MOLECULAR CHARACTERISATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATED AT THE UNIVERSITY TEACHING HOSPITAL, LUSAKA

By

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A Dissertation Submitted to the University of Zambia in Partial Fulfilment of the Requirements for the Degree of Master of Science in Medical Microbiology

The University of Zambia
Lusaka
July, 2014
Declaration

I, Mulemba Tillika Samutela, do declare that this dissertation represents my own work. This work has not been done in Zambia before and neither has it been submitted for any qualification at the University of Zambia or any other university.

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Signature: ............................................

Date: .................................................
Certificate of Approval

Dissertation Title: Molecular Characterisation of Methicillin-Resistant Staphylococcus Aureus Isolated at the University Teaching Hospital, Lusaka

This dissertation of Mulemba Tillika Samutela has been approved in partial fulfilment of the requirements for the degree of Master of Science in Medical Microbiology at the University of Zambia.

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Month/Date/Year

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Examiner 2
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Abstract

Methicillin-resistant *Staphylococcus aureus* is one of the major causes of nosocomial infections worldwide. It is endemic in hospitals and prevalent in the community and amongst livestock. Morbidity and mortality amongst patients with Methicillin-resistant *Staphylococcus aureus* infections are high due to resistance to many antibiotics. In Zambia, there has been an increase in the number of cases of Methicillin-resistant *Staphylococcus aureus*, from 23% in 2003 to 30.7% in 2010, but its molecular characteristics were unknown. In addition, its antibiotic resistance patterns are not clearly defined. Therefore, the objective of this study was to characterise Methicillin-resistant *Staphylococcus aureus* isolated from the University Teaching Hospital in Lusaka, Zambia, using molecular tools. The antimicrobial susceptibility pattern of the Methicillin-resistant *Staphylococcus aureus* was also determined. This was a laboratory-based cross-sectional study. Ninety-five clinical isolates of *Staphylococcus aureus*, collected between June 2009 and December 2012 at the University Teaching Hospital in Lusaka, Zambia, were analysed by SCCmec and spa typing. Antibiotic susceptibility testing was performed using the Kirby-Bauer disc diffusion method. The results demonstrated that, of the 95 *S. aureus* isolates, 43% were Methicillin-resistant *Staphylococcus aureus* strains. Antibiotic resistance to common anti-staphylococcal drugs ranged from 68% to 100%. Multi-drug resistance rates ranged from 17.5 % to 35%. The most prevalent SCCmec types were SCCmec type IV (63%) and SCCmec type III (14.6%). Five spa types, which included a novel type, were detected and the most prevalent spa type was t064 (13%). The prevalence of multidrug resistant Methicillin-resistant *Staphylococcus aureus* was found to be high and has continued to increase. The high prevalence of SCCmec type IV and spa type t064 suggests that the strains circulating are hospital-
acquired and that there may be high genetic exchange amongst the bacterial strains. Regular surveillance and screening is recommended for infection control and treatment guidance.
Dedication

This dissertation is dedicated to my mother Elliet Banda and my daughter Lukundo Nachalwe.
Acknowledgements

First and foremost, I would like to thank and acknowledge the Almighty God for all things and for the gift of life. He has always been faithful to me. Secondly, I would like to thank Dr. Geoffrey Kwenda and Dr. James Mwansa for their unwavering supervision, patience and support without which this work would not have been a success.

A special word of gratitude goes to my classmates, Annie, Grace, Catherine, Mox and Mulowa for their friendship and inspiration in my work throughout the long hours we spent together in the laboratory. Thank you very much. I also would like to extend my gratitude to Angela and Marah for emotional support.

I also owe my indebtedness to Dr. Lukwesa the Unit Head of the UTH Bacteriology Laboratory and the rest of the members of staff. I appreciate their camaraderie and support. They made the laboratory a convivial place to work in.

I would like to thank my parents and siblings for love, guidance, motivation and for being patient with me during my MSc studies. My special gratitude should go to my husband, Kateula, for his enduring love and unwavering support. I also would like to thank the rest of my friends for their support and encouragement throughout my study.

Lastly, I would like to gratefully acknowledge the financial support for my MSc studies. I was supported in part by the SACORE Research Centre, School of Medicine and the University of Zambia Staff Development Office.
# Table of Contents

Declaration ............................................................................................................. ii  
Certificate of Approval ......................................................................................... iii  
Abstract ................................................................................................................. iv  
Dedication ............................................................................................................... vi  
Acknowledgements ............................................................................................... vii  
List of Figures ......................................................................................................... x  
List of Tables .......................................................................................................... xi  
List of Abbreviations ............................................................................................. xii  
List of Appendices ................................................................................................. xiii  
Chapter 1: Introduction ........................................................................................... 1  
1.1 Background ....................................................................................................... 1  
1.2 Statement of the Problem .................................................................................. 2  
1.3 Justification of the Study .................................................................................. 2  
1.4 Literature Review .............................................................................................. 3  
1.4.1 General Characteristics of MRSA ............................................................... 3  
1.4.2 Taxonomy and Phylogenetic Status of Staphylococcus .............................. 4  
1.4.3 Epidemiology of MRSA ............................................................................... 7  
1.4.4 Genomics of MRSA ................................................................................... 11  
1.4.5 Mode of Infection ....................................................................................... 14  
1.4.6 Immune Response ....................................................................................... 15  
1.4.7 Pathogenesis and Virulence of MRSA ...................................................... 18  
1.4.8 Clinical Presentation ................................................................................... 21  
1.4.9 Diagnosis of MRSA Infections ................................................................... 22  
1.4.10 Typing Methods of MRSA ....................................................................... 28  
1.4.11 Treatment and Prevention ........................................................................ 30  
1.5 Research Questions .......................................................................................... 32  
1.6 Objectives ......................................................................................................... 32  
1.6.1 General Objective ...................................................................................... 32  
1.6.2 Specific Objectives..................................................................................... 32  
Chapter 2: Materials and Methods ....................................................................... 33  
2.1 Study Design .................................................................................................... 33
List of Figures

Figure 1.1: Classification scheme for SCCmeC subtype by ccr complex and meC complex type .......................................................... 13

Figure 1.2: Pathogenic factors of Staphylococcus aureus............................... 19

Figure 3.1: Detection of MRSA using the oxacillin and cefoxitin discs .................43

Figure 3.2: MRSA susceptibility profiles ..........................................................44

Figure 3.3: SCCmeC gel picture of controls and selected samples .......................46

Figure 3.4: Distribution of SCCmeC types among the MRSA strains ....................47

Figure 3.5: Spa typing gel picture of controls and selected samples ....................47

Figure 3.6: DNA sequences of the spa gene of representative isolates ................48

Figure 3.7: Phylogenetic relationships of the MRSA strains based on spa types ......50

Figure 3.8: Minimum spanning tree showing the relations of isolates in relation to spa types ..................................................................51
List of Tables

Table 2.1: Primer sequences used for SCC\textit{mec} typing ..................................................39

Table 3.1: Inducible Resistance to Macrolides, Lincosamides and Group B Streptogramins ..........................................................44

Table 3.2: Frequency of multi-drug resistance among MRSA isolates .................45

Table 3.3: Antibiotic resistance patterns of the MRSA isolates.........................45

Table 3.4: Distribution of \textit{spa} types among the MRSA isolates.....................49
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-PCR</td>
<td>Arbitrarily primed polymerase chain reaction</td>
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<tr>
<td>BURP</td>
<td>Based Upon Repeat Pattern</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>Community Acquired or Associated Methicillin Resistant <em>Staphylococcus aureus</em></td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>HA-MRSA</td>
<td>Hospital Acquired or Associated Methicillin Resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>LA-MRSA</td>
<td>Livestock Acquired or Associated Methicillin Resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>MRSA</td>
<td>Methicillin Resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>MSSA</td>
<td>Methicillin Susceptible <em>Staphylococcus aureus</em></td>
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<tr>
<td>MLST</td>
<td>Multilocus Sequence Typing</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PCR-RFLP</td>
<td>Polymerase Chain Reaction-Restriction Fragment Length Polymorphism</td>
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<tr>
<td>PFGE</td>
<td>Pulse-Field Gel Electrophoresis</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random Amplified Polymorphic DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>REA</td>
<td>Restriction Endonuclease Analysis</td>
</tr>
<tr>
<td>SCCmecA</td>
<td>Staphylococcal Cassette Chromosome mecA</td>
</tr>
<tr>
<td>SLST</td>
<td>Single-Locus Sequence Typing</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted-Pair Group Matching Analysis</td>
</tr>
<tr>
<td>UTH</td>
<td>University Teaching Hospital</td>
</tr>
</tbody>
</table>
List of Appendices

Appendix A: Ethics Clearance Letter ................................................................................. 98
Appendix B: Media and Reagent Preparation ....................................................................... 99
Appendix C: List of Publications and Presentations ............................................................ 100
Chapter 1: Introduction

1.1 Background

The genus *Staphylococcus* comprises more than 40 species, most of which are part of the normal flora of the skin and mucous membranes of man and other organisms and are generally harmless (Ghebremedhin *et al.*, 2008). Based on their ability to cause disease, the species have been divided into two groups. The most important group is *Staphylococcus aureus* which is capable of causing a wide range of infections (Gordon and Lowy, 2008). However, *Staphylococcus aureus* is occasionally considered as an opportunistic pathogen and is part of the normal flora (David and Daum, 2010). The second group includes all other species of *Staphylococcus* and are collectively referred to as coagulase negative *Staphylococcus* (CNS) (Bannerman, 2003). Generally, members of the CNS are considered harmless to humans and are usually part of normal flora. However, some members of the CNS such as *Staphylococcus epidermidis* have been increasingly implicated in human disease (Burnie *et al.*, 1997; Rosenthal *et al.*, 2008; Schoenfelder *et al.*, 2010; Castro-Alarcon *et al.*, 2011).

