thus repressing RNA transcription from both *blaZ* and *blaR1-blaI*. In the absence of penicillin, ß-lactamase is expressed at low levels. II. Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autocatalytic activation. III–IV. Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both *blaZ* and *blaR1-blaI* to commence. V–VII. ß-Lactamase, the extracellular enzyme encoded by *blaZ* (V), hydrolyzes the ß-lactam ring of penicillin (VI), thereby rendering it inactive (VII). (**b**) Mechanism of *S. aureus* resistance to methicillin. Synthesis of PBP2a proceeds in a fashion similar to that described for ß-lactamase. Exposure of MecR1 to a ß-lactam antibiotic induces MecR1 synthesis. MecR1 inactivates MecI, allowing synthesis of PBP2a. MecI and BlaI have coregulatory effects on the expression of PBP2a and ß-lactamase. Reference: Lowy (2003).

1.2.3 History and epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA)

Methicillin, introduced in 1961 was the first of the semisynthetic penicillin resistant to destruction by staphylococcal β-lactamase. The discovery of methicillin was an important development as many hospital strains of *S. aureus* had become penicillin resistant in the 1950s through the production of β-lactamase (Reacher *et al.*, 2000). However, its introduction was rapidly followed by reports of methicillin-resistant isolates (Jevons, 1961). First reported in a British hospital, MRSA clones rapidly spread across international borders. Waves of clonal dissemination with different phage types (e.g. 83 complex) were reported in the 1960s and were responsible for a large proportion of staphylococcal infections (Jessen *et al.*, 1969; Parker and Hewitt, 1970). In the United States, the first nosocomial outbreak of MRSA was reported at Boston City Hospital, in 1968 (Barrett *et al.*, 1968). Gentamicin-resistant MRSA (GR-MRSA) was first reported as causing an outbreak in 1976 (Shanson *et al.*, 1976), and subsequently, there was another outbreak of GR-MSRA involving three hospitals (Speller *et al.*, 1976). Data from the National Nosocomial Infections Surveillance System (NNIS) of the United States Centers for Disease Control and Prevention (CDC), which represents a large cross-section of US hospitals, indicated that in the early to mid 1980s, MRSA was detected in tertiary referral hospitals, and in these hospitals, 5-10% of *S. aureus* isolates were MRSA, compared with rates well below 5% at smaller non-referral hospitals (Chamber, 2001). By the 1990s, the proportion of *S. aureus* isolates that were MRSA was 40% at tertiary referral centers and 20% at hospitals with less than 200 beds.

Epidemic MRSA (EMRSA) strains emerged in British hospitals in the late 1980s. Seventeen EMRSA strains are currently recognized and are given the nomenclature EMRSA-1 to 17 (Anonymous, 1994; Aucken *et al.*, 2002). EMRSA-1 has been shown to be similar to Australian eastern seaboard multiresistant MRSA (Townsend *et al.*, 1984). It became prominent in Britain in the mid- to late 1980s (Cookson and Phillips, 1988) and was subsequently replaced by EMRSA-3, EMRSA-15 and EMRSA-16, which became the dominant strains a decade later. EMRSA-15 emerged in 1991 in Southeast England and spread to the Midlands area by early 1992 (Richardson and Reith, 1993). EMRSA-16 emerged in East Northamptoshire, UK, in 1991 (Cox *et al.*, 1995). In 2000, most British MRSA bacteremias were due to EMRSA-15 or EMRSA-16 (Johnson *et al.*, 2001). EMRSA-15 has been reported in New Zealand (Anonymous, 2001), Australia (Pearman *et al.*, 2001), the Czech Republic (Melter *et al.*, 2003) and Spain (Perez-Roth *et al.*, 2004). EMRSA-16 has also been identified in Greece (Aires de Sousa *et al.*, 2003a), Mexico (Aires de Sousa *et al.*, 2001), Canada (Simor *et al.*, 2002) and Spain (Perez-Roth *et al.*, 2003, 2004).

In Australia, the MRSA epidemic occurred in four waves involving distinctly different strains. The first was documented in Sydney between 1965 and 1972 (Vickery *et al.*, 1986). The second, a major epidemic of multi-resistant nosocomial MRSA (mMRSA), involved hospitals in Eastern and Southern Australia beginning in the late 1970s (Pavillard *et al.*, 1982; Turnidge *et al.*, 1989). The third was due to non-multiresistant MRSA (nmMRSA) strains, which were initially detected causing community-acquired infections in remote communities in Western Australia WA (Udo *et al.*, 1993) and which subsequently spread to the capital, Perth (Riley *et al.*, 1995; O'Brien

et al., 1999). The fourth occurred as part of a wider epidemic of community-acquired nmMRSA involving island communities in the South-Western Pacific (SWP), Auckland, New Zealand and major cities in eastern Australia (SWP-MRSA) (Mitchell *et al.*, 1996; Collignon *et al.*, 1998; Riley *et al.*, 1998; Nimmo *et al.*, 2000).

New epidemic MRSA strains susceptible to several or virtually all, non-β-lactam antibiotics have emerged in several European countries. In 1992, a new phenotype arose in French hospitals, characterized by the unexpected reappearance of heterogeneous expression of methicillin resistance and susceptibility to various antibiotics including gentamicin, tetracycline, minocycline, lincomycin, pristinamycin, co-trimoxazole, rifampicin and fusidic acid (Aubry-Damon *et al.*, 1997; Lelievre *et al.*, 1999). The incidence of isolation of strains of this phenotype has increased steadily throughout France, often replacing the classical multidrug-resistant MRSA (Lelievre *et al.*, 1999). A similar clone was recently reported to have replaced the usual multi-resistant MRSA clones and to predominate in a Greek and Spanish hospital with a high incidence of MRSA (Polyzou *et al.*, 2001; Perez-Roth *et al.*, 2004). A marked decrease in the use of gentamicin was suspected to be a factor contributing to the emergence of gentamicin-susceptible MRSA from predominantly gentamicin-resistant MRSA populations in France (Aubry-Damon *et al.*, 1997). However, changes in aminoglycosides consumption alone may not explain the increase in susceptibility to other antibiotics and the reappearance of heterogeneous resistance to methicillin observed with the new MRSA phenotypes. Fitness benefit, namely growth advantage, has been attributed to be an important factor for the spread of these susceptible clones (Laurent *et al.*, 2001).

1.2.3.1 Clinical importance of MRSA

The major reservoir of MRSA in institutions are colonized and infected inpatients, while transient hand carriage of the organism on the hands of health care workers account for the major mechanism for patient-to-patient transmission (Thompson *et al.*, 1982). Patients with MRSA infections have been noted with worse clinical and economic outcomes compared with patients with MSSA infections. MRSA infections are particularly difficult to treat if they are located at anatomical sites, where antibiotic penetration is reduced (Duckworth, 2003). A study by Chang *et al.* (2001) indicated that bacterial meningitis caused by MRSA was associated with a mortality rate of 56%, compared to a mortality rate of 13% in a patient group with meningitis caused by methicillin-susceptible *S. aureus* (MSSA). In addition, cohort studies of patients with MRSA bacteremia have reported higher mortality rates, increased morbidity, longer hospital length of stay, and higher costs compared with patients with MSSA bacteremia (Blot *et al.*, 2002; Melzer *et al.*, 2003; Kopp *et al.*, 2004). In a meta-analysis study, the death rate for patients with MRSA bacteremia was estimated to be about two times higher than the death rate due to bacteremia caused by MSSA (Cosgrove *et al.*, 2003). Moreover, in comparison to patients with MSSA surgical site infections (SSI), patients with MRSA SSI had five additional days of hospitalization, a 1.9-fold increase in hospital charges and a 3.4-fold increase in mortality during the 90-day post-operative period (Engemann *et al.*, 2003).

The higher cost of treating MRSA infections is due to a variety of factors. Firstly, high rates of MRSA infection result in the need for vancomycin, which is more expensive than the drugs normally used to treat *S. aureus* infections. Secondly, it is often necessary

to isolate the patients to keep them from infecting other patients. Finally, patients with MRSA infection stay longer in the hospital (Salyers and Whitt, 2002). A systematic audit of studies describing economic aspects of nosocomial infections revealed that a mean of $35,367 was attributed to MRSA infections (Stone *et al.*, 2002). Studies performed in hospitals in the United States in 1999 showed that the direct medical cost for MRSA infections is $27,083-$34,000 per patient (Abramson and Sexton, 1999; Rubin *et al.*, 1999). Consequently, annual United States health care costs associated with MRSA infections are estimated to be approximately $6 billion. A large fraction of additional costs that are attributed to the development of nosocomial pneumonia caused by *S. aureus* results from prolonged hospital stay, rather than the use of antibiotics to treat these infections (Dietrich *et al.*, 2002). Verhoef *et al.* (1999) also estimated that the costs to bringing an outbreak of MRSA (in which three to five patients are infected) under control in the Utrecht University Hospital, the Netherlands, could amount to $250,000.

1.2.3.2 The *mecA* gene – Mechanism of resistance

The spread of antibiotic resistance among *S. aureus* strains is of great concern in the treatment of staphylococcal infections, since *S. aureus* has quickly acquired resistance to most antibiotics introduced for clinical use. MRSA was 'born' at the moment it acquired the methicillin-resistance gene *mecA*, a 2.1kb exogenous DNA fragment, by horizontal transfer. MRSA was first described in 1961 in England (Jevons, 1961) and, since then, has gradually disseminated, reaching epidemic proportions in some European countries in the 1960s and in the United States in the 1970s (Haley *et al.*, 1982). By the mid-1980s, they emerged as the most important nosocomial pathogens worldwide.

The central genetic component of the resistant mechanism in these bacteria is *mecA*, which – embedded in a larger block of 'foreign' DNA – is not native to *S. aureus* but was imported from an unidentified extraspecies source (Beck *et al.*, 1986; Kuhl *et al.*, 1978). The chromosomally located *mecA* gene encodes a 78-kDa penicillin-binding protein (PBP) 2A, which has unusually low affinity for all β-lactam family of antimicrobial agents (Hartman and Tomasz, 1981; Hartman and Tomasz, 1984; Reynolds and Brown, 1985; Utsui and Yokota, 1985). PBPs are membrane-bound enzymes that catalyse the transpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains (Ghuysen, 1994) (Figure 1.4). Their activity is similar to that of serine proteases, from which they appear to have evolved. PBP2A is a transpeptidase that, assisted by the transglycosidase domain of the native PBP2 of *S. aureus*, takes over the function of the cell wall biosynthesis in the presence of β-lactam antibiotics (Pinho *et al.*, 2001). Thus, resistance to methicillin confers resistance to all β-lactam agents, including cephalosporins. Recent studies determined the crystal structure of a soluble derivative of PBP2a. PBP2a differs from other PBPs in that its active site blocks binding of all β-lactams but allows the transpeptidation reaction to proceed (Lim and Strynadka, 2002). Phenotypic expression of methicillin resistance is variable, and each MRSA strain has a characteristic profile of the proportion of bacterial cells that grow at specific concentrations of methicillin (Tomasz *et al.*, 1991). Expression of resistance in some MRSA strains is regulated by homologues of the regulatory genes for *blaZ*. The *mecI* and *mecR1* genes regulate the *mecA* response to β-lactam antibiotics in a fashion similar to that of the regulation of *blaZ* by the genes *blaR1* and *blaI* upon exposure to penicillin. Deletions or mutations in *mecI* or the promoter region of *mecA* result in constitutive

expression rather than variable expression of *mec* (Niemeyer *et al.*, 1996). Moreover, Rosato *et al.* (2003) recently found that either *mecI* or *blaI* must be functional in all MRSA, and suggested that this may be protective mechanism preventing overproduction of a toxic protein.

Since no homologue of *mecA* exists in methicillin-susceptible staphylococci, it has been assumed that *mecA* was acquired from one of the several coagulase-negative staphylococcal species (Archer and Niemeyer, 1994a). Couto *et al.*, (1996) identified a *mecA* gene in a methicillin-sensitive *S. sciuri* with 88% homology on the amino acid level to MRSA. Transduction of the *S. sciuri mecA* into an MSSA resulted in increased resistance to methicillin coupled with the detection of PBP2a (Couto *et al.*, 2003). This study suggests one possible source of the *mecA* element in *S. aureus*. Hiramatsu *et al.* (2002a) and Okuma *et al.* (2002) have also speculated that the simultaneous detection of the SCC*mec* type IV in different geographical regions of the world potentially reflects its enhanced mobility and multiple simultaneous transmissions from another coagulase-negative staphylococci.

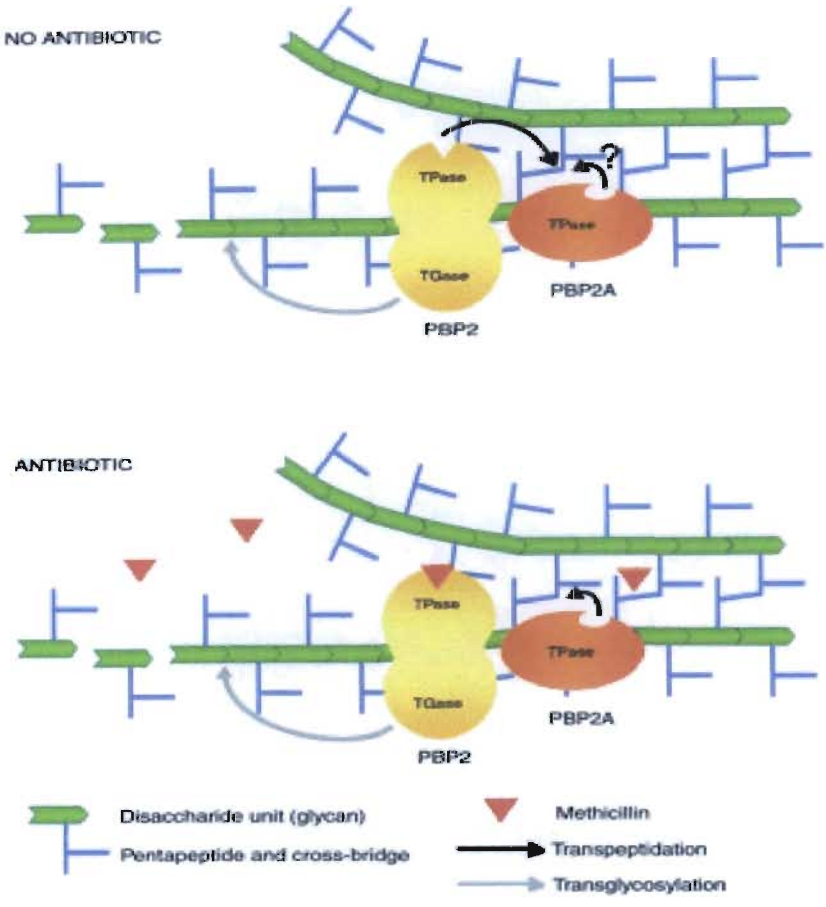

Figure 1.4: Model for the cooperative functioning of the transglycosylase (TGase) domain of PBP2 and the transpeptidase (TPase) activity of PBP2A in methicillin-resistant *S. aureus*. Upper section: In the absence of antibiotic, it is assumed that both the TPase and TGase domains of PBP2 participate in the biosynthesis of staphylococcal peptidoglycan. Lower section: When antibiotic is added to the medium, the TPase domain of PBP is acylated and is no longer capable of performing its peptide crosslinking activity. However, the penicillin-insensitive TGase domain of PBP2 remains functional and cooperates with the TPase activity of the acquired PBP2A for cell-wall synthesis and bacterial growth in the presence of β-lactam antibiotics in the surrounding medium. Reference: Pinho *et al.* (2001).

1.2.3.3 Other factors governing staphylococcal methicillin resistance

The *mecA* gene is the primary determinant of intrinsic methicillin resistance but additional genes are required for a high-level resistance phenotype, besides other environmental factors (Hartman and Tomasz, 1984; Chambers, 1997; Berger-Bachi, 1999; Berger-Bachi and Rohrer, 2002; Katayama *et al.*, 2003a). These genes are native constituents of the *S. aureus* genome and participate mostly but not exclusively in cell-wall biosynthesis and turnover. The *murF*, *fmtA-C*, *sigB*, *hmrA* and *hmrB*, *dlt*, and *ctaA* genes, as well as the auxiliary genes (*aux*) essential for methicillin resistance (genes or mutants of *femA-F*, *femR*, and *femX*) have been described (Chambers, 1997; Berger-Bachi, 1999). Staphylococcal murein hydrolases, like the *lytH* gene product, also play a pivotal role as lytic enzymes in peptidoglycan growth and turnover. Some factors only have a minute effect on methicillin resistance, like those encoded by the global regulators *sar* and *agr*, which control production of virulence factors. However, functions for several genes and gene products like *llm* and *aux16*-encoded protein remain elusive (de Lencastre *et al.*, 1999; Berger-Bachi and Rohrer, 2002; Ray *et al.*, 2003).

1.2.4 Current trends in global prevalence and characterization of MRSA

The prevalence of MRSA in hospitals continues to increase worldwide. Data from the SENTRY surveillance study reported a nosocomial prevalence of 26.3% in Europe and 34.9% in Latin America from 1997-1999 (Diekema *et al.*, 2001), 40.4% in South Africa (Johannersburg), 66.8% in Japan and 22.4% in Australia between 1998-1999 (Bell and Turnidge, 2002). In Central Europe (Austria, Germany and Switzerland), the prevalence of MRSA increased from 1.7% in 1990 to 8.7% in 1995 (Witte *et al.*, 1997).

In the United States, the National Nosocomial Infections Surveillance (NNIS) system reported a 51.3% methicillin rate among *S. aureus* strains from 18,397 intensive care unit patients between January 1998 and June 2002 (NNIS, 2002), which corresponds to an increase of 25% relative to the rates reported for 1995-1999 (NNIS, 2001). Recent reports indicate that MRSA is currently the most commonly identified antibiotic-resistant pathogen in hospitals in the United States with a mean prevalence of 60% among patients in intensive care units (NNIS, 2003; Diekema *et al.*, 2001; Diekema *et al.*, 2004).

An international study of antimicrobial resistance of hospital *S. aureus* comprising 21 worldwide hospital laboratories in 19 countries and states was conducted (Zinn *et al.*, 2004). Resistance patterns varied by region and MRSA occurred in low levels in countries within Northern Europe - Sweden (1%), Finland (2%), increasing levels in Central Europe - Germany (6%) France (12%), United States - Colorado (9%), New Zealand (10%) and Australia (10%) and very high levels in Southern European countries - Belgium (30%), Greece (63%) as well as parts of the United States - California (22%), Asia - Malaysia (49%) and South Africa - Johannersburg (39%) (Zinn *et al.*, 2004). The prevalence of MRSA based on multicentre studies conducted in different regions of the world is presented in Table 1.1. The analysis of the nationwide survey in relation to regions are as follows; Asia-Pacific - Japan (67%), Europe - Greece (44.4%), North America - United States (34.2%), Latin America - Colombia (52%) and Africa - South Africa (34%) (Christiansen *et al.*, 2004).

Table 1.1: Prevalence of MRSA in various regions based on multicentre studies

Region/ Country	No of institutions	Samples	Total number of isolates	MRSA (%)	Year of Study	References
Asia-Pacific						
Japan	3	Clinical	570	67	1999-2001	Christiansen *et al.* (2004)
Korea	8	Clinical	682	64	1999-2001	Kim *et al.* (2004)
Taiwan	3	Clinical	207	60	1999-2001	Christiansen *et al.* (2004)
Australia	21	Clinical	19000	20	1989-1999	Nimmo *et al.* (2003)
Africa						
South Africa (Johannersburg)	1	Clinical	163	34	1999-2001	Christiansen *et al.* (2004)
Nigeria (Lagos)	1	Clinical	142	29.6	1996-1997	Kesah *et al.* (2003)
Kenya	1	Clinical	137	27.7	1996-1997	Kesah *et al.* (2003)
Cote D'Ivoire	1	Clinical	155	16.8	1996-1997	Kesah *et al.* (2003)
Algeria	1	Clinical	208	4.8	1996-1997	Kesah *et al.* (2003)
Latin America						
Colombia	15	Clinical	296	52	2001-2002	Arias *et al.* (2003)
Chile	2	Clinical	428	45.3	1997-1998	Diekema *et al.* (2001)
Argentina	2	Clinical	424	42.7	1997-1998	Diekema *et al.* (2001)
Brazil	12	Clinical	852	34	1997-1999	Sader *et al.* (2001)
North America						
United States	30	Clinical	7169	34.2	1997-1998	Diekema *et al.* (2001)
Canada	8	Clinical	1410	5.7	1997-1998	Diekema *et al.* (2001)
Europe						
Greece	19	Blood	1126	44.4	1999-2002	Tiemersma *et al.* (2004)
United Kingdom	27	Blood	5343	41.5	1999-2002	Tiemersma *et al.* (2004)
Israel	5	Blood	849	38.4	2001-2002	Tiemersma *et al.* (2004)
Croatia	6	Blood	341	36.7	2001-2002	Tiemersma *et al.* (2004)
France	24	Blood	3376	33.1	2001-2002	Tiemersma *et al.* (2004)
Slovenia	8	Blood	657	18.4	2000-2002	Tiemersma *et al.* (2004)
Germany	25	Blood	3757	13.8	1999-2002	Tiemersma *et al.* (2004)
Czech Republic	35	Blood	2426	5.9	2000-2002	Tiemersma *et al.* (2004)
Sweden	54	Blood	6071	0.8	1999-2002	Tiemersma *et al.* (2004)
Netherlands	45	Blood	5359	0.6	1999-2002	Tiemersma *et al.* (2004)

1.2.4.1 Characterization of MRSA based on phenotypic and genetic methods

Monitoring and limiting the intra- and interhospital spread of MRSA strains requires the use of efficient and accurate epidemiologic typing systems that allow the discrimination between unrelated isolates and the recognition of isolates descending from a common ancestor (i.e. belonging to the same clone). During the past four decades, multiple phenotypic and genotypic methods have been developed to type MRSA. The choice of a typing method depends upon the needs, the skills level, resources of the laboratory and the type of question to be answered. An optimal typing method should show high typeability, adequate stability, high technical reproducibility and high discriminatory power. In addition, ease of use, ease of interpretation, rapidity, accessibility and low costs may be considered convenient criteria (Struelens, 1996). Phenotypic typing methods such as phage typing, antimicrobial susceptibility testing and multi-locus enzyme electrophoresis are often limited in reproducibility, as the expression of different genes is often influenced by environmental factors. Moreover, some of these methods lack typeabililty or discriminatory power and, consequently, are not the most adequate approaches for bacterial comparison.

The shortcomings of phenotype-based typing methods have led to the development of typing methods based on the microbial genotype or DNA sequence. The main genotypic techniques used for MRSA typing include (i) plasmid analysis; (ii) Southern hybridization analysis of digested chromosomal DNA, such as ribotyping, *Cla*I-*mecA*::Tn*554* polymorphisms, and binary typing; (iii) polymerase chain reaction (PCR)-based techniques such as random amplified polymorphic DNA (RAPD), repetitive element sequence based-PCR (rep-PCR), amplified fragment length polymorphism, and

SCC*mec* typing; (iv) pulsed-field gel electrophoresis (PFGE) and (v) sequence typing techniques such as *spa* typing and multilocus sequence typing (MLST). These genotyping techniques minimize problems with typeability and reproducibility and, in some cases, enable the establishment of large databases of characterized organisms (Aires de Sousa and de Lencastre, 2004).

1.2.4.2 International surveillance of MRSA and identification of pandemic clones

Based on a combination of different typing methods such as DNA hybridization with the *mecA* and Tn*554* probes, PFGE, RAPD, SCC*mec* typing, *spaA* typing and MLST, seven clonal types have been identified (the Iberian, Brazilian, Hungarian, New York/Japan, Pediatric and the EMRSA-15 and 16 clones) to spread in different regions of the world (Figure 1.5).

The Iberian clone was first identified as the strain responsible for the 1989 outbreak of MRSA disease in a hospital in Barcelona, Spain (Dominguez *et al.*, 1994), but seemed to have been already present in Belgium and France at least since 1984 (Deplano *et al.*, 2000). Subsequently, it was detected in several Portuguese hospitals (Sanches *et al.*, 1995a; Sanches *et al.*, 1995b; Sanches *et al.*, 1996; de Sousa *et al.*, 1998; Oliveira *et al.*, 1998; Sa-Leao *et al.*, 1999) and in many other European countries such as the Czech Republic (Melter *et al.*, 1999), Poland (Krzyszton-Russjan *et al.*, 2002), Sweden (Murchan *et al.*, 2003), Italy and Scotland (Mato *et al.*, 1998). This clone was also associated with epidemics in Germany and the Netherlands (Deplano *et al.*, 2000) and found in some community-acquired MRSA (CA-MRSA) in Finland among persons

who had contact with hospitals (Salmenlinna *et al.*, 2002). Moreover, the Iberian clone was detected in one hospital in New York (Roberts, 1998b).

The Brazilian clone was shown to be widely disseminated in Brazilian hospitals (Teixeira *et al.*, 1995) and to have spread to neighboring countries in South America: Argentina (Corso *et al.*, 1998; Aires de Sousa *et al.*, 2001), Uruguay and Chile (Aires de Sousa *et al.*, 2001), and to Europe: Portugal, the Czech Republic (Sanches *et al.*, 1996; de Sousa *et al.*, 1998; Oliveira *et al.*, 1998; Melter *et al.*, 1999) and one hospital in Greece (Aires de Sousa *et al.*, 2003a), where it displaced the local major clone. It has been detected in other European countries such as Finland, Germany, Ireland, The Netherlands, Poland, Sweden and the United Kingdom (Enright *et al.*, 2002).

The Hungarian clone has been widely spread in Hungarian hospitals since 1993 (de Lencastre *et al.*, 1997; Oliveira *et al.*, 2001a) and was recently described as the major clone in two hospitals in Taiwan and China (Aires de Sousa *et al.*, 2003b).

The New York/Japan clone was identified as the major clone in different states in the United States, namely in New York, Connecticut, New Jersey and Pennsylvania (de Lencastre *et al.*, 1996a; de Lencastre *et al.*, 1996b; Roberts *et al.*, 1998a; Roberts *et al.*, 1998b), in several hospitals across Canada (Simor *et al.*, 2002) and in a hospital in Tokyo (Aires de Sousa *et al.*, 2000). Besides, it has also been detected in Europe: in Finland, Ireland and the United Kingdom (Enright *et al.*, 2002).

Epidemic MRSA (EMRSA-15) is one of the most prevalent MRSA clones in hospitals in the United Kingdom and detected in northern Berlin, Germany, the Czech Republic and Spain (O'Neill *et al.*, 2001; Witte *et al.*, 2001; Moore and Lindsay, 2002; Melter *et al.*, 2003; Perez-Roth *et al.*, 2004). EMRSA-16 is one of the dominant types of

MRSA found in hospitals in the United Kingdom. This clone was widely disseminated in Greece (Aires de Sousa *et al.*, 2003a), Mexico (Aires de Sousa *et al.*, 2001), Canada (Simor *et al.*, 2002) and Spain (Perez-Roth *et al.*, 2003). EMRSA-16 was responsible for the largest single-strain outbreak in Scandinavia that occurred in Sweden during the period 1997-2000 (Seeberg *et al.*, 2002) and in community-acquired MRSA in Finland among persons who had contacts in hospitals (Salmenlinna *et al.*, 2002). This clone was also identified in other European countries such as Denmark, Switzerland and Belgium (Murchan *et al.*, 2003).

The Paediatric clone was first reported in 1991, in a pediatric hospital in Portugal (Sa-Leao *et al.*, 1999) and since then has been identified in Poland (Leski *et al.*, 1998), France and the United Kingdom (Enright *et al.*, 2002), Colombia, Argentina, and the United States (de Lencastre *et al.*, 1996a; Corso *et al.*, 1998; Gomes *et al.*, 2001; Aires de Sousa *et al.*, 2001).

Figure 1.5: Distribution of six pandemic clones in different regions of the world. Reference: Aires de Sousa and de Lencastre (2004).

1.2.4.3 Staphylococcal chromosome cassette *mec* (SCC*mec*)

The genetic element of *mec* has long been known to be localized on the chromosome of *S. aureus* (Sjostrom *et al.*, 1975). It was mapped to a locus between the genes encoding protein A (*spa*) and a protein involved in the biosynthesis of purines (*purA*) (Kuhl *et al.*, 1978). The *mecA* gene, which encodes PBP 2A, and its regulatory genes, *mecI* and *mecR1*, were cloned and sequenced in the 1980s (Matsuhashi *et al.*, 1986; Song *et al.*, 1987). Direct chromosome analysis of MRSA strains revealed that a substantial length of the chromosome DNA segment (greater than 30kb) carrying *mec* had no allelic equivalence in methicillin-susceptible *S. aureus* strains. Therefore, the segment was called additional DNA or *mec*DNA (Beck *et al.*, 1986; Skinner *et al.*, 1988; Dublin *et al.*, 1992). The *mec*DNA and its regulatory genes are widely distributed among many staphylococcal species (Sjostrom *et al.*, 1975; Kuhl *et al.*, 1978; Hiramatsu *et al.*, 1992; Hurlimann-Dalel *et al.*, 1992; Suzuki *et al.*, 1992; Suzuki *et al.*, 1993; Archer *et al.*, 1994b; Luong *et al.*, 2002). The chromosome region surrounding *mecA* gene of the pre-MRSA strain N315 was sequenced and compared to the corresponding region of the methicillin-susceptible *S. aureus* strain NCTC 8325. This procedure demarcated this genomic island (GI) as a specific structure for the genetic trait of methicillin resistance (Ito *et al.*, 1999) and was therefore included into the family of staphylococcal GIs. Subsequent experiments showed that the entire island was precisely excised from the N315 chromosome by the function of two site-specific cassette chromosome recombinases A (*ccrA*) and B (*ccrB*) encoded by the island; thus it was named the staphylococcal cassette chromosome *mec* (SCC*mec*) (Katayama *et al.*, 2000). Therefore, MRSA is produced when methicillin-susceptible *S. aureus* (MSSA) acquires a genetic

element called staphylococcal cassette chromosome *mec* (SCC*mec*). Expression of both *ccr* proteins also promoted site-specific integration at a unique *attBscc* site located at the 3' end of an open reading frame (ORF) of unknown function called *orfX* (Ito *et al.*, 2001). It is located near the replication origin of *S. aureus*, which is 10kb downstream of *purA* and 66-89kb upstream of *spa* gene depending on the size of the integrated copy of SCC*mec* (Kuroda *et al.*, 2001; Baba *et al.*, 2002). This location may provide advantage for the instant utilization of imported antibiotic resistance genes (Ito *et al.*, 2003).

Since the discovery of the first SCC*mec* element from pre-MRSA strain N315 in 1999, several types of SCC*mec* elements have been identified by determining their entire nucleotide sequences (Ito *et al.*, 1999; Ito *et al.*, 2001; Ma *et al.*, 2002; Aires de Sousa and de Lencastre, 2003c). Pre-MRSA is a *mecA* gene-carrying MSSA strain in which *mecA* gene expression is strongly repressed by the presence of an intact *mecI* gene. The SCC*mec* element contains the *mec* gene complex composed of IS*431mec*, *mecA*, and intact or truncated sets of regulatory genes, *mecR1* and *mecI*, and the *ccr* gene complex, which encodes site-specific recombinases responsible for the mobility of SCC*mec* (Katayama *et al.*, 2000).

The *mec* gene complexes are classified into four classes according to their structure (Katayama *et al.*, 2001), i.e. Class A, IS*431-mecA-mecR1-mecI*; Class B, IS*431-mecA-ΔmecR1-mecI*; Class C, IS*431-mecA-ΔmecR1-* IS*431*; and Class D, IS*431-mecA-ΔmecR1*. The *ccr* gene complex contains two site-specific recombinase genes, *ccrA* and *ccrB*, which are responsible for the mobility of SCC*mec* (Katayama *et al.*, 2000; Ito *et al.*, 2001) There are four allotypes in each of the *ccrA* and *ccrB* genes: *ccrA1*, *ccrA2*, *ccrA3*, and *ccrA4* for *ccrA* and *ccrB1*, *ccrB2*, *ccrB3*, and *ccrB4* for *ccrB*. SCC*mec* is classified

into allotypes according to the combination of the *mec* gene complex class and the *ccr* gene complex type that it possesses (Ito *et al.*, 2001; Ma *et al.*, 2002), as follows: type I SCC*mec*, (class B *mec* gene complex and type 1 *ccr* gene complex); type II SCC*mec*, (class A *mec* gene complex and type 2 *ccr* gene complex); type III SCC*mec*, (class A *mec* gene complex and type 3 *ccr* gene complex); and type IV SCC*mec*, (class B *mec* gene complex and type 2 *ccr* gene complex) (Figure 1.6). The region other than the *mec* and *ccr* gene complexes is designated the J (junkyard) region. Each SCC*mec* type is further classified into subtypes on the basis of the J-region sequence (Hiramatsu *et al.*, 2002b). The J regions contain various genes or pseudo genes whose presence does not appear essential or useful for the bacterial cell; the notable exceptions are resistance genes for non-β-lactam antibiotics or heavy metals, some of which are derived from plasmids or transposons. However, the J regions differ greatly among types and subtypes of SCC*mec* leading to variations in their sizes. Type-I SCC*mec* does not contain any antibiotic resistance genes other than *mecA*, and the J-region contains *pls* gene, encoding plasmin-sensitive surface protein (Hilden *et al.*, 1996). Type-II SCC*mec* of N315 and Mu50 contain integrated copy of plasmid pUB110 and transposon Tn*554* in the J region (Ito *et al.*, 1999; Kuroda *et al.*, 2001). Type-III SCC*mec* contains integrated copy of plasmid pT181, transposon Tn*554*, and pseudo Tn*554* that encodes resistance to tetracycline, erythromycin, and cadmium, respectively in its J-region (Ito *et al.*, 2001). Types-IVa and -IVb SCC*mec* do not habour any resistance genes except for *mecA*.

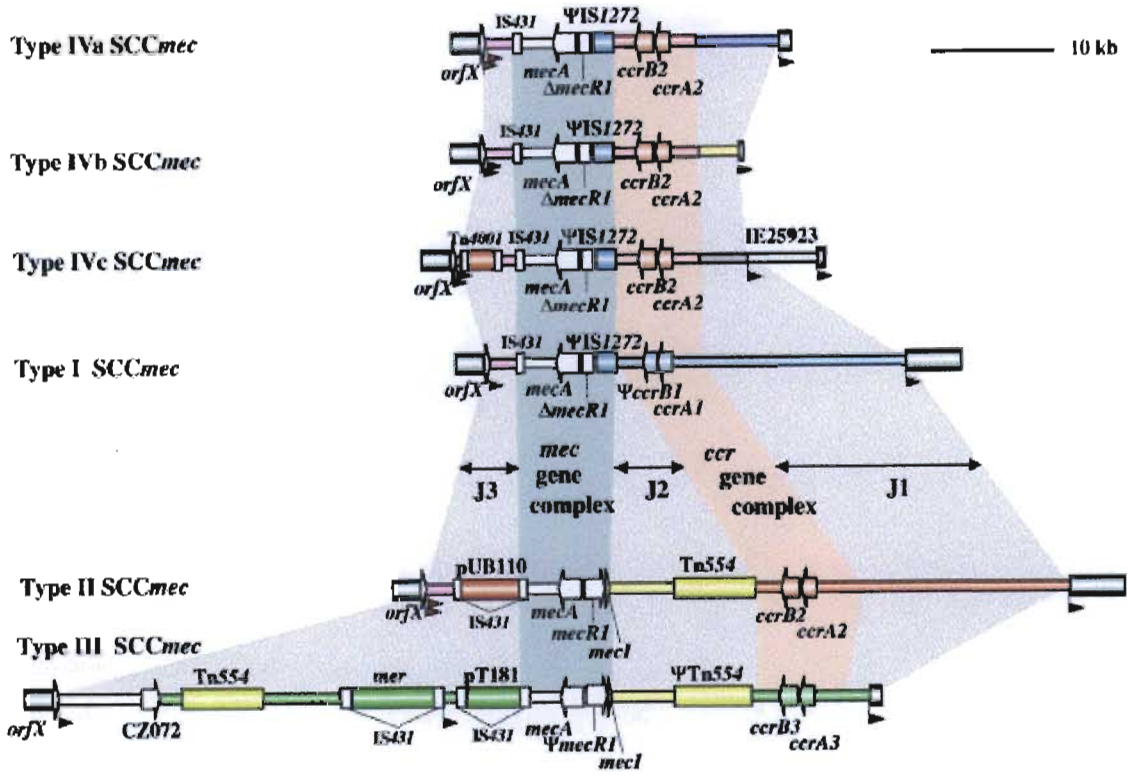

Figure 1.6: Structures of four types of SCC*mec*. SCC*mec* is composed of two essential gene complexes, the *ccr* gene complex (orange) and the *mec* gene complex (gray). *ccr* gene complex is composed of *ccrA*, *ccrB* genes which are responsible for the mobility of the SCC*mec* and some *orfs* surrounding them. The J-region (light gray) of the SCC*mec* is divided into three regions, J1-J3. Reference: Ito *et al.* (2003).

Ito *et al.* (2004) recently identified a novel type V (Figure 1.7). It was found on the chromosome of a community-acquired methicillin-resistant *Staphylococcus aureus* strain (strain WIS - WBG8318) isolated in Australia. The element shared the same chromosomal integration site with the four extant types of SCC*mec* and the characteristic nucleotide sequences at the chromosome-SCC*mec* junction regions. The novel SCC*mec* carried *mecA* bracketed by IS*431* (IS*431*-*mecA*-Δ*mecR1*-IS*431*), which is designated the class C2 mec gene complex; and instead of *ccrA* and *ccrB* genes, it carried a single copy of a cassette chromosome recombinase C (*ccrC*). Type V SCC*mec* is a small SCC*mec*

element (28 kb) and does not carry any antibiotic resistance genes besides *mecA*. Unlike the extant SCC*mec* types, it carries a set of foreign genes encoding a restriction-modification system that might play a role in the stabilization of the element on the chromosome (Ito *et al.*, 2004).

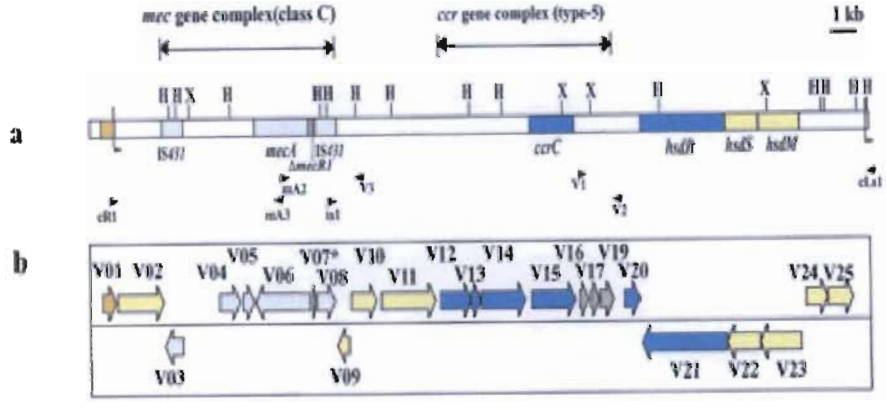

Figure 1.7: Structure of the novel SCC*mec* type V. **a**: Essential structure of type V SCC*mec*. The locations of the essential genes are illustrated. **b**: ORF's in and around type V SCC*mec*. Yellow arrows indicate ORF's unique to type V SCC*mec*, orange arrows, *orfX*; gray arrows, ORF's conserved in the five types of SCC*mec* elements with identities of 48.1 to 93.4%; blue arrows, ORF's commonly found in both the type SCC*mec* and the J region of type III SCC*mec*. Reference: Ito *et al.* (2004).

1.2.4.4 SCC without antibiotic resistance determinants

SCC elements that contain the essential features of SCC*mec*, but lack the *mecA* gene have been recognized in some coagulase-negative staphylococci as well as in *S. aureus* (Luong *et al.*, 2002; Ito *et al.*, 2003; Katayama *et al.*, 2003b). SCC*cap1*, which carries type 1 capsular polysaccharide, was identified in *S. aureus* strain M, a methicillin-susceptible strain (Luong *et al.*, 2002). SCC_{12263} was recently identified in *S. hominis* ATCC 27844. Because it carries a functional pair of *ccr* genes and other sequences with variable homology to those found in SCC*mec* elements, it was proposed that SCC_{12263}

might be an ancestral form of SCC*mec* elements (Katayama *et al.*, 2003b). SCC_{476} was found in MSSA strain 476, which showed great similarity with the previously described *S. homonis* non-*mec* SCC element. It carries a novel gene with homology to the fusidic acid resistance gene *far1* (O'Brien *et al.*, 2002). SCC_{pbp4} (contains *pbp4* and *tagF* genes) was recently discovered in *S. epidermidis* strain ATCC 12228, and has been proposed to represent the primordial genetic element contributing DNA sequences to the SCC*mec* elements found in *S. aureus* (Mongkolrattanothai *et al.*, 2004).

1.2.4.5 Models for MRSA evolution

The key genetic component of methicillin resistance, the *mecA* gene determinant, is not native to *Staphylococcus aureus*. Thus, the evolution of methicillin-resistant *S. aureus* (MRSA) must have begun with the acquisition of the *mecA* determinant from an unknown heterologous source some time before the first reported appearance of MRSA isolates in clinical specimens in the United Kingdom and Denmark (Crisostomo *et al.*, 2001). The first MRSA isolated in England in 1961 were only resistant to β-lactam antibiotics, with resistance conferred by type I SCC*mec*. The relationship between this isolate and modern MRSA has been the subject of some investigations (Enright, 2003). Two models of MRSA evolution were initially proposed in the early 1990s, based on studies using different typing techniques. The first model, described by Kreiswirth *et al.* (1993), was based on analysis of restriction fragment length polymorphisms generated by *Cla*I digestion of chromosomal DNA, followed by hybridization with Tn*554* and *mecA* probes. The limited number of patterns observed in a geographical and temporally diverse sample of MRSA isolates were taken as evidence that *S. aureus* had acquired the

methicillin resistance gene (*mecA*) on only one occasion, and the authors therefore hypothesized that all extant MRSA clones were recent descendants of this prototypical isolate.

Several months before the publication of this study, Musser and Kapur (1992) described MRSA as being polyclonal in a multilocus enzyme electrophoresis analysis of 254 MRSA isolates. The association of *mecA* with divergent genetic backgrounds was taken as strong evidence that the gene is transferred horizontally between *S. aureus* isolates. The explanation from these results was that MRSA isolates had diversified so rapidly between 1961 and 1992 that they had lost any genetic similarity. The existence of modern MRSA lineages that are unrelated to the first MRSA strain by molecular typing methodologies also supported the theory of Musser and Kapur, and was further strengthened by evidence from micro-array analysis (Fitzgerald *et al.*, 2001a) and multilocus sequence typing (MLST) (Enright *et al.*, 2002). These studies indicated larger genetic differences between MRSA lineages than would be expected if they were descended from a strain that emerged after the introduction of methicillin in 1959. It is therefore generally accepted that modern MRSA represent independent acquisitions of SCC*mec* by different genetic lineages of *S. aureus*.

1.2.4.6 Investigation on the epidemiology and evolution of MRSA – Use of epidemiological tools

The recovery of isolates with identical bacteriophage types from different hospitals within and between countries was described in the 1950s by Rountree and Freeman (1955) and Rountree and Beard (1958), in which increased epidemicity was

demonstrated. However, bacteriophage typing has fallen out of favour as a means of characterizing *S. aureus*, because of difficulties in typeability and reproducibility, as well as the cryptic genetic basis upon which characterization relies (Bannerman *et al.*, 1995; Weller, 2000). Molecular typing techniques are commonly used to study the epidemiology of *S. aureus*. The international spread of epidemic clones, such as the Iberian (Sanches *et al.*, 1995a), UK epidemic (Marples and Cooke, 1985; Kerr *et al.*, 1990), New York-Japanese (Ito *et al.*, 1999; Oliveira *et al.*, 2001), German MRSA (Witte, 1999a; Witte *et al.*, 2001) and Viennese (Witte *et al.*, 1999b) have been investigated with a variety of techniques, the most popular of which has been pulsed-field gel electrophoresis (PFGE) (Tenover *et al.*, 1995; Chung *et al.*, 2000).

1.2.4.6.1 Multilocus sequence typing (MLST)

MLST (Maiden *et al.*, 1998) involves sequencing DNA fragments (approximately 500bp) of seven housekeeping genes and comparing these sequences to known alleles at each locus site via the MLST website (http://www.mlst.net). An allelic profile consisting of seven integers that defines a sequence type (ST) is thus obtained. MLST was first applied to *S. aureus* in a study published in 2000 (Enright *et al.*, 2000), in which 155 invasive *S. aureus* isolates were typed. In addition to validating the method against PFGE, the study showed how epidemic clones of MRSA and MSSA could be unambiguously defined by their sequence types (STs). The MLST website currently contains allelic profile of more than 1000 isolates from disease and carriage from about 25 countries.

1.2.4.6.2 SCC*mec* typing

The methicillin resistance structural gene *mecA* is a part (2007bp) of a much larger genetic element, which is inserted precisely into the *S. aureus* chromosome. This staphylococcal chromosomal cassette *mec* (SCC*mec*) varies in size from 20 to 68 kb, but always contains *mecA* and at least part of a regulatory gene *mecR1* and chromosomal cassette recombinase genes (*ccr*). Five main types of SCC*mec* have been described (Ito *et al.*, 2001; Ma *et al.*, 2002; Ito *et al.*, 2004), and although the same types are often associated with divergent lineages, particular MRSA clones are associated with single SCC*mec* elements.

1.2.4.6.3 Origin of the first MRSA

The evolutionary history of the first MRSA clone was clarified by two research groups, using MLST and SCC*mec* typing (Crisostomo *et al.*, 2001; Enright *et al.*, 2002). In the first investigation, a high level of genetic similarity was found between the most prevalent MRSA (ST36) and the methicillin-sensitive *S. aureus* (MSSA; ST30) genotypes (Enright *et al.*, 2000). An evolutionary scenario was therefore proposed describing the emergence of ST36 from ST30 upon acquisition of SCC*mec*. ST36 represents UK epidemic clone 16, one of the major MRSA currently circulating in the country (Anonymous, 1997). In a thorough genetic analysis using several typing technologies, Crisostomo *et al.* (2001) compared the genetic backgrounds and phenotypes of a group of methicillin-susceptible *S. aureus* (MSSA) recovered in the early 1960s, MRSA isolates obtained during the same period, and contemporary epidemic clones of MRSA. The MSSA strains may have been the original recipients of the *mec* element at

the time when the first European isolates of MRSA were identified in Denmark and in the United Kingdom. All the early MRSA isolates resembled a large group of the early MSSA blood isolates in phenotypic and genetic properties, including phage group, antibiotype (resistance to penicillin, streptomycin, and tetracycline), pulsed field gel electrophoresis pattern, and *spaA* type. The early MSSA strains shared a common MLST allelic profile (3-3-1-1-4-4-16; ST 250) with the first MRSA isolates from England, suggesting that the early MSSA examined represented the progeny of a strain that served as the one of the first *S. aureus* recipients of methicillin-resistance determinant in Europe. It was therefore proposed that the first MRSA evolved from this MSSA clone upon acquisition of SCC*mec* I. The close genetic similarity between the first MRSA isolates and the Iberian MRSA clones was also described; and it was suggested that the Iberian clone (ST247-MRSA-I) is a single locus variant (SLV) of ST250. This genotype was proposed as the ancestor of the first member of the Iberian clone, a modern pandemic MRSA that shares the high epidemicity of the 'archaic' clone, a genotype that spread extremely rapidly after its first emergence in 1961 and was identified in 1993. This report highlighted the success of a particular genotype sequentially acquiring resistance determinants.

In 2002, an MLST study was published that provided evolutionary scenarios for all major MRSA clones and a rational nomenclature for *S. aureus* genotypes derived from resistance type (MSSA, MRSA), vancomycin-intermediate and resistant *S. aureus* (VISA or VRSA), ST and SCC*mec* type (Enright *et al.*, 2002). The advantage that this study had over previous investigations was the representative selection of strains chosen (912 isolates from 20 different countries), the use of SCC*mec* typing and MLST to define

clones, and more importantly, the use of BURST (Based Upon Related Sequence Types) algorithm to examine genetic relationships. BURST was developed by Ed Feil (University of Bath, UK) to analyse recent evolutionary events using multilocus datasets such as those produced by MLST. Briefly the algorithm places STs that share five out of the seven MLST alleles into a common clonal complex (CC).

The evolution of the earliest MRSA (ST250) was demonstrated to be more complicated than the scenario outlined by Crisostomo *et al.* (2001). ST-250-MRSA-I was shown to be derived from ST-250-MSSA, which arose from ST8-MSSA, a common cause of epidemic MSSA disease (Figure 1.8). The assignment of ST8-MSSA as the ancestor of ST250-MSSA was supported by the finding that ST8 and ST250 differed at a single locus whose alleles are identical except for a point mutation in *yqil*, unique to ST250 and its descendants (Enright *et al.*, 2002). Further support for this hypothesis was the finding that all isolates of ST250 and its descendants were MRSA with the SCC*mec* class I. ST8-MSSA was therefore proposed to be the ancestral genotype of the first MRSA (Enright *et al.*, 2002). Application of BURST showed that all epidemic hospital MRSA isolates that have been found in more than one country belong to five CCs. (Table 1.2). These CCs were named according to the ST of their proposed ancestor, and included CC8 (archaic MRSA), CC5, (which contains most of the VISA isolates studied to date), and three clonal complexes (CC45, CC30 and CC22) that contain recently emerged international MRSA clones such as UK EMRSA-16 (CC30) (Anonymous, 1997), Berlin epidemic MRSA (CC45) (Witte *et al.*, 1999b) and UK EMRSA-15 or Barnim epidemic MRSA (Witte *et al.*, 2001).

To improve the resolution of MLST and SCC*mec* typing, eight genetic targets were sequenced in a study of five nosocomial MRSA CCs (Robinson and Enright, 2003). In addition to seven MLST genes, those of the seven *S. aureus* surface (*sas*)- associated proteins (Mazmanian *et al.*, 2001) and the IgG binding protein A gene (*spa*) (Shopsin *et al.*, 1999) were sequenced. Phylogenies of concatenated sequences from seven MLST and seven *sas* genes were constructed for 147 genotypically diverse isolates of MRSA and MSSA, in order to examine the patterns of SCC*mec* acquisition within the five major MRSA-containing lineages. This study provided the first estimate of the number of times methicillin resistance had been acquired in this species (at least 20 times). It also indicated that SCC*mec* type IV is the most frequently acquired element within the five major lineages responsible for most hospital-acquired infections (10 acquisitions of SCC*mec* type IV and 10 acquisitions of the other SCC*mec* types). Nearly half (9 of 20) of all acquisitions involved an MSSA clone that acquired SCC*mec* type IV.

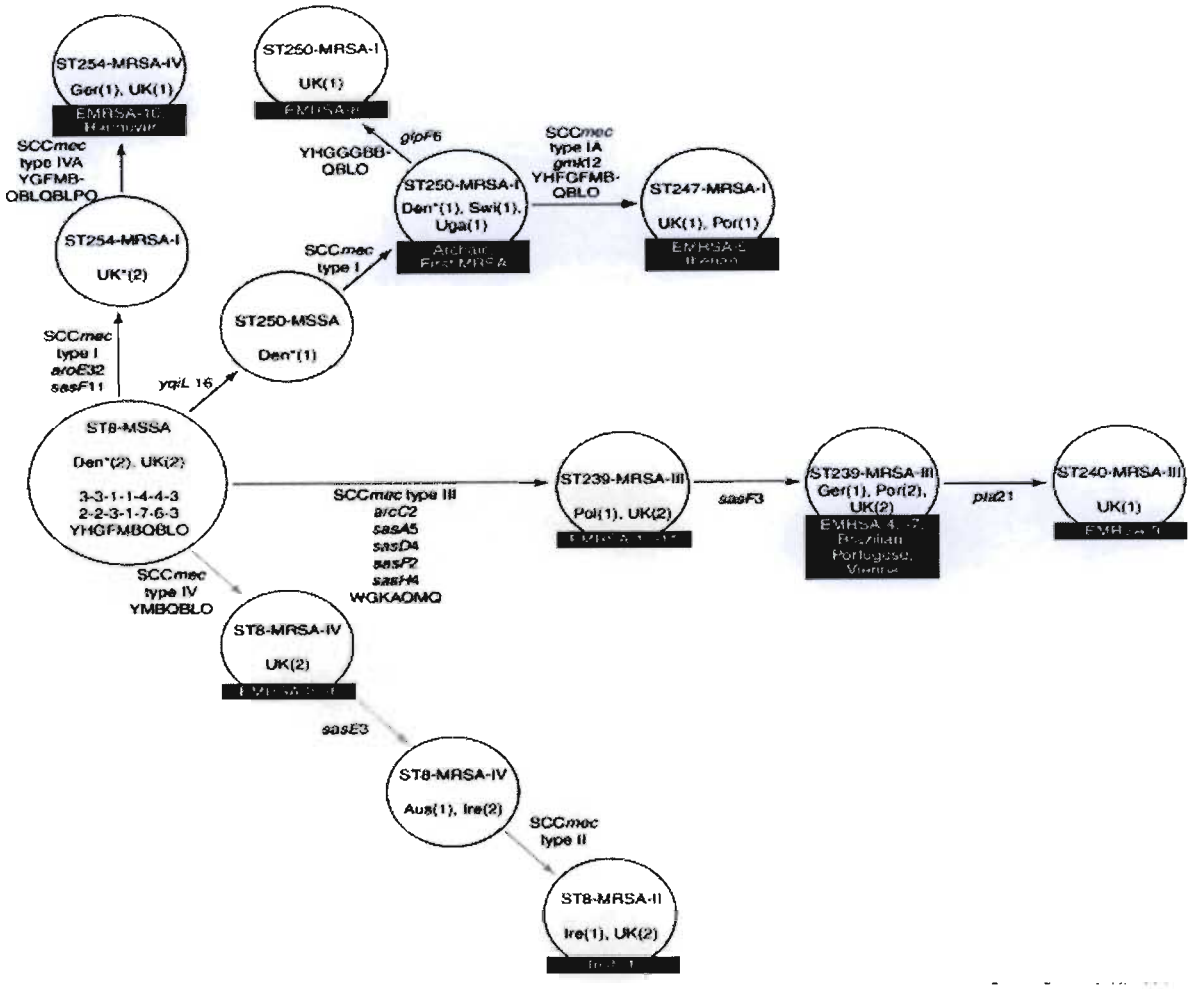

Figure 1.8: Proposed evolutionary models for the emergence of international MRSA in CC8. The large circle represents ST8-MSSA- the presumed ancestral genotype of this CC. Smaller circles represent descendant clones. Arrows indicate the direction and relative amount of change between clones. Names and countries of isolation are given for all clones. Historically early MSSA and MRSA isolates are indicated with asteriks next to their country of isolation. Previously named clones are indicated in black boxes. Reference: Robinson and Enright (2003); Feil and Enright (2004).

Table 1.2: Details of pandemic MRSA clones and their clonal complexes

Clonal Complex	Clone	MLST allelic profile	Previous names of MRSA clones
5	ST5-MRSA-I	1-4-1-4-12-1-10	UK EMRSA-3
5	ST5-MRSA-II	1-4-1-4-12-1-10	New York/Japanese
5	ST-228-MRSA-I	1-4-1-4-12-24-29	Southern German
8	ST8-MRSA-II	3-3-1-1-4-4-3	Irish-1
8	ST8-MRSA-IV	3-3-1-1-4-4-3	UK EMRSA-2,-6
8	ST239-MRSA-II	2-3-1-1-4-4-3	UK EMRSA-1, -4, -11, Portuguese, Brazilian, Viennese
8	ST247-MRSA-I	3-3-1-12-4-4-16	UK EMRSA-5, -17, Iberian
8	ST250-MRSA-I	3-3-1-4-4-16	First MRSA
22	ST22-MRSA-IV	7-6-1-5-8-8-6	UK EMRSA-15, Barnim
30	ST36-MRSA-II	2-2-2-2-3-3-2	UK EMRSA-16
45	ST45-MRSA-IV	10-14-8-6-10-3-2	Berlin

Reference: Enright (2003).

1.2.5 Community-acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA)

For many years, methicillin-resistant *S. aureus* was considered a multi-drug resistant pathogen that has been historically associated with hospitals and health care facilities (Livermore, 2000). However, MRSA besides having established itself as a major hospital pathogen has now been documented in the healthy community and affecting persons without established risk factors for MRSA acquisition (Vandenesch *et al.*, 2003). Until 1980, cases of community-onset MRSA infection were attributed to a history of recent hospitalization, close contact with a person who had been hospitalized,

or similar exposures (Payne *et al.*, 1965; Layton *et al.*, 1995; Gross-Schulman *et al.*, 1998; L'Heriteau *et al.*, 1999). In addition, some cases of community-onset infection were actually due to acquisition of MRSA in long-term settings, an environment that also promotes emergence and spread of resistant staphylococci. However, cases of community-onset MRSA infections occurring in 1980 and 1981 that were reported from Detriot provided the first suggestive evidence that MRSA could spread in communities independent of direct health care exposure. Most of the affected patients were injection drug users, sharing needles was the most likely mode of spread, and previous antimicrobial (especially cephalosporin) use was an important risk factor for infection with the outbreak strain (Saravolatz *et al.*, 1982).

The deaths of four children from rural Minnesota and North Dakota caused by community-onset MRSA infection in 1999 focused attention on this emerging problem (Centers for Disease Control and Prevention, 1999). These children lacked established risks for MRSA infection. In addition, their infections were caused by isolates susceptible to most non-beta-lactam antimicriobials and had identical genotypes distinct from those of MRSA isolates from local hospitals. These reports of infection and colonization by strains of MRSA in children provided compelling evidence that MRSA strains, like penicillinase-producing strains almost 30 years ago, have gained foothold in the community and have emerged as important outpatient pathogens.

Although many authors reported the emergence of community-acquired methicillin resistant *S. aureus* (CA-MRSA) isolates, a standard definition did not exist and at least eight different classifications were observed by Salgado *et al.* (2003) to have been used to classify MRSA infection as community-acquired. The commonly used term

CA-MRSA implies that it is known that the organism was acquired in the community. However, this term is often used to refer to the detection of colonization or infection in the community, rather than to actual acquisition of MRSA in the community. MRSA colonization may persist for months to years, and the acquisition of MRSA frequently goes unrecognized unless clinical infection develops, making it difficult to know with certainty the true site of acquisition (Salgado *et al.*, 2003). Salgado *et al.* (2003) therefore proposed that the presence of risk factors known to be associated with acquisition of MRSA, i.e. recent hospitalization, recent surgery, recent outpatient visit, recent nursing home admission, recent antibiotic exposure, chronic illness, injection drug use and close contact with a person with risk factor(s), should be evaluated before classifying an MRSA isolate as CA-MRSA.

A global analysis that examined the results of 57 studies on the prevalence of CA-MRSA among hospital patients or among community members reported that most persons with CA-MRSA had more than one health care-associated risk, suggesting that the prevalence of MRSA among persons without risk factors still remains very low (<0.24%) (Salgado *et al.*, 2003). However, the high MRSA colonization rates reported among members of "closed populations", such as Australian aboriginal (Udo *et al.*, 1993) and Native American (Groom *et al.*, 2001) communities, may be associated with risk factors for spread in the community, such as overcrowding, high rates of skin infections and frequent use of broad-spectrum antibiotics (Maguire *et al.*, 1998). Salgado *et al.* (2003) observed that the increase in CA-MRSA among non-hospitalized patients seems to be mainly due to the introduction of health-care associated strains into the community. Aires de Sousa and de Lencastre (2003c) found similarities between CA-MRSA and

sporadic nosocomial MRSA isolates, raising the possibility that at least some of the MRSA strains described as community-acquired may actually originate in hospitals. However, other authors reported that clones found in CA-MRSA were different from any of the major hospital-acquired MRSA clones (Dufour *et al.*, 2002; Okuma *et al.*, 2002), suggesting CA-MRSA could have appeared *de novo* through horizontal acquisition of the *mecA* gene (Salmenlinna *et al.*, 2002). Moreover, an Australian study reported the introduction of a strain (originating in the community) into the hospital setting (O'Brien *et al.*, 1999; Saiman *et al.*, 2003).

Three main hypotheses were initially proposed to account for the appearance of CA-MRSA. First, they may actually be health-care acquired pathogens, but the direct link to health care was not detected in the relevant investigations. This hypothesis was consistent with some early investigations of community-onset infections, but recent studies of community MRSA failed to detect health care risks despite detailed investigations. Another hypothesis to account for the emergence of MRSA was that these isolates are direct descendants of health-care acquired strains that are now circulating in the community. The density of health care-acquired MRSA in the community has increased over the past decade, as the prevalence of colonized patients discharged to home or other community settings increased. Hence there are more opportunities for spread to household contacts or others in close proximity to patients in health care settings. However, this hypothesis was inconsistent with the phenotypic (e.g. antimicrobial susceptibility) and genotypic (e.g. genotypic pattern) differences between community-acquired and health care-acquired isolates.

The last hypothesis to explain the appearance of MRSA in communities is that the *mecA* gene, the genetic determinant necessary for the expression of methicillin resistance, has been transferred to one or more penicillin-resistant, methicillin-susceptible strains of *S. aureus* that occupy traditional community niches. This possibility accounts for the distinct phenotypic and genotypic characteristics of CA-MRSA. Horizontal transmission of the plasmid-borne penicillinase gene responsible for penicillin resistance likely played the dominant role in the emergence of community-acquired penicillin-resistant *S. aureus*. *S. aureus* plasmid genes can be transferred by transduction or conjugation, a fact which may explain the enormous genetic diversity among penicillin-resistant methicillin-susceptible *S. aureus* (Gerberding and Chambers, 2001).