The medical importance of *S. aureus* has been heightened by its ability to adapt rapidly to the selective pressure of antibiotics and the resultant emergence and spread of methicillin-resistant *S. aureus* (MRSA) (Deurenberg *et al.*, 2007). MRSA strains do not account for a large proportion of staphylococcal infections compared to their counterparts, methicillin-susceptible *S. aureus* (MSSA) (Miko *et al.*, 2013). In addition, MRSA is not necessarily more virulent than MSSA (Gordon and Lowy, 2009). However, MRSA infections are more difficult and expensive to treat than
MSSA (Weller, 2000; Moodley et al., 2010; Chamchod and Raun, 2012). In the European Union, MRSA is estimated to affect more than 150 000 patients annually, resulting in extra hospital cost of EUR 380 million (Köck et al., 2010).

Zambia has not been spared from the increase in the number of MRSA cases. In recent years nosocomial infections due to MRSA have been reported to be on the increase at the University Teaching Hospital (UTH) (Mwenya, 2003; Kapatamoyo et al., 2010; Mulipilwa, 2012). However, no systematic studies have been carried to understand the extent of this problem in Zambia.

1.2 Statement of the Problem
The increasing prevalence of MRSA worldwide is a growing public health concern. Infections due to MRSA are difficult to treat because of the restricted spectrum of antimicrobial drugs of proven efficacy (Stafeni and Goglio, 2010). The consequence of this is high mortality and morbidity amongst people infected with MRSA strains (Moellering, 2012). In Zambia, there is a paucity of data on the prevalence of MRSA, their antimicrobial susceptibility patterns, circulating genotypes or molecular epidemiology. All the available data was derived from studies using conventional phenotypic methods and clinical presentations of diseases associated with Staphylococci.

1.3 Justification of the Study
Due to the difficulties in treating MRSA infections and the consequent implications of mortality and morbidity, it is important to control the spread of MRSA. Effective control measures are dependent on a thorough knowledge of the organism’s
epidemiology. This is achieved through the use of reliable diagnostic and typing tools for detecting and tracking of sources, pathways of spreading infections and studying population genetics. Careful detection of resistant bacteria provides a fundamental basis for infection control measures and antimicrobial surveillance systems since MRSA are mainly known to be drug resistant (Sundsfjord et al., 2004). This also provides clinicians with knowledge when prescribing appropriate drugs for treating patients. In addition, MRSA molecular diagnostic and typing tools are essential for the detection and monitoring of MRSA infections and in helping provide vital information for the implementation of appropriate control measures. To our knowledge this was the first study of its kind in Zambia to employ molecular typing tools for detecting and typing MRSA.

1.4 Literature Review

1.4.1 General Characteristics of MRSA

MRSA is a well-established nosocomial pathogen that causes a wide range of infections, including minor infections such as skin infections, food poisoning; life-threatening infections such as pneumonia, sepsis, osteomyelitis, and infectious endocarditis (Shopsin and Kreiswirth, 2001; D’Souza et al., 2010; Chamchod and Raun, 2012; Gould et al., 2012; Wang et al., 2012). Severe MRSA infections cause significant morbidity and mortality. Compared to methicillin susceptible Staphylococcus aureus (MSSA), mortality in MRSA infections is twice as high (Moellering, 2012). MRSA pose an economic burden to national health care systems and patients (Moodley et al., 2010; Chamchod and Raun, 2012).
S. aureus is an immobile spherical Gram-positive bacterium that form grape-like clusters and are facultative anaerobes that grow mainly aerobically but can also grow anaerobically (Bannerman, 2003). Growth occurs within 24 to 48 hours of incubation at temperatures of about 35 to 37°C. MRSA like most S. aureus form yellowish colonies although white variants are also common. The biochemical basis for the identification of MRSA includes the production of catalase, coagulase, DNase and the alternative PBP2A which encodes resistance to methicillin (Bannerman, 2003).

1.4.2 Taxonomy and Phylogenetic Status of Staphylococcus

Taxonomically, MRSA belongs to the genus Staphylococcus, within the family Staphylococcaceae, under the order Bacillales, class Bacillus and phylum Firmicutes (Bannerman, 2003). The family Staphylococcaceae has six genera namely Staphylococcus, Gemella, Jeotgalicoccus, Macrococcus, Salinicoccus and Nosocomiicoccus (Garrity and Holt, 2001). The order Bacillales includes a diverse group of microorganisms but Staphylococcus are easily identified on the basis of them being Gram-positive cocci that form clusters, produce catalase, have an appropriate cell wall structure (including peptidoglycan and teichoic acid presence) and low guanine plus cytosine (G + C) content in a range of 30-40 %, whereas members of the genus Micrococcus have a high G + C content of around 70 %. S. aureus has a G + C content of about 28.7% (Gills et al., 2005). Although it was initially believed that all Staphylococcus produced coagulase, it is now known that only six species produce coagulase (Matthews et al., 1997). These include S. aureus, S. delphini, S. hyicus, S. intermedius, S. lutrae and S. schleiferi subsp.coagulans. A seventh species was recently identified as the S. leei (Jin et al., 2004).
A variety of approaches have been used to describe the systematics and taxonomy of Staphylococci. Perhaps one of the important steps in the classification and taxonomy of Staphylococci was the differentiation between Staphylococci and Micrococci. The early classification of Staphylococci was based on the biochemical tests such as anaerobic growth and fermentation of glucose which was proposed by Evans and Kloos in 1972 to distinguish Staphylococci from the genus Micrococci (Evans and Kloos, 1972). However, these methods had several limitations and most importantly that of misclassification of species between the two genera. Based on these tests, Staphylococci were considered anaerobic while Micrococci were aerobic. However, it was observed that some strains of Staphylococci were aerobic. Further studies showed that Staphylococci could be distinguished from Micrococci and other catalase-positive cocci on the basis of their cell wall composition (Schleifer and Kandler, 1972; Endl et al., 1983); cytochrome profile (Faller and Schleifer, 1981) and menaquinone pattern (Collins and Jones, 1981); susceptibility to lysostaphin and erythromycin (Schleifer and Kloos, 1975); bacitracin (Falk and Guering, 1983), and furazolidone (Baker, 1984). Comparative immunochemical studies of catalases (Schleifer, 1986); DNA-DNA and DNA-rRNA hybridization studies (Schleifer et al., 1979; Kilpper et al., 1980); and comparative oligonucleotide cataloguing of 16S rRNA (Ludwig et al., 1981) also clearly demonstrated the epigenetic and genetic difference of Staphylococci and Micrococci. These studies also led to the description of many new staphylococcal species such as S. arlettae, S. equorum, S. kloosii (Schleifer et al., 1984), S. lugdunensis, S. schleiferi (Freney et al., 1988) and S. delphini (Varaldo et al., 1988), and elevation of some subspecies to species level such as S. lentus and S. chromogenes (Hajek et al., 1986).
In recent years there has been an emphasis on the development of molecular tools in the classification and identification of Staphylococci. These methods usually utilise one or several appropriate genes. Besides the 16S rRNA gene (De Buyser et al., 1992; Becker et al., 2004), the 16S-23S rRNA intergenic spacer region (Maes et al., 1997) and the heat shock protein 60 (hsp60) gene (Goh et al., 1996; Goh et al., 1997). Other gene sequences have been used in genetic studies of Staphylococci including: the femA gene (Vannuffel et al., 1999), the sodA gene (Poyart et al., 2001), the tuf gene (Martineau et al., 2001), the rpoB gene (Mollet et al., 1997; Drancourt and Raoult, 2002), and the gap gene (Yugueros et al., 2000; Yugueros et al., 2001). However, each of these has specific applications and advantages. For example, 16S rRNA gene sequencing and PCR-restriction fragment length polymorphism (PCR-RFLP) analysis has been used for species identification but it does not differentiate between *S. lentus* and *S. sciuri* (Ghebremedhin et al., 2008). PCR-RFLP analysis of the 23S rRNA gene with two restriction enzymes is able to discriminate between *Staphylococcus* species, but the interpretation of the results is complicated by intervening sequences (Fujita et al., 2005). In addition, although amplified fragment length polymorphism fingerprinting has proven to be useful for *Staphylococcus* species identification, it is time-consuming and expensive (Taponen et al., 2006). Whole-genome DNA-DNA hybridization analysis (Svec et al., 2004) allows species identification, but is not suitable for routine use. Ghebremedhin and colleagues (2008) proposed the usefulness of sequencing the gap gene for Staphylococci species differentiation and for interfering interspecies phylogenetic relationships (Ghebremedhin et al., 2008).
1.4.3 Epidemiology of MRSA

Resistance to antimicrobial agents is a growing public health concern (Ferreira et al., 2011). There are several ways in which resistance is transmitted between bacteria. These include transformation, conjugation and transduction. Transformation is the direct uptake of free DNA material through the bacterial membrane (Alberts et al., 2002). Conjugation occurs as a result of the transferring of plasmids, while transduction is the transfer of genetic material via viruses called bacteriophages (Waters, 2001). Once the bacteria have acquired the resistance genes, they further spread through clonal expansion.

The emergence of MRSA was first reported in 1961 in the United Kingdom (UK), barely 2 years after the introduction of methicillin in clinical practice (Barber, 1961; Moellering, 2012). Since then, the organism has spread throughout the world with variable geographical prevalence (Vindel et al., 2009; Johnson, 2011; Moellering, 2012). Northern Europe has been shown to have the lowest prevalence rates of about 1%, while North-East Asia shows some of the highest rates of up to over 60% (Ionescu et al., 2010; Stafeni and Goglio, 2010; Johnson, 2011). The European countries showing generally low prevalence rates include Finland, Denmark, Norway, Iceland, Sweden and the Netherlands (van Rijen et al., 2008; Grundmann et al., 2010; Stefani et al., 2012; Monecke et al., 2013). This low prevalence rates has been attributed to major ‘search and destroy’ operations in these countries (Tiemersma et al., 2004; van Rijen et al., 2008; van Cleef et al., 2010). However, some European countries such as Portugal, Greece, Italy and Romania have intermediate rates of 49%, 40%, 37%, 34% respectively (Ionescu et al., 2010; Peres et al., 2013; Drougka et al., 2014; Sanchini et al., 2014). In the United States, the
National Nosocomial Infection Surveillance (NNIS) System data demonstrated that MRSA accounted for over 60% of S. aureus isolates in intensive care units (Boucher and Corey, 2008). East-Asian countries that have shown the highest rates include Sri Lanka (86.5%), South Korea (77.6%), Vietnam (74.1%), Taiwan (65.0%), Thailand (57.0%) and Hong Kong (56.8%) (Lee et al., 1998; Aires de Sousa et al., 2003; Boyce et al., 2005; Song et al., 2011; Zhao et al., 2012; El Amin et al., 2012; INSAR; 2013; Xiao, 2013; Lai et al., 2014).

There has been poor documentation of the prevalence of MRSA in Africa (Breurec et al., 2011; Conceição et al., 2014). Generally, the prevalence rates of MRSA from most African countries have been shown to range from 25% to 50% (Kesah et al., 2003; Bouchillon et al., 2004; Perovic et al., 2006; Stefani et al., 2012; Maina et al., 2013). In South Africa, baseline data from the Pan-European Antimicrobial Resistance Using Local Surveillance (PEARLS) study of 2001 - 2002 showed that the MRSA prevalence was 33.3% (Bouchillon et al., 2004). However, another study involving patients with S. aureus bacteraemia at two academic hospitals in Johannesburg, South Africa, found an MRSA rate of 23% (Perovic et al., 2006). A multicentre study aimed at determining the prevalence and antibiotic susceptibility patterns of MRSA in African countries found the prevalence rates of MRSA in Nigeria, Kenya, and Cameroon to range from 21–30%, while the prevalence was below 10% in Tunisia, Malta, and Algeria (Kesah et al., 2003). A more recent study from Kenya that found an MRSA rate of 84.1% among skin and soft tissue infections which was higher than previous findings in the same region (Maina et al., 2013). This observation would suggest an increase in MRSA prevalence over the years and hence the need to maintain surveillance and control of MRSA infections in Africa.
In Zambia, there is a paucity of data on the burden of MRSA. However, the prevalence of MRSA has been shown to be on the increase at the University Teaching Hospital (UTH). The prevalence of MRSA at the UTH was 23% in 2003; while in 2010 it was found to have risen to about 30% (Mwenya, 2003; Kapatamoyo et al., 2010). A more recent study conducted at the UTH estimated the prevalence of MRSA at 37% (Mulipilwa, 2012). In another study conducted at Kitwe Central Hospital estimated the MRSA prevalence to be at 15% (Kasonde, 2004).

Initially MRSA pathogens were almost exclusively isolated from hospitals, long-term care facilities, or similar institutional settings and were thus called hospital or healthcare-associated or acquired MRSA (HA-MRSA) (David and Daum, 2010; Moellering, 2012). According to the Centers for Disease Control and Prevention (CDC), an infection is considered hospital-associated, if it occurs more than 48 hours after admission (Centers for Disease Control and Prevention, 2003). Thus, an isolate is defined as a HA-MRSA if cultured from a clinical specimen obtained 48 hours after patient's hospital admission or whose sources of isolation were associated with risk factors for HA-MRSA infection within one year of MRSA isolation date.

Risk factors for MRSA colonization or infection in the hospital include prior antibiotic exposure, admission to an intensive care unit or long-term care facility, recent surgery and exposure to an MRSA-colonized patient (Thompson, 1982; Boyce, 1989; Chambers, 2001; Centers for Disease Control and Prevention, 2003). Humans are a natural reservoir for *S. aureus*, and asymptomatic colonization is far more common than infection. Colonization of the nasopharynx, perineum, or skin,
particularly if the cutaneous barrier has been disrupted or damaged, may occur shortly after birth and may recur anytime thereafter. Family members of a colonized infant may also become colonized (Chambers, 2001).

However, the epidemiology of MRSA has changed with the emergence of MRSA infections in the community and amongst livestock, such as pigs (Chambers, 2001; Voss et al., 2005; Huijsdens et al., 2006; Mammina et al., 2010; van Cleef et al., 2011). Community associated or acquired MRSA (CA-MRSA) were first reported in the late 1990s in healthy children and adults in the community without prior exposure to the risk factors associated with MRSA infection (Chambers, 2001; Centers for Disease Control and Prevention, 2003; Eady and Cove, 2003; Vandenesch et al., 2003). A CA-MRSA isolate is defined as one cultured during the first 48 hours of a patient's hospital admission, or from patient's whose sources of isolation were not associated with risk factors for HA-MRSA infection as mentioned above (Centers for Disease Control and Prevention, 2003). CA-MRSA has spread worldwide and is found not only in the community setting but also in healthcare facilities. In fact, some hospitals have reported a predominance of CA-MRSA isolates over HA-MRSA isolates. Thus, the distinction between HA-MRSA and CA-MRSA is slowly becoming unclear (Johnson et al., 2009; Popovich and Weinstein 2009; David and Daum, 2010; Song et al., 2011).

Livestock-associated or acquired MRSA (LA-MRSA) was first associated with human disease in 2003 (Huijsdens et al., 2006; van Cleef et al., 2011). Although LA-MRSA are often associated with asymptomatic colonisation, several cases of infections of variable clinical relevance, ranging from skin and soft tissue infections
to endocarditis, pneumonia and necrotising fasciitis, have been described over the past few years (van Rijen et al., 2008; Pan et al., 2009; Mammina et al., 2010; Schijffelen et al., 2010; Wulf et al., 2012). Risk factors for colonisation include working in direct contact with animals on farms and working with live pigs in slaughterhouses (Köck et al., 2009; van Cleef et al., 2010; Graveland et al., 2011; Gilbert et al., 2012).

1.4.4 Genomics of MRSA

Genomics is the study of all the genes that are present in a genome of an organism. The field of genomics came out of a result of advances in molecular biology which have provided valuable insights into the relationships between the genotypes and phenotypes of microbes (Brosch et al., 2001).

1.4.4.1 Genome structure of *Staphylococcus aureus*

The genome of *S. aureus* was first sequenced in 2001 using Strains N315 and Mu50 by Kuroda and colleagues (2001). The genome size was determined to comprise of approximately 2600 genes (Kuroda et al., 2001). Since then about 18 annotated whole-genome sequences and partial sequences of many more strains have been deposited in GenBank. The data gathered from these sequences have led to the understanding of the genome structure of *S. aureus*. The genome structure has been shown to have three principal components. The first component is a backbone of core genes which are found in all strains and are highly (>97%) conserved (Stefani et al., 2012). The second component is a group of more than 700 core variable (CV) genes distributed throughout the backbone (Stefani et al., 2012). The CV genes are variably distributed and their distribution pattern defines *S. aureus* lineages. The final
component is the mobile genetic elements (MGEs). These are large discrete pieces of DNA that encode mobilisation functions, showing evidence of frequent transfer and to a lesser extent recombination (Lindsay et al., 2006; Lindsay, 2010; Malachowa and DeLeo, 2010).

Epidemiological investigations and typing methods employ molecular tools that make use of the genetic variation between isolates of S. aureus, the population structure and their continued evolution. Despite their wide geographical temporal and presumably selective diversity, S. aureus isolates can be grouped into lineages based on the unique hundreds of CV genes which they contain (Stefani et al., 2012).

1.4.4.2 Genes Responsible for Resistance to Methicillin

1.4.4.2.1 The mecA Gene

Methicillin resistance is mainly due to the acquisition of genes encoding a unique penicillin-binding protein (PBP2’ or PBP2a). PBP2a has decreased affinity for β-lactams and catalyses effective cell wall synthesis even in the presence of penicillins, cephalosporins and carbapenems (Chen et al., 2009; D’Souza et al., 2010; Moellering, 2012). PBP2a is encoded by the mecA gene which is carried on a mobile element known as the staphylococcal chromosome cassette mecA, also called staphylococcal cassette chromosome mecA (SCCmeCA) (Deurenberg et al., 2007; Deurenberg and Stobberingh, 2008; D’Souza et al., 2010; Moellering, 2012). Several SCCmeCA subtypes and their variants have been characterised (Deurenberg et al., 2007; Deurenberg and Stobberingh, 2008; Moellering, 2012). Currently there are eight subtypes of SCCmec (type I-VIII) that have been well studied (Ito et al., 2001;
Jansen et al., 2006; Zhang et al., 2009; Moellering, 2012). Figure 1:1 below show a scheme of the eight SCC\textit{mec} subtypes and some variants.

Figure 1.1: Classification scheme for SCC\textit{mec} subtype by cassette chromosome recombinase (\textit{ccr}) complex and \textit{mec} complex type (Adapted from David and Daum, 2010).

Typical HA-MRSA isolates are characterized by multidrug resistance and the SCC\textit{mec} types I, II and III cassettes (Boyle-Vavra and Daum, 2007). On the other hand, CA-MRSA isolates are generally susceptible to more drug classes, often of SCC\textit{mec} type IV, V and VII genotypes, and frequently carry the Panton-Valentine leukocidin (\textit{pvl}) genes (Boyle-Vavra and Daum, 2007; Afroz et al., 2008).
1.4.4.2 The mecC Gene

The mecC gene is a divergent homologue of the mecA gene which was formerly called mecALGA251 and is 70% homologous to the mecA gene (Garcia-Alvarez et al., 2011; Shore et al., 2011; Ito et al., 2012). It encodes a penicillin-binding protein that differs from that encoded by mecA. This PBP has a higher relative affinity for oxacillin compared to cefoxitin and also different temperature sensitivity (Kim et al., 2012). Although routine culture and antimicrobial susceptibility testing can identify S. aureus isolates encoding the mecC as methicillin-resistant, current diagnostic tests such as PCR assays and latex agglutination tests that detect the mecA and PBP2a respectively, cannot be used to confirm these isolates (Stegger et al., 2012). This further heightens the public health concerns due to MRSA infection as new diagnostics tests have to be developed.