1.2.5.1 Prevalence of CA-MRSA

One of the earliest investigations on CA-MRSA was described in Western Australia (Udo *et al.*, 1993). Most CA-MRSA infections have been reported for other patient populations across many regions of the United States (Moreno *et al.*, 1995; Adcock *et al.*, 1998; Herold *et al.*, 1998; Centers for Disease Control and Prevention, 1999; Gorak *et al.*, 1999; Frank *et al.*, 1999; Suggs *et al.*, 1999; Groom *et al.*, 2001; Naimi *et al.*, 2001; Charlebois *et al.*, 2002; Fey *et al.*, 2003; Baggett *et al.*, 2004; Buckingham *et al.*, 2004; Ellis *et al.*, 2004; Shukla *et al.*, 2004) and Australia (Udo *et al.*, 1993; Gosbell *et al.*, 2001; Nimmo *et al.*, 2001; Munckhof *et al.*, 2002; Munckhof *et al.*, 2003; Coombs *et al.*, 2004; Murray *et al.*, 2004). Other countries include Latvia (Miklasevics *et al.*, 2004), Hong Kong (Ho *et al.*, 2004); Taiwan (Fang *et al.*, 2004; Wang *et al.*, 2004), Switzerland (Liassine *et al.*, 2004), Germany (Witte *et al.*, 2004a;

Witte *et al.*, 2005), United Kingdom (Klein *et al.*, 2003), New Zealand (Adhikari *et al.*, 2002), Saudi Arabia (Bukharie *et al.*, 2001), France (Dufour *et al.*, 2002), Finland (Salmelinna *et al.*, 2002) and recently, in Singapore (Hsu *et al.*, 2005) and in South America (Ribeiro *et al.*, 2005).

Certain ethnic groups are reported to have a propensity for the acquisition of CA-MRSA. These include Native American populations in the Mid-West United States (Groom *et al.*, 2001; Fey *et al.*, 2003) and aboriginals in Canada (Taylor *et al.*, 1990; Embil *et al.*, 1994) and Australia (Maguire *et al.*, 1996; Turnidge *et al.*, 2000). There is accumulating evidence that the virulence of CA-MRSA differs quite considerably, with some isolates having more potential to cause skin infections and others with potential to cause systemic, life-threatening infections. Most infections are mild and limited to skin and soft tissues, but serious infections like pneumonia (Gillet *et al.*, 2002; Boussaud *et al.*, 2003; Klein *et al.*, 2003; Peleg and Munckhof, 2004), endocarditis (Villar *et al.*, 1999; Lin *et al.*, 2000), liver abscess associated with renal disease (Chi *et al.*, 2004a), bacteremia (Chi *et al.*, 2004b), ear infections (Hwang *et al.*, 2002), foodborne illness (Jones *et al.*, 2002a), arthritis (Kallarackal *et al.*, 2000) and brain abscess (Khan *et al.*, 2000) have also been reported.

1.2.5.2 Pathogenesis and Virulence Factors of CA-MRSA

Several genetic factors that may enhance the ability of community-associated MRSA strains to cause disease have been identified.

1.2.5.2.1 Enterotoxins

The sentinel report detailing the cases of four children who died of fulminant MRSA infections in the Northern Plains states acutely raised the awareness of the presence and seriousness of MRSA in the community (Centers for Disease Control and Prevention, 1999). These isolates produced staphylococcal enterotoxins B (SEB) or C (SEC), which are members of the superantigen family implicated in approximately 50% of toxic shock syndrome cases, and were later, reported to produce Panton-Valentine leukocidin (PVL) (Gillet *et al.*, 2002). A larger study of MRSA isolates from American Indians in Nebraska compared community isolates with hospital isolates obtained at an institution, and with a reference collection of isolates causing nonmenstrual toxic shock syndrome (NMTSS) (Fey *et al.*, 2003). It was observed that most of the community MRSA isolates were closely related genotypically to one another (and to MW2) but unrelated to the Nebraska hospital strains or to most of the other reference strains. High levels of SEB or SEC were produced in the CA-MRSA isolates along with 76% of the NMTSS-causing isolates (although none of the hospital MRSA isolates did). The genes for PVL and SEH were found in at least two of the community MRSA isolates. The community isolates were closely related to other community MRSA isolates obtained in the upper MidWest (Groom *et al.*, 2001; Naimi *et al.*, 2001;) and from other sites around United States (Said-Salim, 2003) suggesting that these strains are circulating widely.

They also appeared to be descendants of a methicillin-susceptible NMTSS-causing strain isolated in Alabama in 1986 because their genomes differed only by the apparent insertion of SCC*mec* into the chromosome (Fey *et al.*, 2003).

1.2.5.2.2 Panton-Valentine leukocidin

Panton-Valentine leukocidin is an exotoxin that induces pore formation in neutrophils and monocytes, leading to their activation, degranulation and release of inflammatory mediators (Dinges *et al.*, 2000). Found in both MRSA and MSSA, it has been associated with skin abscesses and furuncles (Cribier *et al.*, 1992), and severe necrotizing pneumonias in children (Gillet *et al.*, 2002). Although it was only identified in 2% to 3% of *S. aureus* strains (Dinges *et al.*, 2000), a study of clinical isolates submitted to a French national reference laboratory found PVL producing strains to be strongly associated with furuncles and community-acquired severe necrotizing pneumonia (Lina *et al.*, 1999). A recent study from the same national laboratory specifically examined PVL-producing MRSA isolates that caused infection in health patients without known MRSA risk factors (Dufour *et al.*, 2002). All isolates carried the genes encoding PVL (*lukS-PV-lukF-PV*) and also genes for another leukocidin (encoded by *lukE-lukD*) that had been found in isolates causing impetigo (Gravet *et al.*, 2001) and other infections (Dufour *et al.*, 2002), but lacked genes encoding for other toxins. A limited number of CA-MRSA clones carrying the PVL genes are currently spreading across several continents (Vandenesch *et al.*, 2003). In Europe, closely related PFGE patterns have been reported for PVL-positive CA-MRSA isolated in Switzerland, France

and the Netherlands, pointing to clonal spread (http://www.eurosurveillance.org/ew/2003/030306.asp).

1.2.5.2.3 Exfoliative toxins

A recent survey of *S. aureus* isolates obtained from Japanese patients with bullos impetigo revealed an unexpected clonal group of MRSA strains producing types A or B exfoliative toxins (ET; exfoliatin, epidermolytic toxin) (Yamaguchi *et al.*, 2002). These toxins, known to cause large blisters at the site of infection, had not been previously detected in MRSA strains. The investigators presented several lines of evidence suggesting that the exfoliative toxin-producing MRSA strains have arisen recently, perhaps by incorporation of a phage carrying the *eta* gene into a new background MRSA, or by acquisition of *mecA* by ET-B producing MSSA strains. Clonal relatedness of exfoliative toxin-positive CA-MRSA strains in Switzerland and Japan has also been reported suggesting clonal spread (Liassine *et al.*, 2004).

1.2.5.2.4 Haemolysins

A study of West Samoan phage pattern (WSSP) -1 and -2 isolates from Australia, New Zealand, and Western Samoa found that they consistently produced higher levels of α and β hemolytic toxins than hospital-endemic MRSA strains (Adhikari *et al.*, 2002). The former lyses red blood cells by pore formation in the cell membranes (and also is known to be dermonecrotic and neurotoxic), whereas the latter is a sphingomyelinase (Dinges *et al.*, 2000). These community strains were also found to be more salt-tolerant and adhered better than nosocomial strains to human epithelial cells.

In summary, there appears to be four different clonal groups of CA-MRSA circulating in geographically defined areas: the exfoliative-producing isolates from Japan, the French PVL-positive isolates, the halotolerant α-toxin-producing isolates from Australia and the enterotoxin-producing isolates from the USA (Eady and Cove, 2003).

1.2.5.3 Genetic characterization of CA-MRSA

Several factors seem to distinguish the community-onset MRSA infections not attributable to health care exposure from those that are health care associated. Community-acquired MRSA tends to cause infections that occur in clusters or small outbreaks that affect unique populations such as young children, Australian Aborigines, Native Americans, Alaskan Natives, prisoners and college athletes. Most CA-MRSA isolates harbor the SCC*mec* type IV that appears to be resistant only to beta-lactam antibiotics and have a heterogenous methicillin resistance phenotype that is consistent with the lack of any antibiotic genes other than *mecA* (Oliveira *et al.*, 2001b; Okuma *et al.*, 2002). This does not, however, preclude chromosomally encoded resistance or the presence of resistance plasmids. Some CA-MRSA strains isolated in Australia contained a 41.4kb plasmid encoding resistance to tetracycline and trimethoprim, as well as resistance to mupirocin and cadmium (Pearman and Grubb, 1993; Udo *et al.*, 1994). Resistance to erythromycin has also been reported in some regions (Embil *et al.*, 1994; Layton *et al.*, 1995; Groom *et al.*, 2001; Fergie and Purcell, 2001; Sattler *et al.*, 2002) and a minority of isolates is multiply resistant (and thus may represent mis-classified hospital MRSA).

CA-MRSA carries a class B *mec* complex (Oliviera *et al.*, 2001b; Daum *et al.*, 2002; Hiramatsu *et al.*, 2002b; Oliviera *et al.*, 2002; Ma *et al.*, 2002;). The SCC*mec* type IV element is found not only in CA-MRSA but also in some hospital MRSA (H-MRSA), notably in EMRSA-15 (Enright *et al.*, 2002), one of the most common hospital MRSA in the United Kingdom that is now spreading globally, and in the paediatric clone that is prevalent in hospitals worldwide, especially among infants and children (Sa-Leao *et al.*, 1999). Type IV SCC*mec* is also responsible for methicillin resistance in gentamicin susceptible MRSA from France (Laurent *et al.*, 2001; Hiramatsu *et al.*, 2002b). Besides the relatively simple antibiotic resistance profile, CA-MRSA strains displaying SCC*mec* type IV were also reported to show several additional differences from most hospital-acquired MRSA strains. CA-MRSA seemed to grow faster in vitro (Okuma *et al.*, 2002) and to carry additional virulence genes (Oliveira *et al.*, 2002). A recent study by Ito *et al.* (2004) reported an SCC*mec* type V, which was recently identified from the chromosome of a community-acquired methicillin-resistant *Staphylococcus aureus* strain (strain WIS WBG8318) isolated in Australia.

Often a large proportion of CA-MRSA strains within one specific region show a high degree of genetic relatedness (usually identified by pulsed field gel electrophoresis typing) and many belong to one or a small number of distinct clonal groups or pulsotypes (Nimmo *et al.*, 2000; Groom *et al.*, 2001; Naimi *et al.*, 2001; Adhikari *et al.*, 2002; Dufour *et al.*, 2002; Salmelinna *et al.*, 2002; Yamaguchi *et al.*, 2002). In Minnesota, more than 80% of CA-MRSA was clonally related (variants of a single pulsotype), yet over 250 PFGE subtypes had been found in the region since 1995 (Naimi *et al.*, 2001). In other regions, this appears not to be the case and isolates demonstrate much more

phenotypic heterogeneity and PFGE profiles (Embil *et al.*, 1994; Layton *et al.*, 1995; Moreno *et al.*, 1995; Hussain *et al.*, 2000). A recent study on the characterization of 117 CA-MRSA isolates from three continents indicated four major findings (Vandenesch *et al.*, 2003). Firstly, two genes are unique to CA-MRSA isolates and shared by isolates from all three continents: a type IV SCC*mec* cassette (further designated IVa by Okuma *et al.* (2002) and the Panton Valentine Leukocidin (PVL) locus. These findings suggested that PVL and SCC*mec* IV might confer a selective advantage for community-based MRSA pathogens. Secondly, CA-MRSA were generally susceptible to most of the antibiotics tested apart from β-lactams, although European isolates appeared more resistant (i.e. to kanamycin, tetracycline and fusidic acid) than the United States and Oceanian isolates. Thirdly, the genetic background of CA-MRSA organisms was different in each of the three continents, although it was predominantly restricted to the *agr3* background, which corresponds to one of the three major phylogenetic lineages of pathogenic MSSA previously described (Baba *et al.*, 2002). Finally, MLST and PFGE analysis showed that within a continent, the genetic background of CA-MRSA strains did not correspond to that of the HA-MRSA in the same continent, suggesting that CA-MRSA did not emerge from local HA-MRSA. However, analysis by MLST indicated that CA-MRSA of each continent shared a common genetic background with HA-MRSA or MSSA of other continents, suggesting that intercontinental exchange of MRSA or MSSA had occurred, possibly followed by the introduction of the SCC*mec* in MSSA and the PVL locus in MSSA or MRSA. The association of SCC*mec* IV (SCCmec IVa according to Baba *et al.*, 2002) with PVL in the CA-MRSA strains most likely did not result from co-acquisition of the two determinants on a single mobile genetic element

because the two loci are widely separated on the *S. aureus* chromosome (Baba *et al.*, 2002).

Using MLST and the SCC*mec* typing as the methods of choice, it has been possible to unravel how CA-MRSA emerged and their relationship to H-MRSA and MSSA. Three key findings have been reported. Firstly, CA-MRSA does not belong to either a distinct or a single clonal lineage. The majority of CA-MRSA isolates for which MLST profiles are known fall into the same clonal complexes as nosocomial MRSA (Tables 1.2 and 1.3), suggesting their derivation from common MSSA ancestors with identical allelic profiles or sequence types (STs) (Enright *et al.*, 2002). Enright and co-workers also defined five of such clonal complexes (based on ST 5, 8, 22, 30 and 45). Fey *et al.* (2003) proposed another sequence type (ST1), regarding isolates from the great plains of North America. In addition, Okuma *et al.* (2002) identified two CA-MRSA isolates with type IV SCCmec belonging to clonal complex 298, indicating a likely seventh group (Table 1.3). Secondly, some CA-MRSA was indistinguishable from widely dispersed H-MRSA clones. The best example is EMRSA-15, a globally disseminated clone that was first identified in the UK (O'Neil *et al.*, 2001). Thus some hospital clones may have moved into the community or conversely may have risen undetected in the community and imported into hospitals. Thirdly, some CA-MRSA clones have already become widely disseminated with identical clones (within ST8) being associated with community-acquired infection in America and Australia (Okuma *et al.*, 2002). Other outbreak strains have been reported (Stemper *et al.*, 2004), and the CA-MRSA strain MW2, which was responsible for the death of a Native American child in North Dakota in 1998, (Centers for Disease Control and Prevention, 1999; Baba *et al.*,

2002) has been linked to several other reports (Groom *et al.*, 2001; Naimi *et al.*, 2001; Fey *et al.*, 2003).

Table 1.3: Multilocus sequence types (STs) of definitive community-acquired MRSA isolates and candidate community-acquired clones

Allelic profile (ST)	Clonal complex	SCC*mec*	Definitive CA-MRSA (number of isolates)	Candidate CA-MRSA (clones)[a]
1-1-1-1-1-1-1	1	Type IV	Minnesota (5), North Dakota (2), Perth (1), NORSA, Adelaide (2), NORSA, Perth (2), NORSA, Brisbane (2)	Nebraska
1-63-1-1-1-1-1	1	Type IV	Adelaide (1)	
1-4-1-4-12-1-10	5	Type IV	None yet found	Paediatric clone[b]
1-4-27-4-12-1-10	5	Type IV	NORSA, Adelaide (1)	
3-3-1-1-4-4-3	8	Type IV	Minnesota (2), NORSA, Perth (1)	Clone V, EMRSA-2, EMRSA-6
3-3-1-1-4-4-3	8	New	NORSA, Adelaide (1)	
3-32-1-1-4-4-3	8	Type IV	None yet found	
7-6-1-5-8-8-6	22	Type IV	Oxford, UK (1)	EMRSA-15[c]
7-6-1-5-8-8-6	22	New	NORSA, Adelaide (1)	
2-2-2-2-6-3-2	30	Type IV	Wooloongabba (8)	
10-14-8-6-10-3-2	45	Type IV	Tennessee (1)	Berlin
10-14-8-6-10-3-2	45	New	Perth (1)	
22-1-14-23-12-53-31	298	Type IV	Perth (2)	
		Type V	Australia (1)	

ST, sequence typing; MRSA, methicillin resistant *S. aureus*; SCC, staphylococcal cassette chromosome; CA-MRSA, community-acquired methicillin-resistant *S. aureus*; NORSA, non-multiresistant oxacillin-resistant *S. aureus*; H-MRSA, hospital associated methicillin resistant *S. aureus*.
[a]H-MRSA clones that contain type IV SCC*mec* that may have arisen in the community but are now associated with nosocomial infections. Alternatively, the clones may initiate infection in the community but the infecting strains were acquired in hospitals. The existence of such clones blurs clonal complex into which they fit has not been published. Taken mainly from Enright *et al.* (2002).
[b]Isolates belonging to the paediatric clone are typically non-multiresistant and thus are phenotypically indistinguishable from CA-MRSA. Until now this clone was considered to be a minor pandemic H-MRSA.
[c]EMRSA-15 is another non-multiresistant clone with evidence of global spread. Reference: Eady and Cove (2003); Ito *et al.* (2004).

1.2.5.4 Treatment of CA-MRSA

The majority of community-acquired skin and soft tissue infections are treated either in the community, or in one of the two hospital settings – accident and emergency departments or dermatological outpatient clinics. Local prescribing guidelines are important in assisting clinicians in their choice of antibiotic treatment. In most cases, such guidelines will specify one or more β-lactamase stable penicillins or second-generation cephalosporin as the treatment of choice where systemic antibiotic therapy is indicated (Eady and Cove, 2003). However, the key issue is to identify patients for whom β-lactams are contra-indicated. The dilemma of clinicians and policymakers is to identify effective alternative treatment options. This is difficult because other possible antibiotics have issues of resistance associated with CA-MRSA (Eady and Cove, 2003).

In contrast to health care-associated MRSA, CA-MRSA is often susceptible to trimethoprim sulfamethoxazole, clindamycin, doxycycline or minocycline, and fluroquinolones although susceptibility to these agents may vary by geographic area (Naimi *et al.*, 2003; Charlebois *et al.*, 2004). For superficial and localized infections, short courses of topical treatment with mupirocin or fusidic acid have been advocated (Eady and Cove, 2003). Linezolid has also been suggested as an alternative to the new fluoroquinolones for the treatment of CA-MRSA infections (Shopsin *et al.*, 2004). Surgical intervention, however, remains an important adjunct to antimicrobial therapy. Perhaps the best policy is to recommend that non β-lactams could be used only in high-risk patients and not to rely on a single alternative agent. Clinicians need to be particularly prudent about using non β-lactams whose selection is not merited by the severity of infection and be aware that increasing use of these agents in emergency and

outpatient departments may encourage an expanding spectrum of resistances among isolates of CA-MRSA. Local monitoring of the prevalence of CA-MRSA is therefore important. Improved hygiene also offers a very reasonable approach to prevent the spread of CA-MRSA (Eady and Cove, 2003).

1.2.6 Antimicrobial Agents effective against MRSA

1.2.6.1 Historical background of vancomycin and teicoplanin

Vancomycin, the first glycopeptide antibiotic, was isolated in the mid-1950s from a strain of *Amycolatopsis orientalis* during the course of a large-scale screening programme promoted by Eli Lilly. This programme was conducted to isolate antistaphylococcal drugs effective against a spate of serious infections caused by penicillinase-producing *S. aureus* strains (Griffith, 1984). The new antibiotic, introduced into clinical practice in 1958, proved to be highly effective in the treatment of staphylococcal infections. However, it soon fell from favour due to its toxicity (especially oto- and nephrotoxicity) and adverse reactions during administration (Woodley and Hall, 1961) and was quickly overshadowed by the novel agents methicillin and cephalothin. Shortly afterwards, ristocetin, another glycopeptide antibiotic, was isolated from *Norcadia lurida* and marketed by Abbott Laboratories but soon had to be withdrawn due to bone marrow toxicity and for causing platelet aggregation during administration (Perkins, 1982). Conversely, vancomycin, though virtually unused for many years, was still kept on the market.

A resurgence of clinical interest in vancomycin began in the late 1970s (Esposito and Gleckman, 1977; Cook and Farrar, 1978; Newsom, 1982; Perkins, 1982; Farber, 1984; Kucers, 1984; Anonymous, 1985; Ingerman, 1989) due to a variety of reasons including: (i) the gradual increase in Gram-positive bacterial infections; (ii) the emergence of highly and often multiply resistant, but vancomycin-susceptible, Gram positive pathogens (e.g. methicillin-resistant staphylococci, enterococci and JK corynebacteria), in hospital-associated infections of compromised patients; (iii) the introduction of novel uses of vancomycin, such as oral administration as a topical agent in the treatment of pseudomembranous colitis or utilization in prophylactic regimens; and (iv) the improved control of vancomycin toxicity resulting from the greater purity of modern drug formulations and the clinical monitoring of serum levels.

The same factors leading to the revival of vancomycin prompted the pharmaceutical industry to seek and develop new glycopeptide antibiotics. Teicoplanin, obtained in the late 1970s in the Lepetit research laboratories by the fermentation of *Actinoplanes teicomyceticus* became commercially available in Europe in the late 1980s (Parenti *et al.*, 1978; Somma *et al.*, 1984). However, glycopeptide antibiotics suffered a major setback during this period, due to the unexpected emergence of the first acquired resistances, in staphylocci and enterococci. Besides their utilization in human chemotherapy, glycopeptides have also been employed as growth promoters in animal husbandry. In particular, avoparcin, a glycopeptide antibiotic isolated in the late 1960s by the fermentation of *Stretomyces candidus* (Kunstmann *et al.*, 1968) became available in the late 1970s as a feed additive in many European countries. Avoparcin feeding of farm animals was suggested to have a selective influence on the emergence of glycopeptide-

resistant enterococci responsible for human infections (Witte and Klare, 1995; Wegener *et al.*, 1999), and, although the matter gave rise to much controversy, in 1997, this practice was banned in the European Community. However, the use of vancomycin and teicoplanin has increased dramatically in the last 20 years, in large because of the increasing prevalence of methicillin resistance in both coagulase-negative staphylococci and *S. aureus*.

1.2.6.2 Mechanism of action

Biochemical studies indicate that glycopeptides inhibit the late stages of peptidoglycan synthesis (Bambeke *et al.*, 2004). The biosynthetic pathway of this polymer involves three steps: (i) the synthesis of cytostolic precursors made of pentapeptides fixed on a disaccharide; (ii) the coupling of these precursors with a lipid carrier and the transfer of the resulting amphiphilic molecule to the outer surface of the membrane; and (iii) the reticulation between individual precursors by transpeptidation and transglycosylation reactions, accompanied by the release of the lipid carrier and its recycling to the inner face of the membrane. Bacteria incubated with vancomycin accumulate cytosolic precursors, suggesting that glycopeptides interfere with the assembly of peptidoglycan and in particular, with transglycosylation reactions (Reynolds, 1989). At the molecular level, the primary target of vancomycin was shown to be the D-Ala-D-Ala terminus of the precursors. Molecular modelling and experimental studies indicate that vancomycin forms a stoechiometric complex with the D-Ala-D-Ala dipeptide via the formation of five hydrogen bonds with the peptidic backbone of the glycopeptide. The formation of this complex prevents the transpeptidation reactions by

steric hindrance (Williams and Waltho, 1988; Reynolds, 1989; Arthur *et al.*, 1996; Loll and Axelsen, 2000).

Vancomycin resistance among staphylococci was developed in laboratories even before the drug was in use clinically (Geraci, 1956; Ziegler, 1956). However, this resistance was so difficult to induce that many felt it would be unlikely to occur in a clinical setting (Moellering, 1998b). Resistance to vancomycin among the organisms encompassed in its spectrum of activity was not observed in the first 30 years of the drug's clinical utilization (Newsom, 1982; Kucers, 1984; Cooper and Given, 1986) and in the mid 1980s, a similarly uniform susceptibility was also believed to be the rule with teicoplanin (Williams and Gruneberg, 1984; Willams and Gruneberg, 1988). Glycopeptide resistance emerged in the second half of the 1980s in coagulase negative staphylococci (CNS) and enterococci. As for enterococci, after initial reports of resistance in clinical isolates (Leclercq *et al.*, 1988; Uttley *et al.*, 1988), a number of phenotypically and genotypically distinct types of acquired glycopeptide resistance were identified. They include *vanA* (inducible resistance to high levels of both vancomycin and teicoplanin), *vanB* (inducible resistance to various levels of vancomycin only), *vanC* and *vanD* (constitutive resistance to vancomycin and teicoplanin) (Dukta-Malen *et al.*, 1992; Evers *et al.*, 1993; Grissom-Arnold *et al.*, 1997; Leclercq and Courvalin, 1997), and *vanE* (biochemically and phenotypically related to *vanC*) (Fines *et al.*, 1999). The *vanG* gene was reported by McKessar *et al.* (2000) but vancomycin-resistant *Enterococcus faecium* (VRE) with those complexes are not as widespread as VRE with the *vanA*, *vanB* and *vanC* gene complexes (Clark *et al.*, 1993; Moulin *et al.*, 1996; Schouten *et al.*, 2000; Lu *et al.*, 2001; Petrich *et al.*, 2001; Kolar *et al.*, 2002). In the *vanA* and *vanB* types, the

resistance genes are usually located on mobile elements, and resistance is transferred to susceptible recipients either by self-transferability of the relevant plasmids or by transposition of the resistance determinants between different replicons, ostensibly in the absence of plasmid DNA (Leclercq *et al.*, 1989; Uttley *et al.*, 1989; Dukta-Malen *et al.*, 1990). The potential of the natural spread of *vanA* or *vanB* resistance to other Gram-positive organisms is illustrated by the detection of *vanA* determinant in previously non-involved *Enterococcus* species (Dukta-Malen *et al.*, 1994) and in *Corynebacterium*, *Arcanobacterium*, *Oerskovia*, *Lactococcus* (French *et al.*, 1992), *Bacillis* species (Fontana *et al.*, 1997), and of a *vanB*-related gene in a faecal isolate of *Streptococcus bovis* (Poyart *et al.*, 1997).

True teicoplanin-resistant CNS was first reported in 1986 in the United States (Del Bene *et al.*, 1986) and the United Kingdom (Wilson *et al.*, 1986), followed by the report of Schwalbe *et al.*, (1987). In these instances, strains of *S. haemolyticus* were implicated. The first *Enterococcus faecium* isolate with transmissible vancomycin resistance was reported in France (Leclercq *et al.*, 1988) and this raised concerns by public health officials and infection control specialists that the *vanA* determinant, which mediated high-level vancomycin resistance in the enterococcal isolate, would be transferred to *S. aureus* (Hospital Infection Control Practices Advisory Committee, 1995; Centers for Disease Control and Prevention, 1997; Wenzel and Edmond, 1998). In-vitro conjugative transfer of the *vanA* determinant from an *E. faecalis* donor to an *S. aureus* recipient by Noble *et al.* (1992), further heightened concerns about the possible spread of *vanA* to *S. aureus*.

1.2.6.3 Definition of vancomycin resistance

Various countries use different breakpoints where vancomycin-resistant staphylococci have been reported. In the United States, the National Committee for Clinical Laboratory Standards (NCCLS) defines *S. aureus* isolates with vancomycin MICs between 8 and 16ug/ml as intermediately sensitive, and isolates with a MIC of 32ug/ml as resistant (NCCLS, 2000) whereas in Japan, the breakpoint for resistance is 8μg/ml (Cosgrove *et al.*, 2004). Similarly, British and Swedish definitions do not include a category for intermediate susceptibility to vancomycin or teicoplanin (Walsh and Howe, 2002). In addition to VISA and vancomycin-resistant *S. aureus*, Hiramatsu *et al.* (1997) described another type of vancomycin resistance called hetero-VISA (hVISA). This strain is susceptible to vancomycin but contains a subpopulation, at a frequency of 10^{-6} or higher with a MIC of vancomycin or more than 4ug/ml. The potential importance of hVISA is that it may be associated with treatment failures (Hiramatsu *et al.*, 1997; Wong *et al.*, 1999; Wong *et al.*, 2000) and a precursor of VISA (Sieradski *et al.*, 1999b; Hussain *et al.*, 2002).

1.2.6.4 Mechanisms of resistance in glycopeptide-resistant *S. aureus*

1.2.6.4.1 Vancomycin-intermediate *S. aureus* (VISA)

The pathogenesis of resistance of *S. aureus* to glycopeptides is not fully understood. Except for rare cases, reduced susceptibility to glycopeptides is not found in methicillin-susceptible *S. aureus* (MSSA) (Bobin-Dubreux *et al.*, 2001; Reverdy *et al.*, 2001). The genetic events resulting in phenotypic expression of resistance appear to be

different in strains with high-level vancomycin resistance compared with strains exhibiting intermediate levels of resistance. *S. aureus* with reduced susceptibility to vancomycin has a thick cell wall in comparison to susceptible *S. aureus* (Cui *et al.*, 2003), whereas vancomycin-resistant *S. aureus* (VRSA) is as a result of the acquisition of the *vanA* gene from vancomycin-resistant enterococcus, which is integrated into the *S. aureus* conjugative plasmid. As reviewed by Hiramatsu *et al.* (2001a), the first clinical *S. aureus* strains with reduced susceptibility (Mu50) had 30-40 layers of peptidoglycan compared to approximately 20 layers in fully susceptible strains. The increased number of layers of vancomycin intermediate *S. aureus* (VISA) contains many D-alanyl-D-alanine targets to which glycopeptides molecules can bind (affinity trapping), thus resulting in reduced access of glycopeptides to their site of action, namely the D-alanyl-D-alanine residues of murein monomers, which are located in the cytoplasmic membranes (Figure 1.9). Penetration of glycopeptides to their site of action in the cytoplasmic membrane is further inhibited by the destruction of the mesh structure of the outer layers of peptidoglycan by the trapped glycopeptide molecules themselves. This event is described as "clogging phenomenon" (Cui *et al.*, 2000).

The overproduction of murein causes detrimental effects on the rapid growth of the cell leading to a prolonged doubling time for cell replication (Cui *et al.*, 2003). This could explain why VRSA strains do not quickly prevail in health care facilities and their detection is mostly confined to patients with MRSA infection undergoing long-term vancomycin therapy (Tenover, 1999; Chesneau *et al.*, 2000; Fridkin, 2001; Tenover *et al.*, 2001). Hiramatsu and his co-workers proposed that the emergence of VRSA would be the result of vancomycin selection exerted upon a hetero-VRSA strain in the hospital,

which returns to hetero-VRSA when vancomycin is not used for a while and the selective pressure lifted. On the other hand, some hetero-VRSA strains are extremely stable and can be disseminated across wards and even across hospitals (Cui *et al.*, 2003).

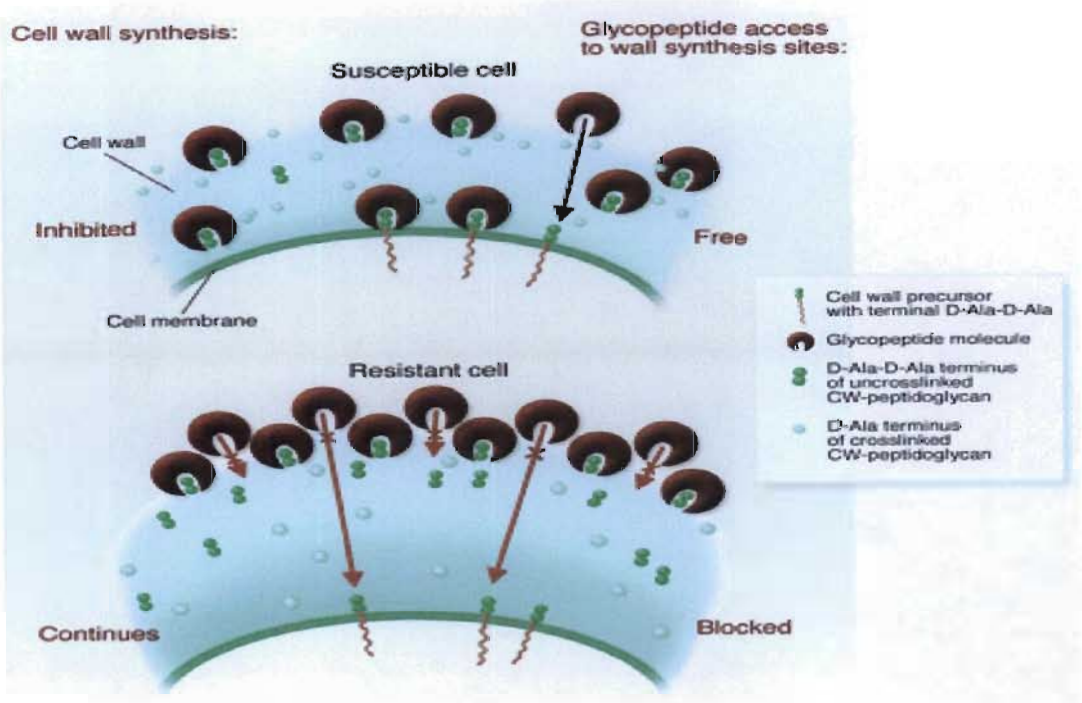

Figure 1.9: Mechanisms of *S. aureus* resistance to vancomycin: VISA strains. VISA strains appear to be selected from isolates that are heterogeneously resistant to vancomycin. These VISA strains synthesize additional quantities of peptidoglycan with an increased number of D-Ala-D-Ala residues that bind vancomycin, preventing the molecule from getting to its bacterial target. Reference: Sieradski *et al.* (1999a) and Lowy (2003).

1.2.6.4.2 Vancomycin-resistant *S. aureus* (VRSA)

The *vanA* gene is carried by a transposon, Tn*1546*, which is integrated in the conjugative plasmid harboured by vancomycin-resistant enterococcus (Hiramatsu *et al*, 2004). Showsh *et al.* (2001) reported that the enterococcal plasmid containing *vanA* also encodes a sex pheromone that is synthesized by *S. aureus*, suggesting a potential facilitator of conjugal transfer. VRSA isolates demonstrate complete vancomycin resistance, with MICs of 128μg/ml. Analysis of the first VRSA isolated in Michigan,

USA, revealed that Tn*1546* is integrated in a *S. aureus* conjugative plasmid designated pLW1043 (Weigel *et al.*, 2003).

pLW1043 is a multi-resistant plasmid with resistance genes against gentamicin, trimethoprim, penicillins, quaternary ammonium compounds, and vancomycin (Weigel *et al.*, 2003). Tn*1546* also carries a set of regulator genes, *vanSR*, that control transcription of *vanA* in a way that allows the expression of vancomycin only when the cell is exposed to vancomycin (Arthur *et al.*, 1992). The *vanA* dependent mechanism activates a cell wall biosynthetic pathway that avoids the vancomycin-sensitive step by producing an abnormal cell wall precursor in which the carboxyl-terminal dipeptide D-Alanyl-D-Alanine is replaced by the vancomycin-insensitive depsipeptide composed of D-Alanyl-D-Lactate (Arthur *et al.*, 1998; Bugg *et al.*, 1991; Arthur and Quintiliani, 2001) (Figure 1.10). This inducible nature of *vanA* genotype is part of the explanation for the successful maintenance of the *vanA* plasmid in methicillin resistant *S. aureus* (MRSA) without selective pressure from vancomycin. Therefore, vancomycin-resistant *S. aureus* could be taken to be an MRSA with a pLW1043-*vanA* plasmid newly added to the useful genetic repertoire for *S. aureus*. The *S. aureus* cell that accidentally takes up genetic material from this repertoire might flourish if the product turned out to be useful in the environment it inhabits (Hiramatsu *et al.*, 2004).

In the second VRSA case reported by Whitener *et al.* (2004), vancomycin was not administered but the patient received courses of topical and oral antimicrobial agents for a right-heel ulcer during the three years before the isolation of VRSA. It therefore appears that the multiple resistance nature of the *vanA* plasmid probably provided the mechanism for the stable maintenance of the plasmid by the MRSA (Hiramatsu *et al.*,

2004). A recent analysis (PCR and DNA sequence analysis) of the Tn*1546* elements from the two clinical isolates of VRSA indicated that while the Michigan VRSA element was identical to the prototype Tn*1546*, the Pennsylvania VRSA showed three distinct modification. This observation and differences in the Tn*1546*-like elements indicate that the first two VRSA isolates were the result of independent genetic events (Clark *et al.*, 2005).

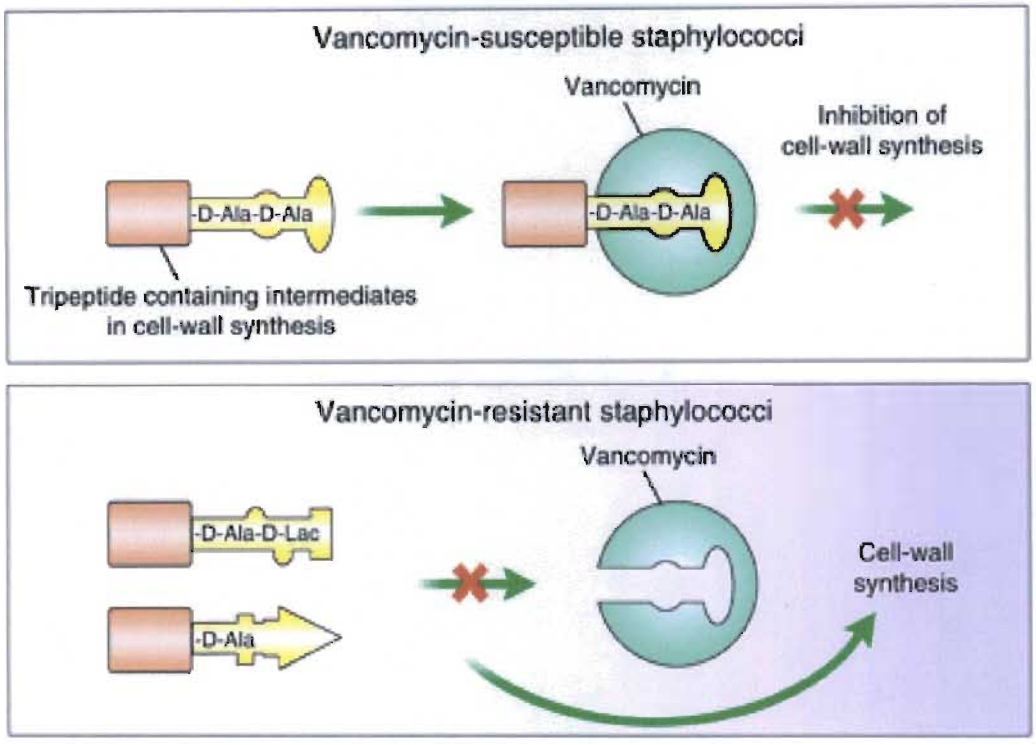

Figure 1.10: Mechanisms of *S. aureus* resistance to vancomycin: VRSA strains. VRSA strains are resistant to vancomycin because of the acquisition of the *vanA* operon from an enterococcus that allows synthesis of a cell wall precursor that ends in D-Ala-D-Lac dipeptide rather than D-Ala-D-Ala. The new dipeptide has dramatically reduced affinity for vancomycin. In the presence of vancomycin, the novel cell wall precursor is synthesized, allowing continued peptidoglycan assembly. Reference: Murray (2000); Lowy (2003).

VRSA strains carry both the chromosomally located *mecA* gene and a plasmid-borne *vanA* gene complex (Weigel *et al.*, 2003). These resistance mechanisms are targeted on the bacterial cell wall by distinct mechanisms. While these two mechanisms coexist in the same VRSA strain and are capable of providing high-level resistance against each class of antibiotics, the mechanisms of expression of the resistant phenotypes are independent and even mutually antagonistic (Severin *et al.*, 2004a). It has been shown that expression of high-level vancomycin resistance does not depend on an intact *mecA*, because selective inactivation of the gene did not reduce the vancomycin MIC of the bacteria (Severin *et al.*, 2004a). Furthermore, vancomycin resistance was suppressed by adding oxacillin to the growth medium under conditions where bacterial growth and cell wall synthesis depends on the transpeptidase activity of PBP2A (de Jonge *et al.*, 1992). A recent study by Severin *et al.*, (2004b) also indicated that while *mecA* is essential for oxacillin resistance, penicillin binding protein 2A, the protein gene product of *mecA*, appeared to be unable to utilize the depsipeptide cell wall precusor produced in the vancomycin-resistant cells for transpeptidation. Severin and his co-workers therefore concluded that penicillin-binding protein 2 (PBP2) could be important for vancomycin resistance and for the synthesis of abnormally structured walls characteristic of vancomycin-resistant *S. aureus*.

1.2.6.5 Epidemiology of Glycopeptide Resistance in *Staphylococcus aureus*

Infections caused by *S. aureus* with high-level resistance to vancomycin are rare. Only three cases have been reported (Centers for Disease Control and Prevention, 2002a; 2002b; 2004; Tenover *et al.*, 2004; Whitener *et al.*, 2004). All three patients had underlying disease, had received antibiotics and were reported from the USA. Coinfection with MRSA and VRE was present. Intermediate resistance of *S. aureus* to vancomycin is clearly more common than high-level resistance and at least 20 cases of VISA infections have been reported from various continents (Walsh and Howe, 2002). Pretreatment with vancomycin was a common feature in many of these patients (Ploy *et al.*, 1998; Rotun *et al.*, 1999). The prevalence of *S. aureus* with heteroresistance to glycopeptides appears to be higher than the corresponding prevalence of VISA. In many areas, the prevalence of vancomycin resistance appears to be low. A recent prevalence survey of more than 1,000 MRSA isolates in Belgium found only one homogeneous vancomycin-resistant *S. aureus* (VISA) and five heterogenous VISA (hVISA) based on population analysis profiling (Pierard *et al.*, 2004). In a Brazilian study of 140 MRSA isolates, five *S. aureus* with reduced susceptibility to vancomycin (8 μg/ml) were detected (Oliveira *et al.*, 2001c). None were positive for *vanA* and all had thickened cell walls. The Netherlands has a very low prevalence of MRSA. As heteroresistance to vancomycin appears to be associated with vancomycin use to treat MRSA infections, heteroresistance would not be expected to be prevalent in countries with low prevalence of MRSA. However, van Griethuysen *et al.* (2003) reported a rate of 6% of heteroresistance to vancomycin in MRSA strains. Epidemiological information on the origin of the affected patients revealed that none of the isolates originated from the

Netherlands. The patients were from Turkey, Greece, Italy, France, Germany, and Cote d'Ivoire.

1.2.7 Approved antimicrobial agents with activity against staphylococci including MRSA

The newest licensed antimicrobials on the United States market as well as many in the pipeline target serious infections caused by Gram-positive bacteria. The most important impetus for developing these agents in the early 1990s, included the intolerance of patients to both beta-lactams and glycopeptide antimicrobial agents, and the emergence and rapid spread of vancomycin-resistant *Enterococcus faecium* (VRE) (Eliopoulos, 2004; Ray and Rice, 2004). While the treatment of VRE infections remains an important therapeutic goal, difficulty enrolling VRE-infected patients in clinical trials and the sharp rise in methicillin-resistant *S. aureus* (MRSA) worldwide caused some pharmaceutical companies developing new agents to eschew United States Food and Drug Administration (FDA) approval for VRE indications in favor of targeting MRSA. The approval of two new antimicrobial agents, in 1999 and 2000, provided additional options for therapy of Gram-positive infections.

1.2.7.1 Quinupristin-dalfopristin

Representatives of the streptogramin A and streptogramin B families of antibiotics occur naturally in combinations that synergistically achieve levels of activity superior to those provided by either antibiotic alone. Quinupristin-dalfopristin are derivatives of pristinamycin, an agent widely used in animal feed as a growth promoter.

Dalfopristin is a streptogramin A and quinupristin is a streptogramin B in a 70:30 ratio, yielding a water-soluble drug suitable for intravenous administration (Batts *et al.*, 2001). Both compounds bind the 23rRNA of the 50S ribosomal subunit and inhibit protein synthesis (Vannuffel *et al.*, 1994; Canu *et al.*, 2001). Alone, each factor displays a moderate bacteriostatic activity while the combination of both factors is often bactericidal (Canu *et al.*, 2001). Quinupristin/dalfopristin was the first antimicrobial on the United States market with clinically important activity against VRE (the vast majority of which are resistant to ampicillin and vancomycin). Approved in September 1999, the components of this combination inhibit protein synthesis by binding to different ribosomal sites, thereby achieving synergy (Barriere *et al.*, 1998; Canu *et al.*, 2001; Ray and Rice, 2004). In the United States, the overwhelming majority (>99%) of *S. aureus*, including both MRSA and oxacillin-susceptible strains, were susceptible to this agent in-vitro (Ballow *et al.*, 2002). The combination is potentially bactericidal against isolates susceptible to both components. Against isolates with constitutive resistance to the macrolide-lincosamide-streptogramin B (MLS_B) class of antibiotics, which is seen frequently in MRSA and less commonly among methicillin-susceptible *S aureus* (Fluit *et al.*, 2001), the combination loses bactericidal activity in vitro (Fuchs *et al.*, 2000). This is because such isolates are resistant to quinupristin, which is a streptogramin B antibiotic. As long as strains remain susceptible to dalfopristin, however, the combination retains inhibitory activity.

In the United States, the only Food and Drug Administration-approved use (Aventis Pharmaceutical, 2000) of quinupristin-dalfopristin as an anti-staphylococcal agent is for treatment of adults with complicated skin and skin structure infections when

the pathogen is a methicillin-susceptible *S aureus*. Otherwise, the drug is approved for complicated skin and skin structure infections caused by *Streptococcus pyogenes* and for serious vancomycin-resistant *Enterococcus faecium* infections associated with bacteremia (the drug is not active against *Enterococcus faecalis*) (Eliopoulos, 2004). While its approval was met with enthusiasm, quinupristin-dalfopristin's clinical utility, has been limited by its intravenous-only formulation. Because this antibiotic is very irritating when given by peripheral vein, a deep catheter is usually required. A syndrome of arthralgias and myalgias, which may become very severe, develops in many patients treated with this combination (Olsen *et al.*, 2001). This syndrome is, however, made reversible with the discontinuation of treatment. Administration of quinupristin-dalfopristin can interfere with clearance of drugs that are eliminated through the cytochrome P450 system, so care is required to avoid potentially serious drug-drug interactions (Aventis Pharmaceutical, 2000).

1.2.7.2 Linezolid

In April 2000, linezolid became the first antimicrobial with a novel mechanism of action to obtain FDA approval in 35 years. The first and only approved oxazolidinone, linezolid inhibits bacterial protein synthesis by interfering with ribosomal initiation of translation (Shinabarger *et al.*, 1997). It has a narrow spectrum of activity against Gram-positive organisms, including staphylococci, streptococci, and enterococci. Essentially all strains of *S. aureus*, including MRSA, from clinical surveys are inhibited by this agent at or lower than the susceptibility breakpoint of 4 μg/ml (Ballow *et al.*, 2002). To date, there are three published reports of clinical isolates of *S. aureus* resistant to linezolid on the

basis of mutations in the 23S ribosomal RNA, in an area central to peptide bond formation (Tsiodras *et al.*, 2001; Wilson *et al.*, 2003; Meka *et al.*, 2004). Single point mutations in the 23 rRNA genes can reduce linezolid binding and the presence of this mutation in two or more of the cellular 23S genes can confer clinically significant levels of resistance (Marshall *et al.*, 2002; Meka *et al.*, 2004). The first point mutation appears to be the important one, thereafter, the bacteria are capable of amplifying the resistance through homologous recombination (Lobritz *et al.*, 2003). Similar mutations, leading to linezolid resistance, have also been encountered among enterococcal strains (Gonzales *et al.*, 2001).

The action of linezolid against *S. aureus* is best described as bacteriostatic, although some bactericidal activities can occur slowly with time (Fuchs *et al.*, 2002). A major advantage of this agent is that it is available both for intravenous and oral use, however, overuse in the community is a concern, as resistance may limit the drug's usefulness (Ray and Rice, 2004). The Food and Drug Administration-approved indications for linezolid use are relatively broad (Pharmacia and UpJohn Corporation, 2001). They include (but are not limited to) complicated skin and skin structure infections caused by MRSA or methicillin-susceptible *S. aureus* or group A or B streptococci, *S. aureus* nosocomial pneumonia, and infections caused by vancomycin-resistant enterococci. With compassionate use protocols, linezolid has been used successfully in many patients who do not respond to treatment with vancomycin (Moise *et al.*, 2002). However, patients with MRSA endocarditis who experience failure in attempts at treatment with linezolid have also been reported, (Ruiz *et al.*, 2002) and the oxazolidinone is not approved for this indication.

Linezolid has the potential to cause myelosuppression (Pharmacia and UpJohn Corporation, 2001). Although all cell lines may be affected (Halpern, 2002), the greatest attention has focused on thrombocytopenia. In comparative clinical trials, there was a small, but not statistically significant increase in occurrence of substantially low platelet counts in patients treated with linezolid (2.4% vs 1.5%), which generally became apparent after approximately 2 weeks of therapy (Gerson *et al.*, 2002). However, in some case series, low platelet counts were observed in ≥20% of patients receiving the drug (Attassi *et al.*, 2002; Orrick *et al.*, 2002). Recommendations for monitoring blood counts are included in the package insert for this agent (Pharmacia and UpJohn Corporation, 2001). Linezolid is a weak monoamine oxidase inhibitor (Batts *et al.*, 2001) and serotonin syndrome has been reported rarely in patients who had also received a selective serotonin reuptake inhibitor (Wigen and Goetz, 2002).

1.2.7.2.1 Drugs being clinically investigated

1.2.7.2.1.1 Daptomycin

Several agents with in-vitro antimicrobial activity against staphylococci have been studied in clinical trials. In September 2003, daptomycin became the first cyclic lipopeptide approved by the United States Food and Drug Administration. Derived from the fermentation of *Streptomyces roseosporus*, it has been known as an antibacterial agent for nearly 20 years (Stratton *et al.*, 1987). Initial clinical studies with this agent commenced in the 1980s, but development was halted. At a time of increasing concern about the rates of resistance to other antimicrobial agents, development of daptomycin resumed in the late 1990s. Daptomycin's bactericidal activity results from disrupting the

cytoplasmic membrane integrity in a reaction that requires sub-physiologic concentrations of calcium and causes depolarization and cell death (Silverman *et al.*, 2003).

The drug exhibits this mechanism against staphylococci, including MRSA, vancomycin-intermediate *S aureus*, coagulase-negative staphylococcal species, and many other Gram-positive bacteria (Barry *et al.*, 2001; Petersen *et al.*, 2002). The drug's activity is typically bactericidal against staphylococci (Fuchs *et al.*, 2002). Though no mechanism of resistance has yet been identified, resistance reported by the sponsor - Cubist Pharmaceuticals, MA, USA - during the Phase II and Phase III clinical trials is very low (<0.2%). The first report of the development of daptomycin resistance in a clinical isolate of MRSA has recently been described (Mangili *et al.*, 2005). Clinical trials using a higher dose (6mg/kg) for treatment of staphylococcal bacteremia is ongoing (Ray and Rice, 2004). The drug has the potential to cause a reversible myopathy which is evident when high doses were given twice daily, however, it appears to be an infrequent event with new dosing regimens (Tally and DeBruin, 2000).

1.2.7.2.1.2 Oritavancin

This agent is a glycopeptide antibiotic derived semi-synthetically from a precursor drug closely related to vancomycin. It has activity in vitro against staphylococci, including MRSA, which is generally comparable with that of vancomycin (Schwalbe *et al.*, 1996; Zeckel *et al.*, 2000). Oritavancin is currently in Phase III clinical trials for skin and soft tissue infection and Phase II for bacteraemia (Ray and Rice, 2004). A striking difference in in-vitro activity between this agent and vancomycin is that

oritavancin can inhibit vancomycin-resistant enterococci, including strains with vancomycin minimum inhibitory concentrations >1000μg/ml, at concentrations of approximately 1.0μg/ml (Zeckel *et al.*, 2000). Oritavancin is bactericidal against *S. aureus*, including MRSA (Zeckel *et al.*, 2000). Another major difference between the new glycopeptide and vancomycin is that the elimination half-life of oritavancin is much longer, in the range of 5 to 15 days compared to vancomycin (Barrett, 2001).

1.2.7.2.1.3 Dalbavancin

Dalbavancin is another semi-synthetic glycopeptide antibiotic that is being clinically investigated. This agent is as much as 16-fold more active than vancomycin against staphylococci tested in vitro (Candiani *et al.*, 1999). Like oritavancin, dalbavancin is eliminated slowly from the serum, with a half-life of several days, even in individuals with normal renal function. The developmental program for this agent has exploited this pharmacokinetic feature, with once-weekly dosing strategies having been used in phase II studies of complicated skin and skin structure infections (Selzer *et al.*, 2003).

1.2.7.2.1.4 Other antibacterial agents

Telithromycin is the first ketolide antibiotic. Ketolides are a subclass of macrolides modified to improve ribosomal binding and thereby retain activity against resistant organisms (Ray and Rice, 2004).

Garenoxacin is a novel des-fluoro (6) quinolone designed to have improved activity against resistant respiratory pathogens. Its structure modification is the absence

of fluorine at C-6; however, its mechanism of action is the same as other in its class (Fung-Tomc *et al.*, 2000). DW286, a naphthyridone, is among several fluoroquinolones in development that has in-vitro activity against MRSA (Kim *et al.*, 2003). Active against MRSA strains that are resistant to other fluoroquinolones, it selects fluoroquinolone-resistant mutants at a lower frequency than older agents (Yun *et al.*, 2002; Firsov *et al.*, 2004).

Glycylcyclines are a subclass of tetracyclines designed to avoid the problems of microbial resistance faced by the traditional tetracyclines. The prototype is tigecycline, a derivative of minocycline. Currently in Phase II studies of intra-abdominal and complicated skin and soft-tissue infections, tigecycline has demonstrated in-vitro activity and early in-vivo efficacy against a wide variety of pathogens, including MRSA. Most of the tetracycline-resistance mechanisms prevalent in clinical bacteria are inactive against tigecycline (Ray and Rice, 2004; Fritsche and Jones, 2004).

Novel β-lactamase-stable cephalosporins with high affinity for PBP2a are in clinical development (Glinka, 2002). The PBP2a affinity of BMS-247243 is 100-fold greater than that of methicillin or cefotaxime, and the drug is bactericidal against MRSA at twice the rate of vancomycin (Fung-Tomc *et al.*, 2002). Other drugs in this class in development include the zwitterionic cephem RWJ-54428 (Malouin *et al.*, 2003), CB-181963 (Huang *et al.*, 2004), BAL5788 (Azoulay-Dupuis *et al.*, 2004), a prodrug of BAL9141 (Enteza *et al.*, 2002; Jones *et al.*, 2002b), and S-3578 (Fujimura *et al.*, 2003). SM-197436, SM-232721, and SM-232724 are novel methylcarbapenems that are also active in-vitro against MRSA (Ueda and Sungawa, 2003).

The lantibiotics nisin, alone and combined with peptidoglycan-modulating antibiotics, shows activity against MRSA and vancomycin-resistant enterococci (Wiedermann *et al.*, 2001; Brumfitt *et al.*, 2002). The lantibiotic gallidermin is as active as nisin and is currently produced and developed for clinical studies (Gotz and Jung, 2001). Other novel antimicrobial agents include ramoplanin (Cudic *et al.*, 2002), muraymycins (Lin *et al.*, 2002), mannopeptimycins (Ruzin *et al.*, 2004), arylalkylidene rhodanines and arylalkylidene iminothiazolidin-4-ones (Zervosen *et al.*, 2004).

Several agents targeting virulence factors have also being investigated. They include RNAIII-inhibiting peptide, which acts against *S. aureus* pathogenesis by disrupting quorum-sensing mechanisms (Dell'Acqua *et al.*, 2004), and a truncated thiolactone peptide found to be a potent inhibitor for all the four *agr*-specificity groups of *S. aureus* (Lyon *et al.*, 2000). *S. aureus* immune globulin intravenous (human) (Altastaph; NABI Biopharmaceuticals) is a hyperimmune, polyclonal, intravenous immunoglobulin product derived from the plasma of human donors who have previously been vaccinated with *S. aureus* polysaccharide conjugate vaccine (StaphVAX: NABI Biopharmaceuticals). This vaccine is a bivalent conjugate capsular polysaccharide covalently bound to recombinant exoprotein A, which has been demonstrated to provide temporary protection against the occurrence of *S. aureus* bactereamia in patients receiving haemodialysis (Shinefield *et al.*, 2002; Robbins *et al.*, 2004). Others include Tefibazumab (Aurexis; Inhibitex), a humanized monoclonal antibody directed at the microbial surface components recognizing adhesive matrix molecule (MSCRAMM) clumping factor A (Hall *et al.*, 2003), INH-A21 (Veronate; Inhibitex), BYSX-A110 (Weisman, 2004), and Aurograb (Neu Tec Pharma) (Burnie *et al.*, 2000; Patti, 2004).

1.2.8 Genome sequencing of *Staphylococcus aureus*

The impact of human health of *S aureus* infections in the community and hospital settings have led to intensive investigation of this organism over recent years. More complete genomes are now available for *S. aureus* than for any other bacterial species, thus providing detailed insight into the evolutionary processes leading to strains of differing virulence and drug-resistance potential (Holden *et al.*, 2004). To date, seven complete genome sequencing of *S. aureus* have been conducted; six have been published, namely an hospital-acquired MRSA (N315) and vancomycin intermediately susceptible *S. aureus* (Mu50) (Kuroda *et al.*, 2001), a community-acquired MRSA (MW2) (Baba *et al.*, 2002), an EMRSA-16 clone (MRSA252), a representative of an invasive community-acquired *S. aureus* clone (MSSA476) (Holden *et al.*, 2004) and recently the *S. aureus* COL strain (Gill *et al.*, 2005). The remaining unpublished but sequenced strains is NCTC 8325 (www.genome.ou.edu/staph.html; ST8) (Table 1.4).

Table 1.4: Details of the seven sequenced *Staphylococcus aureus* strains

Strain	Source	Year	Comments	References
N315	Pharynx, Japan	1982	Hospital-acquired MRSA	Kuroda *et al.* (2001)
Mu50	Wound, Japan	1997	Hospital-acquired VISA, related to N315	Kuroda *et al.* (2001)
MW2	Fatal paediatric bacteremia, North Dakota, USA	1998	Typical USA community-acquired MRSA, PV-toxin positive	Baba *et al.* (2002)
MRSA252	Fatal bacteremia, Oxford, UK	1997	Typical UK hospital-acquired epidemic MRSA (EMRSA-16)	Holden *et al.* (2004)
MSSA476	Osteomyelitis, Oxford, UK	1998	Community-acquired MSSA	Holden *et al.* (2004)
COL	Colindale, UK	1961	Early MRSA	http://www.tigr.org; Gill *et al.* (2005)
NCTC8325	Colindale, UK	<1949	Laboratory strain, parent of non-lysogenic 8325-4	http://www.genome.ou.edu/staph

Adapted from Lindsay and Holden (2004); Gill *et al.* (2005).

1.2.8.1 Phylogenetic relatedness to other organisms

Phylogenetic classification places *S. aureus* in the *Bacillus/Staphylococcus* group. Accordingly, up to 52% of predicted proteins encoded by the N315 genome are similar to those in *Bacillus substilis* and *B. halodurans* (Kuroda *et al.*, 2001). They typically contain housekeeping genes involved in essential functions of the vegetative life of the bacteria such as DNA replication, protein synthesis and carbohydrate metabolism. This finding indicates that about half of the *S. aureus* genome has been vertically transmitted from a common ancestor of the *Bacillus/Staphylococcus* group of bacteria, comprising the backbone of *S. aureus* genome. The rest of the genome encodes proteins similar to those produced by bacterial species ranging from species belonging to *Streptococcus* to far distant eukaroytes including *Homo sapiens* (Kuroda *et al.*, 2001).

The genome of a bacterial species has been proposed to comprise core, auxiliary and lost or foreign genes. Core genes are suggested as those present in more than 95% of species isolates, with auxiliary genes present in 1-95% of isolates, and foreign genes found in less than 1% of isolates (Lan and Reeves, 2000). The differentiation of *S. aureus* core genes and auxiliary genes is facilitated by the identification of regions of the chromosome that are hotspots of variation, have discrete ends, and carry genes predicted to be involved in horizontal gene transfer (Lindsay and Holden, 2004). Preliminary investigation of the genetic diversity of *S. aureus* strains by DNA micro-array analysis suggests that approximately 22% of *S. aureus* genomes are composed of variable regions (Fitzgerald *et al.*, 2001a) and much of the diversity was associated with large scale variations.

The five *S. aureus* sequenced genomes (N315, Mu50, MW2, MSSA476 and MRSA252) range in size from 2.820Mb to 2.903Mb and are predicted to contain 2592 and 2748 protein coding sequences (Holden *et al.*, 2004). Gene order is conserved and the similarity of individual genes between the isolates is typically 98%-100% at the amino acid level. As expected, the majority of genes comprising the core genome are those associated with central metabolism and other housekeeping functions. Supplementing these are genes that are associated with common species functions but are not essential for growth and survival, including virulence genes not carried by other staphylococcal species, surface binding proteins, toxins, exoenzymes and the capsule biosynthetic cluster (Lindsay and Holden, 2004). A summary of the major variable genetic elements in sequenced *S. aureus* strains is presented in Table 1.5.

Subtle variation between orthologous genes (groups of similar genes in different organisms that have the same function and have evolved from a common ancestor) and multilocus sequence typing (MLST) has been used to estimate how closely or distantly related the five sequenced strains are to one another (Lindsay and Holden, 2004). Comparison of the percentage similarities of the DNA of the core genomes showed that N315, Mu50 and MW2, MSSA476 are closely related. Furthermore, the hospital strains N315 and Mu50 and the community-acquired MW2 and MSSA belong to identical but separate sequence types (STs) - (ST5 and ST1 respectively) while COL and NCTC8325 belong to closely related STs (ST250 and ST8). Notably, the level of relatedness inferred by MLST correlates well with the overall genomic divergence over all orthologous gene pairs in the core genome (Lindsay and Holden, 2004). MRSA 252 is the most divergent of the sequenced strains (ST36) and it is a representative of the EMRSA-16 epidemic

clonal group responsible for 50% of the MRSA infections within the UK, and one of the major MRSA clones found in the United States (Johnson *et al.*, 2001; McDougal *et al.*, 2003). About 6% of the MRSA 252 genome was previously undescribed when compared with other published genome, including the hospital-acquired strains. These additional genes fall into the accessory category. The genetic diversity observed was attributed to numerous mechanisms involving the horizontal acquisition of mobile DNA, both on the large and small scale (Holden *et al.*, 2004).

1.2.8.2 Accessory genome

The accessory genome consists of mobile (or once mobile) genetic elements that transfer horizontally between strains. These elements include bacteriophages, pathogenicity islands, chromosomal cassettes, genomic islands and transposons. Many of these genetic elements carry genes with virulence of resistance functions. Therefore, the distribution and horizontal transfer of these elements could have important clinical implications. The identification and characterization of these elements has provided some insights into how *S. aureus* cause disease, and how they are evolving. In particular, several studies have suggested that certain toxin genes are associated with particular lineages of MLST clonal complexes (Moore and Lindsay, 2001; Peacock *et al.*, 2002), including toxic shock syndrome toxin-1 (*tst*), leukocidin DE (*lukDE*), serine protease-like B (*splB*), and superantigens A, G and I (*sea, seg and sei*). The association is not due to vertical transmission alone, but there is evidence of frequent acquisition and loss of particular elements that is restricted to particular clonal complexes (CCs). Although the mechanisms are still unclear, this is a possible explanation for the dominance of certain

CCs in carriage and disease. These observations also suggest the importance of mechanisms that control the distribution of such elements in *S. aureus* populations (Lindsay and Holden, 2004).

1.2.8.2.1 Bacteriophages

S. aureus temperate bacteriophages are common, with most strains carrying at least one phage. The five sequenced strains have nine prophages genomes in their chromosomes and are classified into five families on the basis of intergrase gene homology, which dictates insertion site. None of the sequenced strains appeared to have more than one phage of each family type, presumable owing to phage immunity or competition for insertion sites (Lindsay and Holden, 2004). All the sequenced *S. aureus* strains have prophage ϕSa3 and in each case are integrated into the *hlb* (β-haemolysin) gene (Holden *et al.*, 2004). The prophage contains staphylokinase (*sak*) and enterotoxin type A (*sea*) genes except in N315 which carries a different enterotoxin gene, designated *sep*, that encodes a protein with only 77% amino acid similarity to enterotoxin A (Kuroda *et al.*, 2001). Two additional enterotoxin gene alleles, *seg2* and *sek2,* which encode putative enterotoxin G and K homologues are reported in MW2 and MSSA476 (Table 1.5) (Baba *et al.*, 2002; Holden *et al.*, 2004).

A unique feature on the MW2 chromosome is the ϕSa2mw, which carries the *lukF-PV* and *lukS-PV* genes that encode the Panton-Valentine (PV) leuckocidin components. It has a potent toxic effect on human white-blood cells and is strongly associated with severe forms of pneumonia (necrotic pneumonia) caused by community-acquired *S. aureus* strains (Gillet *et al.*, 2002). In addition, PV toxin strains have been

associated with an increase in severe boils and skin infections among inmates in prisons, and the homosexual community (Anonymous, 2003). The ϕSa2 attachment site is present in all the sequenced strains but they exist in different allelic forms, and only ϕSa2mw has the Panton-Valentine leukocidin genes (Table 1.5) (Baba *et al.*, 2002).

1.2.8.2.2 Transposons

Transposons and insertions sequences can integrate themselves into any chromosome loci by illegitimate recombination (Murphy, 1989). Thus they tend to shuffle genome structure and are thought to contribute much to adaptability of *S. aureus* to the adverse environment (Baba *et al.*, 2002). Tn*554* is a site-specific transposon that encodes resistance to spectinomycin and macrolide-lincosamide-streptogramin B antibiotics. Two copies of Tn*554* were found in N315, Mu50 and MRSA252 genomes. Three additional copies were found in the N315 genome. Another transposon Tn*5801* was uniquely found in Mu50, which carries a gene (*tetM*) encoding tetracycline and minocycline resistance (Kuroda *et al.*, 2001). The MRSA252 chromosome also contained a Tn*552* transposon that encodes the *BlaI*, *BlaR* and *BlaZ* components of the inducible *S. aureus* β-lactamase. There is also an element integrated into the chromosome that contains similarity to the Tn*916* transposon, however, it does not appear to carry any obvious resistance determinants (Holden *et al.*, 2004). Transposons are scarcely recorded in the MW2 and MSSA476 genome (Table 1.5) (Baba *et al.*, 2002; Holden *et al.*, 2004). It would be reasonable to assume that hospitals are a severe environment for microorganisms to survive in, because they are constantly exposed to various antiseptics and new antibiotics. Multiple insertions of transposons and insertions sequences in

hospital-acquired MRSA genomes might be testament to the evolutionary ordeal they have gone through (Baba *et al.*, 2002).

1.2.8.2.3 *S. aureus* pathogenicity islands (SaPIs)

SaPIs often carry superantigen genes, such as toxic shock syndrome (*tst*) and enterotoxins B and C, implicated in toxic shock and food poisoning. Seven SaPIs in human isolates (SaPIn1) (Kuroda *et al.*, 2001), SaPIm1 (Kuroda *et al.*, 2001), SaGIm (Kuroda *et al.*, 2001), vSa3 (MW2) (Baba *et al.*, 2002), SaPI1 (Lindsay *et al.*, 1998), SaPI3 (Yarwood *et al.*, 2002) and SaPI4 (Holden *et al.*, 2004) and two in bovine isolates - SaPIbov (Fitzgerald *et al.*, 2001b) and SaPIbov2 (Ubeda *et al.*, 2003) have been sequenced. Human SaPIs are classified into four groups on the basis of integrase homology and insertion site, and none of the sequenced strains carried more than one copy of each type. The MSSA476 does not have a pathogenicity island in its genome while the MRSA252 carries a SaPI-like element - SaPI4, that contains homologues of pathogenicity island proteins and displays synteny (conserved gene order) with the previously characterized SaPI1, SaPIbov and SaPI3 (Lindsay *et al.*, 1998; Fitzgerald *et al.*, 2001b; Yarwood *et al.*, 2002).

SaPI4 has an integrase gene and insertion site downstream of the ribosomal protein gene *rpsR* but contains several hypothetical proteins with no similarity to characterized virulence genes (Holden *et al.*, 2004). N315 and Mu50 possess the superantigen genes *se1*, *se3* and *tst*. A feature of the toxic shock syndrome toxin (TSST) island family is its close linkage to prophages ϕN315/ϕMu50A and ϕMu50B, respectively, which are integrated in close proximity to these islands. These phages may

be involved in the horizontal transfer of the islands. (Kuroda *et al.*, 2001) A unique feature of Mu50 is the carriage of SaGIm (*fhuD*) gene that possibly encodes a ferrichrome-binding ABC transporter. This iron transporter might confer a selective advantage to Mu50 in human tissue. MW2 also carries two allelic forms of enterotoxin *sel2* and *sel4* (Table 1.5) (Baba *et al.*, 2002; Lindsay and Holden, 2004).

1.2.8.2.4 Genomic Islands

Two types of genomic islands are found in *S. aureus*: vSaα and vSaβ (Kuroda *et al.*, 2001; Baba *et al.*, 2002). Both islands appeared to be extremely stable as they are found in all sequenced isolates in the same location with some genes highly conserved. However, they vary remarkably between strains, each carrying their own number and variants of superantigens, lipoproteins and proteases. Some also carry leukocidins, hyaluronate lyase and lantibiotic genes (Lindsay and Holden, 2004). The genomic island VSaα is distinctive because it carries many putative staphylococcal exotoxin (*set*) genes in all the sequenced strains (Suzek *et al.*, 2001; Williams *et al.*, 2000). They are capable of inducing proinflammatory cytokine production by human peripheral blood mononuclear cells (Williams *et al.*, 2000). The Mu50 and N315 genome have 9 and 10 *set* genes respectively, and the *set* clusters are nearly identical except for one paralogue (*set9*), which is missing in Mu50. The structure of vSaβ differs from strain to strain with the presence or absence of some genes. The superantigen gene cluster (composed of six enterotoxin genes) carried by islands of N315 and Mu50 (type I) are missing from vSaβ of MW2 (type II). Instead, vSaβmw has a novel gene cluster, designated *bsa* (bacteriocin of *S. aureus*), which encodes a putative bacteriocin (toxin or antibiotic to other bacteria)

(Baba *et al.*, 2002). However, the *spl* (staphylococcal serine proteases) and exotoxins were present in N315, Mu50 and MRSA252. The *lukDE* gene (leuckocidin DE) was absent in MRSA252 (carried a *hysA* – hyaluronate lyase gene) and the genomic islands found in MSSA476 matched that of MW2 (Table 1.5) (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Lindsay and Holden, 2004).

1.2.8.2.5 Plasmids

S. aureus isolates, and particularly those from hospitals, often carry one or more free or integrated plasmids. *S. aureus* plasmids can be classified into three types on the basis of size and the genes they carry (Paulsen *et al.*, 1997). All types of *S. aureus* plasmids frequently carry antibiotic resistance genes, or resistance to heavy metals or antiseptics. Some virulence genes are also reported to be carried on plasmid, such as exfoliative toxin B and some superantigens (Zhang *et al.*, 1998;Yamaguchi *et al.*, 2001). Notably, all the sequenced strains carried plasmids. The MW2 strain contained no antibiotic resistance genes apart from *blaZ* (encoding penicillinase) and a *cadD* (cadmium resistance gene) on plasmid pMW2 (20654bp) (Baba *et al.*, 2002; Lindsay and Holden, 2004). The N315 plasmid pN315 (24653bp) contained a cadmium resistance determinant *cadDX* and an arsenate resistance determinant *arsRBC*. On the Mu50 plasmid pMu50 (25107bp), there is a copy of Tn*4001* that carries *aac-aphD* (encoding aminoglycoside resistance). Others include the *qacA* genes that encode resistance to quaternary ammonium compounds (such as diamidines, chlorhexidine, and intercalating dyes) and *traA*, which encodes a probable nicking enzyme for bacterial conjugation. The *blaZ* and *cadD* genes are the only apparent resistance genes within the MSSA476

genome located on a pSAS1 plasmid (Holden *et al.*, 2004). Strain MRSA252 carries an integrated plasmid that confers resistance to heavy metals arsenic (*arsBC*) and cadmium (*cadAC*). Strains N315 and Mu50 also carries a pUB110 plasmid with bleomycin- and kanamycin-resistance genes while MRSA252 carries an integrated pUB110 plasmid (Table 1.5) (Kuroda *et al.*, 2001; Holden *et al.*, 2004).

1.2.8.2.6 Staphylococcal cassettee chromosome (SCC)

SCC elements are mobile genetic elements that integrate at the same site on the *S aureus* chromosome (Ito *et al.*, 2001). These elements carry the *mec* region encoding methicillin resistance, but alternatively they can also carry other sets of genes such as the capsule gene (Hiramatsu *et al.*, 2001b; Luong *et al.*, 2002). At least, four different versions of SCC*mec* are found in *S. aureus*: SCC*mec* types I to IV (Ito *et al.*, 2001; Ma *et al.*, 2002). Recently, the type IV was divided into subtypes IVa, IVb and recently a third subtype IVc was reported in France and Japan (Hiramatsu *et al.*, 2002; Ma *et al.*, 2002; Ito *et al.*, 2003) and a novel type V has been described recently (Ito *et al.*, 2004). The three hospital-acquired MRSA strains (N315, Mu50, MRSA252) possess the type II SCC*mec* elements (Table 1.5). The SCC*mec* encodes resistance to beta lactams, bleomycin, macrolide-lincosamide-streptogramin B, aminoglycosides (tobramycin, amikacin), and spectinomycin (Kuroda *et al.*, 2001). The community-acquired MRSA strain (MW2) has a type IVa element and its structure is smaller in size from that of hospital-acquired MRSA strains. Type IVa SCC*mec* of MW2 comprises two allelic elements - class B *mec*-gene complex (*mec*A and its regulatory genes) and type-2 *ccr A* and *B* genes (Hiramatsu *et al.*, 2001a). Notably, the methicillin-susceptible MSSA476

contained a novel SCC-like element (SCC_{476}), which showed the greatest similarity to a previously described *S. hominis* non-*mec* SCC element (SCC_{12263}) (Katayama, 2003b). SCC_{476} shares the same left and right boundaries (attL and attR, respectively) and similar inverted repeat sequences with the SCC*mec* elements, but does not carry the *mecA* gene. However, it carries a novel gene with homology to the fusidic acid resistance gene *far1* (Table 1.5) (O'Brien *et al.*, 2002).

Table 1.5: Summary of the major variable genetic elements in sequenced *Staphylococcus aureus* strains

	N315	Mu50	MW2	MSSA476	MRSA252
SCC mec type II	*mecA*	*mecA*	-	-	*mecA*
SCC mec type IV	-	-	*mecA*	-	-
SCC_{476}	-	-	-	*farl* homologue	-
Bacteriophage					
ϕSa1	-	NI	-	-	-
ϕSa2	-	-	*lukSF*-PV	-	NI
ϕSa3	*sak, sep*	*sea, sak*	*sea, sak, seg2, sek2*	*sea, sak, seg2, sek2*	*sea, sak*
ϕSa4	-	-	-	NI	-
ϕSa5	-	-	-	-	-
Genomic islands					
Vsaα	*set* [10][c]	*set* [9][c]	*set* [11][c]	*set* [11][c]	*set* [9][c]
Vsaβ	*spl* [5][c], *lukDE*, exotoxin [6][c]	*spl* [5][c], *lukDE*, exotoxin [6][c]	*spl* [4][c], *lukDE, bsa*	*spl* [4][c], *lukDE, bsa*	*spl* [5][c], *hysA*, exotoxin [6][c]
Pathogenicity islands					
SaPI1	-	-	-	-	-
SaPI2	*se1, sec3, tst*	*se1, sec3, tst*	-	-	-
SaPI3	-	*fhuD*	*ear, sel2, sec4*	-	-
SaPI4	-	-	-	-	NI
Plasmids					
I	*ble, kan* (pUB110)[b]	*ble, kan* (pUB110)[b]	-		*ble, kan* (pUB110)[b]
II	*cadDX, arsBC* (pN315B)[b]	-	*blaZ, cadD* (pMW2)[b]	*blaZ, cadD* (pSAS)[b]	*cadAC, arsBC* (Integrated)[b]
III	-	*aacA-aphD, qacA* (pVRSA)[b]	-	-	-
Transposons					
Tn*554*	*ermA, spc* [5][c]	*ermA, spc* [2][c]	-	-	*ermA, spc* [2][c]
Tn*552*	-	-	-	-	*blaZ*
Tn*5801*	-	*tetM*	-	-	-
Tn*916*-like	-	-	-	-	NI

Reference: Lindsay and Holden, (2004).

[a]Abbreviations: NI – virulence or drug resistance genes not identified at present; *aacA-aphD*, aminoglycoside resistance; *arsBC*, arsenic resistance genes; *blaZ*, penicillin resistance; *bsa*, bacteriocin biosynthetic genes; *cadACDX*, cadmium resistance genes; ear, putative β-lactamase type protein; *ermA*, erythromycin resistance; *farl*, fusidic acid resistance; *fhuD*, siderophore transporter; *qacA*, quartenary ammonium compound (antiseptic) resistance; *hysA*, hyaluronate lyase; *lukDE*, two components of the leukocidin DE toxins; *lukSF-PV*, two components of the Panton-Valentine leukocidin toxin; *mecA*, penicillin-binding protein 2a conferring resistance to methicillin; *sak*, staphylokinase; SCC, Staphylococcal cassette chromosome; *sea*, enterotoxin A; *sec3*, enterotoxin C3; *sec4*, enterotoxin C4; *seg2*, enterotoxin G2; *sek2*, enterotoxin K2; *sel*, enterotoxin L; *sel2*, enterotoxin L2; *sep*, enterotoxin P; *set*, staphylococcal enterotoxins; *spc*, spectinomycin resistance; *spl*, staphylococcal serin proteases; *tst*, toxin shock syndrome toxin-1; *tetM*, tetracycline resistance.

[b]The number of homologues, including pseudogenes, located in that region.

1.3 Objectives

Numerous studies have been carried out in most parts of the world on MRSA, which has identified the appearance of multi-drug resistant MRSA clones replacing other MRSA lineages. However, epidemiological information and comparison of *S. aureus* strains in Africa are lacking. The extent of the epidemiological characteristics of antibiotic resistant *S. aureus* in most communities in Africa is largely unknown in spite of the established fact that infections attributed to multidrug-resistant *S. aureus*, in particular MRSA are an important health problem worldwide. At present, there is scarcity of data on antibiotic susceptibility patterns, clonal identities and diversity of *S. aureus* in Africa using traditional and molecular epidemiological techniques.

The main goal of the study was to provide baseline data on the epidemiology of clinical isolates of *S. aureus* strains in Nigeria and South Africa in the development of health intervention strategies. Data from this study would be useful in the establishment of adequate infection control programmes, adjust treatment and national drug policies in the treatment of staphylococcal infections and bring a better understanding on the epidemiology of *S. aureus*.

The specific objectives of the study are: (1) to determine the antimicrobial susceptibility pattern of *S. aureus* from clinical samples in Nigeria and South Africa; (2) determine the prevalence of methicillin resistant *S. aureus* (MRSA) in both countries; (3) understand epidemiologic relationship among MRSA clones and (4) investigate clonal identities and geographic spread of MRSA clones using various genotyping methods.

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CHAPTER TWO

A STUDY ON THE ANTIBIOTIC SUSCEPTIBILITY OF CLINICAL ISOLATES OF *Staphylococcus aureus* OBTAINED FROM SOUTHWESTERN NIGERIA AND KWAZULU-NATAL PROVINCE OF SOUTH AFRICA

2.1 INTRODUCTION

Infectious diseases remain the leading cause of death worldwide in which seventeen million (32%) of the 52 million who die each year succumb to infectious disease or complications arising from infection (ASM, 1997). One of the most important pathogenic microorganisms causing infection in the hospitals and the community all over the world is *Staphylococcus aureus*. It is associated with a variety of clinical infections including septicemia, pneumonia, wound sepsis, septic arthritis, osteomyelitis, and post-surgical toxic shock syndrome (Boyce, 1997; Shopsin and Kreiswirth, 2001). One of the reasons for the success of this human pathogen is its great variability, occurring at different periods and places with different clonal types and antibiotic resistance pattern within regions and countries. In most cases, the strains responsible for these infections are hospital-acquired, where susceptible strains have been largely eliminated and antibiotic resistant strains predominate. This is as a result of selective pressure exerted by the widespread use of antibiotics (Thomas, 1988). Antibiotic resistance is a serious problem in health institutions: treatment failures extend the length of hospital stay, or demand repeated physician visits; hospital beds are blocked to new patients and productive time is lost (Livermore, 2003). The resistance of *S. aureus* to antimicrobials is a worldwide problem

causing substantial rates of morbidity and mortality (Cosgrove *et al.*, 2003; Engemann *et al.*, 2003). Although the infections caused by antibiotic-resistant *S. aureus* cause serious problems in the general population, such infections can be particularly devastating for the very young, the elderly and the immunocompromised (ASM, 1997).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a well-known etiologic agent of a wide variety of infections, and has assumed increasing importance internationally as a cause of both nosocomial and community-acquired infections. MRSA infections are additional to the burden of methicillin susceptible *S. aureus* and are particularly difficult to treat especially if they are located at anatomical sites, where antibiotic penetration is reduced (Duckworth, 2003). Cohort studies of patients with MRSA bacteremia have reported higher mortality rates, increased morbidity, longer hospital length of stay, and higher costs compared with patients with methicillin-susceptible *S. aureus* (MSSA) bacteremia (Blot *et al.*, 2002; Cosgrove *et al.*, 2003; Engemann *et al.*, 2003; Melzer *et al.*, 2003; Kopp *et al.*, 2004; Cosgrove *et al.*, 2005). Studies have also indicated that the direct medical cost for MRSA infections in hospitals in the United States is $27,083-$34,000 per patient (Abramson and Sexton, 1999; Rubin and Samore, 2002). Consequently, annual United States health care costs associated with MRSA infections are estimated to be approximately $6 billion.

Major waves of infectious agents are encountered on the African continent. One of these agents is *S. aureus,* which is implicated as the major etiological agent of wound infections (Kotisso and Aseffa, 1998; Andhoga *et al.*, 2002; Eriksen *et al.*, 2003; Wariso and Nwachukwu, 2003) and life-threatening infections, especially neonatal sepsis, with high mortality rates (Dawodu and Alausa, 1985; Adejuyigbe *et al.*, 2001; Bode-Thomas, 2004).

Studies have also indicated that *S. aureus* is implicated in about 40% of all clinically evident cases of persistent middle ear effusion in otitis media in African children (Cisse *et al.*, 1995; Ndip *et al.*, 1995; Nwawolo *et al.*, 2001). One of the earliest reports of MRSA in Africa was that of Scragg *et al.* (1978) in a Durban hospital in South Africa, while varying reports on the prevalence of MRSA from health institutions in different regions in Africa have been described. MRSA prevalence of 4.8% have been reported in Algeria (Kesah *et al.*, 2003), 17.4% in Tunisia (Ben Jemaa *et al.*, 2004), 27% in Nigeria (Okesola *et al.*, 1999), 39.8% in Kenya (Omari *et al.*, 1997), 39% in South Africa (Zinn *et al.*, 2004), and 66% in Senegal (Sow *et al.*, 1998). MRSA intermediately resistant to vancomycin and teicoplanin in a South African hospital have also been reported (Ferraz *et al.*, 2000).

For practicing physicians, clinical microbiologists and public health officials, knowledge of local antimicrobial resistance patterns of bacterial pathogens is essential to guide empirical and pathogen specific therapy. This information is also critical for optimal decisions regarding hospital formulary and infection control policies. In the African continent, there are relatively few studies on antimicrobial susceptibility of *S. aureus* compared with surveys in other regions of the world. This is in spite of the established fact that multidrug-resistant *S. aureus*, especially MRSA is an important health problem worldwide. Furthermore, there is paucity of data on the resistance profile of *S. aureus* isolates from various regions in the continent. Extensive studies on the prevalence of MSSA and MRSA in Southwestern Nigeria and KwaZulu-Natal province in South Africa are lacking. The importance of monitoring antimicrobial susceptibility pattern of *S. aureus* is important as a first step in understanding its epidemiology and information on drug-resistance pattern could assist clinicians in the selection of empiric antimicrobial

therapy. It is also essential for the rational formulation of public health care policies, and provide useful information on the global surveillance of this pathogen regarding antimicrobial resistance. This study therefore investigated antibiotic susceptibility of *S. aureus* isolates, obtained from clinical samples in Nigeria and South Africa, with various antibiotics used in clinical therapy.

2.2 MATERIALS AND METHODS

2.2.1 Study Areas

Six health institutions in Southwestern Nigeria were included in this study. They comprised of three hospitals located in Ile-Ife, and one health facility in Ilesa, Ipetumodu and Ibadan. The duration of the collection of isolates was from June 2002 to August 2004. A total of 14 hospitals in the KwaZulu-Natal (KZN) province, South Africa, participated in this study from June 2002 to August 2004. The health institutions included four hospitals in the city of Durban, two from Pietermaritzburg, and one health institution from Newcastle, Greytown, Kokstad, Eshowe, Port Shepstone, Scottburgh, Empangeni and Ubombo.

2.2.2 Microbiological analysis and Identification

The *S. aureus* isolates were obtained from the clinical microbiology laboratories of the various health institutions, in both countries. Identification was based on growth and fermentation on mannitol salt agar, colonial morphology on nutrient agar, Gram stain, and positive results for catalase, coagulase and DNase tests. Atypical isolates were identified using API STAPH according to the manufacturer's instructions. The isolates were preserved in MicroBank (Diagnostic Pro-Lab) and stored at -20°C for further characterization.

2.2.3 Antibiotic susceptibility testing

The susceptibility testing of isolates to twenty antibiotics was carried out by the disk diffusion method according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2000, 2003) using antibiotic disks. The antibiotics (Mast Diagnostics, UK) included penicillin (10units), ampicillin (10μg), oxacillin (1μg), gentamicin (10μg), kanamycin (30μg), streptomycin (30μg), neomycin (30μg), erythromycin (15μg), clindamycin (2μg), tetracycline (30μg), minocycline (30μg), trimethoprim (2.5μg), trimethoprim/sulfamethoxazole (25μg), chloramphenicol (30μg), ciprofloxacin (5μg), fusidic acid (10μg), rifampicin (30μg), teicoplanin (30μg), vancomycin (30μg) and mupirocin (5 and 200μg). *S. aureus* ATCC 25923 was the control strain in every test run. Interpretative zone diameters for resistance to fusidic acid, neomycin and streptomycin which are not stated in the NCCLS guidelines were considered as follows; ≤ 18mm – fusidic acid (Skov *et al.*, 2001), ≤ 16mm - neomycin, and ≤ 14mm – streptomycin (Kim *et al.*, 2004). Growth to the edge of the 200μg mupirocin disk indicated high-level resistance, whereas growth within a 14mm zone of inhibition with the 5μg mupirocin disk detected low-level resistance (Udo *et al.*, 1999). In addition, susceptibility testing to a set of ten antibiotics was performed on isolates expressing resistance to oxacillin. The antibiotics included amikacin (30μg), azithromycin (15μg), quinipristin/dalfopristin (15μg), doxycycline (30μg), tobramycin (10μg), methicillin (5μg), cefoxitin (30μg), oleandomycin (15μg), fosfomycin (50μg) and linezolid (30μg). Interpretative zone diameters for resistance (not stated in the NCCLS guidelines) to the following antibiotics were considered as follows: oleandomycin ≤ 17mm and fosfomycin ≤14mm (Members of the SFM Antibiogram Committee, 2003).

The D-test for determining inducible resistance of clindamycin by erythromycin was performed. The erythromycin and clindamycin disks were placed 15-18mm apart. A truncated or blunted clindamycin zone of inhibition (D-shape) indicated inducible resistance. Constitutive resistance was recognized by a clindamycin zone diameter of ≤ 14mm (Fiebelkorn *et al.*, 2003).

2.2.3.1 Determination of reduced susceptibility to vancomycin and teicoplanin and low and high-level resistance to mupirocin

Isolates expressing phenotypic resistance to oxacillin were screened for intermediate resistance to vancomycin and teicoplanin. An inoculum equivalent to MacFarland 2 was swabbed onto Mueller Hinton agar (MHA) before placement of vancomycin and teicoplanin E-test strips (AB Biodisk, Solna, Sweden). The plates were incubated at 35°C and MIC values were noted after 24 hours. Isolates with MIC ≥ 2μg/ml (vancomycin and teicoplanin) were further screened on Brain Heart Infusion Agar (BHIA) using the E-test macrodilution method (Walsh *et al.*, 2001). The plates were incubated at 35°C and MIC values were noted after 48 hours of incubation. Bacterial isolates with MIC values of ≤4μg/ml and ≤8μg/ml were considered as sensitive to vancomycin and teicoplanin respectively. MICs of 8-16μg/ml (vancomycin) and 16μg/ml (teicoplanin) were regarded as intermediately resistance (NCCLS, 2000; Walsh *et al.*, 2001). Isolates exhibiting low and high-level resistance to mupirocin were further assessed by the E-test method as stated above.

2.2.3.2 Susceptibility of MRSA isolates to heavy metals ions and nucleic-acid binding compounds

Susceptibility to heavy metals (cadmium acetate, mercuric chloride) and nucleic-acid binding compounds (ethidium bromide and propamidine isethionate) was performed on a number of MRSA isolates. Disks were prepared in the laboratory with the indicated concentrations (10µl): cadmium acetate (50µg), propamidine isethionate (50µg), mercuric chloride, (109µg) and ethidium bromide (60µg). Interpretative zone diameters were considered according to Udo *et al.* (1999): ≤ 9mm (resistance), 10-12mm (intermediate) for cadmium acetate; ≤ 25mm - (resistance) for mercuric chloride; ≤ 10mm (resistance), 11-14mm (intermediate) for propamidine isethionate; and ≤ 9mm (resistance), 10-14mm (intermediate) for ethidium bromide.

2.2.4 DNA isolation on *S. aureus* isolates

DNA isolation was carried out according to the method of Udo *et al.*, (1999). Isolates were streaked on Brain Heart Infusion Agar (BHIA) and incubated at 37°C overnight. Using a sterile toothpick, six to seven colonies were added to an eppendorf tube containing 50µl lysostaphin (150µg/ml) and 10µl RNase (10mg/ml). It was then incubated at 37°C for twenty minutes. Thereafter, 50µl of diluted Proteinase K (7.5µl of 20mg/ml Proteinase K and 1492µl of sterile distilled water) and 150µl 0.1M Tris were dispensed into the eppendorf tube. It was then incubated in a 60°C water bath for ten minutes and thereafter at 95°C for ten minutes. Centrifugation was carried out at 13000 rpm for ten minutes and a volume of 5µl was used as template DNA for subsequent PCR reactions.

2.2.5 Molecular detection of the *nuc*, *mecA* and *mupA* genes by PCR

Isolates resistant to oxacillin using the disk diffusion technique were confirmed as *S. aureus* by the detection of the *nuc* gene using the polymerase chain reaction (PCR). In addition, the presence of the *mecA* gene was determined, which confirmed the isolates as MRSA. Primers (*nuc*-1) 5'- GCG ATT GAT GGT GAT ACG GTT - 3'; (*nuc*-2) 5'- AGC CAA GCC TTG ACG AAC TAA AGC - 3' and (*mecA*1) 5' - CTC AGG TAC TGC TAT CCA CC; (*mec-A2*) 5' - CAC TTG GTA TAT CTT CAC C - 3' which amplified a 280bp and 449bp segment of the *nuc* and *mecA* genes respectively were employed (Brakstad *et al.*, 1992; Bignardi *et al.*, 1996). The epidemic strain EMRSA-16 served as the positive control for the detection of both genes. In addition, low and high-level mupirocin resistant isolates detected by the disk diffusion method, were confirmed by their MIC values and the detection of the *mupA* gene using the primers (*mupA*-1) 5' - TGA CAA TAG AAA AGG ACA GG - 3' and (*mupA*-2) 5' - CTC TAA TTC AAC TGG TAA GCC - 3' which amplified a 190bp segment of the gene (Woodford *et al.*, 1998).

Each PCR reaction was made up of the following: 25µl of mastermix (Sigma), containing 1.5units of *Taq* DNA polymerase, 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin and 0.2mM dNTPs, 1µl (20pmol) of the forward and reverse primers and 5µl of template DNA. Sterile distilled water was added to make a final volume of 50µl. The thermocycler was programmed with the following parameters: predenaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute and final extension at 72°C for 5 minutes. The PCR conditions were utilized for the detection of the *nucA*, *mecA* and *mupA* genes (Scottish MRSA Reference Laboratory Standard Operating

Procedure, 2004). PCR products were detected by gel electrophoresis using 1.5% w/v agarose (Seakem, Whittaker USA). The agarose gel was run in 1X TBE (0.089M Tris, 0.089M Boric acid, 0.002M EDTA disodium) buffer (pH 8.3) for 2 hours at 80V. Thereafter, the gels were stained with ethidium bromide and visualized under UV light.

2.2.6 Data analysis

The resistance rate to each antibiotic was calculated as the number of intermediate and resistant isolates observed divided by the total number of isolates. Thus, the prevalence of MSSA and MRSA in Nigeria and South Africa was determined. Furthermore, resistance rates of MSSA and MRSA in each country, to the antibacterial agents were ascertained. A comparative analysis of the resistance rates of *S. aureus* isolates, from both countries was also determined, and the student t-test ($p<0.05$) was employed in establishing significant differences.

Antibiotyping of MSSA isolates (in each country) was analysed based on their susceptibility to twenty antibiotics. Isolates with similar resistance profiles were grouped in the same antibiotype and the prevalence in both countries was ascertained.

Antibiotyping of MRSA isolates was based on the susceptibility pattern of isolates to selected antibiotics, representing various classes of antimicrobial agents. They included penicillin (β-lactams), gentamicin (aminoglycosides), erythromycin (macrolide), chloramphenicol (phenicols), tetracycline (tetracyclines), trimethoprim (sulfonamides), rifampicin (ansamycins), ciprofloxacin (fluoroquinolones) and mupirocin.

Antibiotyping of MRSA isolates based on their susceptibility to aminoglycosides, tetracyclines, macrolide-lincosamide group of antibiotics, heavy metals and nucleic acid binding compounds was also determined.

2.3 RESULTS

2.3.1 Recovery and distribution of *S. aureus* isolates from health institutions in Nigeria and South Africa

The distribution of isolates obtained from the various health institutions in both countries, in relation to clinical samples is presented in Table 2.1. Of the 358 isolates identified as *S. aureus* in the various clinical laboratories in Southwestern Nigeria, 200 isolates (56%) were confirmed as *S. aureus* based on growth and fermentation on mannitol salt agar, Gram stain, and positive results for catalase, coagulase and DNase tests. More than one-third of the total number of isolates (from Nigeria) was recovered from wound samples, 42 (21%) from blood cultures, 17 (8.5%) from samples obtained from ocular-related infections, 16 (8%) from urine cultures, and 11 (5.5%) from samples obtained from otitis media. Clinical information was unavailable for 30 *S. aureus* isolates.

A total of 233 isolates were obtained from a study on antibiotic resistance patterns of bacterial isolates from various health institutions in KwaZulu-Natal province, South Africa, in partnership with the Faculty of Pharmacy, University of KwaZulu-Natal. Only six isolates (2.6%) were misidentified. Wound samples were the main source of isolates recovered in KZN province, South Africa (83.7%). Overall, a total of 427 *S. aureus* non-duplicate isolates were obtained from various clinical samples in Nigeria and South Africa.

Table 2.1: Distribution of *S. aureus* isolates in relation to health institutions and clinical samples in Nigeria and South Africa

		Clinical samples								
Country	Health Institutions (Location)	1	2	3	4	5	6	7	8	Total
Nigeria	Hospital A, Ile-Ife	21	27	7	2	1	1	3	10	72
	Hospital B, Ile-Ife	5	0	0	0	0	0	0	0	5
	Hospital C, Ile-Ife	6	0	0	0	0	0	0	0	6
	Hospital D, Ilesa	6	0	3	0	2	0	1	0	12
	Hospital E, Ipetumodu	3	0	0	0	0	0	0	0	3
	Hospital F, Ibadan	36	15	6	0	8	16	1	20	102
	Total	77	42	16	2	11	17	5	30	200
South Africa	Health Institutions (Location)									
	Hospital A, Durban	6	3	1	1	0	0	0	0	11
	Hospital B, Durban	22	2	1	0	0	0	0	0	25
	Hospital C, Durban	2	0	0	0	1	0	2	1	6
	Hospital D, Durban	16	0	0	0	0	0	0	0	16
	Hospital E, Pietermaritzburg	6	0	0	0	0	0	0	0	6
	Hospital F, Pietermaritzburg	16	1	0	1	2	0	1	0	21
	Hospital G, Newcastle	0	0	0	3	0	0	0	0	3
	Hospital H, Greytown	7	0	0	0	1	0	1	0	9
	Hospital I, Kokstad	13	0	0	0	1	1	0	0	15
	Hospital J, Eshowe	4	0	0	0	0	0	0	0	4
	Hospital K, Port Shepstone	8	0	0	1	1	0	0	0	10
	Hospital L, Scottburgh	0	0	1	2	1	0	0	2	6
	Hospital M, Ubombo	2	0	0	0	0	0	0	0	2
	Hospital N, Empangeni	88	0	0	1	0	0	1	3	93
	Total	190	6	3	9	7	1	5	6	227

KEY

Samples from 1- Wound culture; 2 - Blood; 3 - Urine; 4 - Sputum; 5 - otitis media; 6 - eye-related infection; 7 - Other clinical samples (HVS, vaginal swab, endocervical swab, pericardial fluid, endotracheal aspirate); 8 - no clinical information.

2.3.2 Antibiotic susceptibility of *S. aureus* isolates from Nigeria

The antibiotic susceptibility of 200 *S. aureus* isolates obtained from hospitals in Southwestern Nigeria is described in Table 2.2. All the isolates (MSSA and MRSA) were susceptible to teicoplanin, vancomycin, fusidic acid and rifampicin, while less than 3% were resistant to oxacillin, erythromycin, clindamycin, neomycin, minocycline and mupirocin. Most of the MSSA *S. aureus* isolates were resistant to penicillin and ampicillin (90%) followed by tetracycline (51.3%) and trimethoprim (47.2%), while less than 10% of MSSA isolates were resistant to gentamicin, kanamycin, chloramphenicol and ciprofloxacin.

Only three isolates from SouthWestern Nigeria were confirmed as MRSA. The resistance rates are presented in Tables 2.2 and 2.4. Two isolates demonstrated intermediate resistance to oxacillin (zone sizes 12mm) but were confirmed as MRSA by the detection of the *mecA* gene. The MRSA isolates were resistant to tetracycline but susceptible to gentamicin, kanamycin, neomycin, chloramphenicol and mupirocin. Two of the three MRSA isolates were susceptible to streptomycin, trimethoprim, minocycline and ciprofloxacin while one isolate was susceptible to erythromycin and clindamycin. In addition, two MRSA were susceptible to amikacin, tobramycin, fosfomycin, quinipristin/dalfopristin, linezolid, doxycycline, methicillin, cefotixin, ethidium bromide, and propamidine isethionate, but resistant to mercuric chloride and cadmium acetate (Table 2.4).

Table 2.2: Antibiotic susceptibility of *S. aureus* isolates (MSSA and MRSA) from Nigeria

	Nigeria			Nigeria				
	MSSA n=197			MRSA n=3				Total n=200
Antibiotic	Number of isolates that were:		Resistance rate (%)	Number of isolates that were:			Resistance rate (%)	No/Resistance rate (%)
	S	R		S	I	R		
Penicillin	19	178	90.4	0	0	3	100	181 (90.5)
Ampicillin	19	178	90.4	0	0	3	100	181 (90.5)
Oxacillin	197	0	0	0	2	1	100	3 (1.5)*
Erythromycin	194	3	1.5**	1	0	2	66.7	5 (2.5)*
Clindamycin	194	3	1.5**	1	0	2	66.7	5 (2.5)*
Gentamicin	185	12	6.1	3	0	0	0	12 (6.0)*
Streptomycin	175	22	11.2**	2	0	1	33.3	23 (11.5)
Kanamycin	183	14	7.1	3	0	0	0	14 (7.0)*
Neomycin	192	5	2.5**	3	0	0	0	5 (2.5)*
Trimethoprim	104	93	47.2**	2	0	1	33.3	94 (47)*
Trimethoprim/ Sulphamethoxazole	104	93	47.2**	2	0	1	33.3	94 (47)*
Tetracycline	96	101	51.3**	0	0	3	100	104 (52)*
Minocycline	194	3	1.5	2	0	1	33.3	4 (2.0)*
Teicoplanin	197	0	0	3	0	0	0	0
Vancomycin	197	0	0	3	0	0	0	0
Chloramphenicol	179	18	9.1**	3	0	0	0	18 (9.0)
Ciprofloxacin	184	13	6.6**	2	0	1	33.3	14 (7.0)
Fusidic acid	197	0	0	3	0	0	0	0
Rifampicin	197	0	0	3	0	0	0	0*
Mupirocin (5µg)	196	1	0.5	3	0	0	0	1 (0.5)*
Mupirocin (200µg)	196	1	0.5	3	0	0	0	1 (0.5)

KEY

S – sensitive; I – Intermediate; R – Resistant

* - Significant difference ($p < 0.05$) (all *S. aureus* isolates from Nigeria)

** - Significant difference ($p < 0.05$) (MSSA isolates from Nigeria)

2.3.3 Antibiotic susceptibility of *S. aureus* isolates from South Africa

The susceptibility of 227 *S. aureus* isolates from the KwaZulu-Natal province of South Africa to various antibiotics is illustrated in Table 2.3. All the isolates (MSSA and MRSA) were susceptible to vancomycin, teicoplanin and fusidic acid and the proportion of isolates resistant to streptomycin, neomycin, chloramphenicol, ciprofloxacin and mupirocin was less than 10%. About 30% of isolates were resistant to erythromycin, clindamycin, trimethoprim and tetracycline. Resistance rates for rifampicin (20.3%), minocycline (24.2%), gentamicin and kanamycin (28.6%) were also noted. Penicillin and ampicillin were the least effective antibacterial agents. In addition to full susceptibility to vancomycin, teicoplanin and fusidic acid, MSSA isolates from South Africa were susceptible to oxacillin, streptomycin, neomycin and minocycline. Furthermore, less than 1% of MSSA were resistant to chloramphenicol, ciprofloxacin, rifampicin and mupirocin, while resistance to gentamicin, kanamycin and tetracycline was below 10%. Resistance rates for trimethoprim, clindamycin and erythromycin was 10.8% and 11.4%, respectively.

The prevalence of MRSA (confirmed by the detection of the *mecA* gene) was 27% and the susceptibility of MRSA to various antibiotics, heavy metal ions and nucleic-acid binding compounds are presented in Tables 2.3 and 2.4. Among the aminoglycosides, more than 90% of MRSA were resistant to gentamicin, kanamycin and tobramycin. Amikacin was the most active aminoglycoside (16.4%), followed by streptomycin and neomycin (31.1%). Only 9.8% of MRSA isolates were susceptible to tetracycline and minocycline. Resistance levels of MRSA to macrolide-lincosamide antibiotics were high - azithromycin (80.3%), erythromycin (82%), clindamycin (82%) and oleandomycin

(82%). In addition, resistance to trimethoprim and trimethopim/sulfamethoxazole (sulphonamides) was above 80%. Other resistance rates include rifampicin (73.8%), doxycycline (36%), ciprofloxacin (18%) and chloramphenicol (16.4%). Resistance rates for heavy metal ions - mercuric chloride and cadmium acetate - was 46% and 82% respectively, while about 70% of MRSA were susceptible to propamidine isethionate and ethidium bromide.

The ability of the disk diffusion method using oxacillin, methicillin and cefoxitin antibiotic discs to accurately identify MRSA was compared with PCR detection of the *mecA* gene, which is considered the 'gold standard'. Of the 64 MRSA isolates studied, (confirmed by detection of the *mecA* gene), the oxacillin, methicillin and cefoxitin disk diffusion methods accurately detected 62 isolates as MRSA (Tables 2.2, 2.3 and 2.4). Only two MRSA from Nigeria were noted as intermediately resistant to oxacillin (12mm) but sensitive to cefoxitin (20mm; 28mm) and methicillin (15mm; 24mm).

As stated above, all the *S. aureus* isolates from both countries were susceptible to vancomycin and teicoplanin using the disk diffusion technique. All the MRSA studied were further screened for reduced susceptibility to glycopeptides using the E-test macrodilution method (Walsh *et al.*, 2001) on Mueller Hinton Agar and Brain Heart Infusion Agar. The MRSA isolates were susceptible to vancomycin and teicoplanin based on the E-test method. In addition, MRSA from both countries were susceptible to quinipristin/dalfopristin, fosfomycin and linezolid.

Table 2.3: Antibiotic susceptibility of *S. aureus* isolates (MSSA and MRSA) from South Africa

	South Africa			South Africa			
	MSSA n=166			MRSA n=61			Total n=227
Antibiotic	Number of isolates that were: S	R	Resistance rate (%)	Number of isolates that were: S	R	Resistance rate (%)	No/Resistance rate (%)
Penicillin	19	147	88.6	0	61	100	208 (91.6)
Ampicillin	19	147	88.6	0	61	100	208 (91.6)
Oxacillin	166	0	0	0	61	100	61 (26.9)*
Erythromycin	147	19	11.4**	11	50	82	69 (30.4)*
Clindamycin	148	18	10.8**	11	50	82	68 (30.0)*
Gentamicin	160	6	3.6	2	59	96.7	65 (28.6)*
Streptomycin	166	0	0**	42	19	31.1	19 (8.4)
Kanamycin	160	6	3.6	2	59	96.7	65 (28.6)*
Neomycin	166	0	0**	42	19	31.1	19 (8.4)*
Trimethoprim	148	18	10.8**	9	52	85.2	70 (30.8)*
Trimethoprim/ Sulphamethoxazole	148	18	10.8**	9	52	85.2	70 (30.8)*
Tetracycline	153	13	7.8**	6	55	90.2	68 (30.0)*
Minocycline	166	0	0	6	55	90.2	55 (24.2)*
Teicoplanin	166	0	0	61	0	0	0
Vancomycin	166	0	0	61	0	0	0
Chloramphenicol	165	1	0.6**	51	10	16.4	11 (4.8)
Ciprofloxacin	165	1	0.6**	50	11	18.0	12 (5.3)
Fusidic acid	166	0	0	61	0	0	0
Rifampicin	165	1	0.6	16	45	73.8	46 (20.3)*
Mupirocin (5µg)	165	1	0.6	46	15	24.6	16 (7.0)*
Mupirocin (200µg)	165	1	0.6	60	1	1.6	2 (0.9)

KEY
S – sensitive; R – Resistant
* - Significant difference ($p < 0.05$) (all *S. aureus* isolates from South Africa)
** - Significant difference ($p < 0.05$) (MSSA isolates from South Africa)

Table 2.4: Antibiotic susceptibility of MRSA from Nigeria and South Africa

	Nigeria			South Africa			
	MRSA (n=2*)			MRSA (n=61)			
Antibiotic	Number of isolates that were:		Resistance rate (%)	Number of isolates that were:			Resistance rate (%)
	S	R		S	I	R	
Amikacin	2	0	0	51	0	10	16.4
Tobramycin	2	0	0	3	10	48	95.1
Oleandomycin	1	1	50	11	5	45	82.0
Azithromycin	1	1	50	12	0	49	80.3
Fosfomycin	2	0	0	61	0	0	0
Quinipristin/ Dalfopristin	2	0	0	61	0	0	0
Linezolid	2	0	0	61	0	0	0
Doxycycline	2	0	0	39	12	10	36.1
Methicillin	2	0	0	0	0	61	100
Cefoxitin	2	1	33.3	0	0	61	100
Ethidium bromide	2	0	0	42	3	16	31.1
Mercuric chloride	0	2	100	33	0	28	45.9
Cadmium acetate	0	2	100	11	10	40	82
Propamidine Isethionate	2	0	0	43	1	17	29.5

KEY

* Apart from cefoxitin screening test, one MRSA isolate from Nigeria was not included in this analysis

2.3.4 Comparative analysis of resistance rates of *S. aureus* isolates from Nigeria and South Africa

Significant differences (student t-test: $p < 0.05$) were observed between the resistance rates of *S. aureus* isolates from Nigeria and South Africa (Tables 2.2 and 2.3). *S. aureus* resistance to erythromycin, clindamycin, rifampicin, minocycline, gentamicin, neomycin, kanamycin, mupirocin and oxacillin was significantly higher in South Africa compared with Nigeria. This could be ascribed to the marked difference in the prevalence of MRSA in both countries. However, resistance rates of *S. aureus* to trimethoprim, trimethoprim-sulfamethoxazole and tetracycline were significantly higher in Nigeria than in South Africa.

2.3.5 Comparative analysis of resistance profiles of MSSA from Nigeria and South Africa

The in-vitro activities of 20 antimicrobial agents against MSSA in Nigeria and South Africa were analysed (Tables 2.2 and 2.3). As expected, resistance to penicillin and ampicillin were high, exceeding more than 80%, in both countries. Statistical analysis indicated that MSSA resistance to streptomycin, neomycin, trimethoprim, trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, and ciprofloxacin was significantly higher in Nigeria while erythromycin and clindamycin resistance was significantly higher in South Africa.

A total of 15 and 28 antibiotypes were identified among the MSSA isolates in South Africa and Nigeria respectively (Table 2.5). Overall, 4.6% (Nigeria) and 10.8% (South Africa) of MSSA isolates were susceptible to all antibiotics tested. A total of ten MSSA from Nigeria were susceptible to penicillin but resistant to various antibiotics, while one MSSA with that profile was noted in South Africa. Resistance to penicillin was the dominant antibiotype in South Africa whereas in Nigeria, resistance to penicillin, resistance to penicillin, tetracycline and trimethoprim, resistance to penicillin and tetracycline, and resistance to penicillin and trimethoprim accounted for 25.4%, 20.8%, 14.2% and 10.7% of isolates, respectively. Notable differences in the prevalence of MSSA with the following resistance profiles were observed: resistance to penicillin and tetracycline (Nigeria 14.2%; South Africa 1.2%), resistance to penicillin and trimethoprim (10.7%; 1.8%), and resistance to penicillin, tetracycline and trimethoprim (20.8%; 1.8%). Furthermore, the proportion of multi-drug resistant (defined as resistance to penicillin along with at least three classes of antibiotics) MSSA was 13.7% (27 of 197 isolates) and 3.6% (6 of 166 isolates) in Nigeria and South Africa respectively.

Table 2.5: Antibiotypes of MSSA from Nigeria and South Africa

		Nigeria		South Africa
		MSSA (no=197)		MSSA (no=166)
Group	Antibiogram	No (%)	Antibiogram	No (%)
1	Pe^R	50 (25.4)	Pe^R	114 (68.7)
2	$Pe^RTet^RTm^R$	41 (20.8)	Susceptible to all antibiotics	18 (10.8)
3	Pe^RTet^R $Pe^RTet^RMn^R$	28 (14.2) 1 (0.5)	Pe^REry^R	9 (5.4)
4	Pe^RTm^R	21 (10.7)	$Pe^RTm^RGn^RKa^R$	5 (3.0)
5	Susceptible to all antibiotics	9 (4.6)	$Pe^RTet^RTm^REry^R$	4 (2.4)
6	$Pe^RTet^RTm^RCip^RStr^R$ $Pe^RTet^RTm^RCip^RGn^RKan^R$ $Pe^RTet^RTm^RCip^RStr^RGn^RKan^R$	4 (2.0) 2 (1.0) 1 (0.5)	Pe^RTm^R	3 (1.8)
7	$Pe^RTet^RTm^RChl^R$	6 (3.0)	$Pe^RTet^RTm^R$	3 (1.8)
8	Tet^R Tet^RMn^R	3 (1.5) 1 (0.5)	Pe^RTet^R	2 (1.2)
9	$Pe^RTm^RChl^R$	3 (1.5)	$Pe^RTet^REry^R$	2 (1.2)
10	$Pe^RTm^RStr^R$ $Pe^RTm^RGn^RKan^R$ $Pe^RTm^RGn^RStr^RNeo^RKan^R$	1 (0.5) 1 (0.5) 1 (0.5)	$Pe^RTm^REry^R$	1 (0.6)
11	$Pe^RTet^RGn^RTm^RStr^RKan^R$ $Pe^RTet^RGn^RTm^RStr^RNeo^RKan^R$	1 (0.5) 2 (1.0)	Pe^RRf^R	1 (0.6)
12	Tm^R	2 (1.0)	$Pe^RTet^REry^RCip^R$	1 (0.6)
13	$Pe^RTet^RChl^RStr^R$	2 (1.0)	$Pe^RChl^REry^R$	1 (0.6)
14	$Pe^RTet^RKan^R$ $Pe^RTet^RGn^RKan^RMn^R$	1 (0.5) 1 (0.5)	$Tet^RTm^RMu^R$	1 (0.6)
15	$Pe^RTet^RTm^RStr^R$	2 (1.0)	$Pe^RTm^REry^RGn^RKa^R$	1 (0.6)
16	Chl^R	1 (0.5)		
17	Tet^RMu^R	1 (0.5)		
18	Chl^RStr^R	1 (0.5)		
19	$Gn^RCip^RKan^RStr^R$	1 (0.5)		
20	$Pe^RChl^RStr^R$	1 (0.5)		
21	$Pe^RTet^REry^R$	1 (0.5)		
22	$Pe^RTm^RCip^R$	1 (0.5)		
23	$Pe^RTet^RTm^RChl^RStr^R$	1 (0.5)		
24	$Pe^RTm^RChl^RGn^RStr^RKan^R$	1 (0.5)		
25	$Pe^RChl^RCip^RStr^R$	1 (0.5)		
26	$Pe^RTm^RCip^RNeo^RKan^RStr^R$	1 (0.5)		
27	$Pe^RTet^RChl^RCip^REry^R$	1 (0.5)		
28	$Pe^RTm^RTet^RCip^RGn^REry^RStr^RNeo^RKan^R$	1 (0.5)		

KEY

Chl – Chloramphenicol; Cip – Ciprofloxacin; Ery – Erythromycin; Gn – Gentamicin; Ka – Kanamycin; Mu – Mupirocin; Pe – Penicillin; Rf – Rifampicin; Str – Streptomycin; Tet – Tetracycline; Tm – Trimethoprim.

2.3.6 Macrolide-lincosamide resistance in *S. aureus* isolates from Nigeria and South Africa

The phenotypic resistance profile of 427 isolates for the macrolide-lincosamide group of antibiotics is illustrated in Table 2.6. A total of 353 of the 427 isolates studied were susceptible to erythromycin. Resistance to erythromycin and inducible resistance to clindamycin were detected in 2.5% and 29.5% of *S. aureus* isolates from Nigeria and South Africa, respectively. Of the 19 erythromycin-resistant MSSA from South Africa, only one isolate exhibited the constitutive MLS resistance phenotype, which was absent in erythromycin-resistant MSSA/ MRSA from Nigeria, and MRSA from South Africa.

Table 2.6: Phenotypic resistance patterns of *S. aureus* isolates for erythromycin, and clindamycin in Nigeria and South Africa

	Nigeria n=200		South Africa n=227	
	No. (%) of:			
Resistance pattern	MSSA	MRSA	MSSA	MRSA
Erythromycin resistant, clindamycin susceptible	0	0	1 (0.6)	0
Erythromycin resistant, clindamycin resistant (constitutive)	0	0	1 (0.6)	0
Erythromycin resistant, clindamycin resistant (inducible)	3 (1.5)	2 (66.7)	17 (10.2)	50 (82)
Erythromycin susceptible clindamycin resistant	0	0	0	0
Erythromycin susceptible clindamycin susceptible	194 (98.5)	1 (33.3)	147 (88.6)	11 (18)
Total	197	3	166	61

2.3.7 Recovery and distribution of MRSA from various health institutions in Nigeria and South Africa

The distribution of MRSA in relation to clinical samples and health institutions is described in Table 2.7. At least one MRSA was recovered from clinical samples in 13 of the 14 health facilities in the KwaZulu-Natal province of South Africa. Only three MRSA isolates were obtained from hospitals in the Southwestern region in Nigeria. A total of 48 MRSA isolates (78%) were recovered from wound samples, six (9.8%) from sputum, two from otitis media (3.3%), and one isolate each from blood, urine, eye-related infections and endotracheal aspirate from hospitals in KZN province of South Africa. No clinical information was available for one MRSA isolate.

Table 2.7: Distribution of MRSA in relation to health institutions and clinical samples from Nigeria and South Africa

		Clinical samples								
Country	Health institutions and location (total number of *S. aureus* isolates)	1	2	3	4	5	6	7	8	Total
Nigeria	Hospital A, Ile-Ife (72)	1								1*
	Hospital C, Ile-Ife (6)	1								1*
	Hospital F, Ibadan (102)	1								1
	Total	3								3
South Africa	Health institutions and location (total number of *S. aureus* isolates)									
	Hospital A, Durban (11)	4	1	1	1					7
	Hospital B, Durban (25)	8								8
	Hospital C, Durban (6)	2				1		1		4
	Hospital D, Durban (16)	1								1
	Hospital E, Pietermaritzburg (6)	2								2
	Hospital F, Pietermaritzburg (21)	4								4
	Hospital G, Madadeni (3)				2					2
	Hospital H, GreyTown (9)	1								1
	Hospital I, Kokstad (15)	2					1			3
	Hospital J, Eshowe (4)	4								4
	Hospital K, Port Shepstone (10)	3			1					4
	Hospital L, Scottburgh (6)				1	1			1	3
	Hospital N, Empangeni (93)	17			1					18
	Total	48	1	1	6	2	1	1	1	61

KEY
Samples from 1 - Wound culture; 2 - Blood; 3 - Urine; 4 - Sputum; 5 - otitis media; 6 - eye-related infection; 7 - Endotracheal aspirate; 8 - no clinical information
* Isolates exhibiting intermediate resistance to oxacillin, confirmed as MRSA by detection of the *mecA* gene.

2.3.8 Antibiotyping of MRSA from Nigeria and South Africa

The antibiotypes of MRSA based on their resistance pattern to antibiotics representing various classes of antibacterial agents are illustrated in Table 2.8. Multi-drug resistance was defined as resistance to at least four classes of antibiotics. MRSA from South Africa were categorized into 12 antibiotypes, and type VI accounted for about 40% of the total number of isolates. About 87% of MRSA were resistant to at least four classes of antibiotics. Resistance to six classes of antibiotics accounted for more than 40% and four isolates were resistant to eight classes of antibiotics.

The MRSA from Nigeria were grouped into three antibiotypes and only two of the three isolates were multiresistant.

Table 2.8: Antibiotyping of MRSA isolates from Nigeria and South Africa

	South Africa (n=61)		
Type	Antibiogram	No (%)	Resistant to:
I	PEN, GN, ERY, CHL, TET, TS, CIP, MUP5	4 (6.6)	8 antibiotics
II	PEN, GN, ERY, TET, TS, CIP, MUP5	6 (9.8)	7
III	PEN, GN, ERY, CHL, TET, TS, RF	4 (6.6)	7
IV	PEN, GN, ERY, TET, TS, RF, MUP5	3 (4.9)	7
V	PEN, GN, ERY, TET, TS, RF, CIP	1 (1.6)	7
VI	PEN, GN, ERY, TET, TS, RF	25 (40.9)	6
VII	PEN, GN, CHL, TET, TS, RF	2 (3.3)	6
VIII	PEN, GN, TET, TS, RF, MUP5	2 (3.3)	6
IX	PEN, GN, TET, TS, RF	5 (8.2)	5
X	PEN, GN, ERY, TET, RF	1 (1.6)	5
XI	PEN, GN, ERY	6 (9.8)	3
XII	PEN, TET, RF	2 (3.3)	3
	Subtotal	61 (100)	
	Nigeria (n=3)		
XIII	PEN, ERY, TET, CIP	1 (33.3)	4
XIV	PEN, ERY, TET, TS	1 (33.3)	4
XV	PEN, TET	1 (33.3)	2
	Subtotal	3 (100)	
	Total	64	

KEY

PEN – Penicillin (β-lactams)
GN – Gentamicin (aminoglycoside)
ERY – Erythromycin (macrolide)
CHL – Chloramphenicol (phenicols)
TET – Tetracycline (tetracyclines)
TS – Trimethoprim (sulphonamides)
RF – Rifamipicin (ansamycins)
CIP – Ciprofloxacin (fluoroquinolones)
MUP5 – Mupirocin (5μg)

2.3.9 Distribution of MRSA antibiotypes among hospitals in South Africa

The distribution of MRSA antibiotypes in health facilities in KwaZulu-Natal province, South Africa is described in Table 2.9. Multiresistant MRSA (resistance to at least four classes of antibiotics) was identified in 13 of the 14 health institutions in this province. The MRSA isolates with the predominant antibiotype VI was detected in 12 of the 14 hospitals studied. In addition, isolates grouped in antibiotype I which exhibited resistance to eight classes of antibiotics were identified in Hospitals A (Durban), C (Durban), E (Pietermaritzburg) and N (Empangeni), while MRSA in antibiotype II (resistance to seven classes of antibiotics) was noted in Hospitals A (Durban), B (Durban) and N (Empangeni).

Table 2.9: Distribution of MRSA antibiotypes in health institutions of the KwaZulu-Natal province in South Africa

Hospitals (Location) Number of isolates	Antibiotyping (number of isolates) Ant = antibiotype
Durban	
Hospital A (7)	ant I (1); ant II (1); ant VI (1); ant VIII (2); ant IX (1); ant XI (1);
Hospital B (8)	ant II (4); ant III (1); ant IV (1); ant VI (1); ant X (1);
Hospital C (4)	ant I (1); ant IV (1); ant VI (1); ant XI (1);
Hospital D (1)	ant VI (1)
Pietermaritzburg	
Hospital E (2)	ant I (1) ant XI (1);
Hospital F (4)	ant III (1); ant VI (2); ant XI (1)
Newcastle	
Hospital G (2)	ant VI (1); ant IX (1)
GreyTown	
Hospital H (1)	ant VI (1)
Kokstad	
Hospital I (3)	ant VI (2); ant VII (1)
Eshowe	
Hospital J (4)	ant IV (1); ant VI (1); ant XI (1); ant XII (1)
Port Shepstone	
Hospital K (4)	ant III (2); ant VI (2)
Scottburgh	
Hospital L (3)	ant VI (1); ant IX (1); ant XI (1)
Empangeni	
Hospital N (18)	ant I (1); ant II (1); ant V (1); ant VII (1); ant VI (11); ant IX (2); ant XII (1)

2.3.10 Susceptibility profiles of MRSA from Nigeria and South Africa with regards to the aminoglycoside, macrolide-lincosamide-streptogramin B, tetracycline group of antibiotics, heavy metals and nucleic acid binding compounds

The resistance patterns of MRSA in relation to aminoglycosides, macrolide-lincosamide-streptogramin B (MLS_B), tetracycline group of antibiotics, heavy metal ions and nucleic-acid binding compounds is illustrated in Table 2.10. MRSA isolates resistant to gentamicin, kanamycin, and tobramycin but susceptible to amikacin, streptomycin and neomycin (aminoglycosides) was predominant (60%) in South Africa. This phenotype was followed by MRSA resistant to the six antibiotics in this group (16.4%). About 36% and 80% of MRSA were resistant to the tetracyclines and macrolide-lincosamide group of antibiotics respectively. In addition, 14 of MRSA isolates were resistant to heavy metal/nucleic-binding compounds. Overall, six MRSA isolates from Durban, Pietermartizburg and Empangeni were resistant to all the classes of antibacterial agents.

Of the two MRSA isolates from Nigeria, one isolate was resistant to the macrolide-lincosamide group of antibiotics. All the MRSA isolates from the two countries were susceptible to quinipristin/dalfopristin.