1.4.5 Mode of Infection

Direct contact is the main mode of transmission of MRSA infections like those of other S. aureus in both the health care settings and the community (Chambers, 2001). Infection is acquired when one comes in contact with wounds, discharge and soiled areas. Other risk factors include close contact, breaks in the skin due to wounds and in dwelling catheters, poor personal hygiene and living in crowded conditions (Payne et al., 1966; Chambers, 2001; Wertheim et al., 2005; Gordon and Lowy, 2008).

Colonization with S. aureus plays a major role in the acquisition of infection. Generally, most people with S. aureus infections are infected with their colonizing strain (Williams et al., 1959; Kluymans et al., 1997; Wertheim et al., 2005; Chen et al., 2013). For example, in a study on bacteraemia, 82% of patients’ blood isolates
were identical to their nasal isolates (Von Eiff et al., 2001). Approximately 20% of individuals are persistently nasally colonized with *S. aureus*, and 30% are intermittently colonized (Chen et al., 2013). The anterior nares are the main ecological niche for *S. aureus*. Besides the nasal nares, other sites may be colonized, including the axillae, groin, and gastrointestinal tract. Colonization provides a reservoir from which bacteria can be introduced when host defences are breached, whether by shaving, aspiration, insertion of an indwelling catheter, or surgery (Chambers, 2001). It also increases the risk for recurring infection (Fermeiro et al., 2010) and allows *S. aureus* to be transmitted among individuals in both health care and community settings. The basis for *S. aureus* colonization is complex and incompletely understood but appears to involve the host's contact with *S. aureus* (e.g., from other carriers) and the ability of *S. aureus* to adhere to host cells and to evade the immune response (Williams et al., 1959; Chambers, 2001; Miller et al., 2014).

### 1.4.6 Immune Response

Generally, during an infection there is interplay between the microorganisms and the host to determine the outcome of infection. Several strategies of both the innate and adaptive immunity are mounted by the body to combat the infection (Bröker et al., 2014). However, it has been shown that most microorganisms including *S. aureus* have developed ways of evading the immunity and sometimes even use the immune responses to make a safe haven for themselves (Bröker et al., 2014; Löffler et al., 2014).
1.4.6.1 Innate Immunity

The primary defence against *S. aureus* infection is the innate immunity provided by neutrophils (Foster, 2005; Bröker et al., 2014). Other cells such as macrophages are also involved in the innate response to *S. aureus* infections (Foster, 2005; Bröker et al., 2014). Following an infection of the skin by *S. aureus*, there is a strong inflammatory response involving the migration of neutrophils and macrophages to the site of infection (Löffler et al., 2014). These cells attempt to engulf and dispose of the invading organisms with the help of available antibodies that are present in the host’s serum and using the complement (Foster, 2005; Bröker et al., 2014). Thus, the first important internal confrontation between *S. aureus* and the host occurs.

The complement system recruits effector molecules that label cells and target them for destruction by neutrophils and macrophages. While the process of complement fixation occurs by three pathways, the alternative and lectin pathways are components of innate immunity (Foster, 2005). One of the main purposes of complement fixation is opsonisation. Opsonisation promotes phagocytosis of bacterial cells by neutrophils and macrophages (Foster, 2005). Initially, the phagocytes are attracted to the site of infection by chemo-attractant molecules such as small peptide fragments (C3a and C5a) and formylated peptides. The chemo-attractant molecules are released during complement activation and growing bacteria, respectively. The membranes of neutrophils and macrophages have specific receptors for fragments of complement and formylated peptides that enhance the efficiency of phagocytosis. The neutrophils also carry specific receptors that can recognize the Fc region of immunoglobulin G (IgG) and complement proteins bound to the bacterial
surface that facilitate efficient uptake and killing (Foster, 2005; Zipfel, 2009; Song, 2012).

1.4.6.2 Adaptive Immunity

The adaptive immune responses require specific recognition of foreign antigens compared to the innate immune mechanisms which are non-specific. The innate system recognizes an invading pathogen early on and attempts to control its spread. Subsequently, the adaptive immune response is activated to deal with the pathogen in a specific manner and it is effected through the humoral and cell-mediated immune responses (Alberts et al., 2002; Janeway et al., 2005).

1.4.6.2.1 Humoral Immune Response

After the invading bacterium and its products are taken up by macrophages and other antigen-presenting cells such as dendritic cells, it is transported to lymph nodes. In the lymph nodes, these bacterial antigens are presented to the B cells (Foster, 2005; Bröker et al., 2014). This stimulates the B cells and leads to their differentiation into plasma cells by undergoing a class switch recombination, which is necessary to generate IgA and IgG, and antibody affinity maturation by a process known as somatic hypermutation (Peled et al., 2008; Stavnezer et al., 2008). The secreted antibodies neutralize toxins and promote more efficient phagocytosis of bacterial cells. Antibodies to *S. aureus* antigens are present in all humans in low levels, with titres rising following infection (Dryla et al., 2005; Holtfreter et al., 2010; Bröker et al., 2014). However, these antibodies and immunological memory seem to be insufficient to prevent subsequent infections (Roche et al., 2003). This inefficiency has been attributed to immunomodulatory proteins secreted by *S. aureus* that compromise both induced humoral and cell-mediated immunity (Roche et al., 2003;
Stemerding et al., 2013). More all over, the host is unable to respond to re-infection with a robust secondary response owing to depletion of T and B cells (Klein and Dalla-Favera, 2008; Weill et al., 2009).

1.4.6.2.2 Cell mediated Immune Response

For a long time, S. aureus was regarded as a non-invasive pathogen. However, it is now evident that the bacterium can invade many types of host cells (Löffler et al., 2014; Bröker et al., 2014). This is achieved by a mechanism involving the formation of a fibronectin bridge between the bacterial fibronectin-binding proteins and host α5β1 integrin molecules that triggers internalization of the bacterium into the host cell (Peacock et al., 1999; Schwarz-Linek et al., 2003; Schwarz-Linek et al., 2004). The organism can survive in host cells in a semi-dormant form referred to as small colony variants (von Eiff et al., 2000). However, little is known about the role of cell-mediated immunity in combating chronic staphylococcal infections.

1.4.7 Pathogenesis and Virulence of MRSA

Staphylococcus aureus is a versatile pathogen capable of causing a wide range of human diseases due to its extensive armamentarium of virulence factors. MRSA virulence factors are generally not unique to MRSA and MRSA is not necessarily more virulent than MSSA (Gordon and Lowy, 2008). Nonetheless, some MRSA strains contain factors or genetic backgrounds that may enhance their virulence or may enable them to cause particular clinical syndromes (Gordon and Lowy, 2008).

Both structural and secreted products (Figure 1.2) play a role in the pathogenesis of MRSA infections. Two notable features of Staphylococci are that a virulence factor
may have several functions in pathogenesis and that multiple virulence factors may perform the same function (Gordon and Lowy, 2008). Regulation of expression of staphylococcal virulence factors plays a central role in pathogenesis as virulence factors are expressed only when required by the bacterium (Grundmeier et al., 2010; Tuchscherr et al., 2010; Löffler et al., 2014).

![Figure 1.2: Pathogenic factors of *Staphylococcus aureus*, with structural and secreted products both playing roles as virulence factors. A: Surface and secreted proteins. B and C: Cross-sections of the cell envelope. TSST-1 is toxic shock syndrome toxin 1 (Adapted from Gordon and Lowy, 2008).](image)

*S. aureus* initiates an infection by adhering to host tissues through the aid of numerous surface proteins called “Microbial Surface Components Recognizing Adhesive Matrix Molecules” (MSCRAMMs) (Patti et al., 1994; Foster and Hook, 1998). The MSCRAMMs bind molecules such as collagen, fibronectin, and fibrinogen and seem to play a key role in initiation of endovascular infections, bone and joint infections, and prosthetic-device infections (Tung et al., 2000; Menzies, 2003). However, different *S. aureus* strains may have different constellations of MSCRAMMs and so may be predisposed to causing certain kinds of infections (Patti et al., 1994; Foster and Hook, 1998).
After adhering to host tissues or prosthetic materials, the bacterium is able to grow and persist in various ways. One of these ways is the formation of biofilms on host and prosthetic surfaces (Donlan and Costerton, 2002). Biofilms enable *S. aureus* to persist by evading host defences and antimicrobials. In *vitro*, *S. aureus* has been shown to invade and survive inside epithelial cells, including endothelial cells (Ogawa *et al*., 1985; Hamill *et al*., 1986). Theoretically, this may allow *S. aureus* to escape host defences, particularly in endocarditis. *S. aureus* is also able to form small-colony variants (SCVs), which may contribute to persistent and recurrent infection (Kahl *et al*., 1998). In *vitro*, SCVs are able to hide in host cells without causing significant host-cell damage and are relatively protected from antibiotics and host defences (Proctor and Peters, 1998). SCVs can later revert to the more virulent wild-type phenotype, possibly resulting in recurrent infection (Proctor *et al*., 1995).