Table 2.10: Antibiotyping of MRSA for the aminoglycoside, macrolide-lincosamide, tetracycline group of antibiotics, heavy metals and nucleic acid binding compounds

Antibiogram	South Africa	Antibiogram	Nigeria
Classes of antibiotics	MRSA (N=61)	Classes of antibiotics	MRSA (N=2)*
Aminoglycosides**	N (%)	Aminoglycosides	N (%)
$Gn^{R}Kn^{R}Am^{S}Str^{S}Ne^{S}Tob^{R}$	37 (60.6)	Susceptible to all	1 (50)
$Gn^{R}Kn^{R}Am^{R}Str^{R}Ne^{R}Tob^{R}$	10 (16.4)	$Gn^{S}Kn^{S}Am^{S}$**Str^{R}**$Ne^{S}Tob^{S}$	1 (50)
$Gn^{R}Kn^{R}Am^{S}Str^{R}Ne^{R}Tob^{R}$	7 (11.5)		
$Gn^{R}Kn^{R}Am^{S}Str^{S}Ne^{R}Tob^{R}$	2 (3.3)		
Susceptible to all	2 (3.3)		
$Gn^{R}Kn^{R}Am^{S}Str^{R}Ne^{S}Tob^{R}$	2 (3.3)		
$Gn^{R}Kn^{R}Am^{S}Str^{S}Ne^{S}Tob^{S}$	1 (1.6)		
Macrolide-Lincosamide***		Macrolide-Lincosamide**	
$Ery^{R}Ol^{R}Azm^{R}Cc^{Ri}$	49 (80.3)	**$Ery^{R}Ol^{R}Azm^{R}Cc^{Ri}$**	1 (50)
Susceptible to all	11 (18.0)	Susceptible to all	1 (50)
$Ery^{R}Ol^{R}Azm^{S}Cc^{Ri}$	1 (1.6)		
Tetracyclines		Tetracyclines	
$Tet^{R}Mn^{R}Do^{S}$	33 (54.1)	**$Tet^{R}Mn^{R}Do^{S}$**	1 (50)
$Tet^{R}Mn^{R}Do^{R}$	22 (36.1)	**$Tet^{R}Mn^{S}Do^{S}$**	1 (50)
Susceptible to all	6 (9.8)		
Heavy metals/ nucleic acid binding agents		Heavy metals/nucleic acid binding agents	
$Eb^{S}Pi^{S}Hg^{S}$**Cd^{R}**	21 (34.4)	$Eb^{S}Pi^{S}$**$Hg^{R}Cd^{R}$**	2 (100)
$Eb^{R}Pi^{R}Hg^{R}Cd^{R}$	14 (22.9)		
$Eb^{S}Pi^{S}$**$Hg^{R}Cd^{R}$**	9 (14.8)		
Susceptible to all	6 (9.8)		
$Eb^{S}Pi^{S}$**Hg^{R}**Cd^{S}	5 (8.2)		
$Eb^{R}Pi^{R}$ Hg^{S}**Cd^{R}**	3 (4.9)		
Eb^{R} $Pi^{S}Hg^{S}$**Cd^{R}**	2 (3.3)		
Eb^{S}**Pi^{R}**Hg^{S}**Cd^{R}**	1 (1.6)		

KEY

*One MRSA isolate from Nigeria was not included in this analysis

** Aminoglycoside modifying enzyme analysis was not conducted

*** All were sensitive to quiniprisitin/dalfoprisitin

Aminoglycosides: Am – Amikacin; Gn – Gentamicin; Ka – Kanamycin; Ne – Neomycin; Tob – Tobramycin; Str – Streptomycin

Macrolide-Lincosamide – Azm – Azithromycin; Cc – Clindamycin; Ery – Erythromycin; Ol – Oleandomycin

Tetracyclines – Do – Doxycycline; Mn – Minocycline; Tet – Tetracycline

Heavy metals – Cd – Cadmium acetate; Hg – Mercuric chloride

Nucleic-acid binding compounds - Eb – Ethidium bromide; Pi – Propamidine isethionate

The superscript S/R represent susceptibility/resistance.

Cc^{Ri} – inducible resistance to clindamycin

2.3.12 Mupirocin resistance in *S. aureus* isolates from Nigeria and South Africa

Susceptibility to mupirocin (5μg and 200μg) by the disk diffusion method was performed. A total of 17 isolates exhibited resistance to mupirocin, of which 14 MRSA isolates (from South Africa) exhibited low-level resistance. This resistance phenotype was confirmed by E-test, with MICs values ranging from 8 to 24μg/ml. High-level resistance to mupirocin was confirmed by E-test (>1024μg/ml) and detection of the *mupA* gene by PCR. High-level resistance was detected in two (MRSA and MSSA) isolates from South Africa, and one MSSA from Nigeria. Low-level resistant isolates were *mupA* negative (Appendix 2).

2.4 DISCUSSION

Staphylococcus aureus is one of the most common causes of both endemic and epidemic infections acquired in hospitals, which result in substantial morbidity and mortality. In Africa, it is one of the most frequently encountered microorganisms, obtained from various clinical samples, in the microbiology laboratory (Ako-Nai *et al.*, 1995; Aseffa and Yohannes, 1996; Omari *et al.*, 1997; Mulholland *et al.*, 1999; Gebreselassie, 2002; Odusanya, 2002; Ben Jemaa *et al.*, 2004). In this study, 38.5% and 83.7% of *S. aureus* isolates from Nigeria and South Africa were obtained from wound samples (Table 2.1). Studies in African countries including Tanzania (Eriksen *et al.*, 2003), Nigeria (Kolawole and Shittu, 1995; Oni *et al.*, 1997; Emele *et al.*, 1999; Shittu *et al.*, 2003a; Wariso and Nwachukwu, 2003), Kenya (Andhoga *et al.*, 2002), Sudan (Mahdi *et al.*, 2000) and Ethiopia (Kotisso and Aseffa, 1998) have indicated that *S. aureus* is the most frequently isolated pathogen implicated in various wound infections.

Bacterial infections of the skin and underlying soft tissues are one of the most common presentations in patients visiting emergency room clinics in hospitals and office-based practices (Dykhuizen *et al.*, 1994; Tice, 1995; Nathwani *et al.*, 1998). Wound infection is a major concern among health care practitioners, not only in terms of increased trauma to the patient, but also in view of its burden on financial resources and the increasing requirements for cost-effective management within the healthcare system (Bowler, 2001). Nasal carriage of *S. aureus* has been identified as a key factor in the epidemiology and pathogenesis of *S. aureus* wound infection. Three sets of observations have indicated that carriers have higher rates of infection than non-carriers (Weinstein, 1959; Kluytmans *et al.*, 1995; Kalmeijer *et al.*, 2000); the strain causing infection is

usually the carriage strain in a given individual (Wenzel and Perl, 1995; Toshkova *et al.*, 2001); and eradication of carriage reduces nosocomial infection (Kluytmans, 1998; Wilcox *et al.*, 2003). The postulated sequence of events leading to infection is initiated with *S. aureus* nasal carriage, which is then disseminated via the hands to other body sites where infection can occur through breaks in the dermal surfaces. Another important mode of transmission is via transiently colonised hands of health care workers who acquire the organism after close contact with colonised patients or contaminated equipment (Peacock, 1980; Toshkova *et al.*, 2001).

The resistance rates of *S. aureus* isolates from Nigeria and South Africa (Tables 2.2 and 2.3) were compared with data from an international multi-centre study, in which 21 laboratories in about 18 countries participated. The frequency of antibiotic resistance was ranked into four groups; low-level resistance (0-10%), concern (11-40%); major concern (41-80%) and antibiotic rarely useful (>80%) (Zinn *et al.*, 2004). As expected, *S. aureus* resistance to penicillin (88-90%) observed in the two countries studied was in agreement with data from Zinn *et al.* (2004), which reported high levels ranging from 73-97%. In addition, full susceptibility of isolates (MSSA and MRSA) from Nigeria and South Africa to fusidic acid was similar with data from Luthuania, the USA (Colorado, California, New Jersey), France and Poland. This study also confirmed the report of Zinn *et al.* (2004) that fusidic acid showed excellent activity against *S. aureus* isolates in South Africa. All the isolates from Nigeria were susceptible to rifampicin (similar with data from Denmark, Norway, France, Sweden, Lithuania, USA-Colorado, Western Australia), and resistance to ciprofloxacin (7.0%) was comparable with data from Finland, Lithuania and the USA (Colorado). The proportion of *S. aureus* isolates resistant to ciprofloxacin from South Africa

(5.3%) was similar to the report from Sweden, while the resistance rate for rifampicin (20.3%) observed in this study was similar with the survey by Zinn *et al.* (2004) on *S. aureus* isolates from South Africa. The prevalence of *S. aureus* resistance to erythromycin and clindamycin in Nigeria was low (2.5%) but similar with data from Norway and Sweden (erythromycin), Malaysia and Sweden (clindamycin), while rates in South Africa (30%) were comparable with data from Kuwait (erythromycin) and Spain (clindamycin).

Based on the grouping stated by Zinn *et al.*, (2004), low-level resistance of *S. aureus* to fusidic acid, ciprofloxacin, chloramphenicol and mupirocin, was observed in both countries. *S. aureus* resistance to rifampicin, oxacillin, erythromyin, clindamycin, gentamicin, kanamycin, neomycin and minocycline in Nigeria could also be considered as low-level. Resistance to rifampicin, oxacillin, gentamicin, tetracycline, erythromycin, clindamycin, trimethoprim, kanamycin and minocycline of *S. aureus* isolates in South Africa could be of concern while resistance to tetracycline, trimethoprim and or trimethoprim-sulfamethoxazole is considered to be of major concern in Nigeria. Penicillin is considered as rarely useful in both countries. In this study, statistical analysis indicated a higher level of resistance to oxacillin, erythromycin, clindamycin, gentamicin, kanamycin, neomycin, rifampicin, minocycline and mupirocin in *S. aureus* isolates from South Africa compared with Nigeria. This trend was attributed to the higher prevalence of MRSA in South Africa.

All the MSSA isolates from Nigeria and South Africa were susceptible to oxacillin, teicoplanin, vancomycin and fusidic acid. Moreover, MSSA from South Africa did not exhibit resistance to streptomycin, neomycin and minocycline. The resistance rates of MSSA from both countries (Tables 2.2 and 2.3) were compared with data from a SENTRY

survey (Diekema *et al.*, 2001), and a study in Korea (Kim *et al.*, 2004). Notable similarities and differences in resistance trends were observed. Susceptibility of MSSA from Nigeria and South Africa to rifamipicin (>99%) was similar with data from the USA, Canada, Latin America, Europe, the Western Pacific, (SENTRY survey) and in Korea. However, the proportion of MSSA resistant to trimethoprim/trimethoprim-sulfamethoxazole in Nigeria and South Africa were higher (47.2%; 10.8%) compared with the SENTRY (<3%) and Korean (1.2%) surveys. Another comparative analysis of data from this study and that of Jones *et al.* (2003a), under the TSN database program, indicated that trimethoprim-sulfamethoxazole was generally effective (< 2% resistance) against most of the MSSA isolates (in-patients and ICU patients) from the United States, France, Germany, and Spain. Although data from the SENTRY and TSN surveys are greater in scale than this study, however, it appears that there is a high prevalence of MSSA resistant to trimethoprim and or trimethoprim-sulfamethoxazole in Nigeria. Trimethoprim is an antimicrobial agent used extensively in combination with sulfamethoxazole for the treatment of urinary, enteric, and respiratory infections in developing countries. Trimethoprim-sulfamethoxazole is listed among antibacterial agents that have been rendered ineffective or for which there are serious concerns regarding bacterial resistance, in many developing countries, including Nigeria (Okeke, 2003). It appears that misuse and overuse of these antibiotics in Nigeria could have contributed to this trend in Nigeria. A single amino acid substitution, Phe98 to Tyr98, in dihydrofolate reductase (DHFR) is the molecular origin of trimethoprim resistance in *S. aureus* (Dale *et al.*, 1997) and plasmid mediated high-level trimethoprim resistance is dominated by the ubiquitous Tn*4003*-mediated S1 DHFR (Rouch *et al.*, 1989; Burdeska *et al.*, 1990).

A comparative analysis of the susceptibility patterns of MSSA isolates (in both countries) indicates that resistance of MSSA may have evolved under different antibiotic pressure. Most of the MSSA (98.5%) from Nigeria were susceptible to erythromycin, clindamycin and minocycline, whereas chloramphenicol and ciprofloxacin was effective against the majority (99.4%) of MSSA from South Africa. Moreover, significant differences in the susceptibility of MSSA in Nigeria and South Africa to some antibiotics were observed. MSSA resistance to erythromycin and clindamycin was significantly higher in South Africa, whereas, resistance to streptomycin, neomycin, trimethoprim, trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, and ciprofloxacin was significantly higher in Nigeria (Tables 2.2 and 2.3). About 68% of MSSA from South Africa were resistant only to penicillin, but 25% of MSSA isolates from Nigeria exhibited that resistance phenotype. Furthermore, multi-drug resistant MSSA was about four-fold higher in Nigeria compared with South Africa (13.7%; 3.6%).

Epidemiological investigations of *S. aureus* at the moment have observed four major trends. In many countries, infections caused by multiresistant strains (especially methicillin-resistant *S. aureus* – MRSA) are of great concern, whereas in other countries, the frequency of MRSA is low. The third trend is the emergence of MRSA in the community and the fourth trend are the recent reports of vancomycin-intermediate and resistant *S. aureus* in hospitals. The emergence of MRSA has posed challenges in the treatment of infections especially their characteristic multidrug resistance, which restricts the options to treat infections caused by these pathogens (Van Belkum and Vertburgh, 2001; NNIS, 2003; Subedi and Brahmadathan, 2005).

One of the earliest reports on MRSA in Africa was that of Scragg *et al.*, (1978). Thereafter, MRSA has been reported in South Africa (van den Ende and Rotter, 1986; Peddie *et al.*, 1988; Gardie and Kirby, 1993), Ethiopia (Geyid and Lemeneh, 1991), Kenya (Omari *et al.*, 1997), Senegal (Sow *et al.*, 1998), Sudan (Musa *et al.*, 1999) and Nigeria (Rotimi *et al.*, 1987; Okesola *et al.*, 1999). In this study, most of the MRSA were obtained from wound samples, and a marked variation in the prevalence of MRSA in both countries was observed (1.5% - Southwestern Nigeria; 27% - KZN province, South Africa) (Tables 2.2, 2.3 and 2.7). At least one MRSA isolate was recovered from clinical samples in 13 out of the 14 health facilities in the KwaZulu-Natal province of South Africa (Table 2.7). The prevalence rates in this study were lower than reports from previous studies, in both countries. Investigations on the prevalence of MRSA in various health institutions in Southwestern Nigeria ranged from 9% to 50% (Rotimi *et al.*, 1987; Ako-Nai *et al.*, 1991; Okesola *et al.*, 1999; Kesah *et al.*, 2003), while the prevalence of MRSA reported in other major cities such as Johannesburg and Cape Town in South Africa was between 34 and 42% (Diekema *et al.*, 2001; Bell *et al.*, 2002; Christiansen *et al.*, 2004; Zinn *et al.*, 2004). It should be noted, however, that most of these investigations were conducted in one health facility. However, analysis of the proportion of MRSA isolates obtained from hospitals within the city of Durban indicated that the prevalence increased to 34%, which is comparable with data from the major cities in South Africa. The low prevalence of MRSA observed in this study in Southwestern Nigeria is unexpected based on previous reports in this region. More studies are needed in understanding the current status on antibiotic resistance among *S. aureus* isolates in both countries.

Susceptibility results of MRSA from South Africa were compared with data from multicentre investigations conducted in the Czech Republic (Melter *et al.*, 2003), Belgium (Denis *et al.*, 2004) and South Korea (Kim *et al.*, 2004). The high proportion of MRSA from South Africa resistant to gentamicin, kanamycin, tetracycline, erythromycin and clindamycin (Table 2.3), was similar with data from the Czech Republic and South Korea. In addition, susceptibility to fusidic acid observed in this study was similar with data from the Czech Republic and Belgium. Apart from the glycopeptides (vancomycin and teicoplanin), all the MSRA in this study were susceptible to quinipristin/dalfopristin, fosfomycin and linezolid. There is evidence that differences in the prevalence of MRSA occur within countries as well as from hospital to hospital (Fluit *et al.*, 2001). Multi-centre investigations have illustrated the geographical variation for the worldwide prevalence of MRSA, with low levels in the Northern European countries and high levels in the Southern European countries (Fluit *et al.*, 2001; EARSS, 2002; Tiemersma *et al.*, 2004) as well as parts of Australia, United States, Asia and South America (Diekema *et al.*, 2001; Zinn *et al.*, 2004). In this study, the geographic variation within the two countries could not be determined due to the varying and low numbers of MRSA obtained in the various health institutions. Analysis of the MRSA isolates from Southwestern Nigeria also could not be accomplished due to the number of isolates identified in this study.

Multi-drug resistance in MRSA is frequently due to the successive acquisition of plasmids and transposons with resistance determinants (Witte, 1999) or to the spread of a few clonal resistant lineages. There was an obvious relationship between methicillin resistance and resistance to other antibiotics as previously noted in literature (Diekema *et al.*, 2001; Fluit *et al.*, 2001; Kim *et al.*, 2004; Zinn *et al.*, 2004). More than 80% of MRSA

obtained from South Africa were resistant to erythromycin, clindamycin, trimethoprim, tetracycline, minocycline, gentamicin and kanamycin whereas the rates for MSSA was less than 12% (Table 2.3). About 87% of MRSA from South Africa were resistant to at least four classes of antibiotics and these multiresistant isolates were identified in 13 of the 14 health institutions in the KwaZulu-Natal province of South Africa (Table 2.8 and 2.9). MRSA belonging to antibiotype VI was identified in 12 of the 14 hospitals studied, indicating that isolates with this resistance phenotype are widespread in KZN. Furthermore, multiresistant MRSA exhibiting resistance to seven classes of antibiotics (antibiotype II) was detected in Hospitals A and B located in Durban and Hospital N in Empangeni, while MRSA with antibiotype I (resistance to eight classes of antibiotics) was identified in Hospitals A and C (Durban), E (Pietermaritzburg) and N (Empangeni) respectively (Table 2.9). These cities (especially Durban) are the most densely populated areas in KZN province of South Africa. Multi-resistant MRSA has been reported to be relatively high in African countries; including Morocco, Kenya, Nigeria and Cameroun (Kesah *et al.*, 2003), but their antibiotypes were not determined. This study confirms previous investigations from various regions of the world on the multiresistant nature of MRSA (Schmitz *et al.*, 1999a; Santos Sanches *et al.*, 2000; Fluit *et al.*, 2001; Kim *et al.*, 2004). It also indicates that treatment of infections caused by MRSA may be difficult in South Africa, as there are reduced antimicrobial options, which could lead to substantial rates in morbidity and mortality in hospital patients and increased health cost.

Accurate detection of MRSA is clinically important and errors in the detection of methicillin resistance could have adverse clinical consequences (Ribeiro *et al.*, 1999). False-susceptibility results may result in treatment failure and the spread of MRSA if

appropriate infection control measures are not applied. Conversely, false-resistance results may increase health care costs following unnecessary isolation procedures, and may lead to overuse of glycopeptides (Gerberding *et al.*, 1991). Detection of methicillin resistance by phenotypic methods relies on the modification of culture conditions to improve expression, and thus detection, of resistance. Modifications have included lowering the incubation temperature, adding NaCl to agar or broth, and increasing the incubation time (Pottumarthy *et al.*, 2005). The difficulty in detecting methicillin resistance by routine phenotypic susceptibility test methods is primarily due to the genetic and regulatory organization of the *mecA* gene apparatus that encodes the foreign PBP2A (Berger-Bachi and Rohrer, 2002). Transcription of *mecA* is regulated by two distinct but analogous sets of regulatory genes, *mec*R1-*mec*1 and a second homologous regulatory element blaR-bla1 of the staphylococcal penicillinase, *blaZ*. Mec1 and Bla1 are repressors of transcription of *mecA* and *blaZ* genes. MecR1 and BlaR1 are transmembrane signal transducer molecules, which upon binding of the inducer molecule result in the activation of the molecule and cleavage of the repressors Mec1 and Bla1, relieving *mecA* and *blaZ* repression (Berger-Bachi and Rohrer, 2002).

A phenotypic test recently published by the National Committee for Clinical Laboratory Standards (NCCLS, 2004) for the confirmation of MRSA isolates is the cefoxitin disk diffusion method using standard susceptibility testing conditions for the prediction of *mecA*-mediated resistance in staphylococci. All *S. aureus* isolates showing a zone of inhibition of ≤19mm are considered *mecA* positive, while isolates showing zones of inhibition of ≥20mm are considered *mecA* negative. In this study, the oxacillin, methicillin and cefoxitin disk diffusion method accurately detected all the MRSA

(confirmed by detection of the *mecA* gene) from South Africa and one MRSA from Nigeria (Tables 2.3 and 2.4). However, based on the NCCLS guidelines (NCCLS, 2000, 2004), two of the three MRSA from Nigeria were intermediately resistant to oxacillin (12mm) but sensitive to cefoxitin (20mm; 28mm) and methicillin (15mm; 24mm). This observation differs with recent reports on the superiority of cefoxitin over oxacillin in the detection of methicillin resistance (Boubaker *et al.*, 2004; Pottumarthy *et al.*, 2005; Sharp *et al.*, 2005; Velasco *et al.*, 2005). However, this study is in agreement with the observation of previous investigators that phenotypic methods are not completely reliable for the detection of MRSA (Cavassini *et al.*, 1999; Louie *et al.*, 2000; Brown and Walpole, 2001; Nicola *et al.*, 2000; Sakoulas *et al.*, 2001; Boubaker *et al.*, 2004). The application of the disk diffusion method (oxacillin, methicillin, cefoxitin) is still considered useful but confirmation (detection of the *mecA* gene) is recommended, especially for detecting strains exhibiting heterogeneous resistance to methicillin.

Two primary mechanisms are attributed to resistance to macrolides in staphylococci (Leclercq, 2002). The first involves macrolide efflux and is relatively common in *S. aureus* in some geographic areas. A specific efflux pump is encoded by the gene *msr (A)* in staphylococci (Ross *et al.*, 1990). Notably, this resistance does not create resistance to lincosamides (e.g. clindamycin and lincomycin), but only to macrolides, azalides (azithromycin), and group B streptogramins (e.g. quinipristin) (Ross *et al.*, 1990). The second mechanism of resistance to macrolides in staphylococci involves modification of the drug-binding site on the ribosome. This results in resistance to macrolides (and azalides), lincosamides and group B streptogramin and is commonly referred to as the MLS_B resistance (Ross *et al.*, 1989; Roberts *et al.*, 1999). An *erm* gene, usually *ermA* or *ermC*,

encodes methylation of the 23S rRNA-binding site that is shared by these three drug classes. Phenotypically, expression of MLS resistance in staphylococci is either constitutive or inducible. Strains with inducible MLS_B resistance (MLS_Bi) demonstrate in-vitro resistance to 14- and 15 membered-ring macrolides (e.g. erythromycin), while appearing susceptible to 16-membered-ring macrolides, lincosamides, and type B streptogramins. Strains with constitutive MLS_B resistance (MLS_Bc strains) show in-vitro resistance to all of these agents (Roberts *et al.*, 1999). This dissociated resistance arises from differences in the inducing capacities of MLS antibiotics, 14- and 15- membered-ring macrolides being better inducers than the other groups of drugs (Leclercq and Courvalin, 1991). Inducible resistance of clindamycin by erythromycin is demonstrated by the D-test. It involves the placement of an erythromycin disk in close proximity to a disk containing clindamycin, during antibiotic susceptibility testing, using the disk diffusion method. A truncated or blunted clindamycin zone of inhibition (D-shape) indicated inducible resistance. Constitutive resistance is recognized by a clindamycin zone diameter of ≤ 14mm (Fiebelkorn *et al.*, 2003). The D-test has been shown to be a reliable indicator of MLS_Bi strains that harbour either the *ermA* or *ermC* genes (Fiebelkorn *et al.*, 2003). The *erm* (A) genes are mostly spread in methicillin-resistant strains and are borne by transposons related to Tn*554*, whereas *erm* (C) genes are mostly responsible for erythromycin resistance in methicillin-susceptible strains and are borne by plasmids (Leclercq, 2002).

Clindamycin represents a useful option for therapy for various MRSA infections, including musculoskeletal infections, skin and soft tissue infections and even pneumonia with empyema (Martinez-Aquilar *et al.*, 2003). It is also of particular importance as an alternative antibiotic in the penicillin-allergic patient (Fiebelkorn *et al.*, 2003). However,

the use of clindamycin for the treatment of an infection due to an inducibly resistant strain of *S. aureus* has been somewhat hampered by concerns over possible inducible resistance to clindamycin and its impact on clinical outcome. These concerns have been raised especially for deep-seated infections or with large bacterial burden, such as endocarditis, abscesses, and osteomyelitis. The available clinical data are limited and somewhat conflicting, with some patients appearing to respond clinically to clindamycin therapy despite the presence of the MLS_Bi phenotype (Frank *et al.*, 2002; Martines-Aquilar *et al.*, 2003). However, the risk of constitutive mutants selected in-vitro at frequencies of approximately 10^{-7} colony forming units in the presence of these antibiotics, leading to treatment failures has been reported in patients with inducibly resistant *S. aureus* infections (Watanakunakorn, 1976; Drinkovic *et al.*, 2001; Siberry *et al.*, 2003; Levin *et al.*, 2005). The use of clindamycin for uncomplicated cellulitis due to MLS_Bi community-acquired MRSA also remains an unanswered question, because this represents the most widespread presentation associated with this organism, and clindamycin represents an attractive therapeutic option.

In this study, about 80% of *S. aureus* isolates susceptible to erythromycin and clindamycin were MSSA (Table 2.6), while the MLS_Bi phenotype was detected in 72 isolates (20 MSSA and 52 MRSA). Furthermore, *S. aureus* isolates from South Africa accounted for about 93% with this resistance phenotype and 69% were MRSA from South Africa. The overall frequency of *S. aureus* resistance to MLS_B in Nigeria was lower compared with South Africa and this difference was associated with the prevalence of MRSA in both countries. A recent survey in Pennsylvania (USA) reported that 68% of methicillin-susceptible and 12.3% of methicillin-resistant *S. aureus* were D-test positive (Levin *et al.*, 2005). The constitutive MLS_B phenotype was detected in one MSSA from

South Africa, but absent in MRSA from Nigeria and South Africa. This observation is in contrast with that of Kim *et al.* (2004), in which 24% of MSSA and 86% of the MRSA isolates in Korea, exhibited constitutive resistance. Furthermore, constitutive resistance is known to be a common occurrence among MRSA isolates in Belgian hospitals (Denis *et al.*, 2004) and in a Greek hospital (Fokas *et al.*, 2005). These findings indicate that the incidence of constitutive and inducible MLS_B resistance in staphylococcal isolates varies by geographic region.

Of the 61 MRSA from South Africa tested against MLS_B antibiotics, resistance to erythromycin, oleandomycin, azithromycin and inducible resistance to clindamycin was the dominant phenotype (Table 2.10). The proportion of MRSA with the MLS_Bi phenotype (82%) indicates that clindamycin may not be a theraupeutic option for the treatment of an infection due to an inducibly resistant MRSA. If clindamycin is used for treatment of infections with MLS_Bi-producing isolates, close follow-up and monitoring of failure or relapse is needed. However, in more severe infections, the presence of the MLS_Bi phenotype should preclude the use of clindamycin. In patients with non-MLS_Bi *S. aureus* infection, clindamycin can be used safely and effectively. Clindamycin could also be considered for the treatment of infections caused by MSSA in Nigeria, based on the low resistance rate observed.

In staphylococci, in-vitro susceptibility testing for clindamycin may indicate false susceptibility by the broth microdilution method and disk diffusion testing with erythromycin and clindamycin disks in nonadjacent positions (Fiebelkorn *et al.*, 2003). In this study, the D-test demonstrated, like previous studies, to be a simple and reliable method to detect inducible resistance to clindamycin. The clinical microbiology laboratory should

consider routine testing and reporting of inducible clindamycin resistance in *S. aureus*. This is to ensure that clinicians rely on clindamycin test results and be informed about the possibility of clindamycin treatment failure in patients with infections caused by inducibly resistant isolates. Quinipristin/dalfopristin showed excellent activity against all the MRSA isolates and might provide a valuable option for the treatment of MRSA infections in spite of the high prevalence of the MLS_Bi phenotype among these isolates.

Tetracyclines are broad-spectrum antibiotics that have been used widely in human and veterinary medicine, as growth promoters in animal husbandry and even to treat bacterial infections in plants (Levy, 1992; Falkiner, 1998). It is therefore not surprising that tetracycline resistance is prevalent in a diverse range of bacteria and is encoded by a wide range of determinants. Tetracyclines are relatively inexpensive antibiotics, and in some countries, it is the second most frequently prescribed antimicrobial agents (after the penicillins) for the treatment of a number of bacterial infections, including those caused by staphylococci (Col and O'Connor, 1987; Levy, 1992). Two mechanisms of resistance to tetracyclines have been identified in *Staphylococcus* spp: (i) active efflux resulting from acquisition of the plasmid-located genes, *tetK* (Khan and Novick, 1983; Guay *et al.*, 1993) and *tetL*, and (ii) ribosomal protection mediated by transposon-located or chromosomal *tetM* or *tetO* determinants (Nesin *et al.*, 1990; Schwarz *et al.*, 1998). *S. aureus* carrying *tetK* only have been described as resistant to tetracycline, but susceptible to minocycline (Bismuth *et al.*, 1990; Warsa *et al.*, 1996). The *tetM* gene is believed to confer resistance to all available drugs of this group, including tetracycline and minocycline (Bismuth *et al.*, 1990). Most *tetM*-positive isolates also carry the *tetK* gene and MRSA isolates are typically of *tetM* or *tetKM* genotype (Bismuth *et al.*, 1990). The

tetL gene has been found only in *S. aureus* isolates already carrying the *tetM* gene (Bismuth *et al.*, 1990). There are no reports of *tetO*-positive *S. aureus* strains. Both drug efflux and ribosomal protection are inducible in *S. aureus* in-vitro (Mojumdar and Khan, 1988; Nesin *et al.*, 1990).

Three antibiotypes were observed in the susceptibility testing of MRSA (from South Africa), with the tetracycline group of antibiotics (Table 2.10). More than half of the isolates (54%) were resistant to tetracycline, minocycline but susceptible to doxycycline, and about one-third were resistant to all the antibiotics in this group. Although induction of doxycyline and minocycline resistance and PCR of the respective resistance genes was not investigated, the resistance profile of the MRSA suggests that the *tetM* and *tetKM* genes may be the dominant genes present in these isolates.

Aminoglycosides play an important role in the therapy of serious staphylococcal infections. They are potent bactericidal agents, inhibiting protein synthesis by binding to the 30S ribosomal subunit. Gentamicin and tobramycin are often used with either a β-lactam or a glycopeptide, especially in the treatment of staphylococcal endocarditis, as these drugs act synergistically (Schmitz and Jones, 1997). The main mechanism of aminoglycoside resistance in staphylococci is drug inactivation by cellular aminoglycoside-modifying enzymes. Several gene loci encoding such modifying enzymes have been characterized in staphylococci. Clinically, the most important of these encode acetyltransferase (AAC), adenylyltransferase (ANT) or phosphotransferase (APH) activity. Resistance to gentamicin and concomitant resistance to tobramycin and kanamycin in staphylococci are mediated by a bifunctional enzyme displaying AAC (6') and APH (2'') activity (Matsumara *et al.*, 1984; Ubukata *et al.*, 1984). The *aac (6')-Ie-aph (2'')* genes encode this bifunctional enzyme

which is encoded on composite transposon Tn*4001*. Resistance to neomycin, kanamycin, tobramycin and amikacin in staphylococci is mediated by an ANT (4')-I enzyme encoded by *ant (4')-Ia*. This gene is often carried on small plasmids, and then integrated into larger conjugative plasmids, and subsequently into the *mec* region of the chromosome of some *S. aureus* isolates, probably as a result of IS*257*-mediated recombination events (Byrne *et al.*, 1991; Archer and Niemeyer, 1994; Stewart *et al.*, 1994). Resistance to neomycin and kanamycin conferred by an APH (3')-III enzyme has also been described for staphylococci. The *aph (3')-IIIa* gene responsible for this phenotype is carried on the transposon of Tn*5405*, which may be located on both the chromosome and plasmids (Derbsie *et al.*, 1996). The genetics of streptomycin resistance is somewhat more complex, being associated with an *ant (6)-Ia* gene, a resistance gene called *str*, chromosomal mutations (*strA*), an *aph (3')-III* gene, and an *ant (4')-Ia* gene (Courvalin and Fiandt, 1980; Phillips and Shanson, 1984; Projan *et al.*, 1988).

The MRSA isolates from South Africa exhibited higher rates of resistance to aminoglycosides than MSSA (Table 2.3). It also supports the observation of a relationship between oxacillin and aminoglycoside resistance (Schmitz *et al.*, 1999b; Kim *et al.*, 2004). Amikacin was the most active of the aminoglycosides against MRSA in this study. However, a limitation of this study was that aminoglycoside modifying enzyme analysis was not conducted on gentamicin-resistant, amikacin susceptible isolates. Considering the high rates of resistance to aminoglycosides, the inclusion of members of this group in conjunction with a β-lactam or a glycopeptide may not be advisable for the treatment of MRSA infections in South Africa. Seven antibiotypes were observed among the isolates and resistance to gentamicin, kanamycin, tobramycin and susceptibility to amikacin,

streptomycin and neomycin was predominant (Table 2.10). This was followed by isolates resistant to all the aminoglycosides tested. The phenotypic resistance patterns of the MRSA indicate that most of them seemed likely to produce AAC (6') and APH (2''), with or without ANT (4')-I, among the five-modifying enzymes.

Parenteral glycopeptide (vancomycin and teicoplanin) are the mainstay of therapy for systemic infections. However, not all infections are life threatening, and oral antibiotics provide an alternative mode of therapy (Brumfitt and Hamilton-Miller, 1989), particularly when long-term therapy is required e.g. in the presence of prosthetic material. Rifampicin, fusidic acid, ciprofloxacin and trimethoprim-sulfamethoxazole are the widely used oral agents that have demonstrated consistent in-vitro activity and have been recommended in the therapy of MRSA infections (Gottlieb and Mitchell, 1998; Kim *et al.*, 2004). In addition to the advantage of oral administration, these agents have also demonstrated better tissue penetration than the glycopeptide agents. Resistance may occur to one or more of the oral agents, hence susceptibility must be demonstrated by in-vitro testing before clinical use of these agents can be considered. Combination therapy with two oral agents is thought to be important to decrease the risk of selecting for mutants during therapy of MRSA infections (Brumfit and Hamilton-Miller, 1989; Maple *et al.*, 1989; Shanson, 1990). Hence existing resistance to two or more of these agents practically excludes oral therapy from consideration. Rifampicin and fusidic acid is the usual combination used to treat MRSA infections in Australasia (Turnidge and Grayston, 1993). In view of the high rates of resistance of MRSA (South Africa) to rifampicin and trimethoprim-sulfamethoxazole (Table 2.3), combination treatment with these antibacterial agents would be unreliable. Only fusidic acid showed excellent activity against the MRSA studied.

Treatment of *S. aureus* infections is becoming increasingly more complicated due to the emergence of vancomycin resistance in *S. aureus*. The first strain of *S. aureus* with reduced susceptibility to vancomycin (MIC, 8μg/ml) (strain Mu50) was reported in Japan in 1997 (Hiramatsu *et al.*, 1997a). Since then, vancomycin-intermediate *S. aureus* (VISA) isolates have been reported in the United States, Europe and the Far East (Kim *et al.*, 2000; Walsh and Howe, 2002). There are increasing reports of *S. aureus* strains showing heterointermediate resistance to vancomycin (hVISA) since the first report of the prototype strain (Mu3) by Hiramatsu *et al.*, (1997b). In addition, three reports of isolates of vancomycin-resistant *S. aureus* (VRSA) (MIC, ≥32μg/ml) in 2002 and 2004 from the United States have added more serious concern on this emerging trend (CDC 2002a, 2002b, 2004). Different laboratory methods for the detection of vancomycin resistance have been proposed in order to understand the prevalence of *S. aureus* with reduced susceptibility to glycopeptides. These include the vancomycin agar-screening test, agar dilution, E-test, and population analysis (Hiramatsu *et al.*, 1997a; Walsh *et al.*, 2001; Howe *et al.*, 2000). The disk diffusion technique has been regarded as unreliable due to its low sensitivity (Tenover *et al.*, 1998), and the modified population analysis profile (PAP-AUC) is reported as the most reliable and reproducible method for defining population heterogenicity (Walsh and Howe, 2002).

All the MRSA isolates studied did not exhibit reduced susceptibility to vancomycin and teicoplanin using the E-test method on Mueller Hinton Agar and Brain Heart Infusion Agar (BHIA). Although isolates were sensitive to vancomycin and teicoplanin based on their MIC values, higher MIC (E-test) values (between 1-2μg/ml) for some MRSA isolates on Brain Heart Infusion Agar (BHIA) after incubation for 48 hours was observed. It appears that E-test on BHIA and prolonged incubation could be an appropriate and sensitive method for screening reduced susceptibility to glycopeptide in MRSA. This observation supports the report of Walsh *et al.* (2001) and Midolo *et al.* (2003). The detection of two MRSA isolates intermediately resistant to vancomycin in South Africa by Ferraz *et al.* (2000) indicate that continuous surveillance of reduced susceptibility to vancomycin is important and strategies for managing patients with infections caused by resistant strains should be established.

Mupirocin is produced by *Pseudomonas fluorescens* (Fuller *et al.*, 1971) and has in-vitro activity against a wide range of Gram-positive and some Gram-negative bacteria such as *Haemophilus* and *Neisseria* (Thomas *et al.*, 1999). The 2% (20,000μg/ml) mupirocin ointment has been an effective antibacterial agent for the treatment of staphylococcal colonization and superficial wound infections such as impetigo, infected eczema and wound infections. This followed an increase in colonization and infections with MRSA (Kaufmann *et al.*, 1993; Poupard, 1995; Harbath *et al.*, 1999). It has proved extremely effective in eradicating nasal carriage of MRSA from hospital patients and staff and is widely used as an infection control measure (Casewell, 1997; Eltringham, 1997). However, staphylococcal isolates resistant to mupirocin have been found worldwide (Schmitz *et al.*, 1998; Deshpande *et al.*, 2002).

Staphylococci expressing mupirocin resistance can be divided into two groups: low-level resistance (MuL) with MICs in the range 8-256μg/ml and high-level resistant (MuH) with MICs ≥512μg/ml. Low-level resistance to mupirocin is thought to arise from point mutations within the usual chromosomal staphylococcal isoleucyl-tRNA synthetase gene (*ileS*) (Cookson, 1998). High-level resistance results from acquisition of a transferable plasmid carrying a new gene, *ileS*-2, encoding a second novel isoleucyl-tRNA synthetase, which has no affinity for mupirocin. It is also generally agreed that strains with high-level mupirocin resistance cannot be eradicated with mupirocin (Cookson, 1998). Low-level resistance is generally not transferable (Ramsey *et al.*, 1996), and until recently, chromosomal mupirocin resistance was considered clinically unimportant (Cookson, 1998; Henkel and Finlay, 1999). However, low-level mupirocin resistance appears to be more prevalent in clinical isolates than high-level resistance (Alarcon *et al.*, 1998; Schmitz *et al.*, 1998; Deshpande *et al.*, 2002; Fujimura and Watanabe, 2003), and the emergence of low-level mupirocin resistance is thought to increase failure rates for nasal decolonization of MRSA (Harbath *et al.*, 2000; Watanabe *et al.*, 2001; Decousser *et al.*, 2003). Transmission between strains or species remains highly probable for the high-level resistance genes (Woodford *et al.*, 1998), which could evolve into a serious threat to hospital and community infection control (Cookson, 1998).

The prevalence of mupirocin resistance in staphylococcal isolates varies from institution to institution regardless of geographic region monitored (Deshpande *et al.*, 2002; Petinaki *et al.*, 2003). In this study, a total of 17 *S. aureus* isolates exhibited resistance to mupirocin and 94% of these isolates were obtained from South Africa (Table 2.2 and 2.3). The prevalence of mupirocin resistance (South Africa) in this study (7%) was higher than a

previous study (2%) by Zinn *et al.* (2004). Morever, high-level mupirocin resistance in Nigeria (0.5%) and South Africa (0.9%) was lower than reports from Greece (1.6 and 2%) (Maniatis *et al.*, 2001; Petinaki *et al.*, 2004), South Korea (5%) (Yun *et al.*, 2003) and Poland (11.3%) (Leski *et al.*, 1999). Mupirocin resistance has been mainly observed in methicillin resistant staphylococci (Schmitz *et al.*, 1998; Petinaki *et al.*, 2004; Kresken *et al.*, 2004). However, the first report of high-level resistance to mupirocin by Rahman *et al.* (1987) was detected in methicillin-susceptible *S. aureus*. An important observation was that two MSSA from Nigeria and South Africa were *mupA* positive while all the isolates with low-level mupirocin resistance were MRSA from South Africa. It appears that mupirocin resistance in staphylococcal isolates appears to be an emerging trend especially in South Africa. Although few data exists on mupirocin resistance in MSSA isolates (Leski *et al.*, 1999; Jones *et al.*, 2003b; Yun *et al.*, 2003; Chaves *et al.*, 2004; Kresken *et al.*, 2004; Petinaki *et al.*, 2004), it is suggested that MRSA along with MSSA should be routinely tested in both countries so that resistant isolates could be detected early, and to facililate the prompt institution of infection control measures.

Biocides in the form of antiseptics and disinfectants have been useful adjuncts with antibiotics in infection control measures. Increasing apprehension of microbial contamination of everyday living environments have led to increased use of antiseptics and disinfectants both inside and outside health care settings (McDonnell and Russell, 1999; Ug and Ceylan, 2003). Heavy metals such as $AgNO_3$, $CuSO_4$, $HgCl_2$, and $ZnSO_4$ have antimicrobial properties and are used in disinfectant and antiseptic formulations. $AgNO_3$ was administered to prevent gonococcal eye infections, zinc as an antifungal antiseptic, while $CuSO_4$ is used as an algicide (Ronald, 1995). Research reports have

expressed concern that use of biocides may contribute to the development of antibiotic resistance (McDonnell and Russell, 1999; Levy 2000). Many antibiotic resistance genes are plasmid-borne and in some cases, resistance factors for metals are present on the same plasmids, such as mercury and cadmium (Christon *et al.*, 1997). Resistance to acridines, ethidium bromide, quaternary ammonium compounds and propamidine isethionate is mediated by a common determinant on a group of structurally related plasmids. Many of these plasmids carry transposon Tn*4001*, which encodes resistance to the aminoglycosides gentamicin, tobramycin and kanamycin, as well as to the dihydrofolate reductase inhibitor, trimethoprim (Russell, 1997). Cadmium resistance in *S. aureus* is mediated by the *cadA* and *cadB* genes, which have been studied extensively (Shalita *et al.*, 1980; Nucifora *et al.*, 1989; Silver and Phung, 1996). The *cadA* gene confers high-level resistance and is usually located on large plasmids such as pI258 (Shalita *et al.*, 1980; Nucifora *et al.*, 1989) that encode penicillinase production and resistance to other heavy metal ions (Shalita *et al.*, 1980; Udo and Grubb, 1991), aminoglycosides (Udo and Grubb, 1991) and nucleic acid binding compounds (Townsend *et al.*, 1985). The *cadB* gene confers low-level cadmium resistance and has been demonstrated on a large plasmid, pII147 (Shalita *et al.*, 1980) and on small multicopy plasmids (El-Sohl and Ehrlich, 1982). A chromosomal cadmium resistance determinant that is different from *cadA* and *cadB* has also been reported (Witte *et al.*, 1986).

In this study, heavy metal-resistance properties were associated with multi-drug resistance, which supports earlier reports (Schottel *et al.*, 1974; Misra, 1992; Al-Haddad *et al.*, 2001). The high level resistance (>80%) of MRSA in South Africa to cadmium

(Table 2.4) is not unexpected, as bacteria have evolved resistance mechanisms to toxic metals (Rosen, 1996). This trend is similar to an investigation on MRSA in Kuwait (Al-Haddad *et al.*, 2001). However, resistance to mercuric chloride, propamidine isethionate and ethidium bromide, was lower in this study than the Kuwaiti survey.

The identification of bacterial pathogens in human infection plays a key role in the management of patients in health care institutions. More than 40% of isolates obtained from the various clinical laboratories in South Western Nigeria and 2.6% of isolates from South Africa were misidentified as *S. aureus*. Although there is no reason to assume that serious misclassification bias may have affected the prevalence of MRSA in previous studies, the inaccurate reporting serves as an alert for laboratories in Nigeria, to review their procedures on the identification of *S. aureus*.

Atypical isolates were recovered from both countries (Table 2.11). One isolate from Nigeria was catalase-negative while six from South Africa did not ferment mannitol (on mannitol salt agar). These isolates were identified as *S. aureus* based on their positive results with coagulase (slide and tube using rabbit plasma) and DNase tests. Catalase is an oxidoreductase that allows bacteria to inactivate toxic hydrogen peroxide and free radicals formed by the myeloperoxidase system within phagocytic cells (Friedberg *et al.*, 2003). The production of catalase is a more or less a constant feature of *Staphylococcus spp*. It is universally used to distinguish, among Gram-positive cocci, *Staphylococcus* from *Streptococcus*. Most staphylococci are catalase positive with the exception of *S. saccharolyticus* and *S. aureus* subsp. *anaerobius*, which grow more rapidly under anaerobic conditions (Yilmaz *et al.*, 2005).

Reports of catalase-negative *S. aureus* have been recognised as far back as 1955 and from various parts of the world (Lucas and Seely, 1955; Everall and Stacey, 1956; Tu and Palutke, 1976; Carlson and Gorin, 1981; Millar *et al.*, 1986; Crawford *et al.*, 1994; Nice, 1995; Al-Awagi *et al.*, 1996; Klespies *et al.*, 1996; Lee *et al.*, 1996; Over *et al.*, 2000; Bertrand *et al.*, 2002; Friedberg *et al.*, 2003; Yilmaz *et al.*, 2005). Unlike the majority of previous reports of catalase negative *S. aureus* where resistance to only one or two antibiotics was observed (Carlson and Gorin, 1981; Millar *et al.*, 1986; Nice, 1995; Al-Awagi *et al.*, 1996; Klespies *et al.*, 1996; Friedberg *et al.*, 2003), the isolate from Nigeria (C20) was resistant to penicillin, ampicillin, streptomycin, neomycin, kanamycin and ciprofloxacin (Shittu *et al.*, 2003b). Two recent reports on catalase-negative methicillin-resistant *S. aureus* have also been described (Bertrand *et al.*, 2002; Yilmaz *et al.*, 2005). There are still uncertainities regarding the pathogenic role of this organism. However, Bertrand *et al.* (2002) indicated that a catalase-negative isolate belonged to a major epidemic clone in France. Moreover, a recent study has also indicated that septicaemia in an immunocompetent patient was caused by a methicillin-resistant catalase-negative *S. aureus* strain (Yilmaz *et al.*, 2005). It is unclear whether these reports represent the true incidence of this phenotype or whether it is underreported because few laboratories use the catalase test to identify *S. aureus*.

Data on mannitol-negative *S. aureus* are rare though it has been reported that due to genetic variation, some coagulase positive *S. aureus* isolates lack the ability to ferment mannitol, but are nevertheless regarded as *S. aureus* (Tu and Palutke, 1976). However, the fact that API STAPH misidentified two mannitol-negative isolates as *S. lugdunensis* (Table 2.10) suggests that incorrect or delayed identification is possible in the case of

atypical isolates. Interestingly, five of the six isolates, which did not ferment mannitol on MSA, were confirmed as MRSA by the detection of the species-specific *nuc* and *mecA* genes respectively. Laboratories are encouraged to investigate unusual isolates, which may be worth reporting and/or sending to regional or national reference centers. This appears to be the first report of catalase-negative and mannitol-negative *S. aureus* in Nigeria and South Africa respectively.

2.5 CONCLUSIONS AND RECOMMENDATIONS

This study on antibiotic susceptibility of *S. aureus* isolates obtained from various clinical samples in Nigeria and South Africa has shed some light on the trends in the resistance patterns of *S. aureus*, in both countries. Baseline information in assisting physicians, clinical microbiologists and public health officials on critical issues regarding empirical and pathogen specific therapy, have also been highlighted. From this study, it is clear that continuous surveillance on resistance patterns of *S. aureus* in understanding new and emerging trends is of utmost importance.

Inspite of the fact that the prevalence of MRSA in Nigeria was low compared with South Africa, it is clear that multi-resistant MSSA occurred frequently in Nigeria than in South Africa. The acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*) in its different forms by these MSSA isolates, in the hospital environment, could make them resistant to a number of antibiotics, thereby making it extremely difficult to control. This could have serious consequences on infection control measures in health institutions. Therefore strict antibiotic and infection control policies are important factors to be considered in order to forestall the emergence and dissemination of multi-resistant MRSA in Southwestern Nigeria.

In view of the prevalence rates of MRSA in previous studies and this survey, it appears that this pathogen has become established in health institutions in KwaZulu Natal province of South Africa. The isolation of multiresistant MRSA in various hospital centres and the dissemination of isolates with antibiotype VI indicate that adequate steps in limiting spread are urgently needed. Reservoir identification of MRSA is key to controlling MRSA transmission. Culturing samples from hospitalized patients at high risk

of acquiring MRSA can facilitate detection and isolation of colonized patients. Contact precautions and the creation of isolation wards or temporary cohorts within a ward could also to be considered. Hand hygiene has also been credited as the single most effective measure to reduce the transmission of MRSA (Anonymous, 1996). Healthcare workers dealing with wound infections should wear a new pair of gloves, which are discarded after dealing with each patient. Gloves prevent contamination of the hands with microorganisms, and prevent bacteria on the hands of the health worker from inoculating the patient (Anonymous, 1996). Hand antisepsis before and after contact with wound patients could also decrease MRSA transmission. It should be noted that wearing of gloves does not replace the need for hand washing, as gloves can have small defects, and hands could be contaminated when the gloves are removed (Anonymous, 1996). The "search and destroy" policy has been effective in reducing the MRSA incidence rate in the Netherlands (Verhoef *et al.*, 1999). This involves strict antibiotic policy and quarantine of patients until MRSA cultures are negative and screening of all patients and health care workers once a patient is found to carry MRSA. Other precautions include the closure of the ward or ICU when two or more patients or health care worker are found positive with the same MRSA strain. Molecular typing could also be an integral part by helping to confirm epidemiologic associations and routes of spread.

The following observations and recommendations in this study are as follows:

1. The D-test proved to be a simple, reliable method to detect inducible resistance to clindamycin, in *S. aureus*. It is recommended that routine screening on staphylococcal isolates should be standard practice in both countries.
2. Clindamycin may not be a theraupeutic option for the treatment of an infection due to an inducibly resistant strain of *S. aureus* in both countries, especially in South Africa.
3. Detection of methicillin resistance by PCR detection of the *mecA* gene is highly recommended for the confirmation of MRSA.
4. Fusidic acid, rifampicin, minocycline and mupirocin are recommended for the treatment of MSSA infections in both countries.
5. Apart from vancomycin and teicoplanin, fusidic acid, quinipristin/dalfopristin, linezolid and fosfomycin are recommended for the treatment of MRSA infections in South Africa.
6. In view of the high rates of resistance of MRSA isolates to rifampicin and trimethoprim-sulfamethoxazole, combination treatment with these antibacterial agents would be unreliable in the treatment of MRSA infections in South Africa, fusidic acid is recommended.
7. In view of the clinical consequences of high-level mupirocin resistance and the serious threat to hospital and community infection control, MSSA and MRSA isolates should be screened in both countries.
8. Laboratories are encouraged to investigate atypical *S. aureus* isolates, and confirm their identitiy by molecular techniques.

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CHAPTER THREE

MOLECULAR CHARACTERIZATION OF *Staphylococcus aureus* STRAINS FROM SOUTHWESTERN NIGERIA AND KWAZULU-NATAL PROVINCE OF SOUTH AFRICA

3.1 INTRODUCTION

The surveillance, investigation and control of health-care-associated infections have been one of the critical factors in hospital management worldwide. Each year, about two million people acquire nosocomial infections in hospitals in the United States and about 60% of these infections involve antibiotic resistant bacteria (Lowy, 1998). Among pathogens causing hospital infections, Gram-positive cocci such as staphylococci and enterococci have become predominant over the past two decades and the emergence of resistant organisms has added substantially to the burden and cost of health care-related infections (Stefani and Varaldo, 2003; Struelens *et al.*, 2004).

The era of emerging resistant pathogens has necessitated a coordinated multidisciplinary approach to infection management and control in both the individual patient and hospital population, in order to understand the epidemiology of infectious diseases (Zaidi *et al.*, 2003). Infection control practitioners may often notice a cluster of cases with a common pathogen. Laboratory technologists may report a distinctive colony morphology, or biotype. Thus, a common dilemma with multiple isolates of similar organisms is assessing whether they represent the same strain. The analysis of the dynamics of nosocomial infections caused by pathogenic bacterial species depends

heavily on the accurate identification of these taxonomic entities, including the adequate definition of sub-specific strains and clones (van Belkum, 2003). Although a strain is usually defined as a pure microbiological culture obtained in the clinical microbiology laboratory (Dijkshoorn *et al.*, 2000), diagnosing a clone requires additional investigation (Lan and Reeves, 2001). The analysis of multiple bacterial isolates by various typing methods provides a means of characterizing different subgroups within a species. The division of strains into defined subgroups is called bacterial typing.

Strain differentiation is the basis for the study of the epidemiology of infectious diseases (Frenay *et al.*, 1996). Bacterial strain typing distinguishes epidemiologically related or clonal isolates from unrelated clones. A prerequisite for all existing typing schemes is the assumption that strains derived from one clone will share certain characteristics in contrast to strains derived from different clones (Busch and Nitschko, 1999). Thus bacterial typing has several theoretical and practical implications: (i) to analyse the progress of outbreaks and to examine sequential isolates from patients, (ii) to associate unusual pathogenic mechanisms with certain strains and (iii) to increase our knowledge of epidemiology of infectious diseases (Power, 1996).

Methods for typing organisms fall into 2 broad categories: (i) subtyping that is based on the analysis of the phenotype, itself an expression of the genome through translation into structural proteins and enzymes, and (ii) subtyping that is based on the analysis of the genome of an organism. Subtyping by phenotypic methods involves the characterization and differentiation of strains based on products of gene expression. Properties such as biochemical profiles, bacteriophage types, antigens present on a cell's surface, and antimicrobial susceptibility profiles are examples of phenotypic properties

that can be determined in the laboratory. Because they involve gene expression, these properties all have a tendency to vary based on changes in growth conditions, growth phase, and spontaneous mutation.

Genotypic methods are those that are based on analysis of the genetic structure of an organism and include polymorphisms in DNA restriction patterns based on cleavage of the chromosome by enzymes that cleave the DNA into hundreds of fragments (frequent cutters), or into 10 to 30 fragment (infrequent cutters), and the presence or absence of extrachromosomal DNA. Genotypic variations are less subject to natural variation, although they can be affected by insertions or deletions of DNA into the chromosome, the gain or loss of extrachromosomal DNA, or random mutations that may create or eliminate restriction endonuclease sites (Tenover *et al.*, 1997). The choice of a typing method depends upon the needs, the skills level, resources of the laboratory and the type of question to be answered. An optimal typing method should show high typeability, adequate stability, high technical reproducibility and high discriminatory power. In addition, ease of use, ease of interpretation, rapidity, accessibility and low costs may be considered convenient criteria (Struelens, 1996).

Historically, *Staphylococcus aureus* has been an example of a microorganism involved in cross-infection in which carriers among health workers or patients have been identified frequently as the source of outbreaks (Williams *et al.*, 1966). Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important nosocomial pathogens that causes major outbreaks and represents a severe clinical threat to patients worldwide (Fluckiger and Widmer, 1999; Deplano *et al.*, 2000; Stranden *et al.*, 2003). For example, in the United States, MRSA has increased from 2% of all nosocomial infections in National

Nosocomial Infection Surveillance hospitals in 1980 to about 50% in 2004 (Farr, 2004). A recent study also reported that MRSA prevalence have increased significantly in Belgium, Germany, Ireland, the Netherlands and the United Kingdom (Tiemersma *et al.*, 2004). The high mortality and morbidity rates of MRSA infections associated with the potential for intra- and inter-hospital dissemination are of great concern to medical staff and infection control specialists (Tambic *et al.*, 1997; Conterno *et al.*, 1998). In view of this and the consequent implications on health-care costs, many hospitals have attempted to control the spread of MRSA. There is considerable epidemiological interest in the tracking of strains to gain a better picture of the distribution of strains in the population and the dynamics of clonal spread (Crisostomo *et al.*, 2001). Typing plays an important role in understanding the epidemiology of MRSA and evaluating the effectiveness of infection control and antimicrobial prescribing measures (Murchan *et al.*, 2003).

A broad spectrum of technical instruments has been developed and varies from techniques that monitor phenotypic characteristics to those that involve genetic procedures that highlight DNA polymorphisms. The main genotypic techniques used for typing *S. aureus* include (i) plasmid analysis; (ii) Southern hybridization analysis of digested chromosomal DNA, such as ribotyping, *Cla*I-*mecA*::Tn*554* polymorphisms, and binary typing; (iii) polymerase chain reaction (PCR)-based techniques such as random amplified polymorphic DNA (RAPD), repetitive element sequence based-PCR (rep-PCR), amplified fragment length polymorphism, and SCC*mec* typing; (iv) pulsed-field gel electrophoresis (PFGE) and (v) sequence typing techniques such as staphylococcal protein A (*spa*) typing and multilocus sequence typing (MLST). These genotyping techniques minimize problems with typeability and reproducibility and, in some cases,

enable the establishment of large databases of characterized organisms (Aires de Sousa and de Lencastre, 2004).

Molecular typing approaches have been used to a great advantage in identifying and monitoring the international spread of some unique *S. aureus* strains. Using a combination of genotyping such as DNA hybridization with the *mecA* and Tn*554* probes, PFGE, RAPD, SCC*mec* typing, *spa* typing and MLST, seven internationally spread multi-resistant MRSA clones have been identified; the Iberian, Brazilian, Hungarian, New York/Tokyo, EMRSA-15, EMRSA-16 and the paediatric clone (Aires de Sousa and de Lencastre, 2004; Perez-Roth *et al.*, 2004). Enright *et al.* (2002) also proposed a different nomenclature for these clones based on their sequence types (ST) (Enright *et al.*, 2000) and staphylococcal cassette chromosome *mec* (SCC*mec*) types (I through IV) (Hiramatsu *et al.*, 2001; Ito *et al.*, 2001) i.e. the designation of the seven pandemic clones as ST247-IA, ST239-IIIA, ST 239-III, ST5-II, ST 36-II, ST22-MRSA-IV and ST5-IV respectively (Oliveira *et al.*, 2001a; Enright *et al.*, 2002; Enright, 2003).

Although studies have been carried out in many countries on MRSA, which has identified the appearance of multi-drug resistant MRSA clones replacing other MRSA lineages, there is paucity of data on the epidemiology of *S. aureus* (MSSA and MRSA) in Nigeria and South Africa using molecular epidemiological techniques. Recent information on clonal identities and diversity of *S. aureus* does not exist in both countries. Furthermore, data does not exist on the emergence and spread of worldwide multi-resistant MRSA clones in the two countries. Monitoring the geographic expansion of *S. aureus* clones in the global surveillance network in different population is important and this study is expected to provide health personnel and policy makers in both countries,

with baseline information in establishing adequate infection control programmes and health intervention strategies and a better understanding on the global spread of this organism.

Genetic relatedness of methicillin-resistant *S. aureus* obtained in this study was determined using three sets of epidemiological tools. They include antibiotyping, PCR-RFLP of the coagulase gene and pulsed-field gel electrophoresis. Furthermore, selected methicillin-susceptible *S. aureus* (MSSA) from both countries were analysed by PCR-RFLP of the coagulase gene. Carriage of plasmid DNA by MRSA strains was also investigated by plasmid analysis.

3.2 MATERIALS AND METHODS

3.2.1 Plasmid DNA isolation

Isolation of plasmid DNA was based on the cetyl trimethyl ammonium bromide method (CTAB) according to Udo and Grubb (1991). A pure culture of the test strain was inoculated onto an enriched medium (Brain Heart Infusion broth), and incubated in a shaker at 37°C overnight. A 10ml aliquot of the culture was dispensed into oakridge tubes and centrifuged at 4000rpm for 15 minutes. The supernatant was discarded and the pellet was resuspended in 900µl of NE buffer (2.5M NaCl, 10mM EDTA) (pH 8.0) and 100µl lysostaphin (150µg/ml). The cell suspension was incubated at 37°C for 30 minutes. This was followed by the addition of 2ml of lysing solution (0.5% cetrimide; 0.5% sarkosyl), which was carefully dispensed through the sides of the tube. After incubation at 65°C for 20 minutes, the suspension was centrifuged at 12000rpm for 15 minutes, the supernatant dispensed into oakridge tubes containing 5ml of sterile distilled water and centrifuged at 4000rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 600µl E buffer (Tris 40mM, EDTA 2mM) (pH 8.0), 300µl NE buffer (2.5M NaCl, 10mM EDTA) (pH 8.0), 30µl RNase (10mg/ml), and incubated at 37°C for 30 minutes. Chloroform (1ml) was added after the incubation period, the tubes inverted gently for 5 minutes, and centrifuged at 4000rpm for 30 minutes. The aqueous phase was removed and dispensed into a microfuge tube containing 750µl isopropanol, and centrifuged at 13000rpm for 5 minutes. The supernatant was poured off and the tubes were allowed to dry at 37°C for 3 hours. The pellet was then resuspended in 40µl TE (10mM Tris, 0.1mM EDTA) buffer (pH 8.0) and stored at 4°C. Plasmids were analysed by agarose (0.6% w/v)

gel electrophoresis in 1X TAE buffer (Tris 0.04M, Acetic acid 0.02M, EDTA disodium 0.001M) (pH 7.2) at 25V for 16 hours. Plasmid profiles of the strains were based on the number and size of plasmids. The WBG 4483, *Staphylococcus aureus* strain, which has 4 plasmids (40.3kb, 22.5kb, 4.4kb and 3.5kb) served as the plasmid molecular size standard. The approximate plasmid sizes (closed circular forms) were estimated by visual inspection and using the GeneTools program (SynGene Bioimaging System).