Other mechanisms by which *S. aureus* evade the host immune system during an infection include the formation of antiphagocytic microcapsule called the zwitterionic capsule (both positively and negatively charged) which can also induce abscess formation (Foster, 2005). Protein A (a MSCRAMM) binds the Fc portion of immunoglobulin which may prevent opsonisation (Foster, 2005). *S. aureus* may also secrete the chemotaxis inhibitory protein or the extracellular adherence protein. This protein interferes with neutrophil extravasation and chemotaxis to the site of infection (Foster, 2005). In addition, *S. aureus* produces leukocidins that cause leukocyte destruction by the formation of pores in the cell membrane. This seems to be a prominent feature of CA-MRSA strains (Foster, 2005; Gordon and Lowy, 2008).
During infection, *S. aureus* invades and destroys host tissues and metastasises to other sites by producing numerous enzymes, such as proteases, lipases, and elastases (Gordon and Lowy, 2008). *S. aureus* is also capable of producing septic shock by interacting with and activating the host immune system and coagulation pathways. Peptidoglycan, lipoteichoic acid, and α-toxin have been implicated in causing shock (Bhakdi and Tranum-Jensen, 1991; Timmerman et al., 1993; Heumann et al., 1994). Besides septic shock, some *S. aureus* also causes various toxinoses due to production of super antigens (Dinges et al., 2000; Prevost et al., 2003). Examples of such toxinoses include food poisoning and toxic shock syndrome. The super antigens can produce a sepsis-like syndrome by initiating an over production of cytokines sometimes referred to as “cytokine storm” (Dinges et al., 2000; Prevost et al., 2003). Other toxinoses are scalded skin syndrome and bullous impetigo caused by some strains that produce epidermolysins or exfoliative toxins (Dinges et al., 2000; Prevost et al., 2003).

### 1.4.8 Clinical Presentation

MRSA infections affect people of all age groups (Waness, 2010). Children, especially neonates, and the elderly are particularly at risk of acquiring HA-MRSA (Naimi et al., 2003; Waness, 2010). In contrast, CA-MRSA tends to affect healthy children and young adults (David and Daum, 2010; DeLeo et al., 2010; Huang and Chen, 2011). Basically, the clinical manifestations of MRSA infections in both HA-MRSA and CA-MRSA are the same although differences in the intensity of some of the syndromes may be seen (David and Daum, 2010).
Skin and soft tissue infections (SSTIs) are the most common clinical manifestations of MRSA (Fridkin et al., 2005; Tong et al., 2012). Uncomplicated SSTIs typically presents as an abscess that may resemble a spider bite filled with purulent material (Stefani and Goglio, 2010). Abscesses can be found in different anatomical locations such as the breast, vulva, hand after clenched-fist injury and neck (Dryden, 2009; Nalmas et al., 2009; Waness, 2010). Besides abscesses, SSTIs can also present as folliculitis, paronychia, furuncle, felon and cellulitis with drainage or lymphadenitis (Dryden, 2009; Waness, 2010).

MRSA can also cause severe and invasive infections that are life threatening (Weller, 2000; Foster, 2005; Waness, 2010). These infections include necrotising fasciitis, necrotising pneumonia, severe sepsis, and septic thrombophlebitis of large veins such as the iliac or femoral veins (Zetola et al., 2005; David and Daum, 2010; Stefani and Goglio, 2010). Cases of such infections have interestingly increased with the emergence of CA-MRSA (David and Daum, 2010; Tong et al., 2012).

1.4.9 Diagnosis of MRSA Infections

Confirmation of clinical suspicions of MRSA usually involves initially identifying the organism as *S. aureus* and then determining resistance to methicillin. This can involve the use of either phenotypic and/ or genotypic methods. Phenotypic methods are used to detect visible characteristics of the organism such colonial morphology and biochemical reactions. By contrast, genotypic methods detect the genetic characteristics of the organism.
1.4.9.1. Phenotypic Methods

Culture of *S. aureus* on blood agar reveals golden yellow or white colonies within 24 to 48 hours at 37°C (Bannerman, 2003). Various tests can be used to identify *S. aureus*, including production of catalase, protein A, cell-bound clumping factor, extra-cellular coagulase and heat-stable nuclease. The tube coagulase test with rabbit sera and examination of tubes after incubation for 4 hours and 24 hours is the standard test for routine identification of *S. aureus* (Cookson, 1997; Wichelhaus *et al*., 1999). In comparison, the slide agglutination test for clumping factor is very rapid but up to 15% of *S. aureus* strains are negative. Therefore isolates negative in slide tests need to be confirmed with a tube agglutination test (Kuusela *et al*., 1994).

Various commercial latex agglutination tests for identification of *S. aureus* are available (van Griethuysen *et al*., 2001). While earlier versions detected protein A and/or clumping factor only, newer versions also detect other various surface antigens. This has led to improved sensitivity of the tests but at some expense to specificity due to cross reaction with coagulase negative Staphylococci (CNS). There are also many commercial kits and automated instruments which include identification of *S. aureus* (Ligozzi *et al*., 2002; O'Hara *et al*., 2003; Spanu *et al*., 2003; 2004). The performance of these tests is generally good, but they are slower, technically more time-consuming and more expensive than tests such as coagulase and latex agglutination. Hence they are less used for specific identification of *S. aureus*. However, the Staphychrom II a commercial 2 hour chromogenic test based on prothrombin and protease inhibitors has been reported to have a better sensitivity and specificity than the tube coagulase test (Fonsale *et al*., 2004).
In addition, these phenotypic methods have several drawbacks. For example, some other species of Staphylococci such as *Staphylococcus schleiferi* and *Staphylococcus intermedius*, may also give positive results in tube coagulase tests, while *Staphylococcus lugdensis* may give positive results in the slide coagulase test (Peacock *et al.*, 1999; Blake and Metcalfe, 2001). Fortunately, these species are rarely isolated from human infections. The slide coagulase test is also unsuitable for isolates that are not easily emulsified and clumping factor can be obscured by large amounts of capsule. An additional test has to be done to confirm DNase positive strains since CNS produce various amounts of DNase (Bannerman, 2003).

**1.4.9.2 Molecular Methods**

Most molecular methods for identification of *S. aureus* are based on the Polymerase Chain Reaction (PCR) (Towner *et al.*, 1998; Kearns *et al.*, 1999). The goal is to amplify a species-specific target which include the nuclease (*nuc*), coagulase (*coa*), protein A (*spa*), femA and femB, Sa442, 16S rRNA and surface-associated fibrinogen-binding protein genes (Mason *et al.*, 2001; Harmsen *et al.*, 2003; Afrough *et al.*, 2013). Several primers have been designed that detect these targets (Greisen *et al.*, 1994; Hammad *et al.*, 2012; Afrough *et al.*, 2013). In recent years, molecular methods for combined identification and susceptibility testing have been developed for possible use in routine diagnostic laboratories (Oliveira and Lencastre, 2002; Grisold *et al.*, 2002). Generally, molecular tests are used when results in coagulase or other biochemical tests are equivocal or with clinical samples where there is a high suspicion of MRSA infection.
1.4.9.3 Antimicrobial Susceptibility Testing

1.4.9.3.1 Phenotypic Methods

1.4.9.3.1.1 Conventional Methods

Expression of methicillin resistance in the clinical laboratory setting is subject to environmental conditions such as temperature, pH, incubation time, and salt concentration in the medium (Annear, 1968; Brown, 2001). Conditional expression of PBP2a antigen may cause ambiguity in susceptibility tests. Furthermore, methicillin resistance is often expressed heterogeneously, in that only $1 \times 10^4$ to $10^7$ cells of the population is phenotypically resistant (Gustafson and Wilkinson, 1989). Literature on methicillin susceptibility testing is extensive, and often conflicting in recommendations regarding the most reliable method for routine use. Several methods are available for detection of resistance to methicillin. These include the minimum inhibitory concentration (MIC) by dilution methods which can be agar dilution or broth microdilution, the E-test methods, breakpoint methods both agar or broth methods and agar screening method coupled with disc diffusion (National Committee for Clinical Laboratory Standards, 2003a; 2003b; 2004; Andrews, 2004).

Among the conventional methods used to detect MRSA in the laboratory, the oxacillin agar screen, disc diffusion using 1µg oxacillin disc as well as oxacillin (MIC) by agar or broth dilution methods are the most popular. Studies have shown that the cefoxitin disc diffusion tests are more reliable than those with oxacillin (Gal et al., 2001; Nadarajah et al., 2006; Banerjee et al., 2010; Ba et al., 2014). In disc diffusion tests, hyper-producers of penicillinase may show small methicillin or oxacillin zones of inhibition, whereas most true methicillin/oxacillin resistant isolates give no zone may be falsely reported as MRSA (Bignardi et al., 1996; Swenson et
al., 2007). Tests with cefoxitin do not appear to be affected to the same extent as oxacillin by hyper-production of penicillinase (Knapp et al., 1996; Rahbar and Safadel, 2006). Positive test results may have to be confirmed by latex or PCR methods.

1.4.9.3.1.2 Latex agglutination

Several latex agglutination tests are commercially available as kits from different suppliers (Nakatomi and Sugiyama, 1998; van Griethuysen et al., 1999; Cavassini et al., 1999; Louie et al., 2001). These tests are based on the detection of PBP2a. They are rapid; results can be obtained within 10 minutes for most of the kits. More all over, these tests require no special equipment and have been reported to be very sensitive and specific with S. aureus. Thus, they have been deemed suitable for confirmation of resistance or equivocal tests in routine clinical laboratories. Drawbacks of these methods include weak or slow agglutination reactions due to isolates producing small amounts of PBP2a. However, reactions can be enhanced by inducing the production of PBP2a by growing the organism in the presence of a β-lactam antibiotic. Negative reactions are obtained with rare isolates.

1.4.9.3.1.3 Automated methods

There are various automated systems including Vitek/Vitek2 (bioMérieux), Phoenix (Becton Dickinson) and Microscan (Dade Behring) that include tests for methicillin/oxacillin susceptibility (Frebourg et al., 1998; Ligozzi et al., 2002). Generally, they are reliable for S. aureus although difficulties have been reported with some strains (Ribeiro et al., 1999). One of the notable automated systems for methicillin susceptibility is the Crystal MRSA method (Becton Dickinson, Plymouth,
UK) where inhibition of growth of an isolate by oxacillin is indicated by the quenching of fluorescence of an oxygen-sensitive fluorescent indicator by oxygen remaining in the broth. The method is reasonably reliable but requires several hours of incubation.