3.2.2 DNA isolation of *S. aureus* strains

DNA isolation was carried out according to the method of Udo *et al.*, (1999) and as described in Chapter Two (Section 2.2.4).

3.2.3 PCR-RFLP of the coagulase gene

Amplification of the 3' end region of the coagulase gene containing the 81-bp tandem repeats was performed as described by Goh *et al.*, (1992). The gene primers are (COAG 2) 5'- CGA GAC CAA GAT TCA ACA AG3' and (COAG 3) 5'- AAA GAA AAC CAC TCA CAT CA3'. Each PCR reaction was made up of the following: 25µl of mastermix (Sigma), containing 1.5units of *Taq* DNA polymerase, 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin and 0.2mM dNTPs, 1µl (20pmol) of the forward and reverse primers and 5µl of template DNA. Sterile distilled water was added to make a final volume of 50µl. The thermocycler was programmed with the following parameters: 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 2 minutes and extension at 72°C for 4 minutes. The *S. aureus* ATCC 25923 strain served as the positive control in each PCR reaction. The PCR products were detected by gel

electrophoresis using 1.5% w/v agarose (Seakem, Whittaker USA) and run in 1X TBE (0.089M Tris, 0.089M boric acid, EDTA disodium 0.002M) buffer (pH 8.3) at 80V for 2 hours. The gels were stained with ethidium bromide and visualized under UV light.

Restriction fragment length polymorphisms (RFLPs) of the amplicons were determined by digestion with *Alu*I (Fermentas, UK) by a modification of the protocol previously described by Lammler *et al.*, (2001). The reaction mixture was made up of 10µl of the PCR product, 1.0µl enzyme and 2µl of 10X restriction buffer. Sterile distilled water was added to make a final volume of 20µl. It was then incubated at 37°C for 2 hours. The restriction DNA digests was detected by electrophoresis in 2% w/v agarose (Seakem, Whittaker USA) in 1X TBE buffer (0.089M Tris, 0.089M boric acid, EDTA disodium 0.002M) buffer (pH 8.3) at 80V for 2 hours. The gels were stained with ethidium bromide and visualized under UV light.

The sizes of the PCR products and of the restriction DNA digests (RFLPs with respect to the overall number of 81-bp tandem repeats) was estimated by comparison with a 100bp molecular size standard marker, visual inspection and analysis using the GeneTools program (SynGene Bioimaging System). The strains were grouped on the basis of three characteristics of their PCR products, i.e. the presence of one or two PCR products, their size (s), and the *Alu*I restriction digest patterns of the PCR products.

3.2.4 PFGE Typing

PFGE typing of *Sma*I (Fermentas, UK) digested DNA was carried out by a modification of the protocol previously described by Bannerman *et al.* (1995). A colony was inoculated in a Brain Heart Infusion broth culture and incubated at 37°C overnight on a shaker. The pellet from 0.4 ml of this culture was washed in 0.8 ml NET buffer (10 mM Tris, 1 mM EDTA, 10 mM NaCl), and re-suspended in 0.2ml of NET buffer. An aliquot of 50µl lysostaphin (400µg/ml) and 150µl of agarose (0.4g in 25ml of 0.5X TBE buffer) (Seakem, Whittaker USA) at 50°C was then added. The cell/agarose suspension was loaded into block molds (Bio-Rad) and allowed to solidify for 30 minutes. Cells were lysed by incubation at 37°C for 3 hours in lysis buffer (6 mM Trizma base, 100 mM EDTA, 1 M NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% lauroyl sarcosine). This was followed by a second overnight incubation at 55°C in 25µl of proteinase K (20mg/ml) and 475µl of proteolysis buffer (1% lauroyl sarcosine, 75µg Proteinase K in 0.5M EDTA). The blocks were washed three times (using a shaker) in 1 ml TE buffer (10 mM Trizma base, 1 mM EDTA) at 37°C for 30 minutes. Thereafter, one quarter of each agarose block was prepared and washed four times (using a shaker) in 1ml of sterile distilled water at 37°C for 30 minutes. It was then digested with 30 units of *Sma*I overnight according to the manufacturer's instructions and loaded into the wells of 1% PFGE certified agarose gel (Bio-Rad).

Electrophoresis was performed in 0.5X TBE buffer (0.045M Tris, 0.045M boric acid, EDTA disodium 0.001M) (pH 8) by the contour-clamped homogenous electric field method using a CHEF MAPPER system (Bio-Rad). The fragments were separated with a linear ramped pulse time of 6.8 s – 63.8 s over a period of 23 hours at 14°C. The gels

were stained with 1μg/ml ethidium bromide (Sigma) solution for 1 hour, visualized under UV and photographed (SynGene Bioimaging System).

The banding patterns were interpreted visually and the relatedness of the strains was determined according to the recommendation of Bannerman *et al.* (1995) and Jorgensen *et al.* (1996). In addition, the GelCompar II software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium) was used to calculate the Dice similarity indices and to construct a dendogram after cluster analysis by unweighted-pair-group-matching-analysis (UPGMA). Band position tolerance was set at 1.5% and DNA fragments below 80kb were not included in the analysis. By definition, two strains belong to the same cluster if their Dice similarity index is 85% or more. Strains showing the same PFGE pattern were grouped as a pulsotype and assigned using an alphabet (e.g. a, b, c etc). Numeric sub-codes were used to represent < 3 band difference (subtypes, e.g. a1, b1, c1 etc). Strains with banding patterns that differed from the main pattern by at least 3 bands were grouped in a different type.

3.3 RESULTS

3.3.1 Plasmid analysis

A total of 16 plasmid profiles were observed among the 60 MRSA strains from South Africa (Table 3.1; Figures in Appendix 2). MRSA that carried at least one large plasmid (approximately 26-39kb), and one or more small plasmids (2.3-4.8kb) accounted for 40% of the total number of strains studied. A total of 47 MRSA (78.3%) strains harboured at least one small plasmid ranging from 1.2-4.8kb, while plasmid DNA could not be obtained from 13 strains (21.7%). One of the MRSA from Nigeria possessed five plasmids ranging from 2.3 to 26.3kb. Analysis of the plasmid profiles and antibiogram of MRSA from South Africa indicated that strains in which plasmid DNA could not be isolated were resistant to three to six classes of antibiotics, while 45 of 47 MRSA strains that harboured at least one plasmid were resistant to six or more classes of antibiotics.

Table 3.1: Plasmid profiles of MRSA strains from South Africa and Nigeria

Plasmid Profile	Approximate size (kb)	Number of strains
South Africa		
1	26, 4.2, 3.3	2
2	26, 23, 3.3	2
3	28.5, 2.8	6
4	No plasmid	13
5	32, 4.2, 2.8	1
6	37, 2.3	5
7	34, 4.8, 2.3	1
8	37, 3.7, 2.3	3
9	39.3, 3.7, 2.8	1
10	1.2	1
11	2.8	6
12	38.3, 15.4, 2.3	3
13	2.3	12
14	3.7, 2.8	2
15	2.4, 2.3	1
16	4.8, 2.3	1
Nigeria		
17	26, 19, 7.4, 4.2, 2.3	1
18	5.2, 2.8	1

3.3.2 PCR-RFLP analysis of the coagulase gene (*S. aureus* strains from South Africa)

Typing based on PCR-RFLP of the coagulase gene of strains from South Africa is illustrated in Table 3.2. PCR products of 98 strains (37 MSSA and 61 MRSA) from South Africa were analyzed (Figures in Appendix 2). Due to the wide range of sizes of the PCR products, a cut-off value was determined with a limit of ± 20bp. Among the MRSA strains, a single amplicon of 750bp was detected in one strain (1.6%), of 850bp in two strains (3.3%), of 800bp in 14 (23%), and of 650bp in 43 strains (70.5%). No PCR product was detected in one MRSA strain (Figures in Appendix 2).

Eleven differently sized PCR products were identified in MSSA strains. PCR amplification of the 3' end of the coagulase gene revealed a single amplicon in 34 of the 37 strains, which ranged between 480bp and 950bp. Two PCR amplicons of 400bp, 760bp were detected in one strain and of 400bp, 1000bp in two strains. A single amplicon of 480bp was detected in one strain and of 600bp, 650bp, 700bp and 950bp in two strains respectively. PCR products of 850bp (five strains), of 750bp and 900bp (six strains), and 800bp (seven strains) were also identified. No PCR product was detected in one MSSA strain (Figures in Appendix 2). Overall, a single fragment of 650bp was detected in 45 *S. aureus* strains (MSSA and MRSA), followed by 800bp in 21 strains, of 750bp and 850bp in seven strains and 900bp in six strains.

A total of 11 distinct RFLP patterns (types 1-10 and 13) were observed among the 98 strains examined after *Alu*I digestion of the PCR products (Table 3.2; Figures in Appendix 2). Two strains (one MRSA and one MSSA) failed to yield a product with the primers and were therefore classified as twelfth type (14a). The strains belonging to type 7 were subdivided into seven subtypes; group 8 into five subtypes, types 3 and 5 into four subtypes and types 2 and 9 into two subtypes respectively. The 61 MRSA strains were classified into five main RFLP patterns (types 3, 5, 7, 8 and 14) and most of the strains (67.2%) were grouped in subtype 3a. The 37 MSSA strains were categorized into 12 genotypes, and two MSSA in subtype 5b shared similar RFLP patterns with one of the MRSA strains (Table 3.2).

Table 3.2: PCR-RFLP of the coagulase gene in MSSA and MRSA strains from South Africa

					*Alu*I restriction fragments									
Type	Molecular weight (±20bp)	Total number of strains	*mecA*-positive	*mecA*-negative	81	162	243	324	405	486	567	648	729	810
1a	480	1	0	1	+				+					
2a	600	1	0	1	+					+				
2b*		1	0	1		+			+					
3a	650	41	41	0	+						+			
3b		2	2	0							+			
3c		1	0	1	+					+				
3d		1	0	1				+	+					
4a*	700	1	0	1	+	+			+					
4b		1	0	1		+	+		+					
5a*	750	1	0	1	+	+			+					
5b*		3	1	2	+		+		+					
5c		1	0	1	+		+			+				
5d		2	0	2	+			+	+					
6a	750, 400	1	0	1	+			+	+					
7a**	800	5	5	0	+	+				+				
7b***		7	7	0	+			+	+					
7c		2	2	0				+	+					
7d		2	0	2	+	+						+		
7e*		3	0	3		+	+		+					
7f		1	0	1				+		+				
7g*		1	0	1										+
8a	850	1	1	0	+	+				+				
8b*		1	0	1	+			+	+					
8c		2	0	2		+	+		+					
8d		2	0	2			+			+				
8e		1	1	0				+	+					
9a	900	5	0	5	+	+						+		
9b		1	0	1		+		+		+				
10a*	950	2	0	2		+		+		+				
13a*	1000, 400	2	0	2		+		+	+	+				
14a	No product	2	1	1										
Total		98	61	37										

*Similar PCR-RFLP patterns with MSSA strains from Nigeria
**Similar PCR-RFLP patterns with MRSA strains from Nigeria
***Similar PCR-RFLP patterns with MSSA strains from Nigeria

3.3.2.1 Correlation between antibiotyping and PCR-RFLP of the coagulase gene among MRSA strains from South Africa

The association between antibiotyping and PCR-RFLP of the coagulase gene in MRSA from South Africa is described in Table 3.3. Nine antibiotypes were noted for MRSA in type 3a (PCR-RFLP: 650bp; 81, 567bp) and 88% of strains in the predominant antibiotype VI were grouped in PCR-RFLP subtype 3a. In addition, five of the six MRSA strains in antibiotype XI and four of the six MRSA assigned to antibiotype II belonged to PCR-RFLP subtypes 7a and 7b respectively. Furthermore, the four strains in antibiotype I was equally shared between subtypes 7b and 7c.

Table 3.3: Correlation between PCR-RFLP of the coagulase gene and antibiotyping of MRSA strains from South Africa

Type	(PCR-RFLP coagulase gene) ± 20bp (number of strains)	Antibiogram (number of strains)/(antibiotype)
3a	650 (81, 567) (41)	PEN, GN, ERY, TET, TS, RF (22) - (antibiotype VI) PEN, GN, ERY, CHL, TET, TS, RF (4) - (III) PEN, GN, ERY, TET, TS, RF, MU5 (3) - (IV) PEN, TET, RF (2) - (XII) PEN, GN, TET, TS, RF, MU5 (2) - (VIII) PEN, GN, CHL, TET, TS, RF (2) - (VII) PEN, GN, TET, TS, RF (4) - (IX) PEN, GN, ERY, TET, RF (1) - (X) PEN, GN, ERY, TET, TS, RF, CIP (1) - (V)
3b	650 (567) (2)	PEN, GN, TET, TS, RF (1) - (IX) PEN, GN, ERY, TET, TS, RF (1) - (VI)
5b	750 (81, 243, 405) (1)	PEN, GN, ERY, TET, TS, RF (1) - (VI)
7a	800 (81, 162, 486) (5)	PEN, GN, ERY (5) - (XI)
7b	800 (81, 324, 405) (6)	PEN, GN, ERY, TET, TS, CIP, MU (4) - (II) PEN, GN, ERY, CHL, TET, TS, CIP, MU5 (2) - (I)
7c	800 (324, 405) (2)	PEN, GN, ERY, CHL, TET, TS, CIP, MU5 (2) - (I)
8a	850 (81, 162, 486) (1)	PEN, GN, ERY (1) - (XI)
8b	850 (81, 324, 405) (1)	PEN, GN, ERY, TET, TS, CIP, MU5 (1) - (II)
8e	850 (324, 405) (1)	PEN, GN, ERY, TET, TS, CIP, MU5 (1) - (II)
14a	No product (1)	PEN, GN, ERY, TET, TS, RF (1) - (VI)

3.3.3 PCR-RFLP of the coagulase gene (*S. aureus* strains from Nigeria)

A total of 54 strains (3 MRSA and 51 MSSA) from Nigeria were analyzed (Table 3.4; Figures in Appendix 2). PCR amplification of the 3' end of the coagulase gene yielded a single amplicon in 47 of the 54 strains studied, which ranged between 600bp and 1000bp. Two PCR products of 750bp, 840bp were detected in two MSSA and of 400bp, 1000bp in five MSSA strains. Furthermore, a PCR product of 650bp and 950bp was detected in one MSSA strain, of 600bp, 850bp and 900bp in two strains, and of 1000bp in three strains. Single fragments of 700bp in six strains, of 800bp in nine strains, and of 750bp in 18 strains were also identified. A single amplicon of 750bp and 800bp was noted in one and two MRSA strains respectively.

After the restriction analysis of PCR product using *Alu*I, 11 different restriction fragment length polymorphism (RFLP) patterns were distinguished among the strains (Table 3.4; Figures in Appendix 2). The RFLP patterns are illustrated in Table 3.4. The strains belonging to type 7 were subdivided into five subtypes and types 4 and 5 into four subtypes respectively. Subtype 5b was predominant (13 strains) among the MSSA strains, followed by subtype 7b (6 strains), and type 13a (5 strains). MRSA and MSSA strains sharing the same RFLP pattern were observed in genotype 5b and 7h. One of the MRSA strains from Nigeria shared the same PCR-RFLP pattern (type 7a) with five MRSA strains from South Africa while six MSSA strains from Nigeria shared a similar pattern (type 7b) with seven MRSA strains from South Africa. MSSA and MRSA strains from the two countries were identified in subtype 5b (Tables 3.2 and 3.4).

Table 3.4: PCR-RFLP of the coagulase gene in MSSA and MRSA strains from Nigeria

					*Alu*I restriction fragments									
Type	Molecular weight (±20bp)	Total number of strains	*mecA*-positive	*mecA*-negative	81	162	243	324	405	486	567	648	729	810
2b*	600	1	0	1		+			+					
2c		1	0	1	+		+		+					
3e	650	1	0	1			+		+					
4a*	700	2	0	2	+	+			+					
4c		2	0	2	+		+		+					
4d		1	0	1		+			+					
4e		1	0	1			+		+					
5a*	750	3	0	3	+	+			+					
5b*		14	1	13	+		+		+					
5e		1	0	1			+		+					
5f		1	0	1									+	
7a**	800	1	1	0	+	+				+				
7b***		6	0	6	+			+	+					
7e*		1	0	1		+	+		+					
7g*		1	0	1										+
7h		2	1	1	+	+					+			
8b*	850	2	0	2	+			+	+					
9c	900	2	0	2	+			+		+				
10a*	950	1	0	1		+		+		+				
11a	1000	3	0	3		+		+		+				
12a	840, 750	2	0	2	+	+		+	+					
13a*	1000, 400	5	0	5		+		+	+	+				
Total		54	3	51										

*Similar PCR-RFLP patterns with MSSA strains from South Africa
**Similar PCR-RFLP patterns with MRSA strains from South Africa
***Similar PCR-RFLP patterns with MRSA strains from South Africa

3.3.3.1 Comparative analysis of antibiogram of *S. aureus* strains from Nigeria and South Africa with similar PCR-RFLP patterns

The antibiotic resistance profile of MSSA and MRSA strains from both countries with similar PCR-RFLP patterns of the coagulase gene is presented in Table 3.5. Of the 37 and 51 MSSA strains from South Africa and Nigeria analyzed by PCR-RFLP of the coagulase gene, 14 and 29 strains with similar PCR-RFLP patterns were noted from South and Nigeria respectively, and seven groups were identified. Different resistance profiles of MSSA strains from Nigeria and South Africa with similar PCR-RFLP patterns were observed. Multi-resistant (defined as resistance to penicillin along with at least three classes of antibiotics) strains were noted among MSSA strains from South Africa in types 2b and 4a (with identical resistance profiles) and 7e while multi-resistant MSSA from Nigeria was mainly identified in types 5b (10 strains) and 7e (one strain). MSSA from both countries with similar resistance profiles was noted only in type 5a. There was no correlation between antibiogram of the MRSA strain from Nigeria, which shared the same PCR-RFLP pattern (subtype 7a) with the five MRSA strains from South Africa.

Table 3.5: Antibiogram and PCR-RFLP of the coagulase gene in related MSSA and MRSA strains from Nigeria and South Africa

			South Africa (MSSA/MRSA strains)	Nigeria (MSSA/MRSA strains)
Type	Size (±20bp)	RFLP pattern	Antibiogram (number of strains)	Antibiogram (number of strains)
2b	600	162, 405	PEN, ERY, TM, TET (1)	PEN, TM (1)
4a	700	81, 162, 405	PEN, ERY, TM, TET (1)	PEN, TM, TET (2)
5a	750	81, 162, 405	PEN, TM, TET (1)	PEN, TM, TET (3)
5b	750	81, 243, 405	PEN (2)	PEN, TM, TET (2), PEN, CHL, CIP, STR (1), PEN, TM, CIP, TET, STR (4) PEN, TM, GN, TET, KAN, STR (1) PEN, TM, GN, NEO, KAN, STR (1) PEN, TM, GN, CIP, TET, KAN, STR (1) PEN, TM, GN, TET, NEO, KAN, STR (2) PEN, ERY, TM, GN, CIP, TET, NEO, KAN, STR (1)
7a*	800	81, 162, 486	PEN, GN, ERY (5) (MRSA)	PEN, ERY, TET, CIP (1) (MRSA)
7e	800	162, 243, 405	PEN (1) PEN, TM, GN, KAN (1) PEN, ERY, TM, GN, KAN (1)	PEN, TM, GN, CIP, TET (1)
7g	800	810	PEN, ERY (1)	PEN (1)
8b	850	81, 324, 405	Susceptible to all antibiotics (1)	PEN, TM, TET (2)
10a	950	162, 324, 486	PEN, ERY (2)	PEN, TM, TET (1)
13a	1000, 400	162, 324, 405, 486	PEN, RF (1) PEN, ERY (1)	PEN, TM, TET (5)

*Similar PCR-RFLP patterns of MRSA strains from Nigeria and South Africa

3.3.4 PFGE typing

The *Sma*I macrorestriction profiles of the MRSA strains from Nigeria and South Africa are presented in Figures 3.2 to 3.7. Visual interpretation and cluster analysis by Gel Compar grouped the 61 MRSA strains from South Africa into eight types, which comprised of pulsotypes a (21 of 61 strains; 34.4%), b (17 of 61; 27.8%), c (4 of 61; 6.6%), d (10 of 61; 16.4%), and g (6 of 61; 9.8%). Types e, f and h were represented by single strains only. Four subtypes were identified in PFGE type a; five in type b and two in type d (Table 3.7). Discrepancies between visual and computer-aided interpretation was observed in one strain. However, it was assigned to type g (Figure 3.4; Lane 11). The PFGE patterns of MRSA strains in types a and b differed by two to four bands, indicating that the strains were related. In addition, comparison of the banding patterns of MRSA strains in the main type a1 with one of the strains from Ile-Ife, Nigeria (THCD) indicated a two to three band difference, suggesting that these strains are related (Figure 3.8; Lanes 10 and 14).

The distribution of MRSA PFGE types in health institutions within the KwaZulu-Natal province of South Africa is described in Table 3.6 and Figure 3.1. Type a was noted in two hospitals in Durban, and one health institution in Pietermaritzburg, Newcastle, Greytown, Kokstad, Port Shepstone and Empangeni. Type b was identified in two health facilities in Durban and one in Pietermaritzburg, Kokstad, Eshowe, Scottburg and Empangeni. Type c was detected in hospitals located in Empangeni and Scottburgh, and type d in three of the four health facilities in Durban and one in Pietermaritzburg and Empangeni. Types e, f and h represented by single MRSA strains were noted in health

facilities in Newcastle, Durban and Pietermaritzburg respectively, while type g was observed in two hospitals in Durban and one in Pietermaritzburg, Eshowe and Scottburg.

Table 3.6: Distribution of PFGE types of MRSA strains obtained from health institutions of the KwaZulu-Natal province in South Africa

Hospitals (Location) Number of strains	Type	Subtypes	Number of strains
Durban			
Hospital A (7)	a	a1	1
	b	b2, b4	3
	d	d1	2
	g	g	1
Hospital B (8)	a	a1, a2, a3	4
	d	d1, d2	4
Hospital C (4)	b	b1, b4	2
	d	d1	1
	g	g	1
Hospital D (1)	f	f	1
Pietermaritzburg			
Hospital E (2)	d	d1	1
	g	g	1
Hospital F (4)	a	a4	1
	b	b2	1
	g	g	1
	h	h	1
Newcastle			
Hospital G (2)	a	a1	1
	e	e	1
GreyTown			
Hospital H (1)	a	a3	1
Kokstad			
Hospital I (3)	a	a3	1
	b	b4	2
Eshowe			
Hospital J (4)	b	b1, b2, b3	3
	g	g	1
Port Shepstone			
Hospital K (4)	a	a1	4
Scottburgh			
Hospital L (3)	b	b2	1
	c	c	1
	g	g	1
Empangeni			
Hospital N (18)	a	a1, a3	8
	b	b1, b2, b5	5
	c	c	3
	d	d1	2

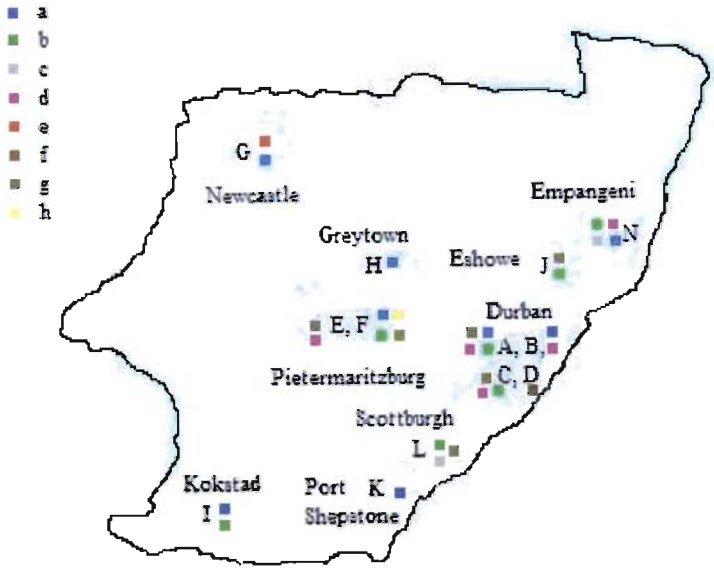
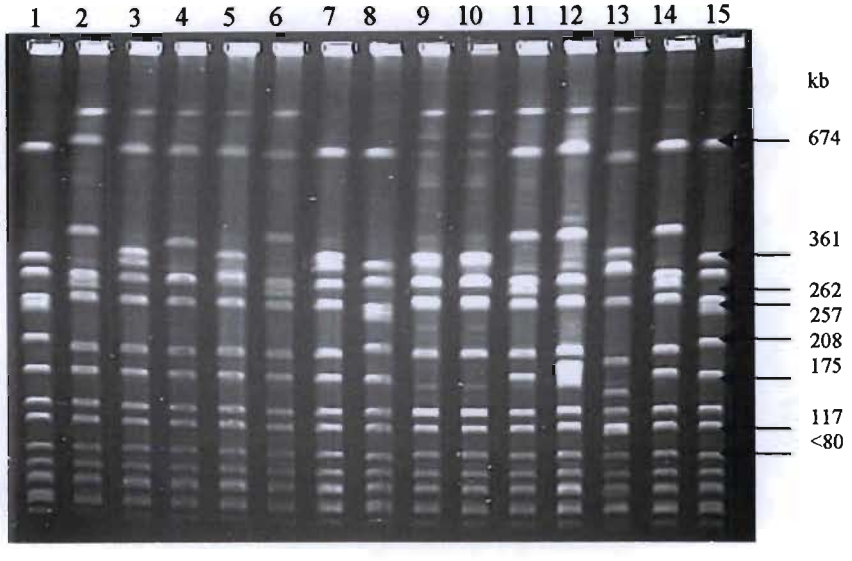
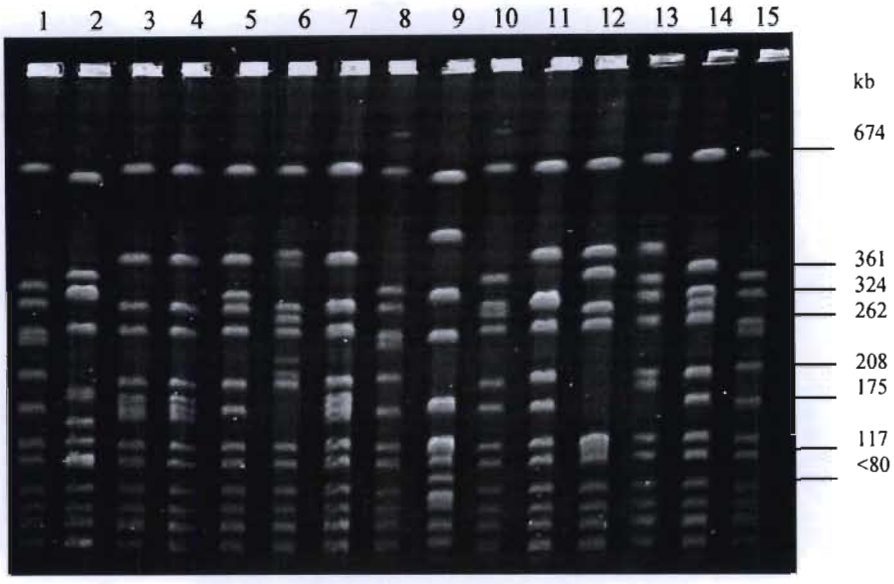


Figure 3.1: Map of KwaZulu-Natal province in South Africa, location of the health institutions (A-N) and the PFGE types (illustrated in colours)



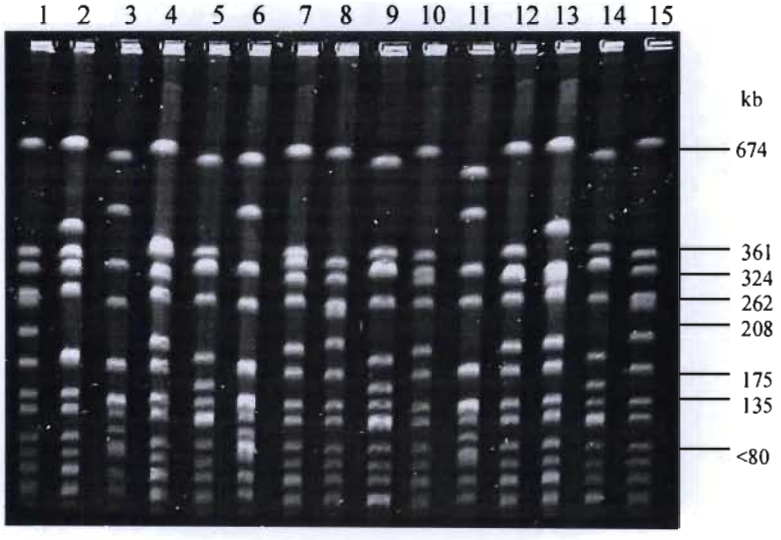
PFGE types a1 b1 a3 b2 a1 b1 b5 b5 a1 c d1 a1

Figure 3.2: PFGE profiles of MRSA from South Africa. Lanes 1, 8 and 15: *S. aureus* NCTC 8325; Lane 2: AC; Lane 3: AD; Lane 4: XQ; Lane 5: XW; Lane 6: 107 (2); Lane 7: ESA; Lane 9: HSA; Lane 10: SSA; Lane 11: 203; Lane 12: 503; Lane 13: 510; Lane 14: 511.



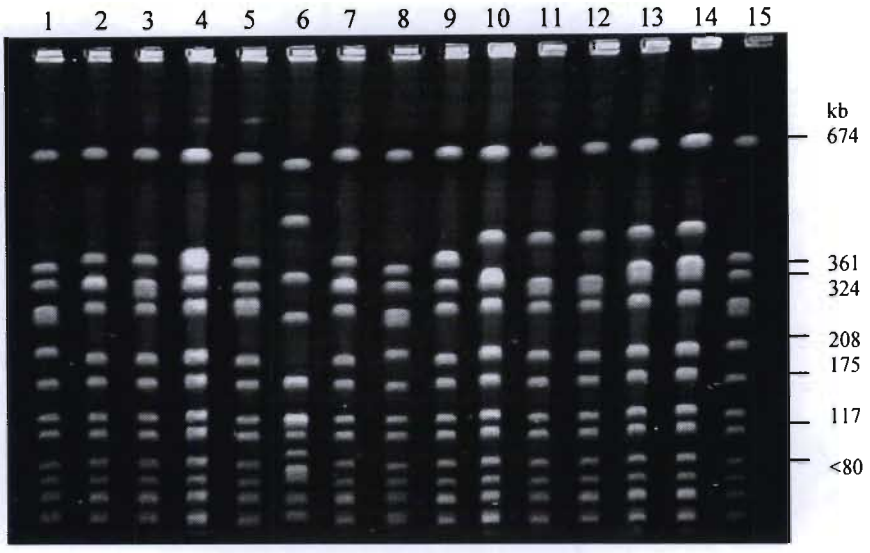
PFGE types d1 c c a1 e c g b2 a3 f a4 b2

Figure 3.3: PFGE profiles of MRSA from South Africa. Lanes 1, 8 and 15: *S. aureus* NCTC 8325; Lane 2: 513; Lane 3: 114XC; Lane 4: 131XB; Lane 5: MD24; Lane 6: MD43; Lane 7: GJC3; Lane 9: GJC7; Lane 10: GJC69; Lane 11: GT33; Lane 12: PM69; Lane 13: EDD52; Lane 14: EDD70.



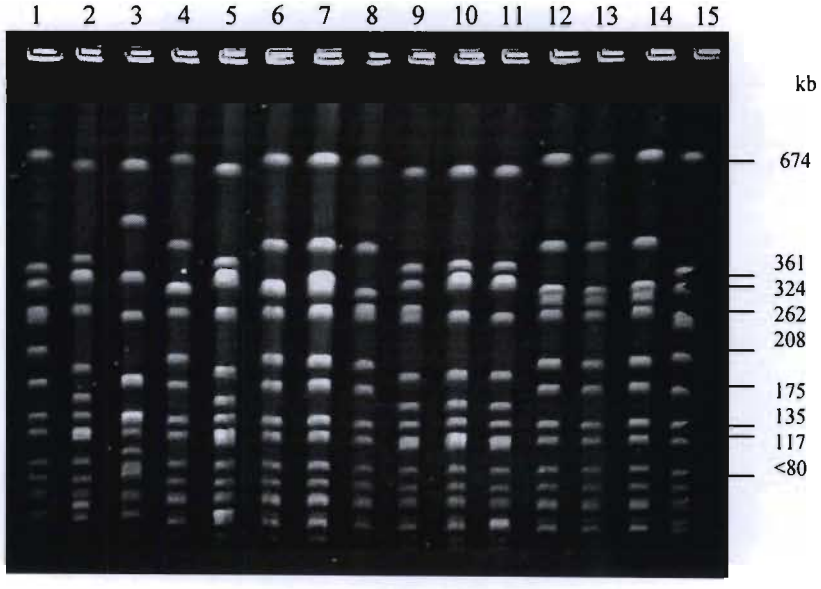
PFGE types h g b4 d1 g b1 d1 b2 g b4 a1 d1

Figure 3.4: PFGE profiles of MRSA from South Africa. Lanes 1, 8 and 15: *S. aureus* NCTC 8325; Lane 2: EDD84; Lane 3: EDD99; Lane 4: KEH12; Lane 5: KEH26; Lane 6: KEH77; Lane 7: KEH88; Lane 9: AD28; Lane 10: AD69; Lane 11: AD77; Lane 12: AD79; Lane 13: AD84; Lane 14: AD87.



PFGE types b4 b2 b1 b3 g b4 b4 a3 a1 a1 a1 a1

Figure 3.5: PFGE profiles of MRSA from South Africa. Lanes 1, 8 and 15: *S. aureus* NCTC 8325; Lane 2: AD98; Lane 3: ESH20; Lane 4: ESH34; Lane 5: ESH37; Lane 6: ESH89; Lane 7: EGU23; Lane 9: EGU28; Lane 10: EGU51; Lane 11: PS33; Lane 12: PS44; Lane 13: PS50; Lane 14: PS94.



PFGE types d1 g a3 d1 a3 a1 a2 d2 d1 d1 a1 a1 a1

Figure 3.6: PFGE profiles of MRSA from South Africa. Lanes 1 and 15: *S. aureus* NCTC 8325; Lane 2: GP11; Lane 3: GP74; Lane 4: RKK6; Lane 5: RKK8; Lane 6: RKK10; Lane 7: RKK52; Lane 8: RKK53 Lane 9: RKK55; Lane 10: RKK56; Lane 11: RKK57; Lane 12: 1KH; Lane 13: 2KH; Lane 14: 4KH.

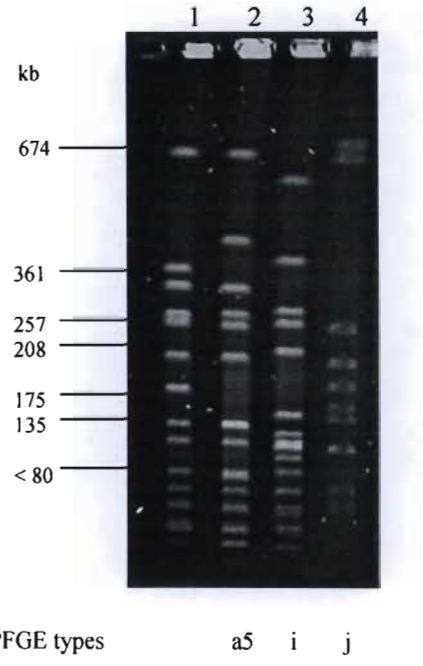


Figure 3.7: PFGE profiles of MRSA from Nigeria. Lane 1: *S. aureus* NCTC 8325; Lane 2: THCD; Lane 3: 15; Lane 4: 28IDA.

3.3.4.1 Clonal relationship between MRSA clones and EMRSA-15 and -16

Analysis of the DNA banding patterns by PFGE of representative MRSA clones in South Africa, MRSA from Nigeria and two worldwide epidemic clones are illustrated in Figure 3.8 and Figures in Appendix 2. No clonal relationship between selected MRSA clones from South Africa and the pandemic clones EMRSA-15 and -16 was observed. However, visual inspection and comparison of the banding patterns of one MRSA obtained in Ibadan, Nigeria (28IDA) revealed that it differed from EMRSA-15 by two to three bands, suggesting that they are closely related (Figure 3.8; Lanes 11 and 13).

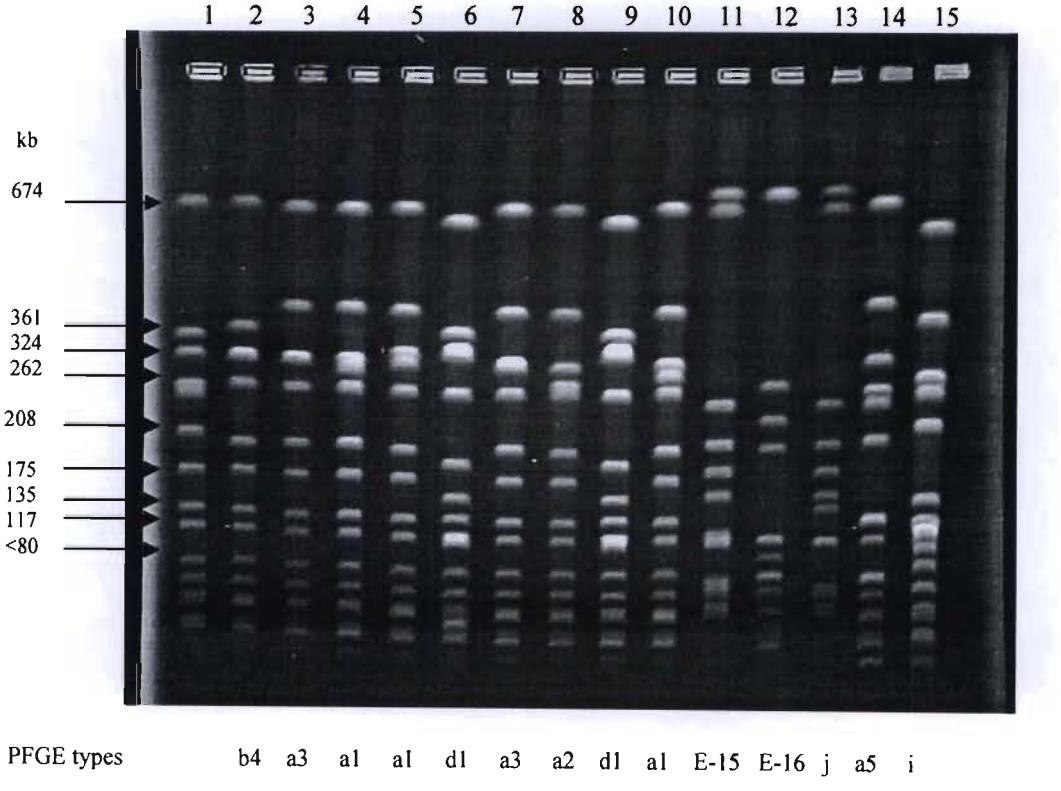


Figure 3.8: PFGE profiles of representative MRSA clones and worldwide clones. Lane 1: *S. aureus* NCTC 8325; Lanes 11 and 12: EMRSA-15 and EMRSA-16; Lane 2: EGU23; Lane 3: EGU51; Lane 4: PS33; Lane 5: PS50; Lane 6: GP11; Lane 7: RKK6; Lane 8: RKK53; Lane 9: RKK56; Lane 10: 1KH; Lane 13: 28IDA (Nigeria); Lane 14: THCD (Nigeria); Lane 15: 15 (Nigeria).

3.3.4.2 Relationships between PFGE and antibiotyping of MRSA strains from South Africa

The PFGE profiles and antibiotyping of 61 MRSA from South Africa are presented in Table 3.7. As stated in Chapter two, antibiotyping of MRSA isolates was based on the susceptibility pattern of isolates to selected antibiotics, representing various classes of antimicrobial agents.

Most of the MRSA strains (87%) were multiresistant (resistance to at least 4 classes of antibiotics) and one MRSA each in subtypes b1 and b3 and six MRSA in type g were non-multiresistant. A total of 14 of the 23 MRSA in the dominant antibiotype VI (Chapter Two) were grouped in type a, which comprised subtypes a1 (ten strains), a3 (two strains), a2 and a4 (one strain) respectively. Other MRSA in antibiotype VI were noted in type b (five strains), made up of subtypes b5 (two strains), b1, b2, b4 (one strain); types c (three strains) e, f and h (one strain) respectively. All the strains assigned to type g (PFGE) belonged to antibiotype XI while MRSA in antibiotype I was grouped in subtype d1. Furthermore, the six MRSA classified in antibiotype II were noted in subtypes d1 and d2. Resistance to rifampicin was a distinct feature of MRSA in types a, b, c, e, f and h, while resistance to ciprofloxacin was a unique character for MRSA in type d, although one MRSA in subtype b1 exhibited that resistance phenotype. In addition, strains in types d and g exhibited 100% susceptibility to rifampicin while susceptibility to tetracycline was also unique for MRSA in type g.

Table 3.7: PFGE patterns and antibiotyping of 3 and 61 MRSA strains from Nigeria and South Africa

PFGE type (number of strains)	Subtypes (number of strains)	Antibiotyping (number of strains)
South Africa		
a (21)	a1 (14)	PEN, GN, ERY, TET, TS, RF (9) PEN, GN, ERY, CHL, TET, TS, RF (3) PEN, GN, TET, TS, RF (1) PEN, GN, ERY, TET, TS, RF (1)
	a2 (1)	PEN, GN, ERY, TET, TS, RF (1)
	a3 (5)	PEN, GN, ERY, TET, TS, RF (2) PEN, GN, ERY, TET, RF (1) PEN, GN, CHL, TET, TS, RF (1) PEN, GN, ERY, TET, TS, RF, MU5 (1)
	a4 (1)	PEN, GN, ERY, TET, TS, RF (1)
b (17)	b1 (4)	PEN, TET, RF (1) PEN, GN, ERY, TET, TS, RF (1) PEN, GN, ERY, TET, TS, RF, CIP (1) PEN, GN, ERY, TET, TS, RF, MU5 (1)
	b2 (5)	PEN, GN, TET, TS, RF (3) PEN, GN, ERY, TET, TS, RF (1) PEN, GN, ERY, CHL, TET, TS, RF (1)
	b3 (1)	PEN, TET, RF (1)
	b4 (5)	PEN, GN, TET, TS, RF, MU5 (2) PEN, GN, CHL, TET, TS, RF (1) PEN, GN, ERY, TET, TS, RF (1) PEN, GN, ERY, TET, TS, RF, MU5 (1)
	b5 (2)	PEN, GN, ERY, TET, TS, RF (2)
c (4)	c (4)	PEN, GN, ERY, TET, TS, RF (3) PEN, GN, TET, TS, RF (1)
d (10)	d1 (9)	PEN, GN, ERY, TET, TS, CIP, MU5 (5) PEN, GN, ERY, CHL, TET, TS, CIP, MU5 (4)
	d2 (1)	PEN, GN, ERY, TET, TS, CIP, MU5 (1)
e (1)	e (1)	PEN, GN, ERY, TET, TS, RF (1)
f (1)	f (1)	PEN, GN, ERY, TET, TS, RF (1)
g (6)	g (6)	PEN, GN, ERY (6)
h (1)	h (1)	PEN, GN, ERY, TET, TS, RF (1)
Nigeria		
a (1)	a5 (1)	PEN, ERY, TET, TS (1)
i (1)	i (1)	PEN, TET (1)
j (1)	j (1)	PEN, ERY, TET, CIP (1)

3.3.4.3 Correlation between various typing methods used in this study

A comparative analysis of antibiotyping, PCR-RFLP of the coagulase gene and PFGE is illustrated in Table 3.8. The dominant genotype (3a) based on PCR-RFLP of the coagulase gene (with 38 strains) was further divided into six types (a, b, c, e, f and h) by PFGE. A total of 18 of the 21 MRSA belonging to type a and 16 of the 17 MRSA in type b were identified in the dominant group by PCR-RFLP of the coagulase gene (650bp; RFLP: 81, 567bp). All the MRSA strains in type d produced the PCR-RFLP pattern (800bp; 850bp, RFLP: 81, 324, 405bp; 324, 405bp) and were split between groups I and II (antibiotyping). MRSA in type g had a unique PCR-RFLP pattern (800bp; 850bp, RFLP: 81, 162, 486bp) and belonged to group XI by antibiotyping.

Table 3.8: Comparative analysis of antibiotyping, PCR-RFLP of the coagulase gene and PFGE in MRSA strains from South Africa

(PCR-RFLP coagulase gene) ± 20bp	Number of strains	PFGE type (number of strains)	Antibiotyping
650 (81, 567)	41	a1 (11)	PEN, GN, ERY, TET, TS, RF (6) PEN, GN, ERY, CHL, TET, TS, RF (3) PEN, GN, TET, TS, RF (1) PEN, GN, ERY, TET, TS, RF (1)
		a2 (1)	PEN, GN, ERY, TET, TS, RF (1)
		a3 (5)	PEN, GN, ERY, TET, TS, RF (2) PEN, GN, ERY, TET, RF (1) PEN, GN, CHL, TET, TS, RF (1) PEN, GN, ERY, TET, TS, RF, MU5 (1)
		a4 (1)	PEN, GN, ERY, TET, TS, RF (1)
		b1 (4)	PEN, TET, RF (1) PEN, GN, ERY, TET, TS, RF (1) PEN, GN, ERY, TET, TS, RF, CIP (1) PEN, GN, ERY, TET, TS, RF, MU5 (1)
		b2 (4)	PEN, GN, TET, TS, RF (2) PEN, GN, ERY, TET, TS, RF (1) PEN, GN, ERY, CHL, TET, TS, RF (1)
		b3 (1)	PEN, TET, RF (1)
		b4 (5)	PEN, GN, TET, TS, RF, MU5 (2) PEN, GN, CHL, TET, TS, RF (1) PEN, GN, ERY, TET, TS, RF (1) PEN, GN, ERY, TET, TS, RF, MU5 (1)
		b5 (2)	PEN, GN, ERY, TET, TS, RF (2)
		c (4)	PEN, GN, ERY, TET, TS, RF (3) PEN, GN, TET, TS, RF (1)
		e (1)	PEN, GN, ERY, TET, TS, RF (1)
		f (1)	PEN, GN, ERY, TET, TS, RF (1)
		h (1)	PEN, GN, ERY, TET, TS, RF (1)
650 (567)	2	a1 (1) b2 (1)	PEN, GN, ERY, TET, TS, RF (1) PEN, GN, TET, TS, RF (1)
750 (81, 243, 405)	1	a1 (1)	PEN, GN, ERY, TET, TS, RF (1)
800 (81, 324, 405)	6	d1 (6)	PEN, GN, ERY, TET, TS, CIP, MU5 (4) PEN, GN, ERY, CHL, TET, TS, CIP, MU5 (2)
800 (324, 405)	2	d1 (2)	PEN, GN, ERY, CHL, TET, TS, CIP, MU5 (2)
850 (81, 324, 405)	1	d2 (1)	PEN, GN, ERY, TET, TS, CIP, MU5 (1)
850 (324, 405)	1	d1 (1)	PEN, GN, ERY, TET, TS, CIP, MU5 (1)
800 (81, 162, 486)	5	g (5)	PEN, GN, ERY (5)
850 (81, 162, 486)	1	g (1)	PEN, GN, ERY (1)
No product	1	a1 (1)	PEN, GN, ERY, TET, TS, RF (1)

3.4 DISCUSSION

Staphylococcus aureus has emerged over the past several decades as a leading cause of hospital- and community-acquired infections (Lowy, 1998). A significant component in the "success" of *S. aureus* has been its acquisition of antibiotic resistance factors (Chambers, 2001). As new antibiotics have come into use, *S. aureus* has responded soon after with resistant strains. This phenomenon has made therapy of staphylococcal diseases a global challenge. Resistance to methicillin was first described for *S. aureus* in 1960, shortly after the introduction of the drug in clinical practice (Jevons, 1961). Since then, methicillin-resistant *S. aureus* (MRSA) has become a widely recognized cause of morbidity and mortality throughout the world (Grubb, 1990). In view of this, considerable epidemiological interest in the tracking of strains has emerged in understanding the population and dynamics of clonal spread of MRSA in many health institutions (Crisostomo *et al.*, 2001). This study characterized MSSA and MRSA strains from Nigeria and South Africa using molecular epidemiological techniques in order to understand the clonal identities and diversity of *S. aureus* in both countries. Concordance between antimicrobial susceptibility patterns and the molecular typing methods was also investigated.

The mechanisms by which *S. aureus* acquire resistance may be classified into two main categories: chromosomal gene mutation and acquisition of resistance genes as a result of conjugation, transduction and transformation. Arguably, the best understood mobile genetic elements are plasmids, which have been widely studied in *S. aureus* and MRSA since the 1970s (Meyers *et al.*, 1976; McGowan *et al.*, 1979; Locksley *et al.*, 1982; Coia *et al.*, 1988). Plasmid analysis, which is based on differentiating strains

according to the number and size of plasmids, was one of the first molecular techniques used for epidemiological investigations of MRSA (McGowan *et al.*, 1979). It is widely accepted that plasmid DNA may confer antibiotic resistance and therefore it might be assumed that multi-resistant MRSA strains would carry additional plasmids. Currently, it is suggested that more than 90% of MRSA strains carry plasmids while numerous studies have supported the important role plasmids play in staphylococcal multi-drug resistance (Lyon *et al.*, 1983; Coia *et al.*, 1988; Morton *et al.*, 1995; Paulsen *et al.*, 1998; O'Brien *et al.*, 2002).

Sixteen plasmid profiles were observed among the 60 MRSA strains studied from South Africa (Table 3.1). The presence of at least one large plasmid (approximately 26-39kb), and one or more small plasmids (2.3-4.2kb) was noted in 40% of strains. In addition, 78.3% of strains carried at least one small plasmid ranging from 1.2-4.8kb. Although the demonstration of a high number of MRSA strains with plasmid DNA was not surprising, however, it differs from the report of Caddick *et al.* (2005) in which resistance determinants in multidrug-resistant MRSA isolates was not associated with plasmid carriage. In this study, all the strains in which plasmid DNA could not be obtained, were resistant to 3-6 classes of antibiotics, while 95.7% (45 of the 47) of MRSA strains that harboured at least a plasmid were resistant to six or more classes of antibiotics.

Coagulase is produced by strains of *S. aureus* (Kloos and Scheifer, 1986). Its production is the principal criterion used in the clinical microbiology laboratory for the identification of *S. aureus* in human infections (Carter *et al.*, 2003). The coagulase gene has been a target for PCR genotyping in which size and DNA restriction endonuclease

site polymorphism within the *coa* gene have been utilized for PCR restriction fragment length polymorphism (PCR-RFLP) analysis (Hookey *et al.*, 1998). The analysis of coagulase-encoding staphylococcal DNA genes have demonstrated variable sequences in the 3'-end coding region of allelic gene forms (Phonimdaeng *et al.*, 1990). DNA sequence analysis of the 3'-end of the coagulase gene revealed heterogeneity in the region containing the 81bp tandem repeats coding repeated 27-amino-acid-residue sequences of the coagulase C-terminal region. PCR amplification of this particular region produced DNA fragments of different sizes, which can be further discriminated by digestion with *Alu*1 (Goh *et al.*, 1992). Coagulase gene typing has been reported to be an attractive method for clinical laboratories because of its ease and speed, and has been widely used in genotyping of clinical *S. aureus* isolates (Goh *et al.*, 1992; Schwarzkopf and Karch, 1994; Tenover *et al.*, 1994; Kobayashi *et al.*, 1995; Lawrence *et al.*, 1996; Nada *et al.*, 1996; Hoefnagels-Schuermans *et al.*, 1997; Hookey *et al.*, 1998; Wichelhaus *et al.*, 2001; Montesinos *et al.*, 2002; Carter *et al.*, 2003). Furthermore, it is widely used for genotypic identification and differentiation of strains isolated from milk of bovine animals affected by mastitis (Aarestrup *et al.*, 1995; Fitzgerald *et al.*, 1997; Annemuller *et al.*, 1999; Raimundo *et al.*, 1999; Schlegelova *et al.*, 2003; Scherrer *et al.*, 2004; Katsuda *et al.*, 2005).

PCR of the coagulase gene yielded four and nine differently sized single PCR products ranging from 600 to 850bp to 480 to 950bp in MRSA and MSSA strains from South Africa respectively (Table 3.2). In addition, amplicons of 400, 750bp and 400, 1000bp were identified in one and two MSSA strains respectively. Results of coagulase gene typing demonstrated that the MRSA and MSSA strains from South Africa were

classified into four and eleven RFLP patterns, respectively. However, two strains (one MSSA and MRSA) did not produce a coagulase gene-derived PCR product and was therefore considered as the twelfth group. The inability for a gene product to be obtained could be due to sequence variations at the sites targeted by the primers, as described by previous investigators (Hookey *et al.*, 1998; Hookey *et al.*, 1999; Montesinos *et al.*, 2002). A total of 67% of MRSA were classified into the subtype 3a, indicating that strains with this profile was predominant in health institutions in KwaZulu-Natal province of South Africa. In contrast, the MSSA strains were diverse and none of the RFLP patterns could be considered as a predominant group (Table 3.2). This finding is similar to previous studies by Goh *et al.* (1992) and Kobayashi *et al.* (1995). It also indicates that genomic variation was lower in MRSA than in MSSA strains. The ability of the PCR-RFLP of the coagulase gene to differentiate between MRSA and MSSA from South Africa was also observed (Table 3.2). The MSSA and MRSA strains did not share similar PCR-RFLP patterns in types 3, 7 and 8, while none of the MRSA strains were identified in types 1, 2, 4, 6, 9, 10 and 13. The RFLP patterns of the MRSA strains were unique and distinct from the MSSA strains, but two MSSA in subtype 5b shared similar PCR-RFLP patterns with one of the MRSA strains (Table 3.2). This trend (similar PCR-RFLP patterns - subtype 5b) was also observed between MSSA and MRSA in Nigeria (Table 3.4). The ability of the PCR-RFLP to distinguish MSSA from MRSA strains has been reported by Lawrence *et al.* (1996), and offers an attractive option to be considered in the epidemiological analysis of *S. aureus*.

Of the 54 strains analyzed from Nigeria, eleven restriction fragment length polymorphism (RFLP) patterns were identified. A dominant RFLP pattern was noted among the MSSA strains (subtype 5b) and one of the MRSA shared the same pattern with MSSA in this group (Table 3.4). The level of concordance between antibiotyping and PCR-RFLP of the coagulase gene among strains with similar RFLP patterns, in both countries was low. Of the 37 and 51 strains from South Africa and Nigeria analyzed by PCR-RFLP of the coagulase gene, 14 and 29 strains exhibited similar PCR-RFLP patterns (Table 3.5). However, apart from strains in subtype 5a, which showed identical resistance profile, correlation between antibiogram and PCR-RFLP of the coagulase gene was low. Although one MRSA strain from Nigeria shared the same PCR-RFLP patterns with five MRSA strains from South Africa, they were assigned to different antibiotypes (XIII and XI) based on their susceptibility patterns.

Schwarz and Cantor (1984) introduced the use of electric field pulsing techniques in conjuction with agarose gel electrophoresis for discrimination of large DNA molecules. PFGE, by far the most widespread molecular typing tool, is considered to be the method of choice for DNA fingerprinting of MRSA and other bacterial pathogens (Kaufmann, 1998). This study demonstrated that MRSA from South Africa belonged to eight types (a to h) as determined by PFGE (Tables 3.6 and 3.7). Moreover, the ability of clones to be transmitted over great distances was mainly observed in PFGE types a and b, which accounted for 62% of the total number of MRSA studied (Figure 3.1). Type a was identified in two hospitals in Durban, and one health institution in Pietermaritzburg, Newcastle, Greytown, Kokstad, Port Shepstone and Empangeni. Type b was noted in two health institutions in Durban and one in Pietermaritzburg, Kokstad, Eshowe, Scottburg

and Empangeni (Table 3.6; Figure 3.1). These findings indicate that inter-hospital spread of PFGE types a and b occur frequently and regularly. Moreover, two to three band difference in the DNA patterns of the MRSA strains in the main PFGE types a and b was observed, indicating that these strains are closely related. These observations signify that there appears to be a major clone circulating in health institutions in KwaZulu-Natal province of South Africa. This is the first study, which has observed this trend, in this province. This development could be due to increased nosocomial transmission within and between hospitals and/or may be attributed to a virulence related property of these strains, which has not been determined. Previous studies in various parts of the world have shown that MRSA strains of identical or very similar clonal types have been recovered from hospitals separated by large geographic distances (Sanches *et al.*, 1995; Teixeira *et al.*, 1995; van Belkum *et al.*, 1997b; Aires de Sousa *et al.*, 1998; Sa-Leao *et al.*, 1999; Coimbra *et al.*, 2000; Coimbra *et al.*, 2003; Norazah *et al.*, 2003; Preney *et al.*, 2005). Epidemiological studies with different molecular typing techniques have also indicated that the massive geographical spread of MRSA results from the wide dissemination of a relatively small number of clones (Crisostomo *et al.*, 2001; Enright *et al.*, 2002; Oliveira *et al.*, 2001b; Stefani and Varaldo, 2003).

Schmitz *et al.* (2000) suggested that the development of higher resistance rates to multiple antibiotics in MRSA is a consequence of the clonal spread of individual multiresistant strains. In this study, antibiotyping of MRSA from South Africa indicated that most of the strains (86.7%) were multiresistant (resistance to at least 4 classes of antibiotics) (Chapter Two). Apart from the non-multiresistant MRSA in subtypes b1, b3 and g, multi-drug resistant MRSA were spread across the different pulsotypes and the

widely disseminated PFGE types a and b were resistant to at least five classes of antibiotics (Table 3.7). Although MRSA strains in type d were more resistant to a number of antibiotics (including low-level resistance to mupirocin) than other clones, dissemination of this clone was mainly observed in three of the four health facilities in Durban (Table 3.6 and 3.7; Figure 3.1). The multi-resistant nature of MRSA strains in the different pulsotypes identified and the dissemination of specific clones have clearly shown that adequate and effective infection control measures are urgently needed in health institutions in the KZN province, South Africa.

Several authors have described the worldwide spread of MRSA clones. The Iberian clone was initially detected in an outbreak in the Bellvitge Hospital in Barcelona, Spain, in 1989 (Dominguez *et al.*, 1994). Later, this clone was found to be widespread in at least eight Portuguese hospitals (Sanches *et al.*, 1995; Aires de Sousa *et al.*, 1996; Sanches *et al.*, 1996; Oliveira *et al.*, 1998), in addition to hospitals in Scotland, Italy, Belgium and Germany, and also in a hospital in New York City, USA (Mato *et al.*, 1998; Roberts *et al.*, 1998). Teixeira *et al.* (1995) first described the Brazilian epidemic clone to be widespread in Brazilian hospitals and also found to be disseminated in other countries in South America (Argentina, Paraguay and Chile) (Coimbra *et al.*, 2000) and in Europe (Portugal and the Czech Republic) (Aires de Sousa *et al.*, 1998; Oliveira *et al.*, 1998; Melter *et al.*, 1999). Sa-Leao *et al.* (1999) described the intercontinental spread of another unique MRSA clone among pediatric patients in Portugal, Poland, Argentina, Colombia and New York. The Brazilian and Hungarian epidemic MRSA clones have been reported in Taiwan and China (Aires de Sousa *et al.*, 2003a), while the New York/Japanese clone initially verified in the USA (Coimbra *et al.*, 2003) has been

discovered in Brazil (Melo *et al.*, 2004). The predominance of specific MRSA clones have also been described in Germany (Witte *et al.*, 1997), Turkey (van Belkum *et al.*, 1997a), Saudi Arabia (van Belkum *et al.*, 1997b), Malaysia (Norazah *et al.*, 2003), France (Preney *et al.*, 2005), Greece (Aires de Sousa *et al.*, 2003b) and Poland (Leski *et al.*, 1998).

In this study, clonal relatedness between MRSA clones in South Africa and two worldwide epidemic clones EMRSA-15 and EMRSA-16 was investigated (Figure 3.34). There was no clonal relationship by PFGE analysis of selected MRSA strains from South Africa with the pandemic clones. However, comparison of banding patterns of one MRSA obtained in Hospital F, Ibadan, Nigeria (Chapter Two) revealed that it differed from EMRSA-15 by two to three bands, suggesting that they are closely related (Figure 3.34; Lanes 11 and 13). EMRSA-15 is one of the most prevalent MRSA clones in hospitals in the United Kingdom and has been detected in northern Berlin, Germany, the Czech Republic and Spain (O'Neill *et al.*, 2001; Witte *et al.*, 2001; Moore and Lindsay, 2002; Melter *et al.*, 2003; Perez-Roth *et al.*, 2004). Susceptibility patterns of the MRSA strain from Ibadan, Nigeria and EMRSA-15 were similar (resistance to erythromycin and ciprofloxacin). In addition, the strain was resistant to tetracycline. This appears to be the first report of the detection of an MRSA closely related to EMRSA-15 in Nigeria. In addition, MRSA in the main type a1 differed by two to three bands with one of the MRSA strains from Ile-Ife (THCD), indicating that these strains are related. This observation suggests that clonal dissemination of MRSA in type a is not restricted to South Africa. Epidemiological studies on the clonal relationship of MRSA in both countries would be useful and important in understanding this trend.

Assays for antibiotic sensitivity are routine standard procedures in all microbiology laboratories, and they represent a commonly used marker for MRSA phenotyping. Resistance markers have proved to be useful in identifying specific clones. The susceptibility of MRSA to trimethoprim-sulfamethoxazole and spectinomycin has proved to be useful in differentiating the Iberian and Brazilian clones (Aires de Sousa *et al.*, 1998). In addition, Norazah *et al.* (2003) reported that resistance of MRSA to fusidic acid and rifampicin was unique in some strains in Malaysia. In this study, the dominant antibiotype was not helpful in discriminating between MRSA as it was detected in PFGE types a, b, c, f and h. However, resistance to ciprofloxacin and susceptibility to rifampicin was a unique character of the multi-resistant MRSA in type d (Table 3.7). Furthermore, all the strains assigned to type g (PFGE) had a unique antibiotype with 100% susceptibility to tetracycline and rifampicin (Table 3.7). These resistance markers could be useful in monitoring the spread of such clones and alert clinical microbiologists on the detection of new clones as and when they arise.

DNA typing techniques have enabled molecular epidemiologists to discriminate bacterial strains belonging to the same species (Aires de Sousa *et al.*, 2001). The evaluation on the use of antibiotyping, PCR-RFLP of the coagulase gene and PFGE as typing tools in understanding the epidemiology of MRSA in South Africa was investigated. PFGE gave a wider spectrum of types and subtypes and the ability of this typing tool to define subclones allowed for greater discrimination among the MRSA strains. This was well demonstrated in the discrimination of MRSA in the dominant group 3a (PCR-RFLP of the coagulase gene) into six types (a, b, c, e, f and h) (Table 3.8). This observation agrees with previous reports that typing by PFGE provides higher

resolution, being able to distinguish subtypes not detected by any of the PCR-based procedures (Saulnier *et al.*, 1993; Struelens *et al.*, 1993; Nada *et al.*, 1996; Hoefnagels-Schuermans *et al.*, 1997; Kumari *et al.*, 1997; Vandenbergh *et al.*, 1999; Montesinos *et al.*, 2002).

Some degree of correlation between the two molecular typing methods was observed. A total of 18 of the 21 MRSA which belonged to PFGE type a and 16 of the 17 MRSA in type b were identified in the dominant subtype 3a (PCR-RFLP of the coagulase gene), indicating that strains in these pulsotypes were genetically related. Furthermore, excellent correlation between antibiotyping, PCR-RFLP of the coagulase gene and PFGE was observed in identifying strains within type g. Only one strain in type g produced a differently sized amplicon by PCR detection of the coagulase gene, but the RFLP pattern was similar with the rest of the strains in the PFGE group. All the strains were grouped in antibiotype XI (Table 3.8; Chapter two). All the ten multi-drug resistant MRSA strains assigned in antibiotype groups I and II produced similar PCR-RFLP patterns indicating that they were closely related. PFGE typing revealed that these strains belonged to a unique type d. Although this study agrees with previous investigators that PFGE is a valuable tool for MRSA typing because of its high discrimatory power (Provost *et al.*, 1992; Struelens *et al.*, 1992; Schlichting *et al.*, 1993; Struelens *et al.*, 1993; Tenover *et al.*, 1994; Nada *et al.*, 1996; Na'was *et al.*, 1998; Schmitz *et al.*, 1998; Olive and Bean, 1999; Montesinos *et al.*, 2002), however, the combination of PFGE, PCR-RFLP of the coagulase gene and antibiotyping provided useful and important information in understanding the epidemiology of MRSA in Nigeria and South Africa.

3.5 CONCLUSION

The surveillance, investigation and control of health care-associated infections are hinged to a large extent on the clinical microbiology laboratory. The multiple roles that the microbiology laboratory play include accurate detection, species identification and susceptibility testing of microorganisms; epidemiologic analysis of clinical and screening test results; targeted microbiological surveys of the hospital environment; and epidemiologic typing of microbial isolates to support outbreak investigations (Struelens *et al.*, 2004). The characterization of *S. aureus* strains from Nigeria and South Africa using epidemiological tools has provided useful data on the clonal identities and diversity of *S. aureus* in both countries. It also provided baseline information on the extent and geographic expansion of MRSA clones. The identification and establishment of some widely disseminated clones in health institutions in KwaZulu-Natal province of South Africa, the genetic relatedness of one MRSA from Nigeria and strains in the widely disseminated clone a in South Africa, and the clonal relatedness of one MRSA from Nigeria with EMRSA-15 indicate that urgent measures are needed in curtailing the spread and establishment of these clones in both countries.

MRSA is usually transmitted by direct contact, therefore preventing contact between infected patients and potential carriers is a principal means of preventing its spread (Dent and Dent, 2005). It is also imperative to ensure that antibiotics are used prudently to minimize the emergence of resistance, and to discourage the spread of MRSA to patients unnecessarily treated with certain antibiotics. Compliance with infection control measures is even more important. These include screening policies, effective hand hygiene and appropriate isolation measures (Cookson, 2005). The "search

and destroy" policy has been effective in reducing the MRSA incidence rate in the Netherlands (Verhoef *et al.*, 1999). This involves strict antibiotic policy and quarantine of patients until MRSA cultures are negative and screening of all patients and health care workers once a patient is found to carry MRSA. In addition, a national registration system of MRSA patients and of hospitals experiencing an MRSA outbreak could be useful adjuncts. This would ensure that patients colonized with MRSA and those who are transferred from a hospital with an MRSA outbreak are tagged, traced and control measures initiated as appropriate.

Although it has not been possible to elucidate why some clones spread rapidly than others, future studies would be important to investigate the genetic relationship of clones in both countries with other pandemic MRSA clones in order to ascertain if these clones were unique and specific to Nigeria and South Africa. There is also the need for more comparative studies on the molecular epidemiology of MRSA in both countries, and closer international collaboration to monitor the spread of current epidemic strains and the emergence of new ones.

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CHAPTER FOUR

CHARACTERIZATION OF MUPIROCIN RESISTANT *Staphylococcus aureus* STRAINS IN SOUTHWESTERN NIGERIA AND KWAZULU-NATAL PROVINCE OF SOUTH AFRICA

4.1 INTRODUCTION

In 1971, pseudomonic acid A was isolated as a metabolite of *Pseudomonas fluorescens* and shown to have antibacterial activity (Fuller *et al.*, 1971). The name was later changed by consensus to mupirocin to avoid any suggestion that it had anti-pseudomonal effect. The spectrum of antibacterial activity includes most Gram-positive and a few Gram-negative bacteria such as *Haemophilus* and *Neisseria* (Thomas *et al.*, 1999). Enterobacteriaceae are intrinsically resistant due to a permeability barrier (Al-Masaudi *et al.*, 1988), and clinical application is, however, directed principally at Gram-positive cocci (Slocombe and Perry, 1991). It is an antibacterial agent with a unique action, binding competitively to bacterial isoleucyl-tRNA synthetase (IRS) and thus, by preventing the incorporation of isoleucine into growing polypeptide chains, arrests protein synthesis (Huges and Mellow, 1980; Morton *et al.*, 1995; Yao and Moellering, 1999). The antibiotic undergoes rapid breakdown in the tissues and can only be used topically. Mupirocin was first marketed for clinical use in the United Kingdom (UK) in 1985 and in the United States in 1988 (Mehtar, 1998) and is now available in more than 90 countries (Kresken *et al.*, 2004). It has potent activity against staphylococci and is used for the treatment of skin and postoperative wound infections and the prevention of nasal carriage of methicillin-resistant

Staphylococcus aureus (MRSA) (Cookson, 1998). Reports of resistance in different parts of the world emerged with the widespread use of mupirocin. Rahman *et al.* (1987) reported the first case of high-level resistance to mupirocin in methicillin-susceptible *S. aureus*. Subsequently, reports of mupirocin resistance have been described in Australia (Riley *et al.*, 1994; Udo *et al.*, 1994), Brazil (Bastos *et al.*, 1999; Ramos *et al.*, 1999), Canada (Miller *et al.*, 1996), Greece (Maniatis *et al.*, 2001; Petinaki *et al.*, 2004), Korea (Yun *et al.*, 2004), Kuwait (Udo *et al.*, 1999; Udo *et al.*, 2001a; Udo *et al.*, 2001b; Udo *et al.*, 2003), Malaysia (Norazah *et al.*, 2001), New Zealand (Hefferman *et al.*, 1995; Skellen *et al.*, 1998), Poland (Leski *et al.*, 1999), Saudi Arabia (Rich *et al.*, 1999), Spain (Alarcon *et al.*, 1998; Perez-Roth *et al.*, 2002; Chaves *et al.*, 2004), United Kingdom (Wise and Johnson, 1991; Connolly *et al.*, 1993), and the United States (Janssen *et al.*, 1993; Bradley *et al.*, 1995; Ramsey *et al.*, 1998; Jones *et al.*, 2003).

Mupirocin resistance in staphylococci is divided into two groups: low- and high-level resistance (MICs 8-256 and >256mg/l) respectively (Cookson, 1998). In most cases, low-level resistance to mupirocin is related to alteration in the host IRS (Farmer *et al.*, 1992; Antonio *et al.*, 2002). Until recently, chromosomal mupirocin resistance was considered clinically unimportant (Cookson, 1998; Henkel and Finlay, 1999). However, low-level mupirocin resistance appears to be more prevalent in clinical isolates than high-level resistance (Alarcon *et al.*, 1998; Schmitz *et al.*, 1998; Deshpande *et al.*, 2002; Fujimura and Watanabe, 2003). Moreover, the emergence of low-level mupirocin resistance is thought to increase failure rates for nasal decolonization of MRSA (Harbath *et al.*, 2000; Watanabe *et al.*, 2001a; Decousser *et al.*, 2003).

The origin of high-level mupirocin resistance (mupH) is still a matter of speculation. Interestingly, it has been described in stored isolates, namely three high-level mupirocin resistant isolates of *S. aureus* and *S. epidermidis* from Nigeria in 1956 (Cookson, 1994), and a coagulase negative staphylococcal isolate from the UK in 1967 (Cookson, 1998), even before mupirocin was used clinically. High-level mupirocin resistant strains cannot be eradicated with mupirocin and constitute a serious clinical problem especially when they are resistant to methicillin (Perez-Roth *et al.*, 2002). The clinical isolates exhibiting high-level resistance to mupirocin contain two distinct IRS enzymes: the endogenous IRS and an additional IRS encoded by the *ileS-2* gene (Morton *et al.*, 1995). This additional enzyme is usually encoded by the *mupA* gene, which is carried on plasmids that vary in size, restriction patterns and their ability to be transferred in conjugation experiments (Rahman *et al.*, 1990; Needlam *et al.*, 1994; Udo *et al.*, 1994; Morton *et al.*, 1995; Udo *et al.*, 1998). However, the *mupA* gene has also been reported in the genomic DNA of a few *S. aureus* isolates expressing low-level resistance, suggesting that the *mupA* gene may also be located in the chromosome (Ramsey *et al.*, 1996; Fujimura *et al.*, 2001). The first report on the chromosomal location of the *mupA* gene in *S. aureus* expressing high-level mupirocin resistance has also been described (Udo *et al.*, 2003). The *mupA* gene, which has been cloned and sequenced, showed a low degree of homology with the staphylococcal *ileS* gene (Dyke *et al.*, 1991; Hodgson *et al.*, 1994). The deduced amino acid sequence indicated homology to the isoleucyl tRNA synthetase of *Escherichia coli* (Webster *et al.*, 1984; Dyke *et al.*, 1991), suggesting that mupirocin resistance might be the result of a modified isoleucyl tRNA synthetase.

Although several investigations have been conducted on mupirocin resistance among *S. aureus* strains in different parts of the world, there is no data on the characterization, genetic relatedness and clonal dissemination of mupirocin-resistant *S. aureus* in Nigeria and South Africa using phenotypic and molecular techniques. Characterization of strains in this study was determined by antibiotyping, resistotypng, PCR-RFLP of the coagulase gene and PFGE. Furthermore, the genetic location of mupH resistance and the ability to transfer the resistance determinant was determined by plasmid analysis, curing and conjugation experiments.

4.2 MATERIALS AND METHODS

The *S. aureus* isolates resistant to mupirocin based on the disk diffusion technique, their MIC values (E-test) and detection of the *mupA* gene by PCR were investigated (See Chapter Two). The protocol for genetic characterization (Plasmid DNA isolation, PCR-RFLP of the coagulase gene, PFGE typing) of strains was performed as described in Chapter three.

The banding patterns were interpreted visually and the relatedness of the strains was determined according to the recommendation of Tenover *et al.* (1995). In addition, the GelCompar II software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium) was used to calculate the Dice similarity indices and to perform a dendogram after cluster analysis by unweighted-pair-group-matching-analysis (UPGMA). Band position tolerance and optimization were set at 2%. By definition, two isolates belonged to the same cluster if their Dice similarity index is 85% or more. DNA fragments below 80kb

were not included in the analysis. Strains showing the same PFGE pattern were grouped in a pulsotype as described in Chapter three.

Two mupH strains (35IBA – Nigeria; RKK6 – South Africa) were selected for subsequent investigations involving curing and conjugation experiments. The resistance profiles of these strains are presented in Table 4.1.

4.2.1 Curing of resistance determinants on plasmids

The loss of resistance determinants (plasmids) was investigated according to the protocol of Udo *et al.* (2001b). The two strains were checked for purity, streaked on Brain Heart Infusion Agar (BHIA), and incubated at 43°C for 24 hours. Sub-culturing onto a freshly prepared BHIA was repeated twice after 24 hours of incubation. Serial dilutions of the cured strain was plated on BHIA plates, and incubated at 37°C for 24 and 48 hours.

4.2.2 Preparation of selection plates and replica plating

Selection plates containing BHIA with mupirocin (10μg/ml) was set up for strain 35IBA, while BHIA plates with erythromycin (5μg/ml) and mupirocin (10 μg/ml) was prepared for RKK6. Single colonies were screened for loss of resistance by the replica plating method. Colonies, which grew on the primary plate but did not grow on the selection plates, were noted and susceptibility testing of these colonies was performed in order to confirm loss of resistance. Colonies that lost antimicrobial resistance were screened by plasmid analysis for the presence or absence of the resistance determinants.

4.2.3 Conjugation Experiments

Plasmid transfer by conjugation was performed according to the protocol of Udo *et al.* (2001b). Strains 35IBA (Nigeria) and RKK6 (South Africa) served as donor strains, while WBG 541 (Townsend *et al.*, 1985) (resistant to fusidic acid and rifampicin), was the recipient strain. A 2ml volume of an overnight broth culture of the donor, recipient strains and mixture of the donor and recipient strains (2ml each) was dispensed into sterile universal bottles. The culture was then pelleted by centrifugation (2000rpm for 10 minutes), the supernatant discarded and 4ml of 40% polyethylene glycol (PEG) was added to each bottle and incubated (with gentle shaking) at 37°C overnight in a water bath. This was followed by centrifugation at 2000rpm for 10 minutes; 1ml of sterile Brain Heart Infusion Broth (BHIB) was added to each universal bottle, and vortexed gently to re-suspend the bacterial culture. Serial dilutions (undiluted, 10^{-1}, 10^{-2}, 10^{-3}) of the mixture (donor and recipient strains) and undiluted culture of the donor and the recipient strains (control experiments) were plated onto BHIA containing appropriate antibacterial agents, and incubated at 37°C for 18-24 hours. Transfer was considered to have occurred when growth was observed on the selection plates from donor-recipient mixtures and not from the control experiments. Based on their resistance profiles, transcipients were screened on BHIA plates containing mupirocin (10µg/ml), and fusidic acid (5µg/ml); erythromycin (5µg/ml) and fusidic acid (5µg/ml) for the experiment involving strain RKK6; mupirocin (10µg/ml) and fusidic acid (5µg/ml); tetracycline (5µg/ml) and fusidic acid (5µg/ml) for strain 35IBA. Single colonies of transconjugants were screened on BHIA containing appropriate antibiotics by the replica plating method. Sensitivity testing with selected

antibiotics was performed on transcipients and transfer frequency was expressed as the number of transconjugants per number of donor cells.

4.2.4 Partial DNA sequencing and analysis of the *ileS*-2 gene

The PCR products obtained in the detection of the *mupA* gene (as described in Chapter Two) were purified using the High Pure PCR purification kit (Roche, USA) according to the manufacturer's instructions. Partial DNA sequencing was performed using the forward primer sequence: *mupA*-1: TGA CAA TAG AAA AGG ACA GG, which amplified a 190bp segment of the *mupA* gene. Automated sequencing was performed using the Spectrumedix SCE2410 genetic analysis system with the BigDye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems). All reactions were performed according to the manufacturer's instructions. Chromatograms were analysed and edited using Chromas (version 2.3). An alignment of the DNA sequences was carried out by the ClustalX program (Thompson *et al.*, 1997) based on the nucleotide sequence of the *ileS*-2 gene in *S. aureus* J2870 (Hodgson *et al.*, 1994) submitted to the Gene Bank (Accession number X75439).

4.3 RESULTS

4.3.1 Antibiogram of low and high-level mupirocin resistant *S. aureus* strains

The results of the disk susceptibility testing with mupirocin disks (5 and 200 μg/ml) revealed that 14 isolates from six health institutions in South Africa expressed low-level resistance to mupirocin. These included 11 strains from wound samples and one strain each from blood, urine specimens and endotracheal aspirate. Low-level mupirocin resistance was confirmed by E-test, with MICs values between 8 and 24μg/ml. Five strains had MICs of 12μg/ml, four with MICs of 8 and 24μg/ml respectively and one strain had an MIC of 16μg/ml (Table 4.1).

The antibiotypes of the mupirocin-resistant strains based on their resistance pattern to antibiotics representing various groups of antibacterial agents are presented in Table 4.1. All the low-level mupirocin resistant strains were MRSA with 100% resistance to gentamicin, tetracycline and trimethoprim. A total of twelve strains were resistant to erythromycin, ten to ciprofloxacin and four to rifampicin and chloramphenicol respectively. Resistotyping revealed that six low-level mupirocin/methicillin resistant strains exhibited resistance to cadmium acetate, propamidine isethionate, mercuric chloride and ethidium bromide, four to mercuric chloride, two to cadmium acetate and mercuric chloride, and one to cadmium acetate. One strain was susceptible to the heavy metals and nucleic-acid binding compounds. The *mupA* gene was not detected in the low-level strains. High-level mupirocin resistance was detected in two strains (one MSSA and one MRSA) from South Africa and one MSSA strain from Nigeria. This resistance phenotype was confirmed by E-test (>1024μg/ml), and detection of the *mupA* gene by PCR (Table 4.1; Figure in Appendix).

The methicillin/mupirocin resistant strain from South Africa was obtained from a wound culture while the mupirocin resistant strain from Nigeria was recovered from a blood culture. Information on the methicillin-susceptible mupirocin-resistant strain from South Africa was not available.

4.3.2 Genotyping (PCR-RFLP of the coagulase gene, PFGE) of low and high-level mupirocin resistant *S. aureus* strains

The PCR-RFLP analysis of the coagulase gene is presented in Tables 4.1 and Figures 4.1 and 4.2. PCR amplification of the coagulase gene revealed a single amplicon of 650bp, 800bp and 850bp among the low-level mupirocin-resistant strains. RFLP patterns of two fragments of 81bp, 567bp were observed in four strains, of 325bp, 405bp in three strains, and of 81bp, 324bp, 405bp in seven strains. The sizes of the PCR products for the high-level mupirocin-resistant strains were 650, 750 and 800bp respectively and three RFLP patterns (81bp, 567bp; 243bp, 486bp and 81bp, 162bp, 567bp) were identified.

The methicillin/low-level mupirocin resistant strains were grouped based on the PFGE types as described in Chapter three. Two main PFGE patterns, designated types b and d were identified among the low-level mupirocin resistant strains (Figure 4.3). The PFGE types included subtype d1 with nine strains; subtype b4 with three strains and one strain in subtypes b1 and d2 (Table 4.1). The high level mupirocin-resistant strains were grouped into three PFGE patterns (a3, k and i1) (Table 4.1; Figure 4.4). DNA patterns of the methicillin/mupH resistant MRSA strain revealed that it differed by two to three bands from the low-level mupirocin resistant strains assigned to PFGE subtypes b1 and

b4, indicating that these strains are closely related. A three-band difference was also observed between one of the MRSA (strain 15) and the high-level mupirocin MSSA strain (35IBA) from Nigeria (Figure 4.4; Lanes 3 and 8).

Table 4.1: Characterization (antibiogram, PCR-RFLP of the coagulase gene and PFGE) of low and high-level mupirocin resistant *S. aureus* from Nigeria and South Africa

Strain No	Resistance pattern (Antibiogram and Resistotyping)	*mupA* gene	MIC E-test (µg/ml)	Molecular weight (± 20bp)	RFLP (bp)	PFGE type
AD 98	PEN, OX, GN, TET, TS, RF, MU (L), Cad, Hg	-	24	650	81, 567	b4
AD 79	PEN, OX, GN, TET, TS, RF, MU (L)	-	24	650	81, 567	b4
ESH 34	PEN, OX, GN, ERY, TET, TS, RF, MU (L), Cad	-	24	650	81, 567	b1
KEH 12	PEN, OX, GN, ERY, TET, TS, RF, MU (L), Cad, Hg	-	24	650	81, 567	b4
513	PEN, OX, GN, ERY, TET, TS, CIP, MU (L), Cad, Pi, Hg, Eb	-	8	800	81, 324, 405	d1
RKK 8	PEN, OX, GN, ERY, TET, TS, CIP, MU (L), Cad, Pi, Hg, Eb	-	8	800	81, 324, 405	d1
RKK 57	PEN, OX, GN, ERY, TET, TS, CIP, MU (L), Cad, Pi, Hg, Eb	-	12	800	81, 324, 405	d1
GP 11	PEN, OX, GN, ERY, CHL, TET, TS, CIP, MU (L), Cad, Pi, Hg, Eb	-	8	800	81, 324, 405	d1
KEH 26	PEN, OX, GN, ERY, CHL, TET, TS, CIP, MU (L), Hg	-	12	800	81, 324, 405	d1
RKK 55	PEN, OX, GN, ERY, TET, TS, CIP, MU (L), Hg	-	12	850	81, 324, 405	d2
AD 28	PEN, OX, GN, ERY, CHL, TET, TS, CIP, MU (L), Hg	-	12	800	324, 405	d1
AD 87	PEN, OX, GN, ERY, TET, TS, CIP, MU (L), Cad, Pi, Hg, Eb	-	16	850	324, 405	d1
510	PEN, OX, GN, ERY, CHL, TET, TS, CIP, MU (L), Cad, Pi, Hg, Eb	-	12	800	324, 405	d1
RKK 56	PEN, OX, GN, ERY, TET, TS, CIP, MU (L), Hg	-	8	800	81, 324, 405	d1
RKK6	PEN, OX, GN, ERY, TET, TS, RF, MU (H), Cad	+	>1024	650	81, 567	a3
P1929*	TM, TET, MU (H)	+	>1024	750	243, 486	k
35 IBA**	TET, MU (H)	+	>1024	800	81, 162, 567	i1

*Strain not screened for resistance to heavy metals and nucleic acid binding compounds
**Strain from Nigeria

KEY
PEN – Penicillin (β-lactams)
OX – Oxacillin (β-lactams)
GN – Gentamicin (aminoglycoside)
ERY – Erythromycin (macrolide)
CHL – Chloramphenicol (phenicols)
MU (L) - Low-level mupirocin resistance
TS – Trimethoprim (sulphonamides)
RF – Rifamipicin (ansamycins)
CIP – Ciprofloxacin (fluoroquinolones)
TET – Tetracycline (tetracyclines)
MU (H) - High-level mupirocin resistance
Heavy metals – Cad: Cadmium acetate; Hg: Mercuric chloride
Nucleic acid binding compounds – Eb: Ethidium bromide; Pi: propamidine isethionate

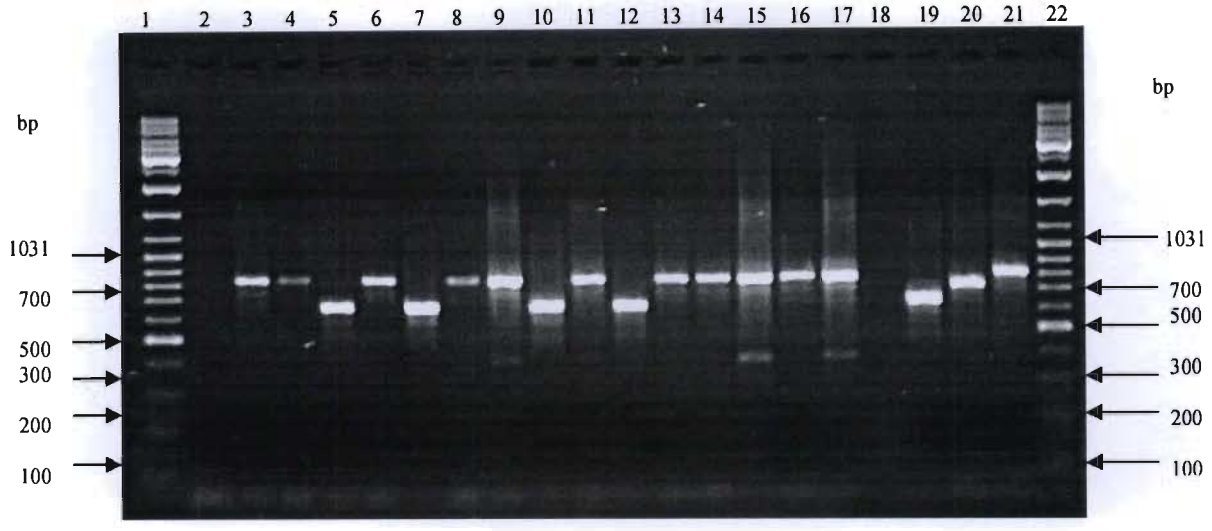


Figure 4.1: PCR detection of the coagulase gene in mupirocin-resistant strains. Lanes 2 and 3: negative and positive controls. Lanes 4-17: Low-level mupirocin (mupL) resistant MRSA strains: AD28, AD79, AD87, AD98, 510, 513, ESH34, GP11, KEH12, KEH 26, RKK8, RKK55, RKK56, RKK57. Lanes 19-21: High-level mupirocin (mupH) resistant strains: RKK6, P1929 (South Africa), 35IBA (Nigeria). Lanes 1 and 22 are molecular weight markers.

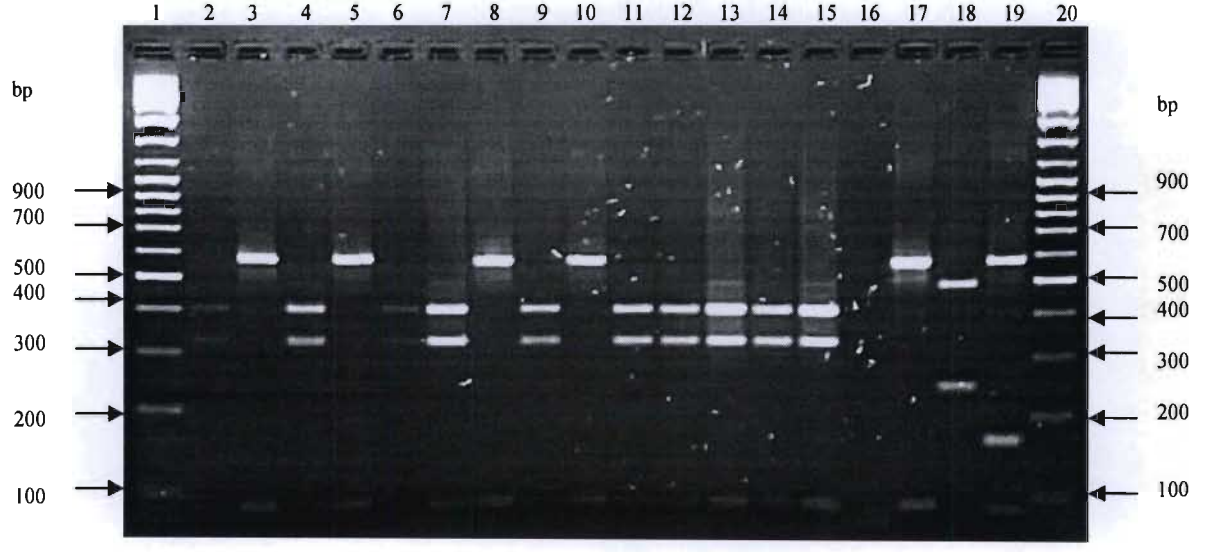
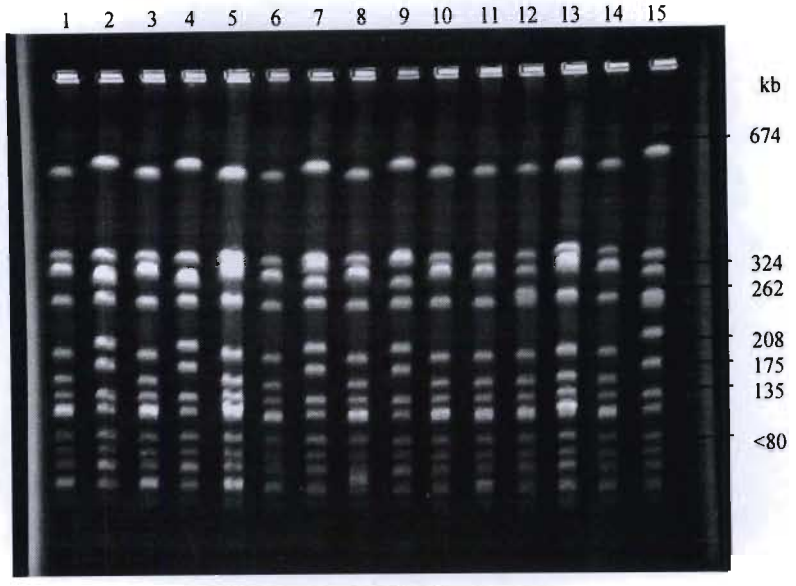
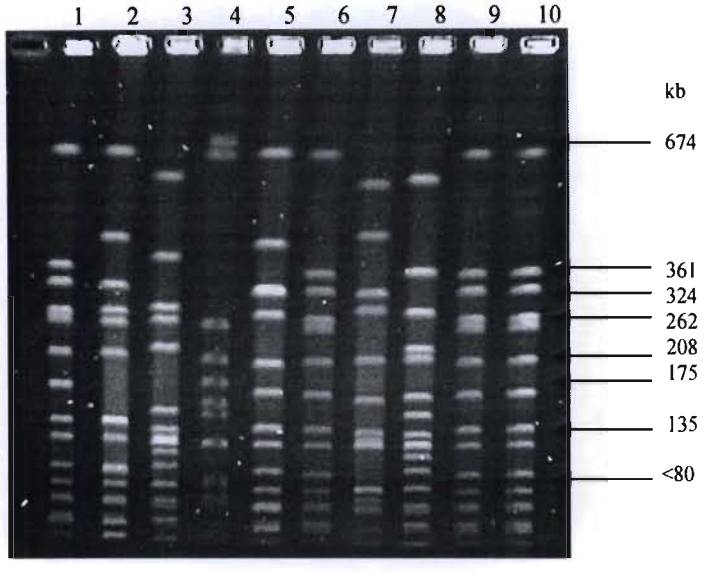


Figure 4.2: PCR-RFLP of the coagulase gene in mupirocin resistant strains. Lanes 2-15: mupL strains: AD28, AD79, AD87, AD98, 510, 513, ESH34, GP11, KEH12, KEH26, RKK8, RKK55, RKK56, RKK57. Lanes 17-19: mupH strains: RKK6, P1929 (South Africa), 35IBA (Nigeria). Lanes 1 and 20 are molecular weight markers.



PFGE types d1 b4 d1 b4 d1 d1 b1 d1 b4 d1 d1 d2 d1 d1

Figure 4.3: PFGE patterns of low-level mupirocin-resistant strains. Lanes 1-14: AD28, AD79, AD87, AD98, 510, 513, ESH 34, GP11, KEH12, KEH26, RKK8, RKK55, RKK56, RKK57. Lane 15: Molecular weight standard *S. aureus* NCTC 8325.



PFGE types a5 i j a3 k i1

Figure 4.4: PFGE profiles of MRSA/High-level mupirocin resistant strains from Nigeria and South Africa. Lanes 1, 6, 9 and 10: *S. aureus* NCTC 8325; Lane 2: THCD; Lane 3: 15; Lane 4: 28IDA; Lane 5: RKK6 (high-level mupirocin resistant strain); Lane 7: P1929 (high-level mupirocin resistant strain); Lane 8: 35IBA (high-level mupirocin resistant strain).

4.3.3 Curing experiment

To isolate the plasmids and determine the location of resistance determinants, two high-level mupirocin resistant strains - 35IBA (Nigeria) and RKK6 (South Africa) were selected and used in curing and conjugation experiments. In the curing experiment, six of the 293 colonies (2.0%) screened for strain 35IBA were found to have lost resistance to mupirocin but exhibited resistance to tetracycline. Plasmid analysis indicated that this was accompanied by the loss of approximately a 33-kb plasmid in the colonies screened by agarose gel electrophoresis (Figure 4.5). Of the 294 colonies screened on mupirocin and erythromycin selection plates for strain RKK6, loss of resistance to erythromycin and mupirocin was observed in three and six colonies respectively. Erythromycin and mupirocin resistance in the cured strains of RKK6 was lost together with a plasmid of approximately 2.3-kb and 37-kb respectively (Figure 4.5). Identical PFGE profiles were observed in the parent and cured strains, indicating that the *mupA* gene was not located on the chromosome of the two strains studied (Figure 4.6).

4.3.4 Conjugation experiment

No transconjugant was obtained on selection plates for strain 35IBA by the polyethylene glycol method. However, 120 colonies were identified from donor-recipient mixtures on BHIA plates containing mupirocin (10μg/ml), and fusidic acid (5μg/ml) for strain RKK6. In addition, two colonies were noted from donor-recipient mixtures on the selection plates containing erythromycin (5μg/ml) and fusidic acid (5μg/ml). Replica plating of 98 colonies (transconjugants) on BHIA selection plates containing cadmium (5μg/ml), erythromycin (5μg/ml) and mupirocin (10μg/ml) yielded one, three and 98

colonies respectively. Susceptibility pattern of the parent and cured strains along with the transconjugants are presented in Table 4.2. The transfer frequency was calculated as 1.2 x 10^{-5} and 2 x 10^{-8} for mupirocin and erythromycin resistance determinants respectively.

Susceptibility testing of transcipients and plasmid analysis indicated that transconjugants resistant only to mupirocin carried a plasmid of approximately 37kb (Figure 4.7). However, seven fragments (12.6, 10.9, 6.3, 4.8, 2.5, 2.3 and 1.7kb) were obtained in the transconjugant (transMup) with the mupirocin plasmid by *Eco*RI restriction analysis, indicating that the size of the mupirocin plasmid was 41.1kb (Figure 4.8). A total of nine fragments (17.3, 12.6, 10.9, 6.3, 4.8, 2.7, 2.5, 2.3, 1.7 kb) were obtained from restriction analysis of the transconjugant with the plasmids associated with resistance to mupirocin and cadmium (transCad). Apart from the seven fragments that were common to the two transconjugants, two fragments of 17.3 and 2.7kb were identified in transCad, indicating that the size of the cadmium plasmid is about 20-kb (Figure 4.8). Furthermore, transcipients exhibiting resistance to erythromycin carried a 2.3-kb plasmid (Figure 4.5).

Table 4.2: Susceptibility profile of parent, cured, recipient strains and transconjugants derived from strain RKK6.

Isolate	Resistance pattern	MIC E-test (μg/ml)	*mupA* gene
35 IBA (parent)	Tet^R Mup^R	>1024	+
35 IBA (yellow) – cured strain	Tet^R	ND	-
RKK 6 (parent)	Ox^R, Ery^R, Cd^{Ri}, Rp^R, Tet^R, Mup^R	>1024	+
RKK 6^C A (cured strain of RKK6)	Ox^R, Rp^R, Tet^R, Mup^R	>1024	+
RKK 6^C D (cured strain of RKK6)	Ox^R, Ery^R, Cd^{Ri}, Rp^R	ND	-
Trans Mup (transconjugant of RKK6)	Rp^R, Fc^R, Mup^R	>1024	+
Trans Ery (transconjugant of RKK6)	Ery^R, Cd^{Ri}, Rp^R, Fc^R	ND	-
Trans Cad (transconjugant of RKK6)	Rp^R, Fc^R, Cad^R, Mup^R	>1024	+
WBG 541 (recipient strain)	Rp^R, Fc^R	ND	-

Tet – Tetracycline; Mup – Mupirocin; Ox – Oxacillin; Ery – Erythromycin;
Cd^{Ri} – Clindamycin (inducible resistance); Rp – Rifampicin; Fc – Fusidic acid; Cad – Cadmium
The superscript R represents resistance.
ND – Not determined

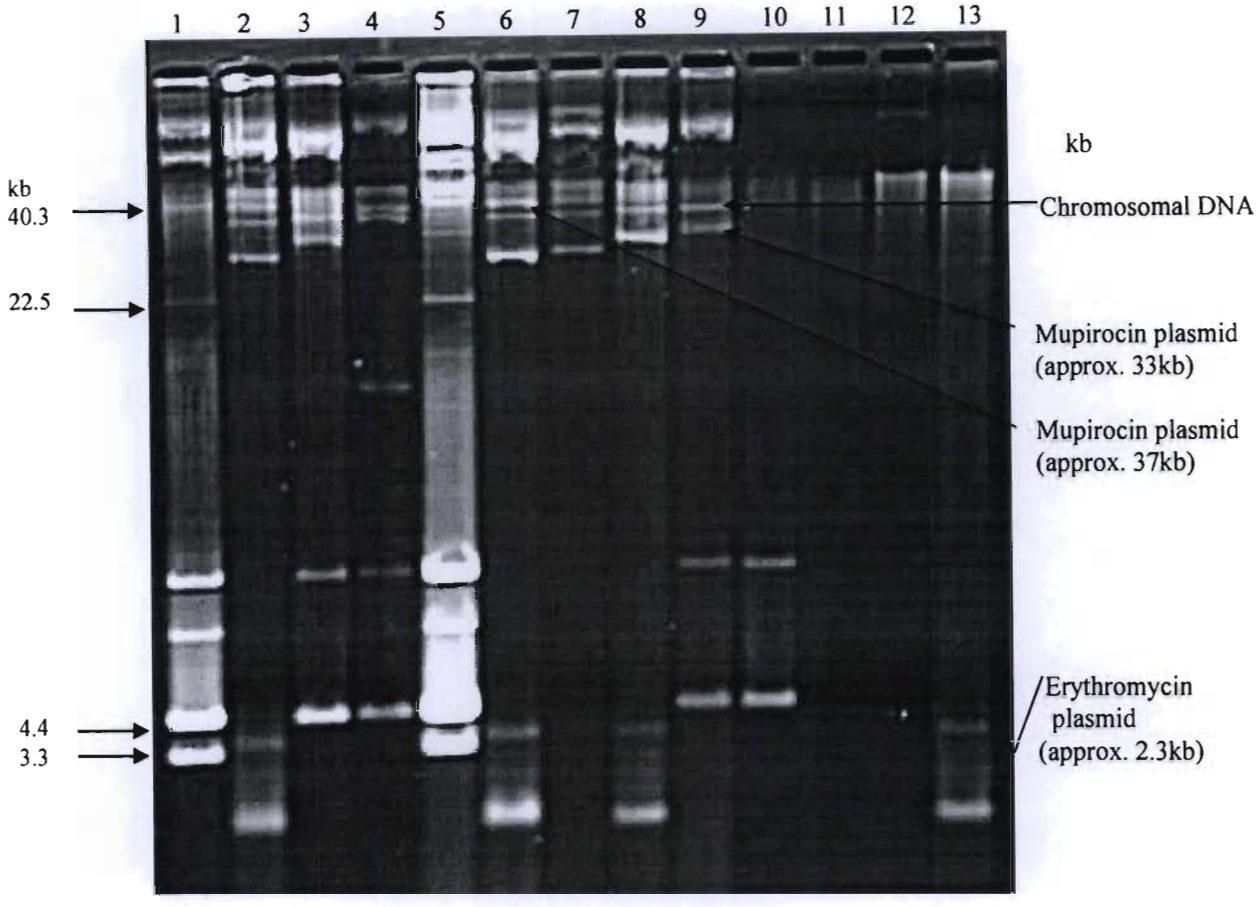


Figure 4.5: Plasmid profile of parent, cured strains and transconjugants: Only closed circular (CCC) DNA are labelled. Lanes 1 and 5: WBG 4483; Lanes 2-4: High level mupirocin strains Lane 2 - RKK6 (South Africa), Lane 3 - 35IBA (Nigeria), Lane 4 - P1929 (South Africa); Lane 6: RKK6 parent strain; Lane 7 - RKK6^{c}A (cured strain of RKK6 – resistant to mupirocin, susceptible to erythromycin); Lane 8 – RKK6^{c}D (cured strain of RKK6 – susceptible to mupirocin, resistant to erythromycin); Lane 9 - 35IBA parent; Lane 10 – 35IBA yellow - cured strain of 35IBA (susceptible to mupirocin, resistant to tetracycline); Lane 11 – Trans (Mup) - transconjugant of RKK6 (resistant to mupirocin only); Lane 12 – Trans (Cad) transconjugant of RKK6 (resistant to mupirocin and cadmium); Lane 13 – Trans (Ery) transconjugant of RKK6 (resistant to erythromycin).

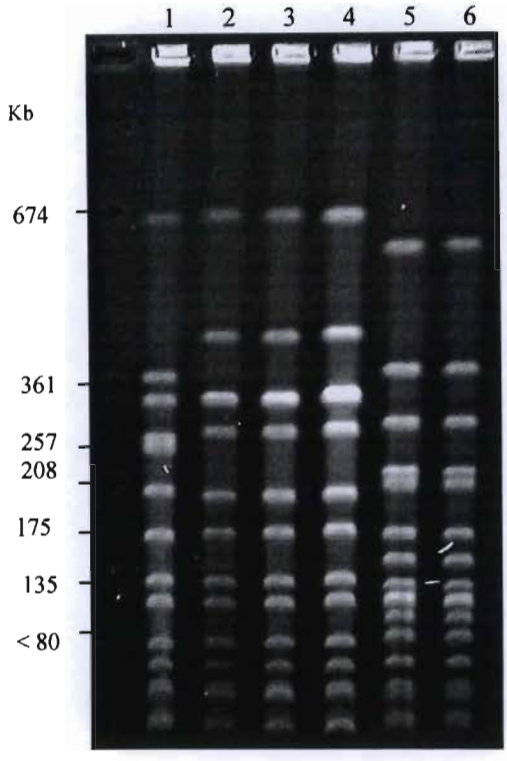


Figure 4.6: PFGE patterns of high-level mupirocin resistant strains - parent and cured strains. Lane 1: *S. aureus* NCTC 8325; Lane 2: RKK6 (parent strain); Lane 3 - RKK6^{c}A (cured strain of RKK6 – resistant to mupirocin, susceptible to erythromycin); Lane 4 – RKK6^{c}D (cured strain of RKK6 – susceptible to mupirocin, resistant to erythromycin); Lane 5 - 35IBA parent strain; Lane 6 – 35IBA yellow - cured strain of 35IBA (susceptible to mupirocin, resistant to tetracycline).

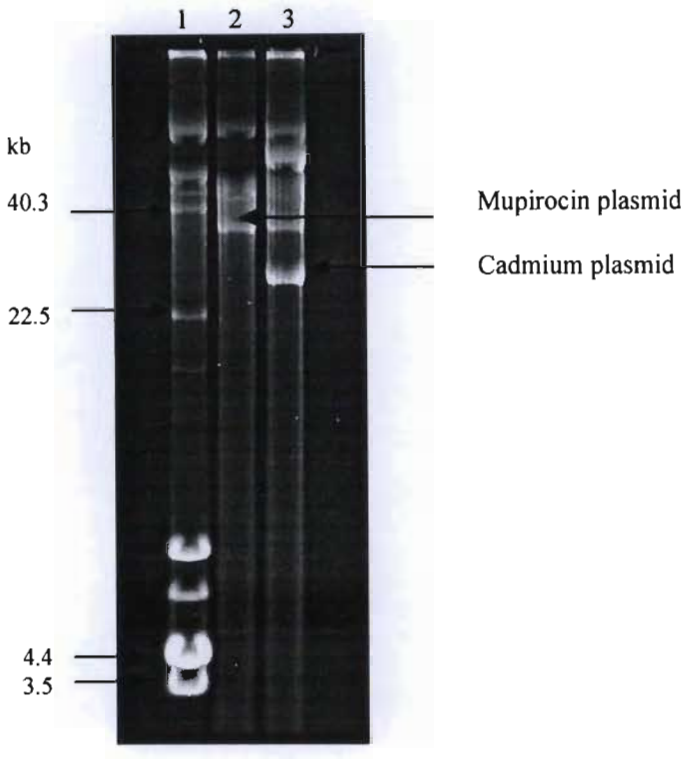


Figure 4.7: Plasmid profile of transconjugants derived from the methicillin/mupirocin resistant *S. aureus* (strain RKK6) from South Africa. Lane 1 - WBG 4483 (only the closed circular forms of the plasmids are labelled); Lane 2 – Trans (Mup) - transconjugant of RKK6 (resistant to mupirocin only); Lane 3 – Trans (Cad) transconjugant of RKK6 (resistant to mupirocin and cadmium).

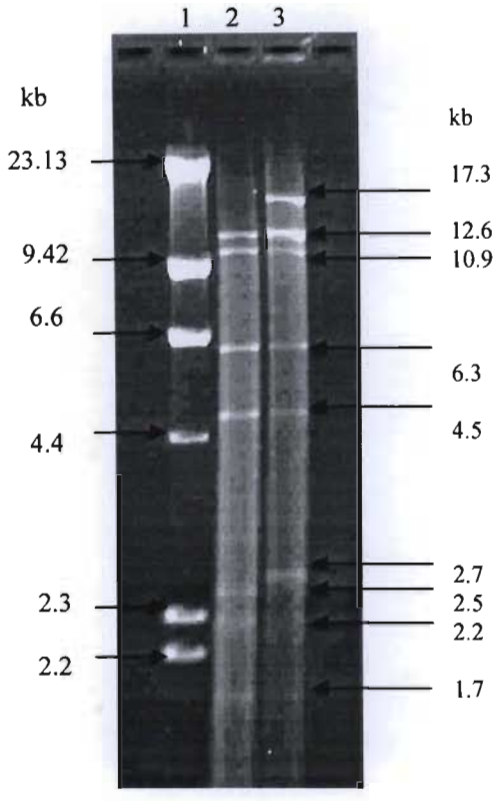


Figure 4.8: *EcoR*I restriction analysis of transconjugants derived from the methicillin/mupirocin resistant *S. aureus* strain from South Africa. Lane 1 – Molecular weight standard, phage lambda DNA digested with *Hind*III; Lane 2 – Trans (Mup) - transconjugant of RKK6 (resistant to mupirocin only); Lane 3 – Trans (Cad) transconjugant of RKK6 (resistant to mupirocin and cadmium).

4.3.5 Partial DNA sequence analysis of the *ileS*-2 gene

The alignment of partial DNA sequences of the *ileS*-2 gene in *S. aureus* J2870, the high-level mupirocin-resistant *S. aureus* strains and one of the transconjugants (transMup) obtained in this study is presented in Figure 4.9. The analysis indicated that the *ileS*-2 gene of *S. aureus* strains from South Africa and the transconjugant was identical to that of *S. aureus* J2890. However, two base substitutions (nucleotide positions $641_{A\ to\ C}$; $671_{A\ to\ T}$) were observed in the MSSA strain 35IBA from Nigeria. The first base substitution (nucleotide positions $641_{A\ to\ C}$; AAG to CAG) did not lead to a change in the amino acid arginine. However, the second base substitution ($671_{A\ to\ T}$; ATT to TTT) indicated a change in the amino acid sequence of the *ileS*-2 gene in *S. aureus* J2870 from leucine to phenylalanine (Figure 4.10).

```
*MUPgene     TGACGGCCCCCCAACTGCAAATGGCCTTCCTCATGCTGGCCATGTTCTTG
RKK6         TGACGGCCCCCCAACTGCAAATGGCCTTCCTCATGCTGGCCATGTTCTTG
TransMUP     ---CGGCCCCCCAACTGCAAATGGCCTTCCTCATGCTGGCCATGTTCTTG
35IBA        TAGCGGCCCCCCAACTGCAAATGGCCTTCCTCATGCTGGCCATGTTCTTG
P1929        TGACGGCCCCCCAACTGCAAATGGCCTTCCTCATGCTGGCCATGTTCTTG

MUPgene      GAAGAGTAATCAAGGATTTA-GTTGCAAGATTAAAAACTATGC-AAGGTT
RKK6         GAAGAGTAATCAAGGATTTA-GTTGCAAGATTAAAAACTATGC-AAGGTT
TransMUP     GAAGAGTAATCAAGGATTTA-GTTGCAAGATTAAAAACTATGC-AAGGTT
35IBA        GCAGAGTAATCAAGGATTTA-GTTGCAAGATTTAAAACTATGC-AAGGTT
P1929        GAAGAGTAATCAAGGATTTA-GTTGCAAGATTAAAAACTATGC-AAGGTT

MUPgene      TTTATGTAGAAAGAAAAGC-AGGATGGGATACCCA-TGGCTTACCAGTTG
RKK6         TTTATGTAGAAAGAAAAGC-AGGATGGGATACCCA-TGGCTTACCA----
TransMUP     TTTATGTAGAAAGAAAAGC-AGGATGGGATACCCA-TGGCTTACCA----
35IBA        TTTATGTAGAAAGAAAAGC-AGGATGGGATACCCA-TGGCTTACCA----
P1929        TTTATGTAGAAAGAAAAGC-AGGATGGGATACCCA-TGGCTTACCA----
```

* Partial DNA sequence of the *ileS*-2 gene of *S. aureus* J2890

Figure 4.9: Alignment of DNA sequences of the *ileS*-2 gene in *S. aureus* J2870 (nucleotide positions 590 to735) and the high-level mupirocin-resistant *S. aureus* strains and transconjugant obtained in this study. The nucleotide sequence coloured black indicate regions of base substitutions in strain 35IBA from Nigeria.

```
Mup gene
G P P T A N G L P H A G H V L G R V I K D L V A R L K T Met Q G F
Y V E R K A G W D T H G L P

RKK 6
G P P T A N G L P H A G H V L G R V I K D L V A R L K T Met Q G F
Y V E R K A G W D T H G L P

35IBA
G P P T A N G L P H A G H V L G R V I K D L V A R F K T Met Q G F
Y V E R K A G W D T H G L P
```

Figure 4.10: Amino acid sequences of the gene products (nucleotide positions 595 to 730). The amino acid sequence coloured black indicate change in amino acid from leucine (*ileS*-2 gene in *S. aureus* J2870 and RKK6) to phenylalanine in strain 35IBA (Nigeria).

4.4 DISCUSSION

This study investigated the emergence and characterization of low and high-level mupirocin resistant *S. aureus* strains in Nigeria and South Africa, using phenotypic and molecular methods. Clinical isolates of mupirocin-resistant *S. aureus* was reported in 1987 (Rahman *et al.*, 1987), and resistance has frequently been attributed to the clinical use of mupirocin over extended periods (Udo *et al.*, 1998) or in areas of highly concentrated application, such as dermatology or burns units (Eltringham, 1997; Poupard, 1995). In this study, a total of 17 strains were resistant to mupirocin and 16 (94%) of these strains were obtained from South Africa (Table 4.1). The low-level resistant strains were MRSA and recovered from six health institutions in the KwaZulu-Natal (KZN) province of South Africa. Mupirocin is prescribed and administered in the treatment of MRSA infections and *S. aureus* nasal colonization among hospital patients in KZN in South Africa, and it appears that selective pressure of mupirocin use could have contributed to what appears to be an emerging trend. The reports on the increase in failure rates for nasal decolonization of MRSA due to the emergence of low-level mupirocin resistance (Harbath *et al.*, 2000; Watanabe *et al.*, 2001a; Decousser *et al.*, 2003) should be of serious concern as no low-level mupirocin resistance MRSA had previously been reported in the KZN province in South Africa. However, the low-level mupirocin resistant MRSA were susceptible to teicoplanin, vancomycin, fusidic acid, linezolid, fosfomycin, and quinipristin-dalfopristin (Chapter Two).

S. aureus strains exhibiting high-level mupirocin resistance were detected in both countries. They consisted of a MSSA and one MRSA in Hospitals B and C in Durban, South Africa, and one MSSA from Hospital F, Ibadan, Nigeria (Chapter Two: Table 2.1;

Table 4.1). An important finding is that mupirocin is not prescribed or administered in the hospital in Ibadan, Nigeria, where the high-level strain was obtained. Data exists that high-level mupirocin resistant isolates of *S. aureus* and *S. epidermidis* have been isolated in Nigeria as early as 1956 (Cookson, 1994), before the introduction of mupirocin in clinical practice. Other workers have also reported mupirocin resistant *S. aureus* without any apparent exposure to the agent (Cookson *et al.*, 1990; Hefferman *et al.*, 1995). This could be due to the fact that mupirocin is a natural product to which microorganisms have presumably been exposed in nature (Chatfield *et al.*, 1994). Although the high-level mupirocin-resistant strains were present in low numbers, they were not detected prior to this study because susceptibility testing for mupirocin resistance was not performed in hospitals in both countries. Few data are available on mupirocin resistance in MSSA isolates (Leski *et al.*, 1999; Jones *et al.*, 2003; Yun *et al.*, 2004; Chaves *et al.*, 2004; Kresken *et al.*, 2004; Petinaki *et al.*, 2004). However, the isolation of MSSA resistant to mupirocin indicates that MSSA should also be routinely tested for mupirocin resistance in both countries. This is to allow for early detection of resistant isolates and to facilitate the early institution of infection control measures.

Characterization of the mupirocin-resistant strains was determined using antibiogram, PCR-RFLP of the coagulase gene and PFGE. PCR of the coagulase gene in low-level mupirocin resistant strains revealed that the dominant type (10 of 14 low-level mupirocin resistant strains) produced a single amplicon of 800 or 850bp with similar RFLP patterns (Table 4.1; Figures 4.1 and 4.2). Two main PFGE types (b and d) were identified in the low-level mupirocin resistant strains studied and type d was identified as the dominant clone (Table 4.1; Figure 4.3). Some degree of correlation was observed in the

analysis of the three typing methods (antibiogram, PCR-RFLP and PFGE). The low-level mupirocin resistant MRSA strains with a PCR product of 650bp (RFLP pattern: 81, 567bp) belonged to the main clone b by PFGE typing. There was good correlation between antibiogram and the two genotyping methods, although an additional resistance character (resistance to erythromycin) was noted in two strains in PFGE type b (Table 4.1). MRSA strains with PCR-RFLP pattern (81, 324, 405bp; 324, 405bp) had similar resistance pattern, except for four strains, in which resistance to chloramphenicol was also observed. PFGE typing indicated that these strains belonged to the main dominant clone d. The low-level mupirocin resistant strains with the PFGE type b were observed in two health institutions in Durban and one in Eshowe while strains with type d were noted in three health facilities located in Durban, one in Pietermaritzburg, Greytown and Empangeni (Chapter two; Table 2.1 and Table 4.1). MRSA strains in clone d were resistant to at least eight classes of antibiotics. The emergence and spread of low-level mupirocin resistance in MRSA has been reported in a community hospital in Japan (Watanabe *et al.*, 2001b). This study has demonstrated that clonal dissemination of multiresistant MRSA strains exhibiting low-level resistance to mupirocin exists in health institutions in KwaZulu-Natal province of South Africa.

Analysis using the three typing methods indicated that the high-level mupirocin-resistant strains did not arise from the same clone (Figure 4.4). However, DNA patterns of the methicillin/mupH resistant MRSA strain showed that it differed by two to three bands from the low-level mupirocin resistant strains assigned to PFGE subtype b1 and b4, indicating that these strains are related (Chapter Three). Moreover, a three-band difference was also detected between one MRSA (strain 15) and the high-level mupirocin

MSSA strain (35IBA) from Nigeria indicating that they are genetically related (Figure 4.4). The ability of MRSA strains in main clone a (in which the mupirocin/methicillin resistant *S. aureus* strain belonged) to be transmitted over great distances (Chapter Three), the establishment of clone d in hospitals in Durban, clearly indicate that urgent measures need to be taken to prevent clonal dissemination of the mupirocin/methicillin resistant *S. aureus* in KZN, South Africa. Furthermore, the discovery of the mupirocin resistant strain from a health facility in Nigeria in which the antibiotic is not administered or prescribed, and the relatedness of one MRSA with the mupH MSSA indicate that more studies are needed in understanding the genetics and evolution of mupirocin resistance of *S. aureus*, in Nigeria.

Plasmid-mediated resistance to antimicrobial agents among pathogenic bacteria constitutes a major clinical and economic problem worldwide, which has been the subject of extensive genetic and biochemical studies. Plasmid transfer *in vitro* is used to characterize plasmids and establish the genetics and spread of plasmid-linked resistance genes. Multiresistant *S. aureus* isolates commonly harbour two or more plasmids that often vary in size and resistance phenotypes. In such circumstances, an effective study of plasmid-linked resistance determinants depends upon the successful transfer of plasmids from resistant isolates to suitably marked sensitive, and plasmid free recipients (Udo *et al.*, 1991). In *S. aureus*, mixed-culture transfer, phage mediated conjugation and conjugation can transfer resistance determinants in vitro (Lacey 1980; Archer and Johnston, 1983; Forbes and Schaberg, 1983; McDonnell *et al.*, 1983; Townsend *et al.*, 1985).

In the late 1970s and early 1980s, studies on the transfer of plasmids mediating gentamicin resistance between strains and between species of staphylococci firmly

established conjugative transfer as a mechanism whereby resistance genes borne on plasmids could be disseminated in staphylococci (Naidoo and Noble, 1978; Naidoo and Noble, 1981; Forbes and Schaberg, 1983; McDonnell *et al.*, 1983). In this study, the genetic location of the high-level mupirocin resistance was determined by plasmid analysis, involving curing and conjugation experiments. Three features were identified in the transfer experiments and plasmid analysis of strain RKK6 that harboured the mupirocin plasmid. The first feature was the transfer of the plasmid mediating mupirocin resistance (Table 4.2; Figure 4.7). A total of seven fragments were detected by *Eco*RI restriction analysis in the transconjugant harbouring the mupirocin plasmid, indicating that the size of the mupirocin plasmid was 41.1kb (Figures 4.8). This was higher than the estimated size of 37kb calculated from the undigested plasmid DNA (Figure 4.5). As the 41.1-kb high-level mupirocin plasmid mediated its own transfer in conjugation experiments, it fits the description of a conjugative plasmid. No transconjugants were obtained on selection plates for strain 35IBA (Nigeria) by the polyethylene glycol method, suggesting that the mupirocin plasmid is not non-self-transmissible or that other conditions are needed. Phage-mediated conjugation was however, not performed on the mupirocin-resistant strain. Some authors have suggested that high-level mupirocin resistance genes reside on transposons (Rahman *et al.*, 1989; Cookson, 1990; Slocombe and Perry, 1991) and the first report of the chromosomal location of the *mupA* gene in *S. aureus* expressing high-level mupirocin resistance have been described (Udo *et al.*, 2003). In this study, PFGE analysis of the parent and cured strains indicated that the *mupA* was plasmid-mediated (Figure 4.6). High-level mupirocin resistance has been found in self-transmissible and non-self transmissible plasmids in different countries (Rahman *et al.*, 1989; Udo *et al.*,

1994; Connolly *et al.*, 1993; Udo *et al.*, 1997), and this study has demonstrated what appears to be the first report of a conjugative mupirocin plasmid in South Africa and a non-conjugative mupirocin plasmid in Nigeria.

Although staphylococcal gentamicin resistance (Townsend *et al.*, 1985; Projan and Archer, 1989) and the cryptic (Udo *et al.*, 1991; Udo *et al.*, 1992) conjugative plasmids have been known to mobilize non-conjugative plasmids, conjugative transfer of plasmids mediating mupirocin resistance have been found to encompass the co-transfer of small non-conjugative plasmids encoding resistance to tetracycline, gentamicin and chloramphenicol (Needham *et al.*, 1994; Udo *et al.*, 1997; Udo *et al.*, 1998; Udo *et al.*, 2001a; Udo *et al.*, 2001b) and large plasmids encoding resistance to penicillin (Pawa *et al.*, 2000). The transfer of resistance determinants mediating mupirocin and triclosan resistance in MRSA has also been reported (Cookson *et al.*, 1991). The second feature observed in this study was the transfer of the 41.1-kb plasmid, which accompanied transfer of the high-level mupirocin resistance and the co-transfer of what appears to be a 20-kb plasmid encoding cadmium resistance. It is proposed that the 41.1-kb plasmid belongs to a class of mupirocin resistance conjugative plasmids that are capable of mobilizing certain staphylococcal non-conjugative and conjugative plasmids (Projan and Archer, 1989; Udo *et al.*, 1991; Udo *et al.*, 1992). The third feature was the transfer of the plasmid mediating erythromycin resistance, indicated by the carriage of a 2.3-kb plasmid. The demonstration of conjugative transfer of the mupirocin plasmid and co-transfer of additional resistance markers clearly support the judicious use of this topical antibiotic in health institutions in South Africa. This should be considered prior to incorporating mupirocin into an infection control program. In hospital settings in which mupirocin might be used on a broad basis, such as in the control

of certain postoperative wound infections, it would be essential to monitor the emergence of mupirocin-resistant strains.