1.4.9.3.2 Molecular Methods

A variety of molecular techniques for the detection of MRSA including conventional PCR and real time PCR including Multiplex PCR assays have been developed (Ito et al., 2001; Louise et al., 2001; Zhang et al., 2004; Zhang et al., 2008; Xu et al., 2012; Panda et al., 2014). These methods have the advantage over conventional identification and antibiotic resistance detection by being rapid and provide for accurate identification and characterisation of MRSA (Silbert et al., 2014). Most of these methods work by amplifying the mecA gene and indeed several other target genes such as the nuc, femA and femB genes (Mason et al., 2001; Harmsen et al., 2003; Afrough et al., 2013). However, the discovery of the mecC gene posed a challenge to these diagnostic techniques as they could not detect the mecC gene (Stefani et al., 2012).

More recently commercial Real-Time PCR assays that provide MRSA results in less than a couple of hours have been developed (Silbert et al., 2014). These assays include the Xpert MRSA® assay (Cepheid, Sunnyvale, CA), which runs exclusively on the GeneXpert® system (Cepheid, Sunnyvale, CA) and, the BD MAX™ MRSA assay (BD Diagnostics, Québec, Canada) performed on the BD MAX System™ (BD Diagnostics, Sparks, MD) (Rossney et al., 2008; Wolk et al., 2009; Hombach et al.,
2010; Dalpke et al., 2012). Some of these newer assays are able to detect both the meca and mecC genes simultaneously (Silbert et al., 2014).

1.4.10 Typing Methods of MRSA

MRSA typing is an essential component of an effective surveillance system to describe epidemiological trends and infection control strategies (Weller, 2001; Trindade et al., 2003; Mehndiratta and Bhalla, 2012). Several phenotypic and molecular typing techniques are available for typing MRSA. In general, phenotypic methods are easier to perform, easier to interpret, cost effective and are widely available (Weller, 2001; Trindade et al., 2003; Mehndiratta and Bhalla, 2012). However, they are less discriminatory than molecular methods (Weller, 2001; Mehndiratta and Bhalla, 2012). Molecular typing results are more likely to remain constant over time compared to phenotypic results (Tenover et al., 1994,). Additionally, molecular typing techniques are cost effective, rapid, reproducible and highly discriminatory (Weller, 2001; Mehndiratta and Bhalla, 2012).

1.4.10.1 Phenotypic Methods

Several phenotypic methods have been employed in the typing of MRSA. These include antibiogram typing (O'Neill et al., 2001; CLSI, 2009; Mehndiratta et al., 2010), phage typing (Blair, 1961; Marples, 1997; Mathur and Mehndiratta, 2000; Amorim et al., 2007), serotyping (Schlichting et al., 1993), biotyping (Coia et al., 1990; O'Neill et al., 2001), whole cell protein typing (Gaston et al., 1988; Coia et al., 1990), immunoblotting (Gaston et al., 1988), multilocus enzyme electrophoresis (Weller, 2000) and zymotyping (Gaston et al., 1988; Schlichting et al., 1993). In the recent past there has been an emphasis on the use of molecular techniques rather than
the phenotypic techniques to type MRSA. However, some of the phenotypic techniques such as antibiogram typing and phage typing have remained useful and are usually performed together with MRSA molecular typing (Mehndiratta and Bhalla, 2012).

1.4.10.2 Genotypic Methods

Diverse molecular typing techniques have been used to type MRSA including plasmid DNA analysis (Gaston et al., 1988; Coia et al., 1990; Zuccarelli et al., 1990), Restriction Endonuclease Analysis (REA) of chromosomal DNA (Jordens and Hall 1988), southern blot analysis of RFLP (Weller, 2000), ribotyping (Prevost et al., 1992), binary typing (van Leeuwen et al., 1990; Zadoks et al., 2000; Shopsin and Kreiswirth 2001), Pulsed-Field Gel Electrophoresis (Schlichting et al., 1993; Hallin et al., 2007; Melles 2007), Arbitrarily Primed Polymerase Chain Reaction or Random Amplified Polymorphic DNA (AP-PCR/RAPD) and PCR-Restric
tion Fragment Length polymorphism (PCR-RFLP) (van Belkum et al., 1995; Mehndiratta et al., 2009), Multilocus Sequence Typing (MLST) and Single-Locus Sequence Typing (SLST) e.g. spa typing (Enright et al., 2000; Strommenger et al., 2006), Staphylococcal Cassette Chromosome mec (SCCMec) typing (Chongtrakool et al., 2006; Zhang et al., 2009); Toxin gene profile typing (Sharma et al., 2000; Kim et al., 2006; Cai et al., 2007). The choice of typing technique depends on the purpose of the study, the facilities available and the utility of data generated to answer a desirable research question.
1.4.11 Treatment and Prevention

Few antibiotics are effective against MRSA since, besides being resistant to β-lactam antibiotics, it is also resistant to other classes of antibiotics (Weller, 2000). Currently, about 13 antimicrobials are available with activity against resistant Gram-positive organisms including MRSA and vancomycin resistant enterococci (VRE) (Rybak, et al., 2013; van Hal and Fowler, 2013). These include commonly utilized or recently developed agents such as vancomycin, trimethoprim—sulfamethoxazole (TMP/SMX), daptomycin, linezolid, ceftaroline and telavancin. Older or less employed agents such as clindamycin, tigecycline, quinupristin—dalfopristin, tetracyclines, fosfomycin and chloramphenicol are also used to treat severe and systemic MRSA infections (Grim et al., 2005; Welte and Pletz, 2010; Rybak, et al., 2013).

Vancomycin and teicoplanin which are glycopeptides are currently considered the mainstay of therapy of most MRSA infections (Weller, 2000; Rybak et al., 2009; van Hal and Fowler, 2013). However, the emergence of isolates with reduced susceptibility to vancomycin namely; vancomycin intermediate S. aureus (VISA) and heterogeneous-vancomycin-intermediate S. aureus (hVISA) has steadily increased leading to increased treatment failure (Oosthuysen, 2007; Vindel et al., 2009; D’Souza et al., 2010; Stafeni and Goglio, 2010; Welte and Pletz, 2010). This has led to development of the new drugs; however, these drugs have limited usage for severe infections due to pharmacokinetic limitations or adverse reactions. Adaptive or cross-resistance to each of these agents has also been documented (Welte and Pletz, 2010; Rybak, et al., 2013).
For example, decreased susceptibility to daptomycin has been attributed to cell wall thickening observed with VISA and hVISA which has also been linked to modifications to the regulatory gene walKR (Jones et al., 2008; Cafiso et al., 2012). Additionally, daptomycin non-susceptible (DNS) *S. aureus* expressing alterations in production of charged surface molecules have been observed due to mutations in the *mprF* gene (Jones et al., 2008). Although resistance to linezolid is rarely observed in clinical practice, local outbreaks attributed to horizontal transfer of the 9-chloramphenicol-florfenicol resistance (*cfr*) methyltransferase genes have been documented (Sanchez et al., 2010). Other linezolid resistance mechanisms involve point mutations in the 23S ribosomal subunit and are commonly encountered at institutions with higher usage of linezolid (Xiong et al., 2000; Locke et al., 2009; Shaw et al., 2011).

There are currently a handful of next-generation agents nearing approval which may represent new options for the treatment of serious infections due to MRSA and other MDR organisms. These include Solithromycin, Tedizolid, Dalbavancin and Oritavancin (Marbury et al., 2009; Rodríguez-Avial et al., 2012; Rodvold et al., 2012). Another treatment strategy that is being encouraged is the exploration of novel combination of the already available drugs. Combination strategies used this far include vancomycin and β-lactam combinations, daptomycin and β-lactam combinations, TMP/SMX combinations and fosfomycin combinations (Climo et al., 1999; Fox et al., 2006; Dhand et al., 2011; Leonard, 2012; Leonard and Rolek, 2012; Linasmita, 2012).
Prevention of MRSA infections usually employs several strategies (van Rijen et al., 2008). These strategies include hand hygiene (Stevenson et al., 2014), screening for colonisation and subsequent decolonisation (Cookson et al., 2011; Chen et al., 2013). Antibiotic prophylaxis is also employed especially pre-surgery (Hsiue et al., 2014). Unfortunately, there is no S. aureus vaccine available at the moment despite many attempts to develop one (Projan et al., 2006; Daum and Spellberg, 2012; Proctor, 2012).

1.5 Research Questions
1.5.1 What is the prevalence of MRSA at UTH?
1.5.2 What are the antimicrobial susceptibility patterns of MRSA at UTH?
1.5.3 What are the molecular characteristics of the MRSA strains at the UTH?

1.6 Objectives
1.6.1 General Objective
To characterise methicillin-resistant Staphylococcus aureus strains isolated from at the University Teaching Hospital using molecular tools.

1.6.2 Specific Objectives
1.6.2.1 To identify Methicillin-Resistant Staphylococcus aureus strains.

1.6.2.2 To determine the drug susceptibility patterns of the Methicillin-Resistant Staphylococcus aureus to commonly used anti-staphylococcal antimicrobials.

1.6.2.3 To determine the genotypes of the methicillin-resistant Staphylococcus aureus strains.
Chapter 2: Materials and Methods

2.1 Study Design

This was a laboratory-based retrospective cross-sectional study on MRSA isolates collected from June 2009 to December 2012.

2.2 Study Site

The study was conducted at the University Teaching Hospital in the Bacteriology Laboratory in the Department of Pathology and Microbiology. The University Teaching Hospital is a tertiary referral and teaching hospital in Lusaka with a bed capacity of approximately 2000 and comprises of several clinics and admission wards. It is the largest referral hospital and the centre for all microbiology diagnostic work in Zambia.

2.3 Sampling Frame

The study utilised a convenient sample of 95 archived suspected clinical MRSA isolates obtained from June 2009 to December 2012 in the Bacteriology Laboratory at the University Teaching Hospital.

2.3.1 Inclusion Criteria

Only one MRSA isolate per patient’s sample was included in the study to avoid duplication of isolates.
2.3.2 Exclusion Criteria

Methicillin Susceptible *Staphylococcus aureus* and coagulase negative *Staphylococcus* were excluded from the study.

2.4 Detection and Identification of MRSA Strains

2.4.1 Bacterial Isolation and Identification

Archived *Staphylococcus aureus* isolates suspected to be MRSA were used in this study. These organisms were isolated from various clinical specimens including pus, blood, urogenital, urine and respiratory specimens submitted at the Bacteriology Laboratory at the UTH. The isolates were recultivated and identified using standard microbiology methods (Koneman, 2005; Garcia and Isenberg, 2010). Using a loop, the isolates were cultured on blood agar plates (with 5% defibrinated sheep blood). The seeded plates were then incubated at 37°C for 18 to 24 hours. Several biochemical tests including the Gram stain, catalase test, coagulase tube test using plasma and DNase test were done according to the standard operating procedure on greyish or golden yellow colonies to identify *Staphylococcus aureus* (Bannerman, 2003).

2.4.2 Gram Stain

The Gram-stain was performed by adding Crystal Violet for one minute on an air dried and heat fixed smear of the suspect colonies (Koneman, 2005). The slide was then washed with distilled water. Lugol’s iodine was applied for 1 minute and washed off using water. Decolourisation was done by adding acetone for about 10-15 seconds and then washing with water immediately. Dilute carbol fuchsin was used to counter stain the slides for about 30 seconds and rinsed thoroughly with water. The
slides were air dried and then examined using a microscope. *Staphylococcus aureus* is Gram-positive cocci in clusters.

### 2.4.3 Catalase Test

Catalase test was performed with 3% hydrogen peroxide (Koneman, 2005). Briefly, a drop of hydrogen peroxide was applied onto a slide and by using an applicator stick a colony was emulsified into the drop of hydrogen peroxide. Formation of bubbles denoted a catalase positive test result. *S. aureus* is catalase positive. This test differentiates Staphylococci from Streptococci.

### 2.4.4 Coagulase Test

The coagulase test was done on the catalase positive isolates (Koneman, 2005). A loopful of colonies of the catalase positive isolates was added to 1 ml of citrated rabbit plasma in a test tube. The test tube was incubated at 37°C and observed for coagulation (formation of a clot) hourly for 4 hours. If there was no coagulation after 4 hours the tube was incubated up to 24 hours. Coagulation denoted a positive result. *Staphylococcus aureus* is coagulase positive. This test differentiates *S. aureus* from *S. epidermidis* and other Micrococci.

### 2.4.5 DNase Test

The DNase test was also used to identify the isolates. This test was performed on DNase agar plates (Koneman, 2005). DNase is an enzyme produced by *S. aureus* that cleaves DNA. The DNA present in the agar is hydrolysed by DNase if this enzyme is produced by the organism. After incubation of the DNA agar plate, the plate is flooded with 1M hydrochloric acid (HCl), which precipitates any unhydrolysed
DNA, producing cloudiness. A zone of clearance is visible where the DNA has been hydrolysed.

Eight to ten isolates were tested for DNase production per DNA plate by drawing horizontal and vertical lines on the agar plate, creating small squares. A known *S. aureus* strain, ATCC *Staphylococcus aureus* 25923, was included on every plate as a positive control. Each square on the plate was inoculated with a single colony of each isolate. The plate was then incubated aerobically overnight at 37°C. After incubation the plate was flooded with 1M HCl and left to stand for a minute after which excess HCl was discarded. A zone of clearing around the inoculum was taken as a positive result. *Staphylococcus aureus* is DNase positive.

Thus, isolates included in the study were Gram-positive cocci in clusters, catalase positive, coagulase positive and DNase positive.

### 2.5 Detection of Methicillin Resistance

#### 2.5.1 Kirby-Bauer Disc Diffusion Antibiotic Susceptibility Method

Resistance to methicillin was detected using the Kirby-Bauer disc diffusion antibiotic susceptibility method according to the 2012 guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). The test was performed using 1µg oxacillin and 30µg cefoxitin antibiotic discs (Mast Diagnostics, Mast Group Ltd, Merseyside, UK) on Mueller-Hinton agar plate (Mast Diagnostics, Mast Group Ltd, Merseyside, UK). The interpretive criteria of the CLSI for *Staphylococcus aureus* was used to establish the antibiotic susceptibility of the isolates.
Briefly, a single colony was suspended in 1ml saline, adjusted to a 0.5 McFarland standard and spread evenly with a sterile cotton swab onto the agar plate surface. After the surface of the agar plate had dried for about 5 minutes, the antibiotic disc was placed on the inoculated plate and incubated for 24 hours aerobically at 35°C. ATCC Staphylococcus aureus 25923 was included as the control strain. The results were interpreted by measuring the zone diameters with the aid of sliding callipers, including the disc diameter.

2.6 Determination of Drug Susceptibility Patterns of MRSA Strains
Antimicrobial susceptibility testing against 30µg vancomycin, 30µg teicoplanin, 5µg ciprofloxacin, 10µg gentamicin, 15µg erythromycin, 10µg clindamycin, 30µg chloramphenicol, 25µg co-trimoxazole, 10 units penicillin G, 30µg tetracycline and 30µg amikacin (Mast Diagnostics, Mast Group Ltd, Merseyside, UK) was also done using the Kirby-Bauer disc diffusion antibiotic susceptibility method as outlined in Section 2.5.1 according to the guidelines of the CLSI to determine the antibiogram of the MRSA strains. Inducible resistance to macrolides, lincosamides, and group B streptogramins, commonly referred to as MLSBi, was also detected using the D-test using erythromycin and clindamycin discs. The control strain Staphylococcus aureus ATCC 25923 was included when testing for antibiotic resistance.

2.7 Determination of the Genotypes of MRSA Strains
2.7.1 DNA Extraction and Estimation
The NucliSENS easyMAG nucleic acid extraction protocol (bioMérieux Inc, Durham, NC, USA), with modifications, was used to extract the genomic DNA. Briefly, the bacterial isolates were cultured overnight on blood agar at 37°C. Using a
swab, several bacterial cells were emulsified in 700µl of NucliSENS easyMAG lysis buffer in an eppendorf tube and left to stand for 30 minutes to one hour at room temperature for maximum off-board lysis. Then 400µl of the bacterial suspension was transferred to the easyMAG disposable sample strip wells and 100µl of undiluted silica were added to the sample-lysis buffer mix. The sample strips were then loaded onto the easyMAG machine 3.2 v3 system (bioMérieux Inc, Durham, NC, USA) and the NucliSENS easyMag off-board lysis procedure was followed according to the manufacturer’s instruction to extract the DNA at a final elution volume of 50µl.

Ultraviolet spectroscopy at 260nm was used to estimate the concentration of the DNA. An Optical density (OD) of 1 at 260nm corresponded to a DNA concentration of 50µg/ml of double-stranded DNA and a DNA/protein absorbance ratio of 260nm/280nm was used to determine the purity of the DNA sample. The DNA was stored at -20°C until required for use.

### 2.7.2 Staphylococcus Cassette Chromosome mec Typing

To determine the SCCmec structural variants of each MRSA isolate a previously described protocol for a SCCmec multiplex PCR (Milheirico et al., 2007a; 2007b) was used. Eight loci (A to H) were included in the multiplex PCR, together with an internal positive control, the mecA gene. Located downstream of the pls gene is locus A, specific to SCCmec type I; internal to the kdp operon is locus B and is specific to SCCmec type II; locus C is specific to SCCmec types II and III and is internal to the mecI gene; locus D is present in SCCmec types I, II and IV and is internal to the dcs region; located in the region between integrated plasmid pT1258 and transposon
Tn554 is locus E and is specific to SCC\textit{mec} type III; locus F is located in the region between Tn554 and the chromosomal right junction in \textit{orfX} and is also specific for SCC\textit{mec} III. To distinguish structural variant IA and IIIA, loci G and H were included, respectively. Locus G is the left junction between IS431 and pUB110, while locus H is the left junction between IS431 and pT181. The oligonucleotide primer sequences that were used are shown in the table below.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CIF2 F2</td>
<td>TTCgAgTTgCTgATgAAgAgAg</td>
<td>495</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CIF2 R2</td>
<td>ATTTACCACAAggACTACCAgC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>KDP F1</td>
<td>AATCACTCTgCCATTggTgATgC</td>
<td>284</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>KDP R1</td>
<td>CgAATgAAgTgAAAgaAAAaTggg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>MEC1 P2</td>
<td>ATCAAgACTTgCATTCAgg</td>
<td>209</td>
<td>II, III</td>
</tr>
<tr>
<td></td>
<td>MEC1 P3</td>
<td>gCggTTTCAATTCACTTgTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>DCS F2</td>
<td>CATCCTATgATAgCTTggTC</td>
<td>342</td>
<td>I, II, IV</td>
</tr>
<tr>
<td></td>
<td>DCS R1</td>
<td>CTAAATCATAgCCATgACCg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>RIF4 F3</td>
<td>gTgATTgTTgCTgAgATAgTgg</td>
<td>243</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>RIF4 R9</td>
<td>CgCTTTATCTgTATCTTACgC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>RIF5 F10</td>
<td>TTCTTAAgTACACgCTgAATCg</td>
<td>414</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>RIF5 R13</td>
<td>gTCACAgTAATTCCATCAATgC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>IS431 P4</td>
<td>CAggCTCCTCTCAgATCTACg</td>
<td>381</td>
<td>IA</td>
</tr>
<tr>
<td></td>
<td>pUB110 R1</td>
<td>gAgCCATAAACACCAATAgCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>IS431 P4</td>
<td>CAggCTCCTCTCAgATCTACg</td>
<td>303</td>
<td>IIIA</td>
</tr>
<tr>
<td></td>
<td>pT181 R1</td>
<td>gAgAAATgggAAAaCTTCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{meca}</td>
<td>MECA P4</td>
<td>TCCAgATTACAACCTTCAgAg</td>
<td>162</td>
<td>Internal</td>
</tr>
<tr>
<td></td>
<td>MECA P7</td>
<td>CCACTTCATATCTTgTAAgC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NB: Primer sequences were those previously reported by Hanssen and Sollid, 2006.