Preliminary investigations using partial sequence analysis revealed that the *ileS*-gene of strains RKK6, P1929, TransMup (transconjugant of RKK6) and *S. aureus* J2870 was identical (Figure 4.9). However, alignment of the *ileS*-gene in 35IBA and *S. aureus* J2870 revealed a base substitution (nucleotide position $671_{A\ to\ T}$; ATT to TTT) in the DNA sequence of the mupH strain from Nigeria, leading to a change in the amino acid sequence from leucine to phenylalanine (Figure 4.9). Future studies involving full sequence analysis of *ileS*-2 gene in strain 35IBA will probably provide new insights on the evolution of the *ileS*-2 gene in *S. aureus*.

4.5 CONCLUSION

The emergence of mupirocin resistance in both countries and the potential loss of one of the major weapons in MRSA infection control emphasize the need that prolonged and widespread use of mupirocin in healthcare facilities should be discouraged. MRSA eradication strategies should be designed carefully with reliable laboratory screening for resistance and periodic antibiotic prescribing and infection control audits. In this way, if resistance does emerge, it is more likely to be detected rapidly, and action taken early and effectively to minimize spread. Any agent should not be used as a substitute for poor infection control and antibiotic prescribing practices, but as part of an overall policy developed, audited and reviewed by the local relevant health care workers. It is also recommended that routine testing of MSSA and MRSA for mupirocin resistance be conducted even in facilities where mupirocin is not being used, because mupirocin-resistant strains can be introduced into such facilities, where it can spread among patients. This will facilitate the early detection of resistance and can help control the spread of mupirocin-resistant MRSA.

Although the possibility of horizontal transfer of this conjugative plasmid among *Staphylococcus spp* was not established, there is evidence that a larger pool of mupirocin resistance exists in coagulase-negative staphylococci than in *S. aureus* in many countries (Deshpande *et al.*, 2002; Yun *et al.*, 2004; Kresken *et al.*, 2004; Petinaki *et al.*, 2004). The former may act as a reservoir for high-level resistance in patients treated with mupirocin. Management of MRSA infections may also require screening for mupirocin resistance among coagulase negative staphylococci.

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CHAPTER FIVE

CONCLUDING REMARKS AND FUTURE RESEARCH STUDIES

This study on the susceptibility patterns and epidemiology of *S. aureus* strains from Nigeria and South Africa has provided baseline information for physicians, clinical microbiologists and public health officials in the establishment of adequate infection control programmes, and national drug policies in the treatment of staphylococcal infections. The multi-resistant nature of some MSSA isolates from Nigeria, multidrug-nature of MRSA strains from South Africa, the emergence of mupirocin-resistant *S. aureus* and isolation of atypical *S. aureus* strains from both countries clearly indicate the need for continuous surveillance in understanding new and emerging trends in the susceptibility patterns of *S. aureus*. Future studies include the characterization of multi-drug resistant *S. aureus* isolates, which involves the detection of genes conferring resistance to macrolides, lincosamides, aminoglycosides and tetracycline.

Bacterial strain typing, or subspeciation, has become an important clinical tool to investigate nosocomial transmission. The characterization of *S. aureus* strains from both countries using various epidemiological tools has identified some widely disseminated MRSA clones, establishment of multi-drug resistant clones in health institutions in KwaZulu-Natal province of South Africa and the genetic relatedness of one MRSA strain from Nigeria with the pandemic EMRSA-15. An epidemic clone is known for its ease of transmission, long-term persistence, rapid intra- and inter-hospital spread and ability to cross geographical and continental boundaries. Future studies would be important in investigating the genetic relationship of clones from the two countries with other

pandemic MRSA clones in order to ascertain if these clones were unique and specific to Nigeria and South Africa. More studies on the comparative analysis of the molecular epidemiology of MRSA in both countries are of great importance. There is also the need for closer international collaboration to monitor the spread of current epidemic strains and the emergence of new ones. Further characterization of MRSA strains using SCC*mec* typing and MLST would clearly assist in understanding the evolution of MRSA clones, and the behaviour and fitness of this successful pathogen under hospital conditions. Although it was not been possible to elucidate why some MRSA clones spread rapidly than others, urgent measures are needed in curtailing the spread and establishment of these clones. In this regard, future studies in understanding virulence related property such as increased adherence resulting in colonization and infection could be of major importance with respect to control of MRSA dissemination. In addition, future studies involving full sequence analysis of the *ileS*-2 gene in the mupirocin-resistant strain from Nigeria would probably provide new insights on the evolution of the *ileS*-2 gene in *S. aureus*.

Antibiotic susceptibility patterns of S. aureus isolates from Nigeria

No	PG	AM	E	TM	GM	C	CIP	RP	CD	T	NE	FC	TC	VA	MU	MN	K	S	TS	OX	KF	CX	Type of specimen
D26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
D54	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Blood
D45	1	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	1	1	1	0	0	0	Wound swab
D21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Urine
D36	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Wound swab
D9	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	Wound swab
D19	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Urine
D24	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	Urine
D12	1	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	1	1	1	0	0	0	NA
D32	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Sputum
D37	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Ear swab
D28	1	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	Sputum
D53	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
D23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Semen
D15	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
D18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
D14	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Blood
D29	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
D16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Urine
D4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
D35	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
D20	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
D40	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	Wound swab
D7	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	HVS
D51	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
D8	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
D52	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
J6	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	NA
E	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
K	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
A27	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
A1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
THK	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
THW	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
THE	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
THF	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
2IB	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
C1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	NA
C34	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	NA
C20	1	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	NA
C35	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	NA
C4	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	NA
C5	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	NA
C22	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	NA
C25	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	NA
C49	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	NA
C40	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
C48	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	NA
4IB	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
5IB	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	Wound biopsy
C32	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA
3IB	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ear swab
B6	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Ear swab
B35	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	HVS
B1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound aspirate
B8	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Ear swab
B21	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Breast aspirate
B10	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Urine
B3	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Wound aspirate
B5	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Wound aspirate
B19	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Urine
B9	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Aspirate
B26	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Aspirate
B31	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Urine
F	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
10	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Blood
13	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Blood
O	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Blood
19 (1)	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
5	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
G	1	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Blood
R	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	Blood
H	1	1	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Blood
C	1	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	Blood
P	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Blood
E1	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	Blood
20	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Blood
Y	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Blood
V	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Blood
X	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
Q	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
W	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
29	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Blood
30	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
36	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
19	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
41	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
13C	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Wound swab
34	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Wound swab
38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
32	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
11	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
Boil	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Wound swab
18	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
40	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
6 IBA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
9 IBA	1	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Urine
11 IBA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	Wound swab
4 IBA	1	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Blood
7 IBA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	Blood
5 IBA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
12 IBA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Blood

2 IBA	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound biopsy
13-I IBA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Blood
4MXD	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	HVS
28 IFE	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	Urine
48 IFE	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
34 IFE	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
40 IFE	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	Wound swab
36 IFE	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Eye swab
15 IFE	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Blood
48? IBA'	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Urine
1* IBA*	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	NA
20 IBA*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
30 IBA*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
23 IBA*	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	Blood
8 IBA*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Eye swab
14 (1) IB	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Eye swab
31 IBA*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Urine
**7 IBA*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
**2 IBA*	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA
42 IBA*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
**6 IBA*	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	NA
14 IBA*	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Eye swab
19 IBA*	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
**3 IBA	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
**8 IBA	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	NA
40 IBA*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Eye swab
35 IBA	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	Blood
16 IBA*	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Blood
18 IBA*	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Ear swab
15 IBA*	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
50 IBA*	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Urine
21 IBA*	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Eye swab
17 IBA*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
2 IBA*	1	1	1	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
**4 IBA*	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	NA
1 IBA	1	1	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	Wound swab
18 IF	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Urine
8 IDA	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
17* IDA	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound aspirate
2 IDA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Blood
7 IDA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Eye swab
15 IDA	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound aspirate
24 IDA	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Eye swab
21 IDA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Eye swab
26 IDA	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
23 IDA	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
3 IDA	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
4 1DA	1	1	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	Wound swab
27 IDA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
10 IDA	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Eye swab
6 IDA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
32 IDA	1	1	1	1	1	0	1	0	1	1	1	0	0	0	0	0	0	1	1	1	0	0	0	Blood
14 IF	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA
22 IDA	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Ear sweb
13 IF	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Urine
20 IDA	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	Wound swab
12 IDA	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Ear swab
14IDA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound aspirate
25 IDA	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
18 IDA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Eye swab
11 IDA	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Ear swab
19* IDA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Eye swab
31 IDA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Eye swab
30 IDA	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Blood
1 IDA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
15 IF	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA
12 IF	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Blood
13 IDA	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
16 IDA	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
1 IF	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	ECS
17 IDA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound aspirate
5 IDA	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Skin swab
9 IDA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Urine
29 IDA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Ear swab
29 NEW	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
3 NEW	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
23 NEW	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
27 NEW	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
28 NEW	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Eye swab
30 NEW	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Ear swab
39 NEW	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Urine
6 NEW	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Eye swab
36 NEW	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Ear swab
21 NEW	1	1	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
40 NEW	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	Wound swab
18 NEW	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Eye swab
10 NEW	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
1 NEW	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Blood
5 NEW	1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Eye swab
11 NEW	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	Wound swab
7188	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
7731	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
7396	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
7428	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	NA
NO	178	178	3	93	12	18	13	0	3	101	5	0	0	0	1	3	14	22	93	0	0	0		NA
%	90	90	1.5	47	6.1	9.1	6.6	0	1.5	51	2.5	0	0	0	0.5	1.52	7	11	47	0	0	0		

No	PG	AM	E	TM	GM	C	CIP	RP	CD	T	NE	FC	TE	VA	MU	MN	K	S	TS	OX	KF	CX	Type of spacimen
MSSA - Nigeria - 112 isolates intermediately resistant to oxacillin																							
Susceptibility patterns of MRSA isolates from Nigeria																							
No	PG	AM	E	TM	GM	C	CIP	RP	CD	T	NE	FC	TE	VA	MU	MN	K	S	TS	OX	KF	CX	Type of specimen
28 IDA	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0	Wound swab
THCD	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	0	0	Wound swab
16	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	Wound swab

Antibiotic susceptibility patterns of MSSA isolates from South Africa

No	PG	AM	E	TM	GM	C	CIP	RP	CD	T	NE	FC	TC	VA	MU	MN	K	S	TS	OX	KF	CX	Type of specimen
GJC 39	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Urine
GJC 74	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Sputum
GJC 93	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
EDD 11	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 15	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 20	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 30	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ear swab
EDD 33	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	HVS
EDD 38	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 41	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 45	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 49	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 54	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
EDD 55	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 56	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 64	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EDD 79	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ear swab
EDD 87	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
EDD 96	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Sputum
GT 6	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
GT 7	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
GT 30	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
GT 34	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
GT 41	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Vaginal swab
GT 67	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ear swab
GT 68	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
GT 85	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
BS 35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
BS 66	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 4	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 39	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
RKK 49	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Urine
RKK 54	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
RKK 63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 65	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
RKK 66	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 68	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 82	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 83	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 84	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 85	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 86	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 88	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
RKK 99	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EGU 13	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EGU 17	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EGU 42	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EGU 47	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EGU 59	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EGU 60	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ear swab
EGU 63	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EGU 70	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EGU 72	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EGU 75	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	Wound swab
EGU 81	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
EGU 83	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
KEH 59	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Endotracheal aspirat
GP 85	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
GP 86	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
GP 99	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
GP 98	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PS 27	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PS 28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PS 46	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PS 47	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PS 61	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ear swab
PS 90	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
AD 59	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	Blood
AD 68	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
AD 80	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Blood
AD 93	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
MD 22	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Sputum
PM 13	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 25	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 57	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 66	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 70	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 71	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 72	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 73	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 74	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 76	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 77	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 79	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
PM 80	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
102	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
103	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
103'	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
104	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
104..	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	Wound swab
105	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
106	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
107 (1)	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab

110	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
113	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
119	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
202	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
204	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
205	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
212	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
302	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
319	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
405..	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
406..	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
501	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
501..	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
512	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
514	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
515	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
Bayo4	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	Wound swab
6 KH	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
9 KH	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
11 KH	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
ASA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
BSA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
CSA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
DSA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
FSA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
GSA	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
ISA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
JSA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
LSA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
MSA	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	Wound swab
NSA	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	Wound swab
OSA	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	Wound swab
PSA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
QSA	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
RSA	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
TSA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
USA	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
VSA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
WSA	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XSA	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XD	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XE	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XF	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
XG	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XH	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XI	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XJ	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XK	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XM	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XN	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XP	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XR	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XS	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XT	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Pericardial fluid
XU	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XV	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XX	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XY	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
XZ	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Wound swab
AA	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
AB	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
AE	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
AF	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
NG 24	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
NG 36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Wound swab
P1929	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	NA
TOTAL	147	147	19	18	6	1	1	1	18	13	0	0	0	0	0.6	0	3.6	0	10.8	0	0	0		
%	89	89	11	11	3.6	0.6	0.6	0.6	11	7.8	0	0	0	0										

No	PG	AMP	E	TM	GM	C	CIP	RP	CD	T	NE	FC	TEC	VAN	MUP	MN	K	S	TS	OX	KF	CXM	Type of specimen
GJC 3	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Sputum
GJC 7	1	1	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	1	Ear swab
GJC 69	1	1	0	1	1	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	0	0	
EDD 52	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
EDD 70	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	Wound swab
EDD 84	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
EDD 99	1	1	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	1	Wound swab
GT 33	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
RKK 6	1	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	1	1	0	0	Wound swab
RKK 8	1	1	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	Wound swab
RKK 10	1	1	1	0	1	0	0	1	1	1	1	0	0	0	0	1	1	0	0	1	0	0	Wound swab
RKK 52	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
RKK 53	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
RKK 55	1	1	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	Wound swab
RKK 56	1	1	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	Wound swab
RKK 57	1	1	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	Wound swab
EGU 23	1	1	0	1	1	1	0	1	0	1	0	0	0	0	0	1	1	1	1	1	0	0	Wound swab
EGU 28	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
EGU 51	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Eye swab
KEH 12	1	1	1	1	1	0	0	1	1	1	0	0	0	0	1	1	1	0	1	1	0	0	Wound swab
KEH 26	1	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	Endotracheal aspirate
KEH 77	1	1	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	1	Wound swab
KEH 88	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Ear swab
GP 11	1	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	Wound swab
GP 74	1	1	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	1	Wound swab
PS 33	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Sputum
PS 44	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
PS 50	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
PS 94	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
AD 28	1	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	Wound swab
AD 69	1	1	0	1	1	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
AD 77	1	1	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	1	Wound swab
AD 79	1	1	0	1	1	0	0	1	0	1	0	0	0	0	1	1	1	0	1	1	0	0	Blood
AD 84	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	1	Sputum
AD 87	1	1	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	Wound swab
AD 98	1	1	0	1	1	0	0	1	0	1	0	0	0	0	1	1	1	0	1	1	0	0	Catheter tip
MD 24	1	1	0	1	1	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	0	0	Sputum
MD 43	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	1	1	Sputum
PM 69	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
ESH 20	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
ESH 34	1	1	1	1	1	0	0	1	1	1	0	0	0	0	1	1	1	0	1	1	0	0	Wound swab
ESH 37	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	Wound swab
ESH 89	1	1	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	1	Wound swab
107-2	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
203	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
503..	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
510	1	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	Wound swab
511	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
513	1	1	1	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1	Wound swab
ESA	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab (burns)
HAS	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
SSA	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
1KH	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
2KH	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
4KH	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
AC	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0	1	1	1	1	1	0	0	Wound swab
AD	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	Wound swab
XB	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
XC	1	1	0	1	1	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
XQ	1	1	0	1	1	1	0	1	0	1	0	0	0	0	0	1	1	0	1	1	0	0	Wound swab
XW	1	1	0	1	1	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	0	0	Sputum
NO	61	61	50	52	59	10	11	45	50	55	19	0	0	0	15	55	59	19	52	61	17	18	
%	100	100	82	85.2	97	16.4	18	74	82	90	31	0	0	0	24.6	90	97	31	85	100	28	29.51	%

	Aminoglycosides						MLS antibiotics					Tetracyclines	Heavy Metals				
No	GM	KM	AM	SM	NE	TOB	EM	OL	AZM	CC	QD	TE	MH	DO	No	EB	Pi	Hg	Cd
GJC 3	1	1	0	0	0	int	1	1	1	1	0	1	1	0	GJC	0	0	0	int
GJC 7	1	1	0	1	1	int	1	1	0	1	0	0	0	0	GJC	int	1	1	1
GJC 69	1	1	0	0	0	1	0	0	0	0	0	1	1	0	GJC	0	0	0	0
EDD 52	1	1	0	0	0	1	1	1	1	1	0	1	1	0	EDD	0	0	0	1
EDD 70	1	1	0	1	0	1	1	1	1	1	0	1	1	int	EDD	0	0	0	1
EDD 84	1	1	0	0	0	0	1	1	1	1	0	1	1	int	EDD	0	0	0	1
EDD 99	1	1	0	1	1	int	1	1	1	1	0	0	0	0	EDD	1	1	1	1
GT 33	1	1	0	0	0	1	1	1	1	1	0	1	1	0	GT	0	0	0	int
RKK 6	1	1	0	0	1	1	1	1	1	1	0	1	1	int	RKK	0	0	0	1
RKK 8	1	1	1	1	1	1	1	int	1	1	0	1	1	1	RKK	1	1	1	1
RKK 10	1	1	0	0	1	1	1	1	1	1	0	1	1	0	RKK	0	0	0	1
RKK 52	1	1	0	0	0	1	1	1	1	1	0	1	1	0	RKK	0	0	0	1
RKK 53	1	1	0	0	0	1	1	1	1	1	0	1	1	0	RKK	1	1	0	int
RKK 55	1	1	1	1	1	1	1	1	1	1	0	1	1	1	RKK	0	0	1	0
RKK 56	1	1	1	1	1	1	1	int	1	1	0	1	1	1	RKK	0	0	1	0
RKK 57	1	1	1	1	1	1	1	1	1	1	0	1	1	1	RKK	1	1	1	1
EGU 23	1	1	0	1	0	1	0	0	0	0	0	1	1	0	EGU	int	0	0	int
EGU 28	1	1	0	0	0	1	1	1	1	1	0	1	1	0	EGU	int	0	0	int
EGU 51	1	1	0	0	0	1	1	1	1	1	0	1	1	0	EGU	0	0	1	1
KEH 12	1	1	0	0	0	1	1	1	1	1	0	1	1	int	KEH	0	0	1	1
KEH 26	1	1	1	1	1	1	1	int	1	1	0	1	1	1	KEH	0	0	1	0
KEH 77	1	1	0	1	1	int	1	1	1	1	0	0	0	0	KEH	1	1	1	1
KEH 88	1	1	0	0	0	1	1	1	1	1	0	1	1	int	KEH	0	0	1	1
GP 11	1	1	1	1	1	1	1	int	1	1	0	1	1	1	GP	1	1	1	1
GP 74	1	1	0	1	1	1	1	1	1	1	0	0	0	0	GP	1	1	1	1
PS 33	1	1	0	0	0	1	1	1	1	1	0	1	1	0	PS	0	0	1	1
PS 44	1	1	0	0	0	1	1	1	1	1	0	1	1	int	PS	0	0	1	0
PS 50	1	1	0	0	0	1	1	1	1	1	0	1	1	int	PS	0	0	1	1
PS 94	1	1	0	0	0	1	1	1	1	1	0	1	1	0	PS	0	0	0	1
AD 28	1	1	1	1	1	1	1	int	1	1	0	1	1	1	AD	0	0	1	1
AD 69	1	1	0	0	0	1	0	0	0	0	0	1	1	0	AD	0	0	1	1
AD 77	1	1	0	1	1	int	1	1	1	1	0	0	0	0	AD	1	1	1	1
AD 79	1	1	0	0	0	1	0	0	0	0	0	1	1	0	AD	0	0	0	0
AD 84	1	1	0	0	0	1	1	1	1	1	0	1	1	0	AD	0	0	0	1
AD 87	1	1	1	1	1	1	1	1	1	1	0	1	1	1	AD	1	1	1	1
AD 98	1	1	0	0	0	1	0	0	0	0	0	1	1	0	AD	0	0	1	1
MD 24	1	1	0	0	0	int	0	0	0	0	0	1	1	0	MD	0	0	0	0
MD 43	1	1	0	0	0	int	1	1	1	1	0	1	1	0	MD	0	0	0	int
PM 69	1	1	0	0	0	1	1	1	1	1	0	1	1	0	PM	0	0	0	0
ESH 20	1	1	0	0	0	1	1	1	1	1	0	1	1	0	ESH	0	0	0	1
ESH 34	1	1	0	0	0	1	1	1	1	1	0	1	1	0	ESH	0	0	0	1
ESH 37	0	0	0	0	0	0	0	0	0	0	0	1	1	0	ESH	0	0	1	0
ESH 89	1	1	0	1	1	int	1	1	1	1	0	0	0	0	ESH	1	1	1	1
SSA1	1	1	0	0	0	1	1	1	1	1	0	1	1	int	SSA	0	0	0	1
1KH	1	1	0	0	0	1	1	1	1	1	0	1	1	int	1KH	1	1	1	1
2KH	1	1	0	0	0	1	1	1	1	1	0	1	1	0	2KH	1	1	0	1
4KH	1	1	0	0	0	1	1	1	1	1	0	1	1	int	4KH	1	1	1	1
ESA	1	1	0	0	0	1	1	1	1	1	0	1	1	int	ESA	1	1	0	1
HAS	1	1	0	0	0	1	1	1	1	1	0	1	1	int	HAS	0	0	1	1

	Aminoglycosides						MLS antibiotics					Tetracyclines	Heavy Metals				
No	GM	KM	AM	SM	NE	TOB	EM	OL	AZM	CC	QD	TE	MH	DO	No	EB	Pi	Hg	Cd
AC	1	1	0	1	1	int	1	1	1	1	0	1	1	0	AC	0	0	0	0
XQ	1	1	0	0	0	int	0	0	0	0	0	1	1	0	XQ	0	0	0	int
AD	0	0	0	0	0	0	0	0	0	0	0	1	1	0	AD	0	0	0	0
107-2	1	1	0	0	0	1	1	1	1	1	0	1	1	0	107	0	0	0	int
XW	1	1	0	0	0	1	0	0	0	0	0	1	1	0	XW	0	0	0	int
203	1	1	0	0	0	1	1	1	1	1	0	1	1	0	203	0	0	0	1
503 Red	1	1	0	0	0	1	1	1	1	1	0	1	1	0	503	0	Int	0	1
511	1	1	0	0	0	1	1	1	1	1	0	1	1	0	511	0	0	0	Int
513	1	1	1	1	1	1	1	1	1	1	0	1	1	1	513	1	1	1	1
510	1	1	1	1	1	1	1	1	1	1	0	1	1	1	510	1	1	1	1
114XB Re	1	1	0	0	0	1	1	1	1	1	0	1	1	0	114	0	0	0	1
131 XC Re	1	1	0	0	0	1	0	0	0	0	0	1	1	0	131	0	0	0	1
TOTAL	59	59	10	19	19	48	50	45	49	50	0	55	55	10	TOT	18	17	28	40
															%				
	GM	KM	AM	SM	NE	TOB	EM	OL	AZM	CC	QD	TE	MH	DO		EB	Pi	Hg	Cd
						10		5						12		3	1		10
						INT		INT						INT		INT	INT		INT

Susceptibility pattern of two MRSA from Nigeria

	Aminoglycosides						MLS antibiotics					Tetracyclines	Heavy Metals				
No	GM	KM	AM	SM	NE	TOB	EM	OL	AZM	CC	QD	TE	MH	DO	No	EB	Pi	Hg	Cd
THCD	0	0	0	1	0	0	1	1	1	1	0	1	1	0	THC	0	0	1	1
15	0	0	0	0	0	0	0	0	0	0	0	1	0	0	15	0	0	1	1

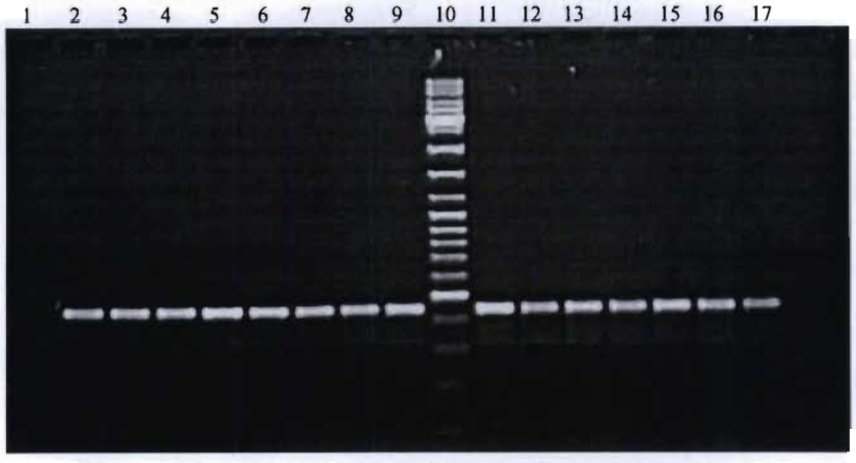
Susceptibility patterns of MRSA isolates from South Africa

No	MET	PEN	GM	KM	AM	SM	EM	CC	CM	TE	W	MH	RF	SH	FD	AZM	CIP	NV	DO	OL	QD	FOX	TOB	EB	Pi	Hg	Cd	MUP200	FOS	LZD	MUP5	VAN	TEIC	MUP	VAN	TEICO	Type of specimen
GJC 3	1	1	1	1	0	0	1	1	0	1	1	1	1	0	0	1	0	0	0	1	0	1	int	0	0	0	int	0	0	0	0	1.5	1.5		0	0	Sputum
GJC 7	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	1	int	int	1	1	1	0	0	0	0	2	2		0	0	Ear swab
GJC 69	1	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1.5	1.5		0	0	NA
EDD 52	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	1.5	1.5		0	0	Wound swab
EDD 70	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	int	1	0	1	1	0	0	0	1	0	0	0	0	2	1.5		0	0	Wound swab
EDD 84	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	int	1	0	1	0	0	0	0	1	0	0	0	0	2	1.5		0	0	Wound swab
EDD 99	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	1	int	1	1	1	1	0	0	0	0	2	2		0	0	Wound swab
GT 33	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	int	0	0	0	0	2	1.5		0	0	Wound swab
RKK 6	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	int	1	0	1	1	0	0	0	1	1	0	0	1	1.5	1	>1024	0	0	Wound swab
RKK 8	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	0	1	int	0	1	1	1	1	1	1	0	0	0	1	1.5	1.5	8	0	0	Wound swab
RKK 10	1	1	1	1	0	0	1	1	0	1	0	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	1.5	1.5		0	0	Wound swab
RKK 52	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	1.5	2		0	0	Wound swab
RKK 53	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	1	1	0	int	0	0	0	0	1	1		0	0	Wound swab
RKK 55	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	0	1	1	0	1	1	0	0	1	0	0	0	0	1	1	1	12	0	0	Wound swab
RKK 56	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	0	1	int	0	1	1	0	0	1	0	0	0	0	1	2	2	8	0	0	Wound swab
RKK 57	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	0	1	1	0	1	1	1	1	1	1	0	0	0	1	1.5	1	12	0	0	Wound swab
EGU 23	1	1	1	1	0	1	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	1	1	int	0	0	int	0	0	0	0	2	2		0	0	Wound swab
EGU 28	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	int	0	0	int	0	0	0	0	2	1.5		0	0	Wound swab
EGU 51	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	1	1	0	0	0	0	1.5	1.5		0	0	Eye swab
KEH 12	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	int	1	0	1	1	0	0	1	1	0	0	0	1	2	1.5	24	0	0	Wound swab
KEH 26	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	int	0	1	1	0	0	1	0	0	0	0	1	2	3	12	0	0	Endotracheal asprate
KEH 77	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	1	int	1	1	1	1	0	0	0	0	1.5	1.5		0	0	Wound swab
KEH 88	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	int	1	0	1	1	0	0	1	1	0	0	0	0	1.5	1.5		0	0	Ear swab
GP 11	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	int	0	1	1	1	1	1	1	0	0	0	1	1.5	1.5	8	0	0	Wound swab
GP 74	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	2	2		0	0	Wound swab
PS 33	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	1	1	0	0	0	0	1.5	1.5		0	0	Sputum
PS 44	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	int	1	0	1	1	0	0	1	0	0	0	0	0	1.5	2		0	0	Wound swab
PS 50	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	0	0	int	1	0	1	1	0	0	1	1	0	0	0	0	1.5	1.5		0	0	Wound swab
PS 94	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	1.5	1		0	0	Wound swab
AD 28	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	int	0	1	1	0	0	1	1	0	0	0	1	2	1.5	12	0	0	Wound swab
AD 69	1	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	1.5	1		0	0	Wound swab
AD 77	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	1	int	1	1	1	1	0	0	0	0	3	2		0	0	Wound swab
AD 79	1	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	2	1.5	24	0	0	Blood
AD 84	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	2	1.5		0	0	Sputum
AD 87	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	0	1	1	0	1	1	1	1	1	1	0	0	0	1	2	2	16	0	0	Wound swab
AD 98	1	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	1	2	1	24	0	0	Catheter tip
MD 24	1	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	1	int	0	0	0	0	0	0	0	0	1.5	1.5		0	0	Sputum
MD 43	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	int	0	0	0	int	0	0	0	0	0.75	0.75		0	0	Sputum
PM 69	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	1.5	1.5		0	0	Wound swab
ESH 20	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	2	1.5		0	0	Wound swab
ESH 34	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	1	1.5	1.5	24	0	0	Wound swab
ESH 37	1	1	0	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	2	1		0	0	Wound swab
ESH 89	1	1	1	1	0	1	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	1	int	1	1	1	1	0	0	0	0	2	2		0	0	Wound swab
SSA1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	int	1	0	1	1	0	0	0	1	0	0	0	0	1	1		0	0	Wound swab
1KH	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	int	1	0	1	1	1	1	1	1	0	0	0	0	1.5	1.5		0	0	Wound swab
2KH	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	1	1	1	1	0	0	0	0	1.5	1.5		0	0	Wound swab
4KH	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	int	1	0	1	1	1	1	1	1	0	0	0	0	1	2		0	0	Wound swab
ESA	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	1	0	int	1	0	1	1	1	1	0	1	0	0	0	0	1.5	1.5		0	0	Wound swab (burns)
HAS	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	int	1	0	1	1	0	0	1	1	0	0	0	0	1.5	1.5		0	0	Wound swab
AC	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	int	0	0	0	0	0	0	0	0	1	1		0	0	Wound swab
XQ	1	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	1	int	0	0	0	int	0	0	0	0	1.5	1		0	0	Wound swab
AD	1	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1.5	1.5		0	0	Wound swab
107-2	1	1	1	1	0	0	1	1	0	1	1	1	1	0	0	1	0	0	0	1	0	1	1	0	0	0	int	0	0	0	0	1.5	1		0	0	Wound swab
XW	1	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	int	0	0	0	0	1.5	1.5		0	0	Sputum
203	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	2	1		0	0	Wound swab
503 Red	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	int	0	1	0	0	0	0	1.5	1.5		0	0	Wound swab
511	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	int	0	0	0	0	2	2		0	0	Wound swab
513	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	0	1	1	0	1	1	1	1	1	1	0	0	0	1	2	2	8	0	0	Wound swab
510	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	0	1	1	1	1	1	1	0	0	0	1	2	2	12	0	0	Wound swab
114XB Rec	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	3	2		0	0	Wound swab
131 XC Re	1	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	3	2		0	0	Wound swab
TOTAL	61	61	59	59	10	19	50	50	10	55	52	55	45	58	0	49	11	0	10	46	0	61	48	16	17	28	40	1	0	0	15	TOTAL					
%	100	100	96.7	98.7	16	31.1	82	82	16	90	85	90	73.8	95.1	0	80	18	0	16	75	0	100	78.69	26.23	27.9	45.9	65.6	1.63934	0	0	24.59	%					

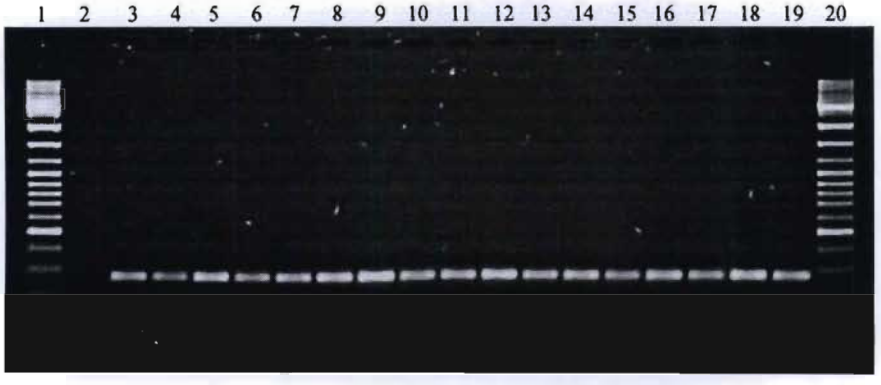
Susceptibility pattern of two MRSA from Nigeria

No	MET	PEN	GM	KM	AM	SM	EM	CC	CM	TE	W	MH	RF	SH	FD	AZM	CIP	NV	DO	OL	QD	FOX	TOB	EB	Pi	Hg	Cd	MUP200	FOS	LZD	MUP5	VAN	TEIC	MUP	VAN	TEICO	Type of specimen
THCD	0	1	0	0	0	1	1	1	0	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1.5	1.5		0	0	Wound swab
15	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	2	1.5		0	0	Wound swab

APPENDIX 2



PCR detection of the *mecA* gene (449bp). Lane 10: molecular weight marker; Lanes 2-9; 11-17: MRSA strains.

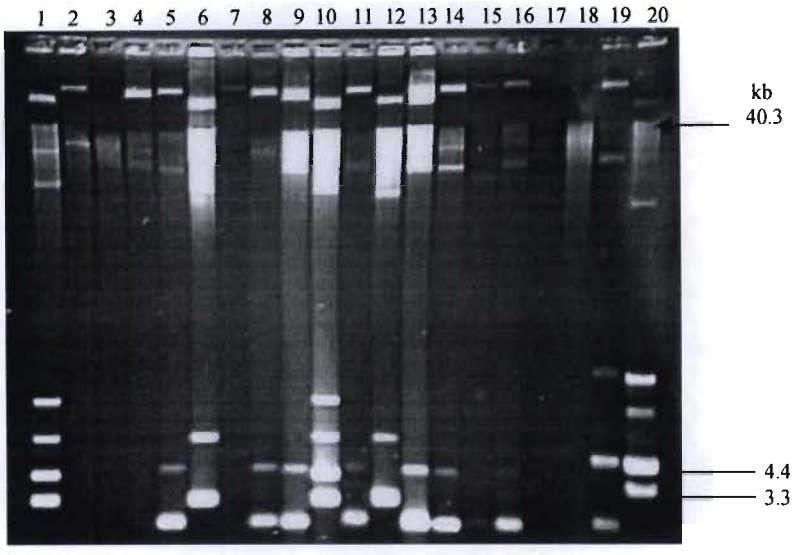


PCR detection of the *nuc* gene (280bp). Lanes 1 and 20: molecular weight markers; Lane 2: negative control; Lane 3-19: positive *S. aureus* strains

PCR detection of the *mupA* gene (190bp) in low and high-level mupirocin resistant strains. Lanes 3-16: low-level mupirocin resistant strains; Lane: 17-19: high-level mupirocin resistant strains RKK6, P1929 (South Africa), 35IBA (Nigeria).

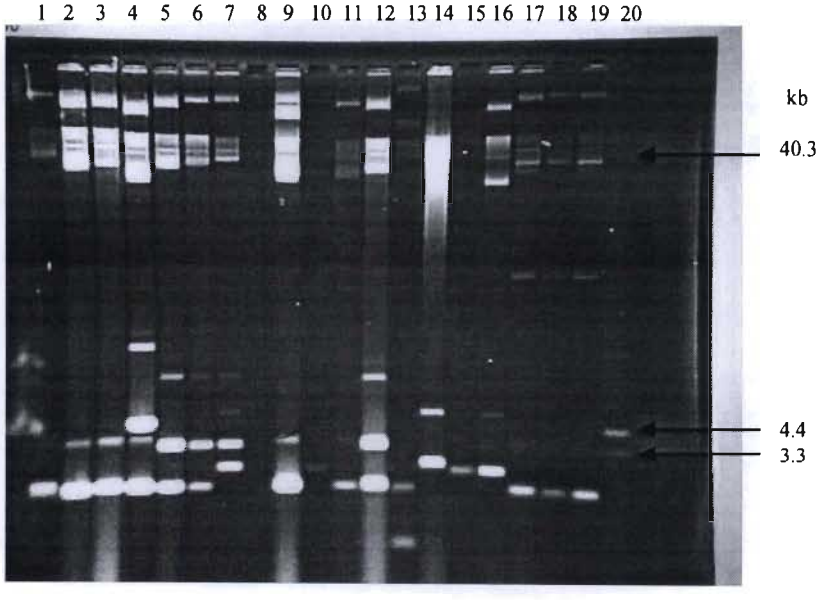
Plasmid profiles of MRSA strains from South Africa and Nigeria

Plasmid Profiles	Approximate size (kb)	Strain Numbers
1	26, 4.2, 3.3	510, AD28
2	23, 3.3	AD87, 513
3	28.5, 2.8	503, AD84, 114XC, 131XB, ESH34, RKK57
4	No plasmid	AD69, AD77, AD98, ESH37, GP74, AD, ESH89, XW, MD24, GJC7, GJC69, KEH77, AD79
5	32, 4.2, 2.8	EGU23
6	37, 2.3	EGU28, EGU51, PS33, RKK6, RKK10
7	34, 4.8, 2.3	PS44
8	37, 3.7, 2.3	PS50, PS94, RKK52
9	39.3, 3.7, 2.8	GP11
10	1.2	RKK53
11	2.8	RKK56, 203, 511, ESH20, RKK8, RKK55
12	38.3, 15.4, 2.3	1KH, 2KH, 4KH
13	2.3	AC, GJC3, KEH88, EDD84, KEH12, GT33, PM69, EDD52, ESA, HSA, SSA, 107(2)
14	3.7, 2.8	XQ, KEH26
15	2.4, 2.3	MD43
16	4.8, 2.3	EDD70
17	26, 19, 7.4, 4.2, 2.3	THCD*
18	5.2, 2.8	15*



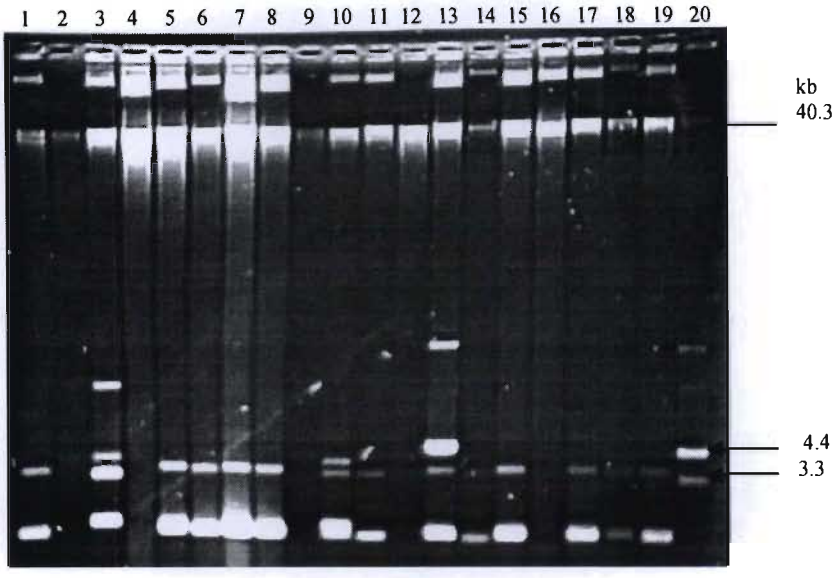
Plasmid profiles 1 4 4 4 3 2 4 11 3 1 11 2 3 3 11 3 4 4 5

Plasmid profiles of MRSA strains from South Africa. Lane 1: AD28; Lane 2: AD69; Lane 3: AD77; Lane 4: AD79; Lane 5: AD84; Lane 6: AD87; Lane 7: AD98; Lane 8: 203; Lane 9: 503; Lane 10: 510; Lane 11: 511; Lane 12: 513; Lane 13: 114XC; Lane 14: 131XB; Lane 15: ESH20; Lane 16: ESH34; Lane 17: ESH37; Lane 18: ESH89; Lane 19: EGU23; Lane 20: WBG 4483. Only closed circular (CCC) DNA of WBG 4483 are labelled.



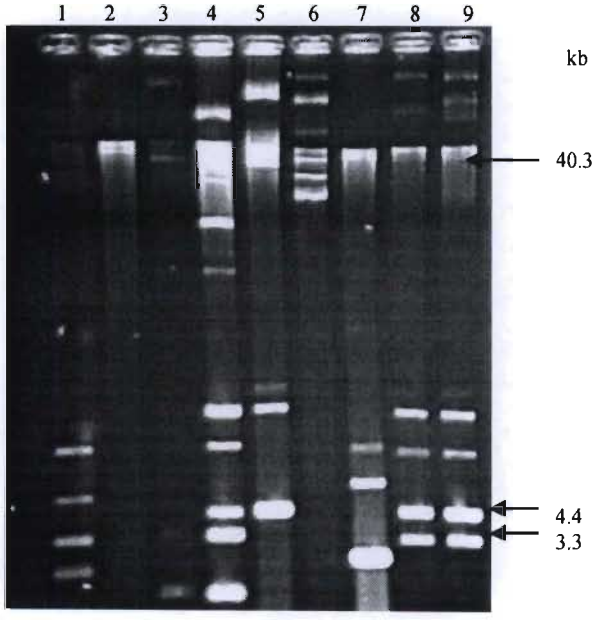
Plasmid profiles 6 6 6 7 8 8 9 4 6 11 6 8 10 11 11 3 12 12 12

Plasmid profiles of MRSA strains from South Africa. Lane 1: EGU 28; Lane 2: EGU51; Lane 3: PS 33; Lane 4: PS44; Lane 5: PS50; Lane 6: PS94; Lane 7: GP11; Lane 8: GP74; Lane 9: RKK6; Lane 10: RKK8; Lane 11: RKK10; Lane 12: RKK52; Lane 13: RKK53; Lane 14: RKK55; Lane 15: RKK56; Lane 16: RKK57; Lane 17: 1KH; Lane 18: 2KH; Lane 19: 4KH; Lane 20: WBG 4483. Only closed circular (CCC) DNA of WBG 4483 are labelled.



Plasmid profiles 13 4 14 4 13 13 13 13 4 15 13 4 16 13 13 4 13 13 13

Plasmid profiles of MRSA strains from South Africa. Lane 1: AC; Lane 2: AD; Lane 3: XQ; Lane 4: XW; Lane 5: 107(2); Lane 6: ESA; Lane 7: HSA; Lane 8: SSA; Lane 9: MD24; Lane 10: MD43; Lane 11: GJC3; Lane 12: GJC7; Lane 13: EDD70; Lane 14: EDD84; Lane 15: KEH12; Lane 16: GJC69; Lane 17: GT33; Lane 18: PM 69; Lane 19: EDD 52; Lane 20: WBG 4483. Only closed circular (CCC) DNA of WBG 4483 are labelled.



Plasmid profiles 14 4 13 17 18

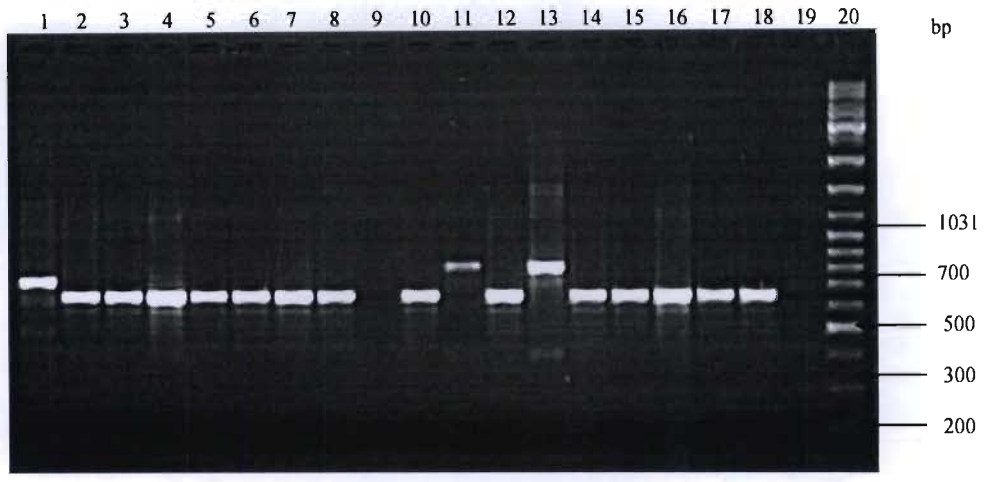
Plasmid profiles of MRSA strains (South Africa) and MRSA and MSSA strains from Nigeria. Lane 1: KEH26; Lane 2: KEH77; Lane 3: KEH88 (MRSA strains from South Africa); Lane 4: THCD (MRSA - Nigeria); Lane 5: 35IBA; Lane 6: 1IBA (MSSA strains - Nigeria); Lane 7: 15 (MRSA - Nigeria); Lane 8: WBG 4483, Lane 9: WBG 4483. Only closed circular (CCC) DNA of WBG 4483 are labelled.

PCR-RFLP of the coagulase gene (MSSA and MRSA strains from South Africa)

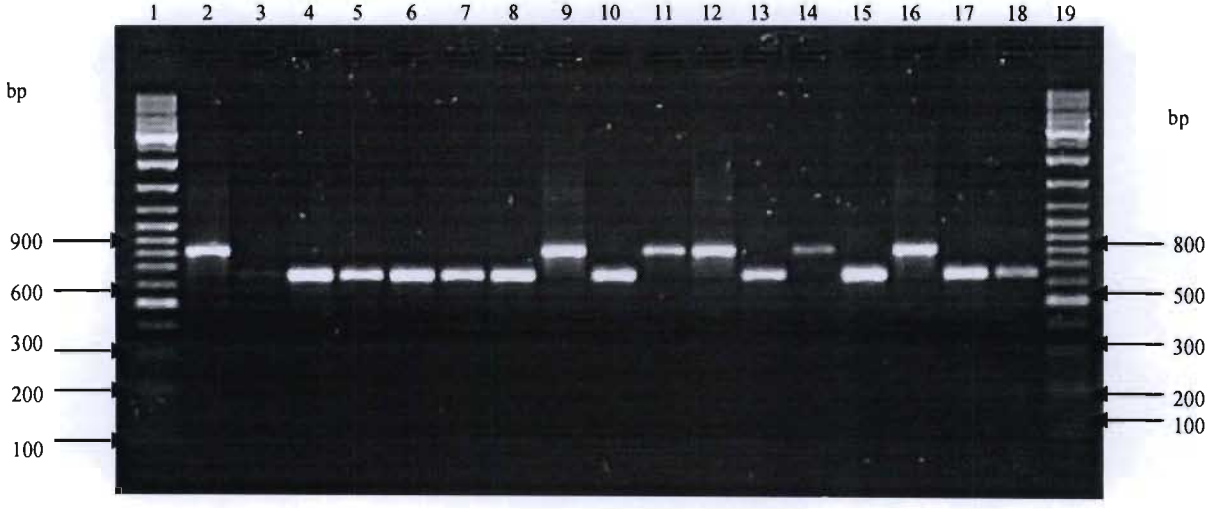
Strain Number	Molecular weight (bp)	RFLP (bp)	Strain Number	Molecular weight (bp)	RFLP (bp)
*GT 6	480	81, 405	*GSA	690	81, 162, 405
*RKK 54	580	162, 405	*AD 68	720	162, 243, 405
*EDD 79	600	81, 486			
			*EGU 60	740	81, 243, 405
XW	640	81, 567	*KEH 59	740	81, 324, 405
AD 69	630	81, 567	*GJC 39	750	81, 324, 405
EDD 84	640	81, 567	*EDD 30	730	81, 243, 486
AD	650	81, 567	*GT 30	730	81, 162, 405
XQ	640	81, 567	*RKK 65	730	81, 243, 405
107 (2)	650	81, 567	AC	730	81, 243, 405
ESA	640	81, 567			
HAS	650	81, 567	*AD 59	760, 380	81, 324, 405
SSA	640	81, 567			
503	650	81, 567	510	810	324, 405
511	650	81, 567	513	810	81, 324, 405
114XC	650	81, 567	GJC 7	820	81, 162, 486
131XB	650	81, 567	EDD 99	780	81, 162, 486
MD 24	650	81, 567	KEH 26	790	81, 324, 405
MD 43	650	81, 567	KEH 77	780	81, 162, 486
GJC 3	650	81, 567	AD 28	810	324, 405
GT 33	630	81, 567	AD 77	780	81, 162, 486
PM 69	650	81, 567	GP 11	790	81, 324, 405
EDD 52	650	81, 567	GP 74	790	81, 162, 486
EDD 70	650	81, 567	RKK 8	820	81, 324, 405
KEH 12	640	81, 567	RKK 56	820	81, 324, 405
KEH 88	630	81, 567	RKK 55	820	81, 324, 405
AD 79	640	81, 567	RKK 57	790	81, 324, 405
AD 84	640	81, 567	*AD 80	810	81, 162, 648
PS 33	650	81, 567	*EDD 38	820	81, 162, 648
PS 44	650	81, 567	*GT 67	820	162, 243, 405
PS 50	640	81, 567	*EGU 75	800	162, 243, 405
PS 94	650	81, 567	*EDD 49	810	810
RKK 6	640	81, 567	*XF	810	324, 486
RKK 10	640	81, 567	*MSA	810	162, 243, 405
RKK 52	640	81, 567			
RKK 53	650	81, 567	ESH 89	840	81, 162, 486
1 KH	630	81, 567	AD 87	830	324, 405
2 KH	640	81, 567	*RKK 50	830	81, 324, 405
4 KH	650	567	*RKK 39	830	162, 243, 405
GJC 69	660	567	*RKK 82	850	243, 486
AD 98	660	81, 567	*PS 61	840	243, 486
ESH 20	660	81, 567	*GJC 74	840	162, 243, 405
ESH 34	660	81, 567			
ESH 37	660	81, 567	*EDD 11	900	162, 324, 486
EGU 23	660	81, 567	*AB	910	81, 162, 648
EGU 28	660	81, 567	*AE	910	81, 162, 648
EGU 51	670	81, 567	*XN	900	81, 162, 648

*EDD 33	660	324, 405	*PM 13	880	81, 162, 648
*GT 41	660	81, 486	*EDD 87	890	81, 162, 648
*GP 99	950	162, 324, 486			
*XSA	930	162, 324, 486			
*XZ	980, 400	162, 324, 405, 486			
*WSA	1000, 420	162, 324, 405, 486			
*EDD 96	No product				
203	No product				

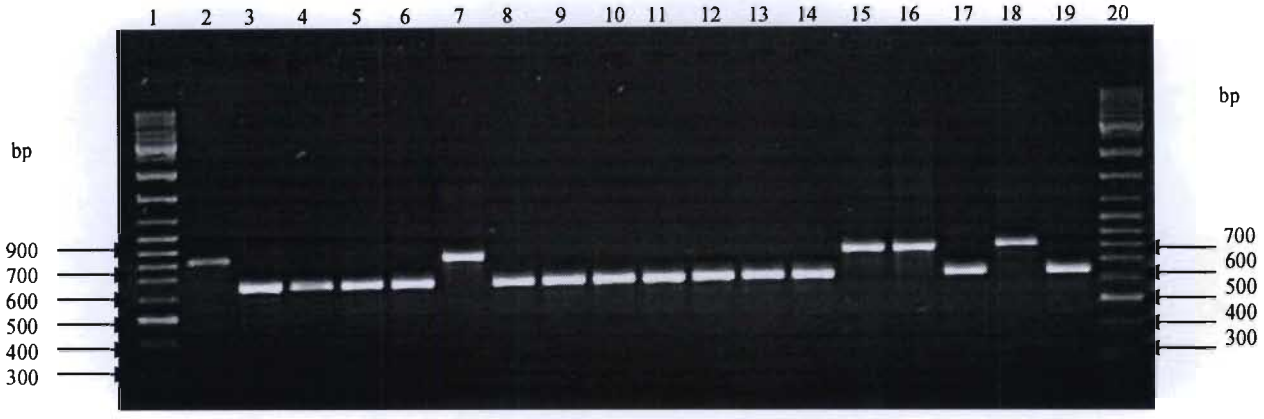
*MSSA strains



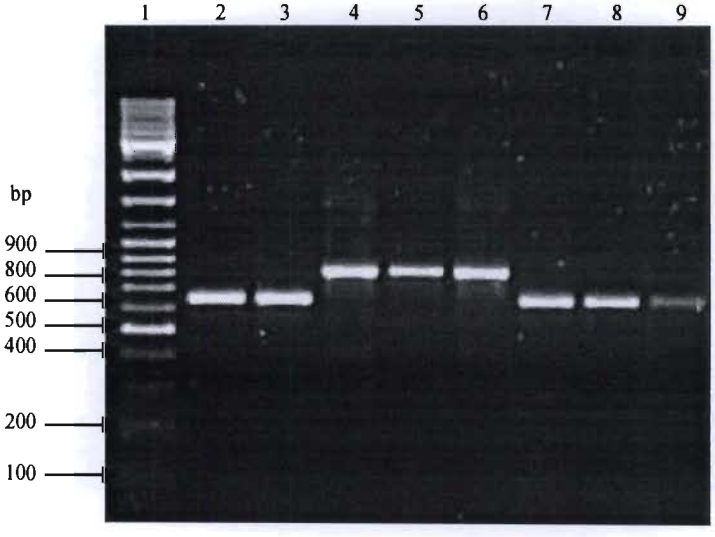
PCR detection of the coagulase gene in MRSA strains from South Africa. Lane 1: AC; Lane 2: AD; Lane 3: XQ; Lane 4: XW; Lane 5: 107(2); Lane 6: ESA; Lane 7: HSA; Lane 8: SSA; Lane 9: 203; Lane 10: 503; Lane 11: 510; Lane 12: 511; Lane 13: 513; Lane 14: 114XC; Lane 15: 131XB; Lane 16: MD24; Lane 17: MD43; Lane 18: GJC3; Lane 19: negative control; Lane 20: molecular weight marker.



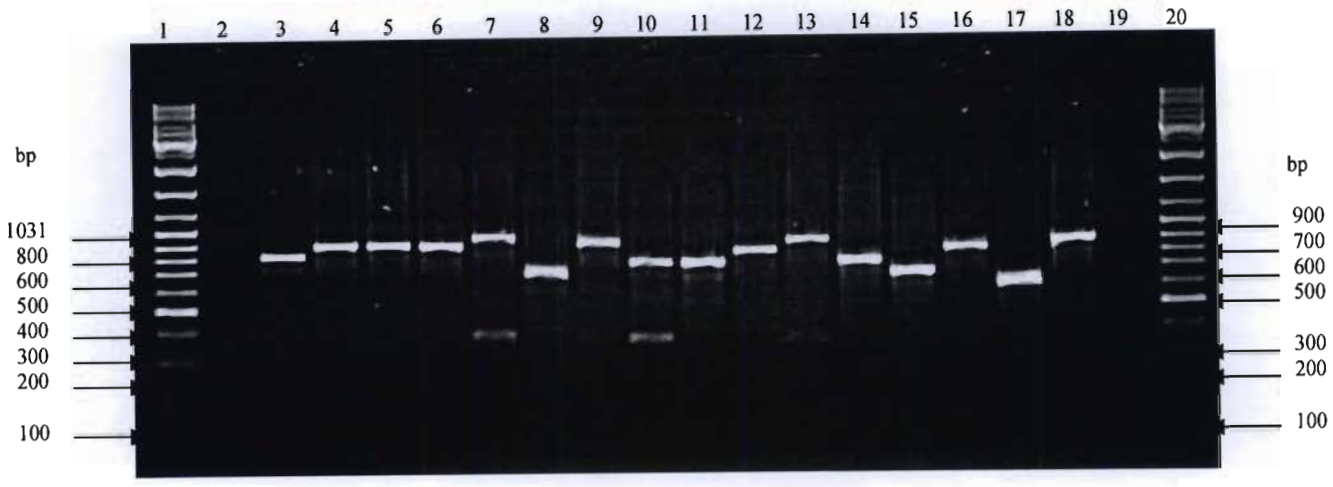
PCR detection of the coagulase gene in MRSA strains from South Africa. Lanes 1 and 19 are molecular weight markers. Lane 2: GJC7; Lane 3: GJC69; Lane 4: GT33; Lane 5: PM69; Lane 6: EDD52; Lane 7: EDD70; Lane 8: EDD84; Lane 9: EDD99; Lane 10: KEH12; Lane 11: KEH26; Lane 12: KEH77; Lane 13: KEH 88; Lane 14: AD28; Lane 15: AD69; Lane 16: AD77; Lane 17: AD79; Lane 18: AD84.



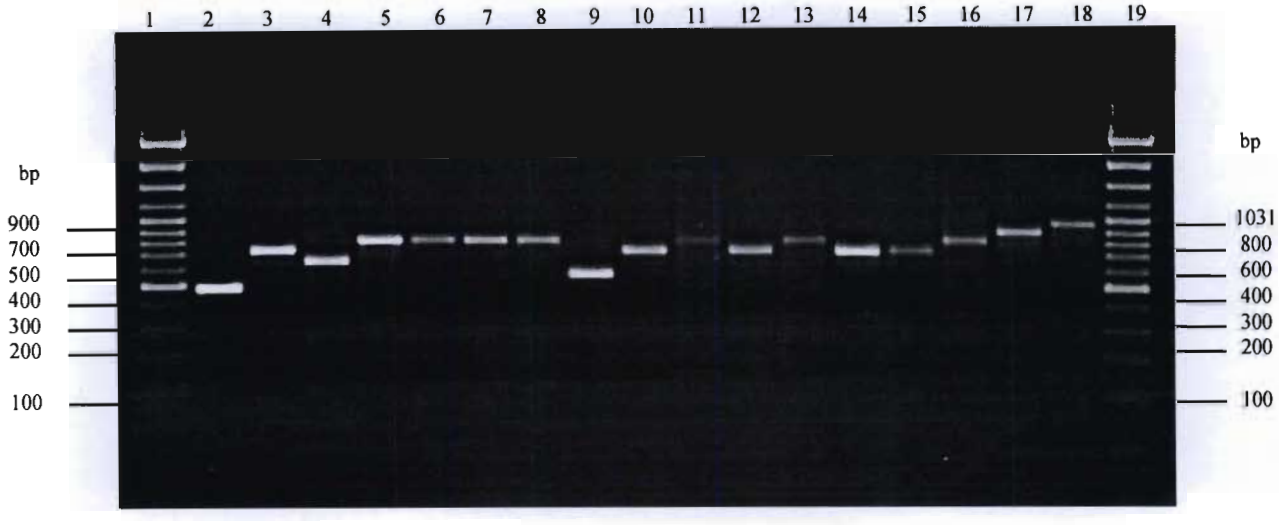
PCR detection of the coagulase gene in MRSA strains from South Africa. Lanes 1 and 20 are molecular weight markers. Lane 2: AD87; Lane 3: AD98; Lane 4: ESH20; Lane 5: ESH34; Lane 6: ESH37; Lane 7: ESH89; Lane 8: EGU23; Lane 8: EGU28; Lane 9: EGU51; Lane 10: PS33; Lane 11: PS44; Lane 12: PS50; Lane 13: PS94; Lane 14: GP11; Lane 15: GP74; Lane 16: RKK6; Lane 17: RKK8; Lane 18: RKK10.



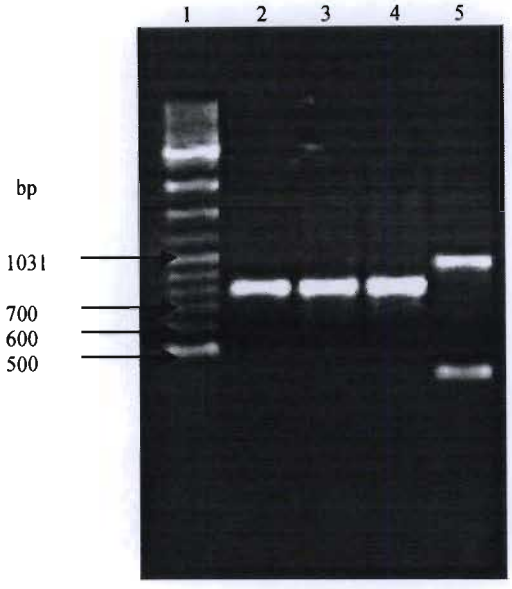
PCR detection of the coagulase gene in MRSA strains from South Africa. Lane 1 is the molecular weight marker. Lane 2: RKK52; Lane 3: RKK53; Lane 4: RKK55; Lane 5: RKK56; Lane 6: RKK57; Lane 7: 1KH; Lane 8: 2KH; Lane 9: 4KH.



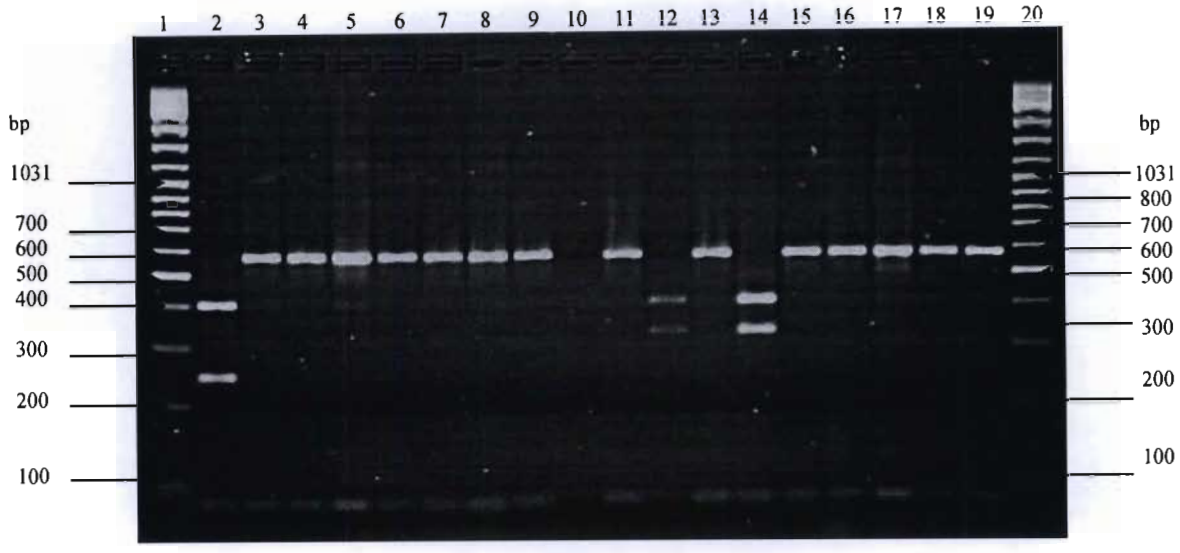
PCR detection of the coagulase gene in MSSA strains from South Africa. Lanes 1 and 20 are molecular weight markers. Lanes 2 and 3 are negative and positive controls. Lane 4: AB; Lane 5: AE; Lane 6: XN; Lane 7: XZ; Lane 8: GSA; Lane 9: XSA; Lane 10: AD 59; Lane 11: AD68; Lane 12: AD80; Lane 13: EDD 11; Lane 14: EDD30; Lane 15: EDD33; Lane 16: EDD38; Lane 17: EDD79; Lane 18: EDD87; Lane 19: EDD96.



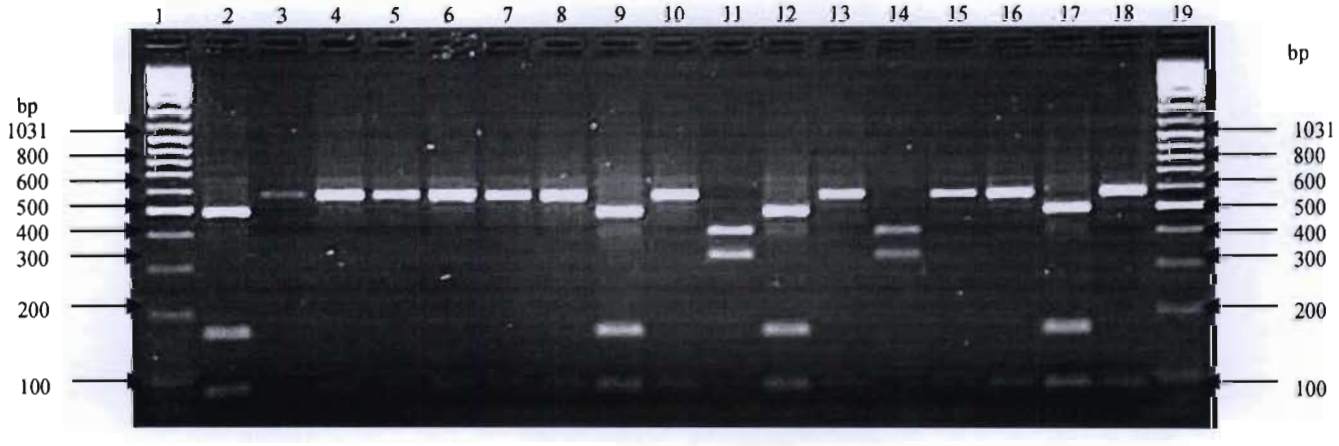
PCR detection of the coagulase gene in MSSA strains from South Africa. Lanes 1 and 19 are molecular weight markers. Lane 2: GT6; Lane 3: GT30; Lane 4: GT41; Lane 5: GT67; Lane 6: PS 61; Lane 7: RKK39; Lane 8: RKK50; Lane 9: RKK54; Lane 10: RKK65; Lane 11: RKK82; Lane 12: EGU60; Lane 13: EGU75; Lane 14: KEH 59; Lane 15: GJC39; Lane 16: GJC74; Lane 17: PM13; Lane 18: GP99.



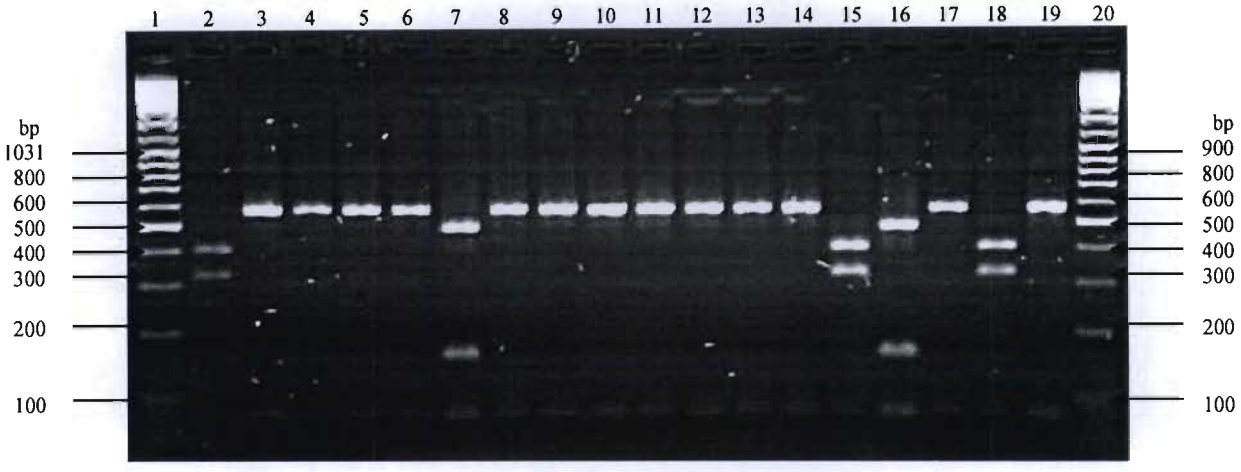
PCR detection of the coagulase gene in MSSA strains from South Africa. Lane 1: Molecular weight marker. Lane 2: EDD49; Lane 3: XF; Lane 4: MSA; Lane 5: WSA.



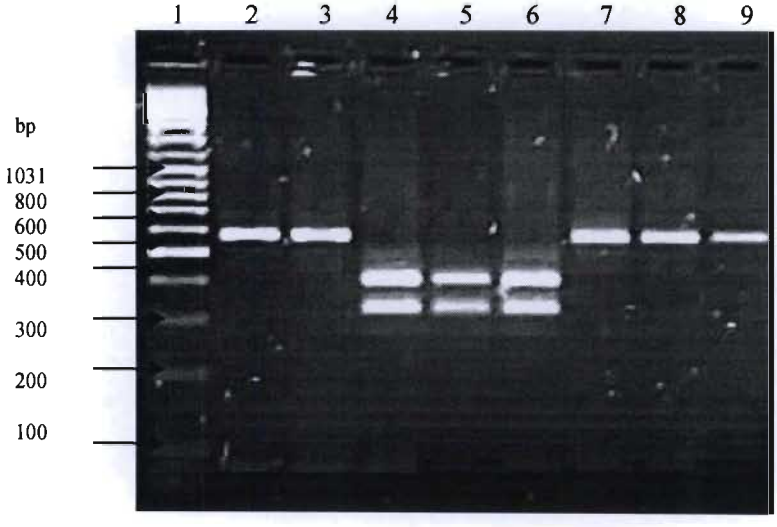
RFLPs of the coagulase gene in MRSA strains from South Africa. Lanes 1 and 20 are molecular weight markers. Lane 2: AC; Lane 3: AD; Lane 4: XQ; Lane 5: XW; Lane 6: 107(2); Lane 7: ESA; Lane 8: HSA; Lane 9: SSA; Lane 10: 203; Lane 11: 503; Lane 12: 510; Lane 13: 511; Lane 14: 513; Lane 15: 114XC; Lane 16: 131XB; Lane 17: MD24; Lane 18: MD43; Lane 19: GJC3.



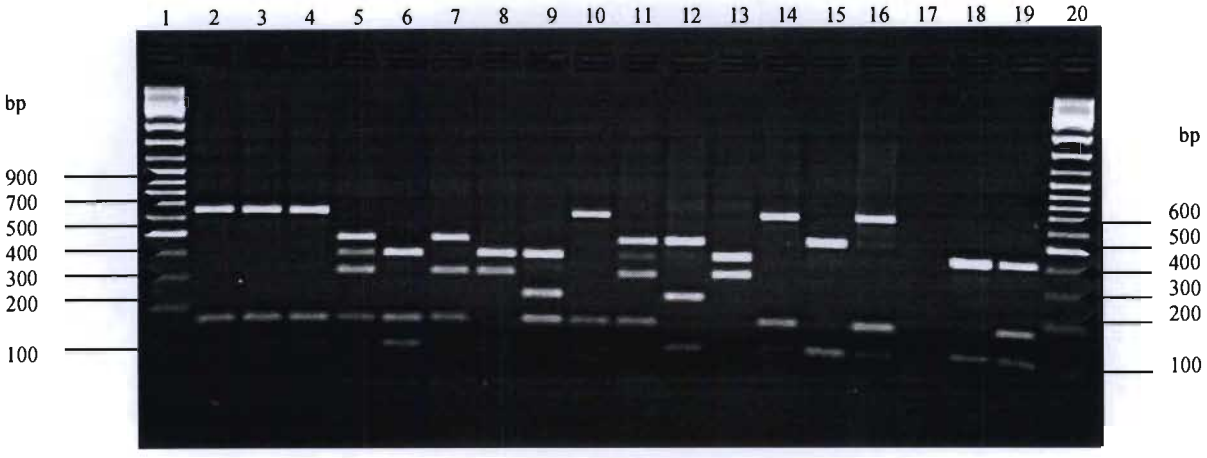
RFLPs of the coagulase gene in MRSA strains from South Africa. Lanes 1 and 19 are molecular weight markers. Lane 2: GJC7; Lane 3: GJC69; Lane 4: GT33; Lane 5: PM 69; Lane 6: EDD52; Lane 7: EDD70; Lane 8: EDD84; Lane 9: EDD99; Lane 10: KEH12; Lane 11: KEH26; Lane 12: KEH77; Lane 13: KEH88; Lane 14; AD28; Lane 15: AD84; Lane 16: AD79; Lane 17: AD77; Lane 18: AD69.



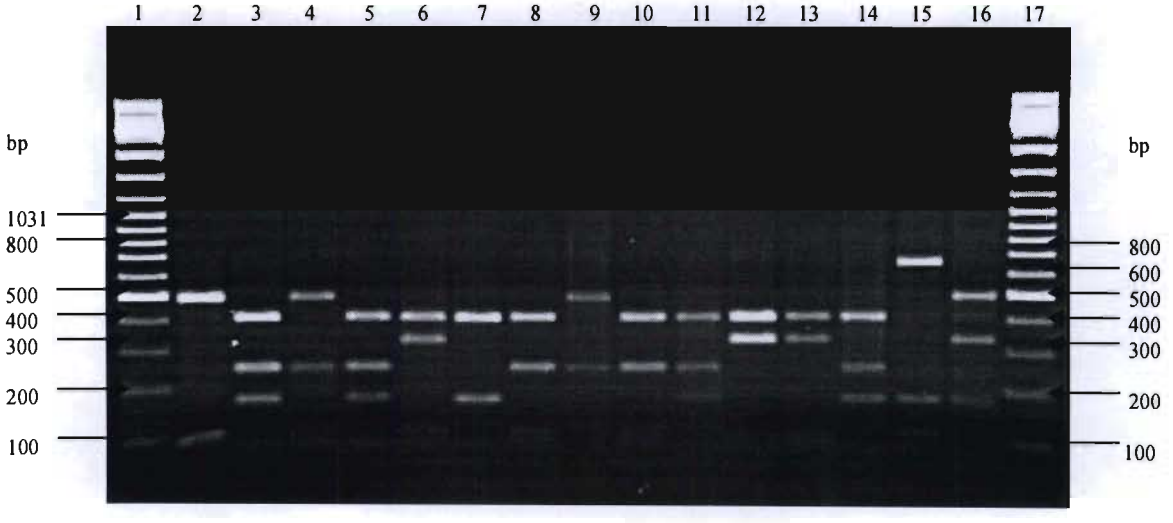
RFLPs of the coagulase gene in MRSA strains from South Africa. Lanes 1 and 20 are molecular weight markers. Lane 2: AD87; Lane 3: AD98; Lane 4: ESH20; Lane 5: ESH34; Lane 6: ESH37; Lane 7: ESH89; Lane 8: EGU23; Lane 9: EGU28; Lane 10: EGU51; Lane 11: PS 33; Lane 12: PS44; Lane 13: PS50; Lane 14: PS94; Lane 15: GP11; Lane 16: GP74; Lane 17: RKK6; Lane 18: RKK8; Lane 19: RKK10.



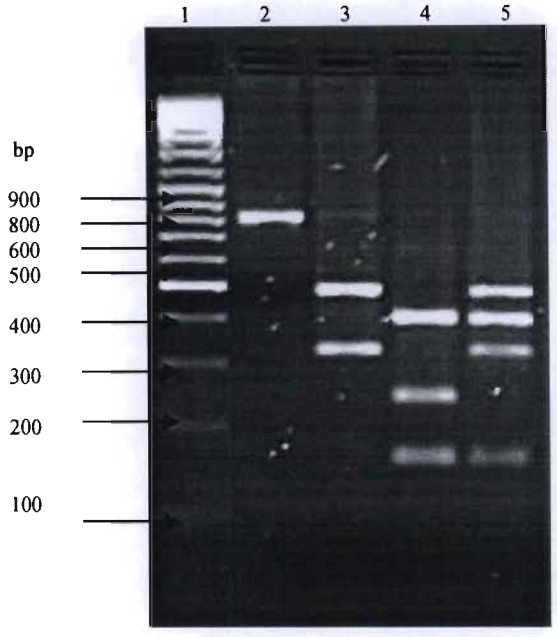
RFLPs of the coagulase gene in MRSA strains from South Africa. Lane 1 is the molecular weight marker. Lane 2: RKK52; Lane 3: RKK53; Lane 4: RKK55; Lane 5: RKK56; Lane 6: RKK57; Lane 7: 1KH; Lane 8: 2KH; Lane 9: 4KH.



RFLPs of the coagulase gene in MSSA strains from South Africa. Lanes 1 and 20 are molecular weight markers. Lane 2: AB; Lane 3: AE; Lane 4: XN; Lane 5: XZ; Lane 6: GSA; Lane 7: XSA; Lane 8: AD59; Lane 9: AD68; Lane 10: AD80; Lane 11: EDD11; Lane 12: EDD30; Lane 13: EDD33; Lane 14: EDD38; Lane 15: EDD79; Lane 16: EDD87; Lane 17: EDD96; Lane 18: GT 6; Lane 19: GT30.



RFLPs of the coagulase gene in MSSA strains from South Africa: Lanes 1 and 17 are molecular weight markers. Lane 2: GT41, Lane 3: GT67; Lane 4: PS61; Lane 5: RKK39; Lane 6: RKK50; Lane 7: RKK54; Lane 8: RKK65; Lane 9: RKK82; Lane 10: EGU60; Lane 11: EGU75; Lane 12: KEH59; Lane 13: GJC39; Lane 14: GJC74; Lane 15: PM13; Lane 16: GP99.

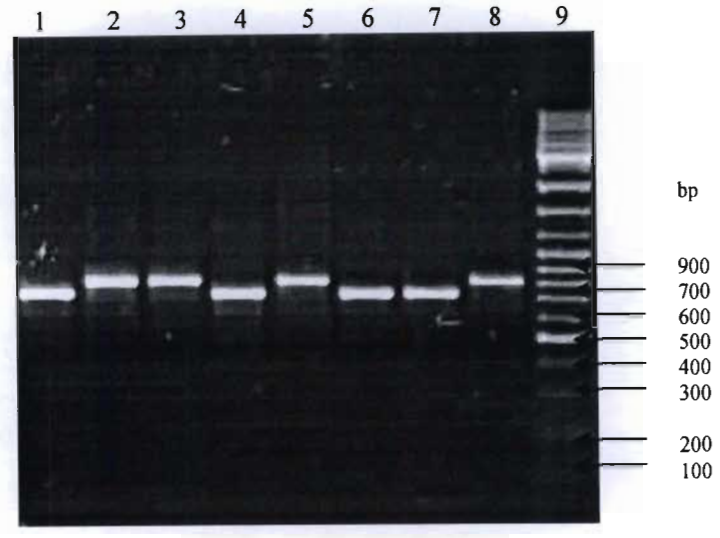


RFLPs of the coagulase gene in MSSA strains from South Africa. Lane 1: Molecular weight marker. Lane 2: EDD49; Lane 3: XF; Lane 4: MSA; Lane 5: WSA.

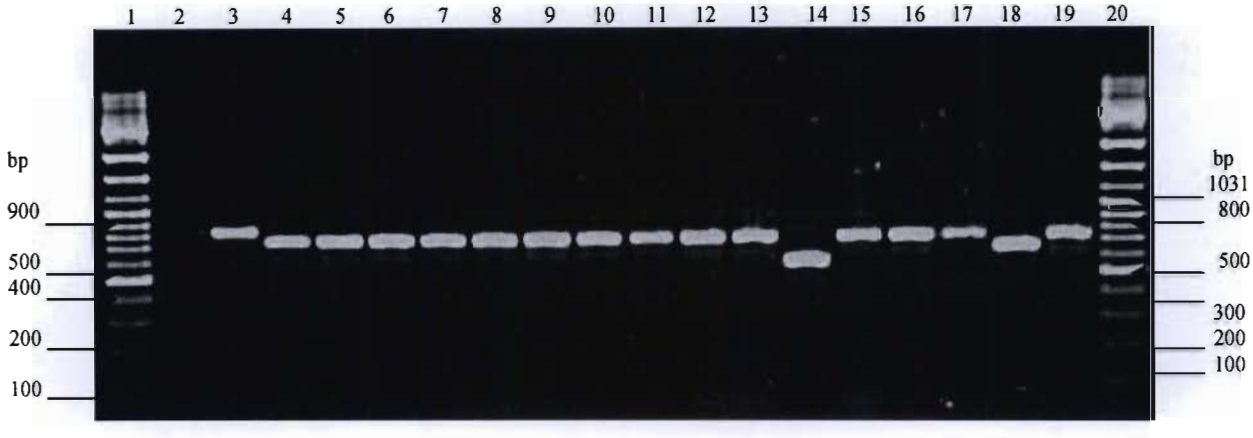
PCR-RFLP of the coagulase gene (MSSA and MRSA strains from Nigeria)

Strain No	Molecular weight (bp)	RFLP (bp)	Strain No	Molecular weight (bp)	RFLP (bp)
P	570	162, 405	4 IDA	820	162, 243, 405
B31	570	81, 243, 405	35 IBA	810	81, 162, 567
			28* Ife	810	81, 324, 405
2 IBA	660	243, 405	E1	810	81, 324, 405
			5IB	820	81, 324, 405
23* IBA	720	81, 243, 405	11IBA	820	81, 324, 405
C20	720	243, 405	12IBA	820	81, 324, 405
C35	720	81, 162, 405	13(1) IBA	810	81, 324, 405
B6	720	81, 162, 405	40*IBA	810	810
B10	720	81, 243, 405	*THCD	810	81, 162, 567
B35	720	162, 405	*28 IDA	820	81, 162, 486
32 IDA	730	81, 243, 405	19	830	81, 324, 405
1 IBA	730	81, 243, 405	4IB	830	81, 324, 405
22 IDA	730	81, 243, 405			
D12	760	81, 243, 405	16* IBA	900	81, 324, 486
D28	760	81, 243, 405	19* IBA	880	81, 324, 486
D37	760	81, 162, 405			
D45	750	81, 243, 405	7IBA	970	162, 324, 486
10	750	81, 243, 405			
29	750	81, 162, 405	1*IBA	1000	162, 324, 486
H	750	81, 243, 405	D40	1000	162, 324, 486
R	740	81, 162, 405	11	1010	162, 324, 486
G	730	81, 243, 405			
C	740	81, 243, 405	32	840, 750	81, 162, 324, 405
Y	740	729	20	840, 750	81, 162, 324, 405
15 Ife	730	81, 243, 405			
40 Ife	740	243, 405	18*IBA	980, 400	162, 324, 405, 486
4 IBA	740	81, 243, 405	50IBA	990, 400	162, 324, 405, 486
9 IBA	730	81, 243, 405	THK	1000, 410	162, 324, 405, 486
*15	730	81, 243, 405	THW	980, 410	162, 324, 405, 486
			B9	980, 400	162, 324, 405, 486

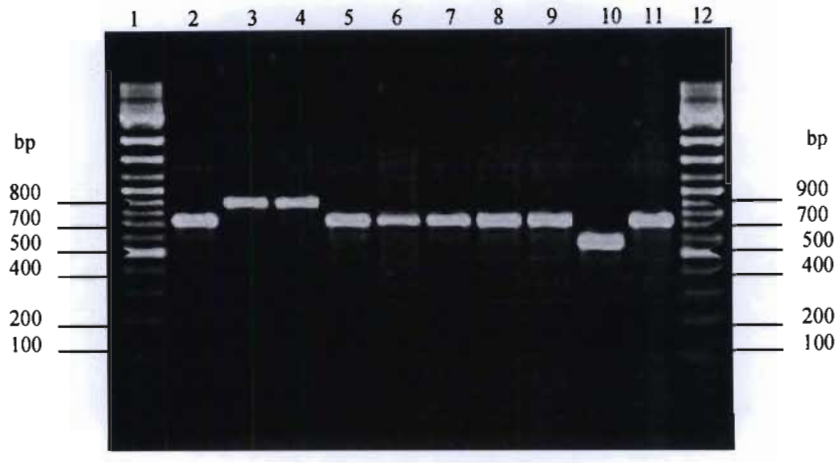
*MRSA strains from Nigeria



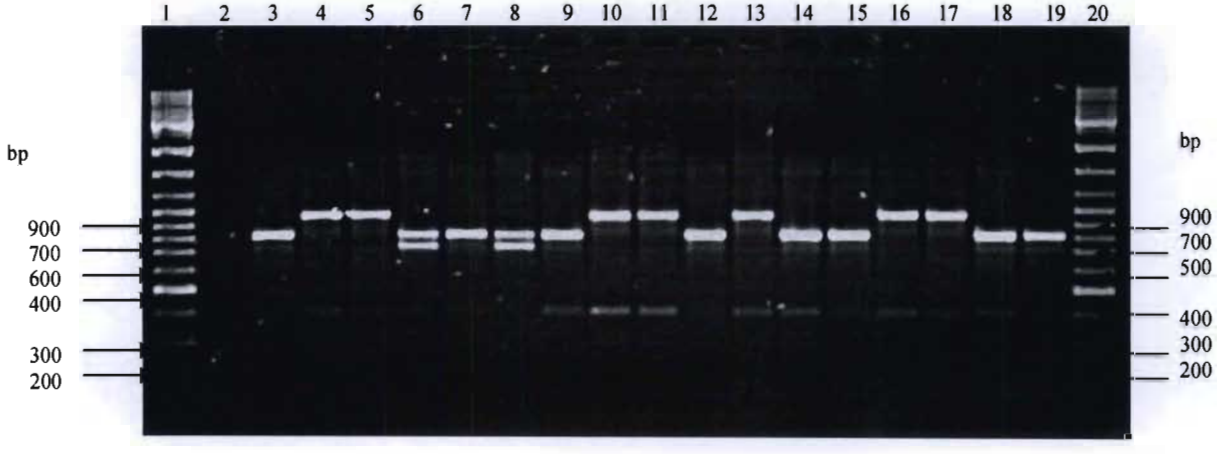
PCR detection of the coagulase gene. MRSA and MSSA strains from Nigeria. MRSA strains from Nigeria. Lane 1: strain No. 15 – (Hospital C); Lane 2: THCD (Hospital A) and Lane 5: 28 IDA (Hospital F). MSSA strains: Lane 3: 41DA; Lane 4: 22IDA; Lane 6: 32IDA; Lane 7: 11BA; Lane 8: 35IBA. Lane 9 is the molecular weight marker.



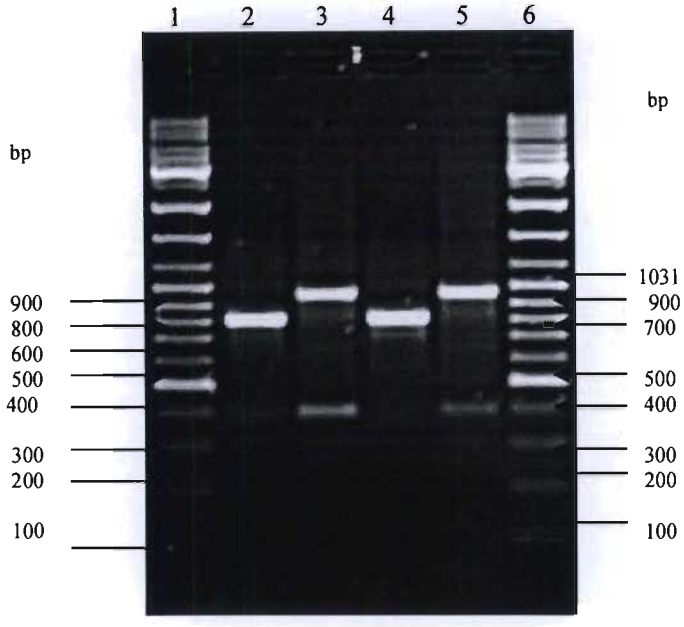
PCR detection of the coagulase gene in MSSA strains from Nigeria. Lanes 1 and 20 are molecular weight markers. Lanes 2 and 3 are negative and positive controls. Lane 4: D12; Lane 5: D28; Lane 6: D37; Lane 7: D45; Lane 8: 10; Lane 9: 29; Lane 10: H; Lane 11: R; Lane 12: G; Lane 13: C; Lane 14: P; Lane 15: Y; Lane 16: 15Ife; Lane 17: 40Ife; Lane 18: 2IBA; Lane 19; 4IBA.



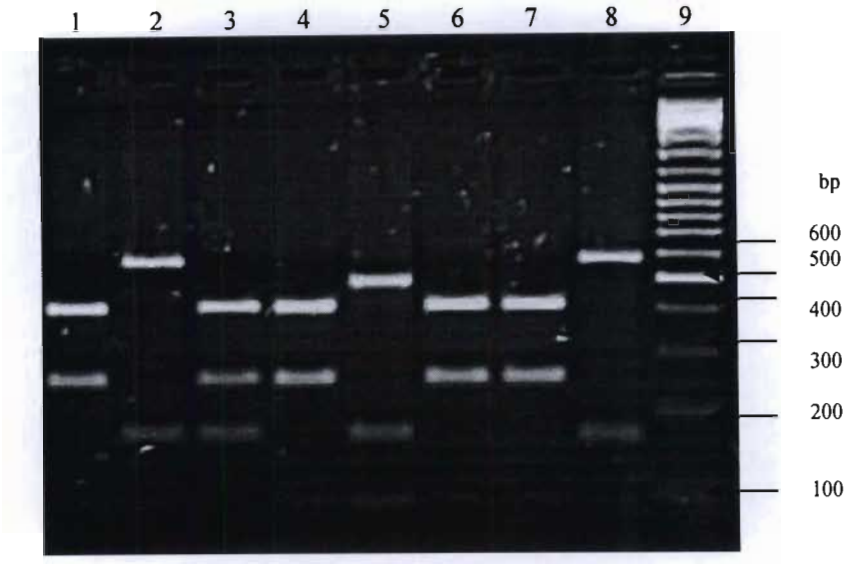
PCR detection of the coagulase gene in MSSA strains from Nigeria. Lanes 1 and 12 are molecular weight markers. Lane 2: 9IBA; Lane 3:16*IBA; Lane 4: 19*IBA; Lane 5: 23*IBA; Lane 6: C20; Lane 7: C35; Lane 8: B6; Lane 9: B10; Lane 10: B31; Lane 11: B35.



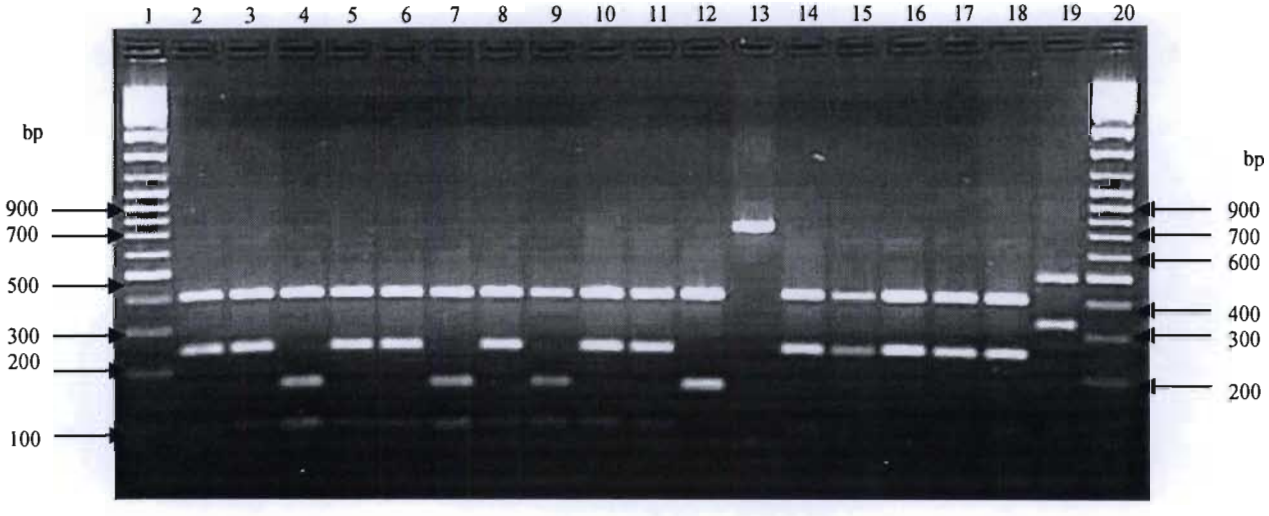
PCR detection of the coagulase gene in MSSA strains from Nigeria. Lanes 1 and 20 are molecular weight markers. Lanes 2 and 3 are negative and positive controls; Lane 4: D40; Lane 5: 11; Lane 6: 32; Lane 7: 19; Lane 8: 20; Lane 9: E1; Lane 10: THK; Lane 11: THW; Lane 12: 20*Ife; Lane 13: B9; Lane 14: 4IB; Lane 15: 5IB; Lane 16: 1*IBA, Lane 17: 7IBA; Lane 18: 11IBA; Lane 19: 12IBA.



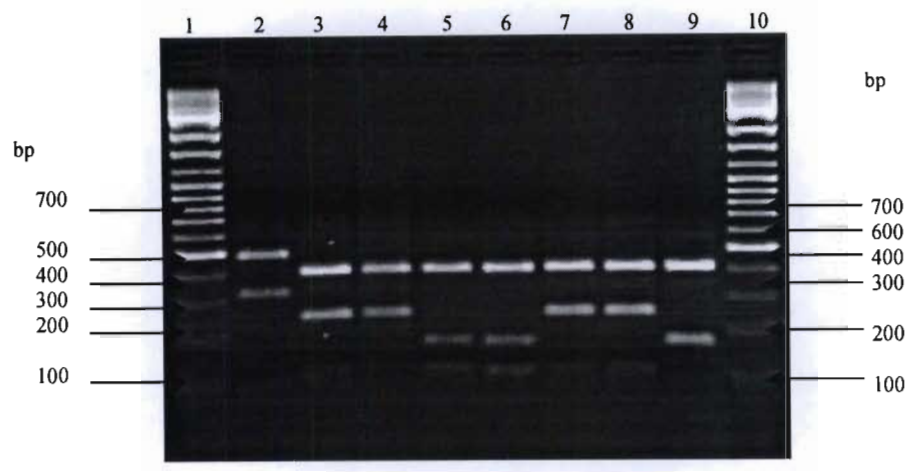
PCR detection of the coagulase gene in MSSA strains from Nigeria. Lanes 1 and 6 are molecular weight markers. Lane 2: 13(1) IBA; Lane 3: 18*1BA; Lane 4: 40*IBA; Lane 7: 50IBA.



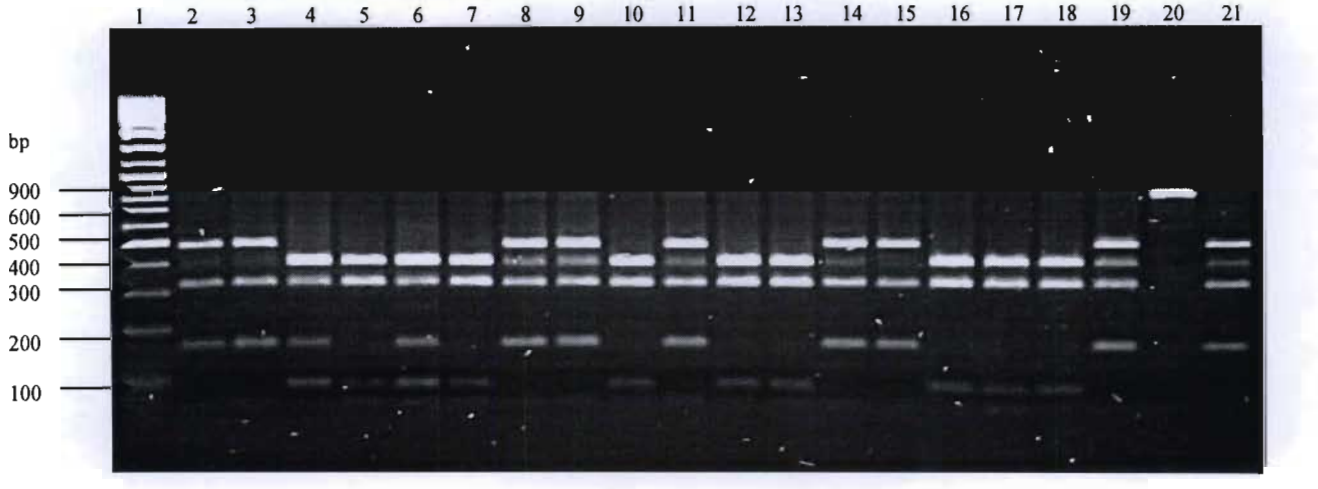
RFLPs of the coagulase gene in MRSA and MSSA strains from Nigeria. MRSA strains Lane 1: strain No. 15 – (Hospital C); Lane 2: THCD (Hospital A) and Lane 5: 28 IDA (Hospital F). MSSA strains Lane 3: 4IDA; Lane 4: 22IBA; Lane 6: 32IDA; Lane 7: 1IBA; Lane 8: 35IBA. Lane 9 is the molecular weight marker.



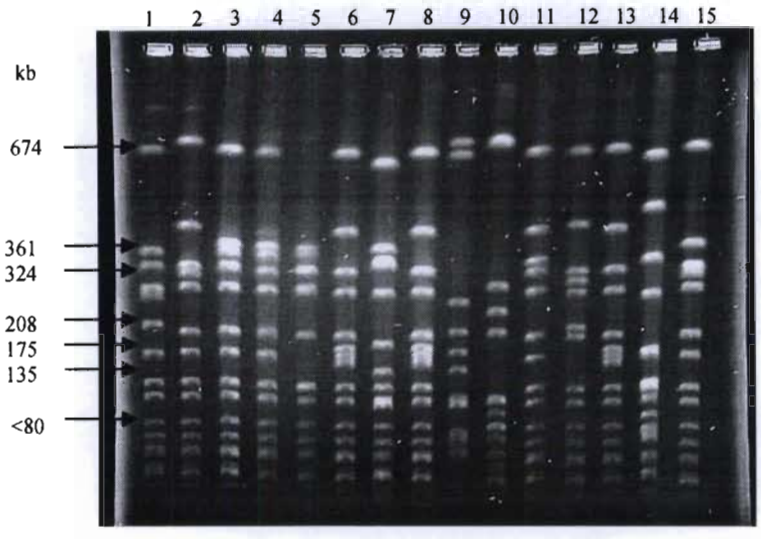
RFLPs of the coagulase gene in MSSA strains from Nigeria. Lanes 1 and 20 are molecular weight markers. Lane 2: D12; Lane 3: D28; Lane 4: D37; Lane 5: D45; Lane 6: 10; Lane 7: 29; Lane 8: H; Lane 9: R; Lane 10: G; Lane 11: C; Lane 12: P; Lane 13: Y; Lane 14: 15Ife; Lane 15: 40Ife; Lane 16: 2IBA; Lane 17: 4IBA; Lane 18: 9IBA; Lane 19: 16*IBA.



RFLPs of the coagulase gene in MSSA strains from Nigeria. Lanes 1 and 10 are molecular weight markers. Lane 2: 19*IBA; Lane 3: 23*IBA; Lane 4: C20; Lane 5: C35; Lane 6: B6; Lane 7: B10; Lane 8: B31; Lane 9: B35.

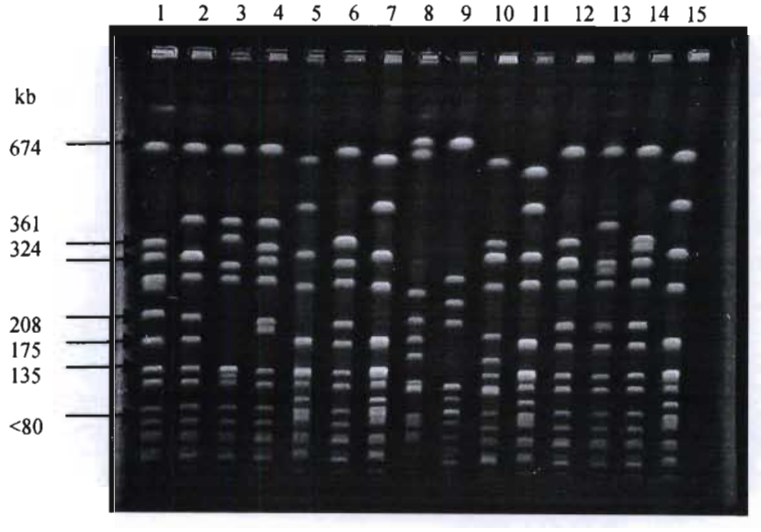


RFLPs of the coagulase gene in MSSA strains from Nigeria. Lane 1 is molecular weight marker. Lane 2: D40; Lane 3: 11; Lane 4: 32; Lane 5: 19; Lane 6: 20; Lane 7: E1; Lane 8: THK; Lane 9: THW; Lane 10: 20*Ife; Lane 11: B9; Lane 12: 4IB: Lane 13: 5IB; Lane 14: 1*IBA; Lane 15: 7IBA; Lane 16: 11IBA; Lane 17: 12IBA; Lane 18: 13(1) IBA; Lane 19: 18*1BA; Lane 20: 40*IBA; Lane 21: 50IBA.



PFGE types a1 b1 b1 b5 c d1 c E15 E16 a1 e c g b2

PFGE profiles of representative MRSA clones and worldwide clones. Lane 1: *S. aureus* NCTC 8325; Lanes 9 and 10: EMRSA-15 and EMRSA-16; Lane 2: AC; Lane 3: AD; Lane 4: ESA; Lane 5: HSA; Lane 6: 503Red; Lane 7: 513; Lane 8: 114XC; Lane 11: MD24; Lane 12: MD43; Lane 13: GJC3; Lane 14: GJC7; Lane 15: GJC69.



PFGE types a3 f a4 g b4 g E15 E16 d1 g b4 a1 b1 g

PFGE profiles of representative MRSA clones and worldwide clones. Lane 1: *S. aureus* NCTC 8325; Lanes 8 and 9: EMRSA-15 and EMRSA-16; Lane 2: GT33; Lane 3: PM69; Lane 4: EDD52; Lane 5: EDD99; Lane 6: KEH12; Lane 7: KEH77; Lane 10: AD28; Lane 11 AD77; Lane 12: AD79; Lane 13: AD84; Lane 14: ESH34; Lane 15: ESH89.

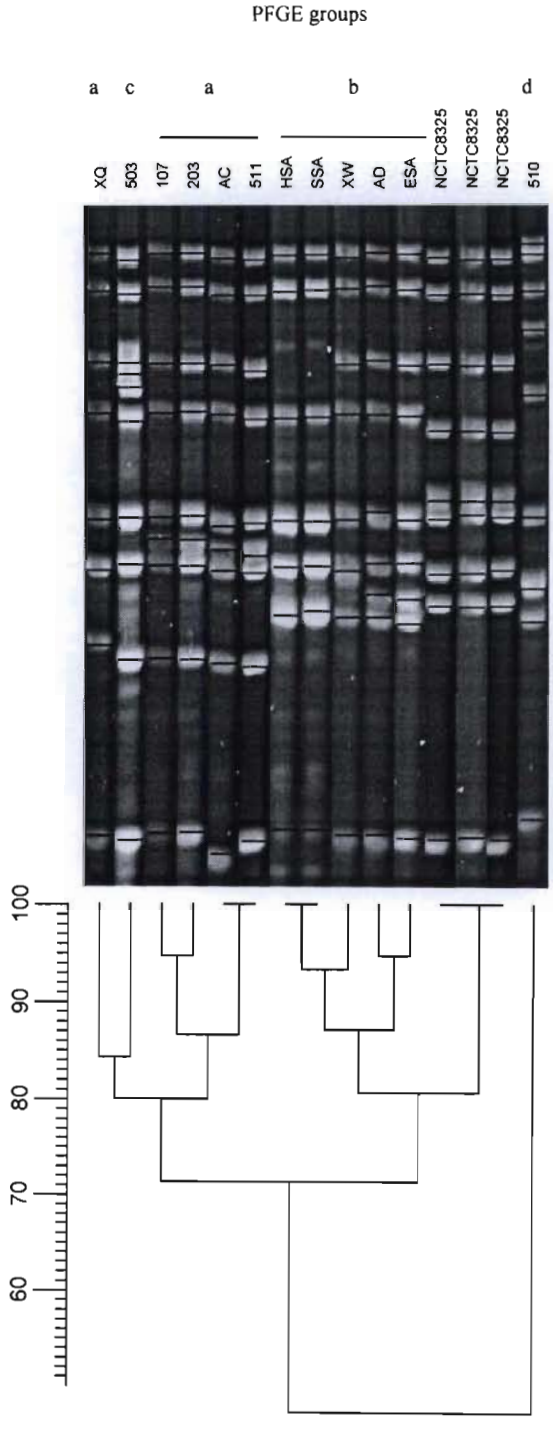
Antibiotyping and PFGE of MRSA strains from Nigeria and South Africa

Strain no	PFGE type	Antibiogram	Strain no	PFGE type	Antibiogram
AC	a1	PEN, GN, ERY, TET, TS, RF	510	d1	PEN, GN, ERY, CHL, TET, TS, CIP, MU5
107-2	a1	PEN, GN, ERY, TET, TS, RF	513	d1	PEN, GN, ERY, TET, TS, CIP, MU5
203	a1	PEN, GN, ERY, TET, TS, RF	KEH26	d1	PEN, GN, ERY, CHL, TET, TS, CIP, MU5
511	a1	PEN, GN, ERY, TET, TS, RF	AD28	d1	PEN, GN, ERY, CHL, TET, TS, CIP, MU5
MD24	a1	PEN, GN, TET, TS, RF	AD87	d1	PEN, GN, ERY, TET, TS, CIP, MU5
AD84	a1	PEN, GN, ERY, TET, TS, RF	GP11	d1	PEN, GN, ERY, CHL, TET, TS, CIP, MU5
PS33	a1	PEN, GN, ERY, TET, TS, RF	RKK8	d1	PEN, GN, ERY, TET, TS, CIP, MU5
PS44	a1	PEN, GN, ERY, TET, TS, RF	RKK56	d1	PEN, GN, ERY, TET, TS, CIP, MU5
PS50	a1	PEN, GN, ERY, CHL, TET, TS, RF	RKK57	d1	PEN, GN, ERY, TET, TS, CIP, MU5
PS94	a1	PEN, GN, ERY, CHL, TET, TS, RF	RKK55	d2	PEN, GN, ERY, TET, TS, CIP, MU5
RKK52	a1	PEN, GN, ERY, CHL, TET, TS, RF			
1KH	a1	PEN, GN, ERY, TET, TS, RF	MD43	e	PEN, GN, ERY, TET, TS, RF
2KH	a1	PEN, GN, ERY, TET, TS, RF			
4KH	a1	PEN, GN, ERY, TET, TS, RF	PM69	f	PEN, GN, ERY, TET, TS, RF
RKK53	a2	PEN, GN, ERY, TET, TS, RF			
XQ	a3	PEN, GN, CHL, TET, TS, RF	GJC7	g	PEN, GN, ERY
EGU51	a3	PEN, GN, ERY, TET, TS, RF	EDD99	g	PEN, GN, ERY
GT33	a3	PEN, GN, ERY, TET, TS, RF	KEH77	g	PEN, GN, ERY
RKK6	a3	PEN, GN, ERY, TET, TS, RF, MU5	AD77	g	PEN, GN, ERY
RKK10	a3	PEN, GN, ERY, TET, RF	ESH89	g	PEN, GN, ERY
EDD52	a4	PEN, GN, ERY, TET, TS, RF	GP74	g	PEN, GN, ERY
			EDD84	h	PEN, GN, ERY, TET, TS, RF
AD	b1	PEN, TET, RF			
ESA	b1	PEN, GN, ERY, TET, TS, RF, CIP	THCD*	a5	PEN, ERY, TET, TS
KEH88	b1	PEN, GN, ERY, TET, TS, RF			
ESH34	b1	PEN, GN, ERY, TET, TS, RF, MU5	15	i	PEN, TET
XW	b2	PEN, GN, TET, TS, RF			
GJC69	b2	PEN, GN, TET, TS, RF	281DA	j	PEN, ERY, TET, CIP
EDD70	b2	PEN, GN, ERY, CHL, TET, TS, RF			
AD69	b2	PEN, GN, TET, TS, RF			
ESH20	b2	PEN, GN, ERY, TET, TS, RF			
ESH37	b3	PEN, TET, RF			
KEH12	b4	PEN, GN, ERY, TET, TS, RF, MU5			
AD79	b4	PEN, GN, TET, TS, RF, MU5			
AD98	b4	PEN, GN, TET, TS, RF, MU5			
EGU23	b4	PEN, GN, CHL, TET, TS, RF			
EGU28	b4	PEN, GN, ERY, TET, TS, RF			
HSA	b5	PEN, GN, ERY, TET, TS, RF			
SSA	b5	PEN, GN, ERY, TET, TS, RF			
503	c	PEN, GN, ERY, TET, TS, RF			
114XC	c	PEN, GN, ERY, TET, TS, RF			
131XB	c	PEN, GN, TET, TS, RF			
GJC3	c	PEN, GN, ERY, TET, TS, RF			

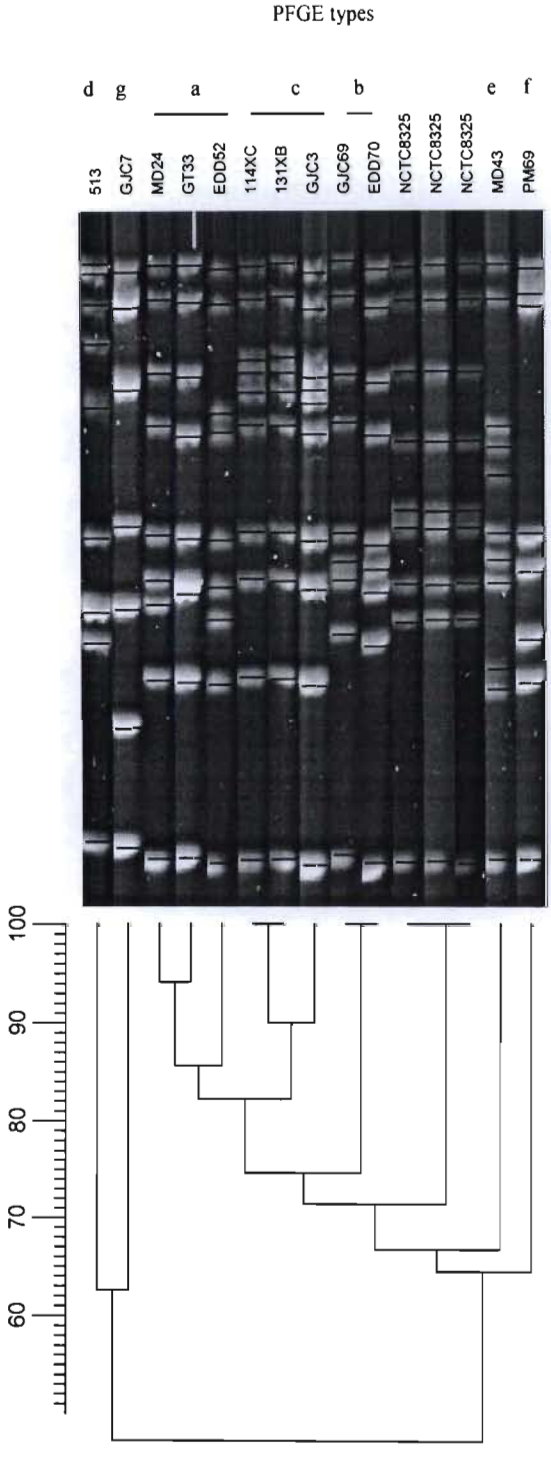
PCR-RFLP of the coagulase gene and PFGE patterns of MRSA strains from South Africa

Strain Number	Molecular weight (± 20bp)	RFLP (bp)	PFGE type	Strain Number	Molecular weight (bp)	RFLP (bp)	PFGE type
				AC	730	81, 243, 405	a1
XW	640	81, 567	b2				
AD 69	630	81, 567	b2	GJC 7	820	81, 162, 486	g
EDD 84	640	81, 567	h	EDD 99	780	81, 162, 486	g
AD	650	81, 567	b1	KEH 77	780	81, 162, 486	g
XQ	640	81, 567	a3	AD 77	780	81, 162, 486	g
107 (2)	650	81, 567	a1	GP 74	790	81, 162, 486	g
ESA	640	81, 567	b1				
HSA	650	81, 567	b5	RKK 8	820	81, 324, 405	d1
SSA	640	81, 567	b5	RKK 56	820	81, 324, 405	d1
503	650	81, 567	c	RKK 57	790	81, 324, 405	d1
511	650	81, 567	a1	GP 11	790	81, 324, 405	d1
114XC	650	81, 567	c	KEH 26	790	81, 324, 405	d1
131XB	650	81, 567	c	513	810	81, 324, 405	d1
MD 24	650	81, 567	a1	AD 28	810	324, 405	d1
MD 43	650	81, 567	e	510	810	324, 405	d1
GJC 3	650	81, 567	c				
GT 33	630	81, 567	a3	ESH 89	840	81, 162, 486	g
PM 69	650	81, 567	f	AD 87	830	324, 405	d1
EDD 52	650	81, 567	a4	RKK 55	830	81, 324, 405	d2
EDD 70	650	81, 567	b2				
KEH 12	640	81, 567	b4				
KEH 88	630	81, 567	b1	203	No product		a1
AD 79	640	81, 567	b4				
AD 84	640	81, 567	a1				
PS 33	650	81, 567	a1				
PS 44	650	81, 567	a1				
PS 50	640	81, 567	a1				
PS 94	650	81, 567	a1				
RKK 6	640	81, 567	a3				
RKK 10	640	81, 567	a3				
RKK 52	640	81, 567	a1				
RKK 53	650	81, 567	a2				
1 KH	630	81, 567	a1				
2 KH	640	81, 567	a1				
AD 98	660	81, 567	b4				
ESH 20	660	81, 567	b2				
ESH 34	660	81, 567	b1				
ESH 37	660	81, 567	b3				
EGU 23	660	81, 567	b4				
EGU 28	660	81, 567	b4				
EGU 51	670	81, 567	a3				
4 KH	650	567	a1				
GJC 69	660	567	b2				

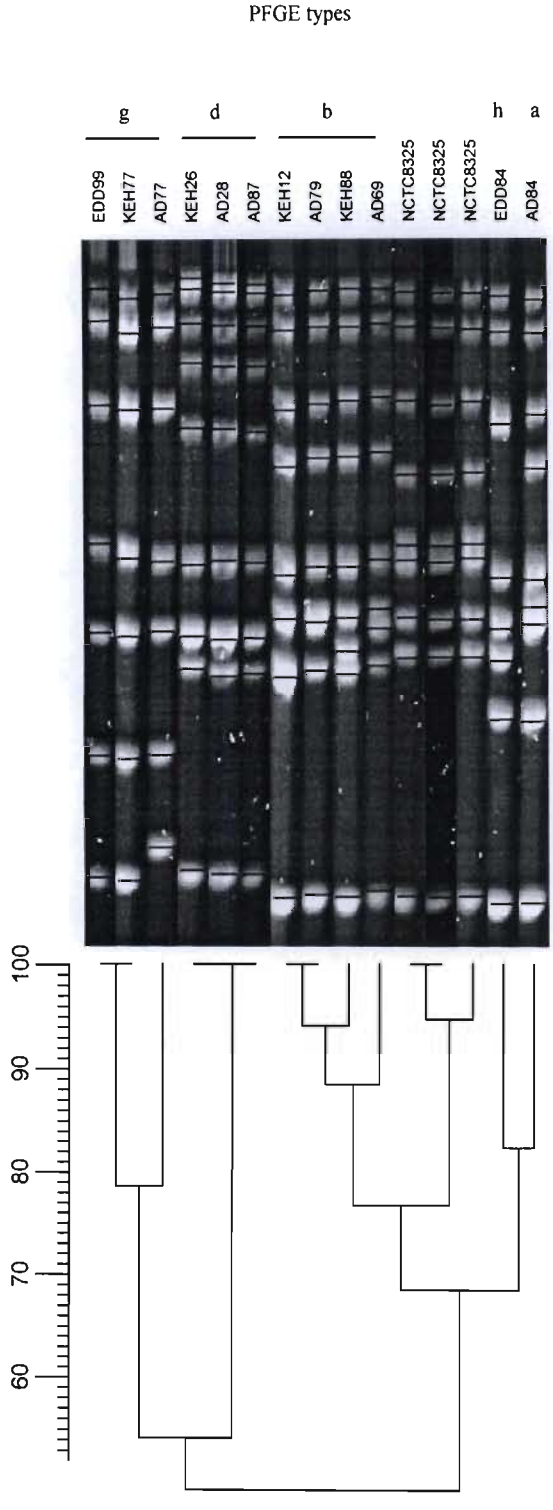
PFGE of MRSA strains (South Africa) - Dendogram



PFGE of MRSA strains (South Africa) - Dendogram



PFGE of MRSA strains (South Africa) - Dendogram



PFGE of MRSA strains (South Africa) - Dendogram

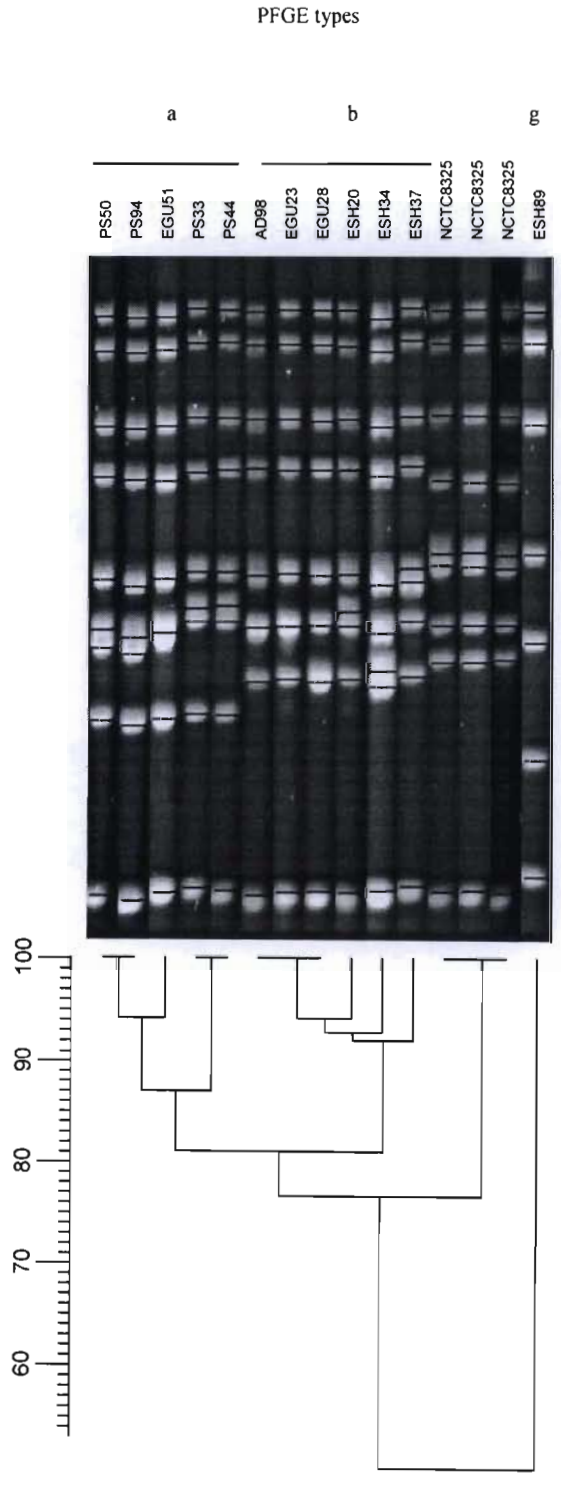
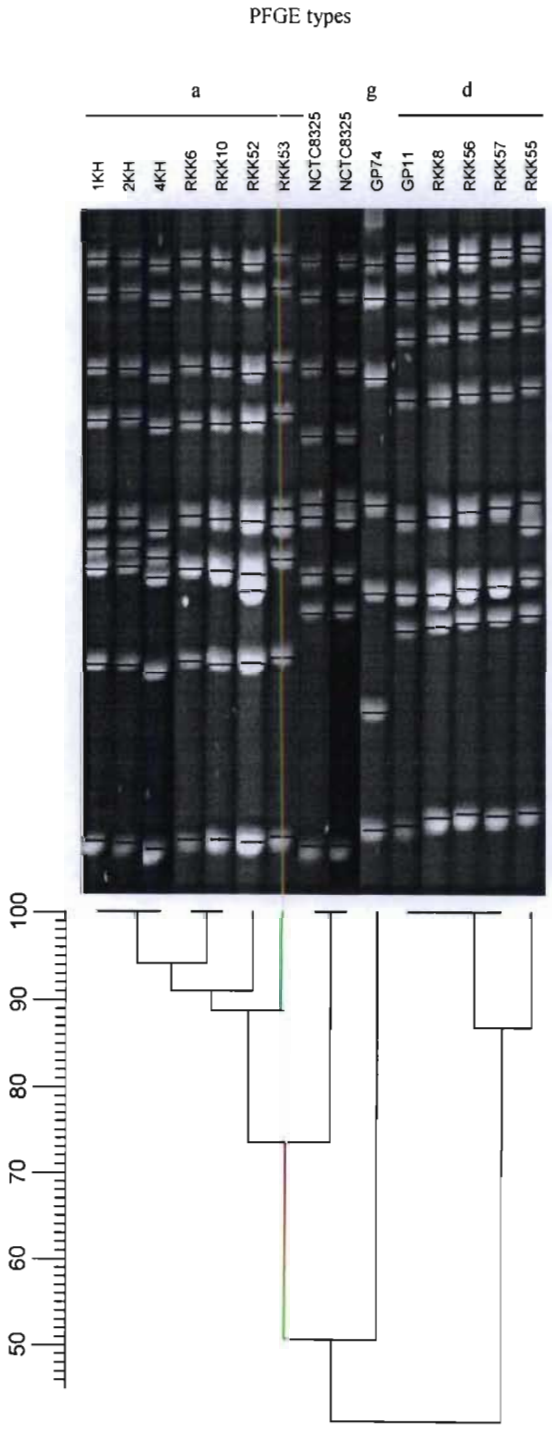
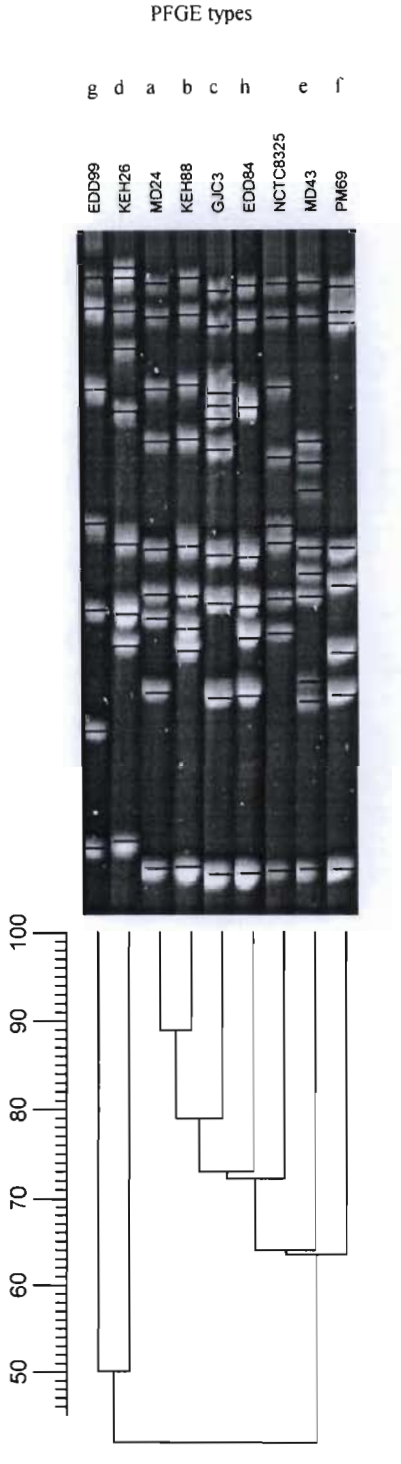


Figure 3.31: PFGE of MRSA strains (South Africa) – Dendogram



Dendogram of representative MRSA clones (South Africa)



PFGE of MRSA strains (Nigeria) - Dendogram

PFGE patterns of low-level mupirocin resistant strains - Dendogram