Briefly, PCR reactions were performed on a Gene Amp 2700 PCR thermal cycler (Applied Biosystems, CA, USA). A final volume of 25µl was used, containing 2µl bacterial DNA template, primer and 2X PCR Master Mix (Thermo Scientific, Hanover, MD, USA), with a final MgCl$_2$ concentration of 3mM. The cycle conditions used were as follows: initial denaturation at 94°C for 4 minutes, followed
by 30 cycles of 94°C for 30 seconds (denaturation), 53°C for 30 seconds (annealing) and 72°C for 1 minute (extension), 4 minutes at 72°C as the final extension time. Electrophoresis of 5µl of the PCR product was performed on a Tris-Borate-EDTA (TBE) agarose gel (wt/vol) (100V) containing 1µl ethidium bromide (10 mg/ml). A 50bp ladder (Thermo Scientific, Hanover, MD, USA) was used as a molecular weight standard and all gels were visualised using a Biotop Biosens SC - 645 Gel Documentation System (Biotech Co. Ltd, Shanghai, China).

2.7.3 Spa Typing

To determine the spa types of the MRSA strains spa typing was done following a previously described protocol (Shopsin et al., 1999). Briefly, the spa gene was amplified by PCR using the primers 1095F (5’-AgACgATCCTTCggTgAgC-3’) and 1517R (5’-gCTTTTgCAATgTCATTTACTg-3’) which result in amplicons of variable sizes ranging from about 240 bps to above 320bps (Shopsin et al., 1999). The PCR reactions were performed on a Gene Amp 2700 PCR thermal cycler (Applied Biosystems, CA, USA). A final volume of 25µl was used, containing 2µl bacterial DNA template, primer and 2X PCR Master Mix (Thermo Scientific, Hanover, MD, USA), with a final MgCl₂ concentration of 3mM. DNA isolated from ATCC 25923 Staphylococcus aureus was used as a PCR control strain. The cycle conditions used were as follows: initial denaturation at 95°C for 4 minutes, followed by 30 cycles of 95°C for 30 seconds (denaturation), 60°C for 30 seconds (annealing) and 72°C for 45seconds (extension). The final extension time of 10 minutes at 72°C was used. Electrophoresis of 5µl of the PCR product was performed on a Tris-Borate-EDTA (TBE) agarose gel (wt/vol) (100V) containing 1µl ethidium bromide (10 mg/ml). A 50bp ladder (Thermo Scientific, Hanover, MD, USA) was used as a
molecular weight standard and all gels were visualised using a Biotop Biosens SC-645 Gel Documentation System (Biotech Co. Ltd, Shanghai, China).

2.7.3.1 DNA Sequencing

DNA fragments for sequencing were prepared from PCR positive samples using the QIA quick Gel Extraction Kit (Qiagen Inc. Valencia, CA, USA) according to the manufacturers’ recommendations. Sequencing reactions were set-up as follows: 100ng of PCR product, 2µl Big Dye Terminator Reaction Mix (Applied Biosystems, Foster City, CA, USA), 1µl 5×sequencing buffer (Applied Biosystems) and 2µl of 5µM forward primer. The reaction was made up to 10µl with distilled water. The cycle conditions were 96°C for 1 minute, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The reaction was kept at 4°C until it was purified using ethanol precipitation.

Ethanol precipitation was done as follows: The reaction was made up to 100µl with distilled water, where after 10µl of 3M Sodium acetate (NaAOc) (Sigma-Aldrich) (pH 4.6) and 250µl of 100% ethanol was added. The samples were then centrifuged at 14 000rpm for 20 minutes. The supernatant was aspirated and 250µl of 70% ethanol was added. The samples were centrifuged at 14 000rpm for 8 minutes. The supernatant was aspirated and the samples were left to air dry for 15 minutes. Sequencing was performed on the forward strand at the Inqaba Biotechnical Industries Sequencing Facility (Pretoria, South Africa) by the dye terminator method using an ABI PRISM 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA). The DNA sequence reads were edited using the Ridom TraceEdit Software (Ridom Bioinformatics GmbH, Würzburg, Germany).
2.7.3.2 DNA Sequence Analysis

The chromatograph sequence files of the isolates were analysed with the software package Bionumerics Spa typing plug in version 7.1 (Bionumerics, GmbH, Würzburg, Germany). After providing the input sequence (SCF format), the software automatically detected the repeat units and assigned a spa type. To ensure that no beginning or end repeat is excluded the software searches for the 5’ and 3’ signature sequences at the correct distance.

2.8 Data Analysis

The data was analysed using the GraphPad Prism Software Version 6.0 for Windows (GraphPad, San Diego, California, USA). Descriptive data was presented in frequency tables and graphs as percentages. Categorical variables were compared using the Chi-squared test and these variables included susceptibility to oxacillin and cefoxitin. Tests were interpreted at 5% significance level (two-sided) and 95% Confidence Interval. A p-value of < 0.05 was considered significance.

2.9 Ethics Considerations

Ethics approval for this study was granted by the University of Zambia Biomedical and Research Ethics Committee (UNZABREC). The ethics Clearance Certificate Number was 014-09-12 (Appendix A). Permission to use the archival MRSA isolates was sought from the head of the Department of Pathology and Microbiology at the University Teaching Hospital. To ensure confidentiality and anonymity the bacterial isolates were assigned project identification numbers.
Chapter 3: Results

3.1 Identification of Methicillin-Resistant *Staphylococcus aureus* Strains

A total of 95 stored isolates of suspected Methicillin-resistant *Staphylococcus aureus* from various clinical specimens collected from June 2009 to December 2012 at the University Teaching Hospital were included in this study. All isolates were confirmed as *S. aureus* using the tube coagulase and DNase tests. Of the 95 *S. aureus* isolates, 43% (41/95 isolates) were identified as methicillin-resistant *Staphylococcus aureus* strains. The cefoxitin disc was superior in detecting methicillin resistance compared to the oxacillin disc (40% vs. 28.4%; p = 0.0388). However, two isolates resistant to oxacillin were susceptible to cefoxitin. Figure 3.1 shows the susceptibility of the isolates to oxacillin and cefoxitin.

![Figure 3.1: Detection of MRSA using the oxacillin and cefoxitin discs.](image-url)
3.2 Determination of Drug Susceptibility Patterns of MRSA Strains

The MRSA strains were resistant to co-trimoxazole (100%), ciprofloxacin (95%), penicillin G (95%), erythromycin (78%), tetracycline (78%) and gentamicin (68%) as shown in Figure 3.2.

Inducible resistance to macrolides, lincosamides, and group B streptogramins (MLSBi) was detected in 68.3% (28/41) isolates as shown in table 3.1.

Table 3.1: Inducible resistance to macrolides, lincosamides, and group B streptogramins (MLSBi)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Proportion of Isolates % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLSBi Positive</td>
<td>68.3 (28)</td>
</tr>
<tr>
<td>MLSBi Negative</td>
<td>31.7 (13)</td>
</tr>
</tbody>
</table>

Multi- drug resistance to a combination of four, five, six and seven antibiotics was observed in 17.5 %, 27.5 %, 35%, and 17.5 % of the MRSA isolates, respectively as shown in Table 3.2. Isolates were classified as multidrug resistant (MDR) if, in addition to the β-lactams, they were resistant to ≥3 classes based on susceptibility to
erythromycin, clindamycin, chloramphenicol, ciprofloxacin, tetracycline, trimethoprim/sulfamethoxazole.

Table 3.2: Frequency of multi-drug resistance among MRSA isolates

<table>
<thead>
<tr>
<th>No. of drugs</th>
<th>Proportion of Isolates % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>17.5 (7)</td>
</tr>
<tr>
<td>6</td>
<td>35 (14)</td>
</tr>
<tr>
<td>5</td>
<td>27.5 (11)</td>
</tr>
<tr>
<td>4</td>
<td>17.5 (7)</td>
</tr>
</tbody>
</table>

To assess whether antibiotic resistance phenotypes clustered together, antibiotic resistance patterns were assigned using the designation PG + TS + Gen + Ery + CD + Cip + C + T (Table 3.3). Isolates were grouped into 13 antibiotic resistance patterns. Five predominant groups were found: PG + TS + Gen + Ery + Cip + T (23%), PG + TS + Gen + Ery + Cip + C + T (18%), PG + TS + Ery + Cip (15%), PG + TS + Gen + Cip + T (13%) and PG + TS + Ery + Cip + T (10%).

Table 3.3: Antibiotic resistance patterns of the MRSA isolates (n=41)

<table>
<thead>
<tr>
<th>Resistance Pattern</th>
<th>Proportion of Isolates % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG + TS + Gen + Ery + Cip + C + T</td>
<td>18 (7)</td>
</tr>
<tr>
<td>PG + TS + Gen + Ery + CD + C + T</td>
<td>3 (1)</td>
</tr>
<tr>
<td>PG + TS + Gen + Ery + Cip + T</td>
<td>23 (9)</td>
</tr>
<tr>
<td>PG + TS + Gen + Cip + C + T</td>
<td>5 (2)</td>
</tr>
<tr>
<td>PG + TS + Gen + Ery + Cip + C</td>
<td>3 (1)</td>
</tr>
<tr>
<td>PG + TS + Ery + Cip + C + T</td>
<td>3 (1)</td>
</tr>
<tr>
<td>PG + TS + Gen + Cip + T</td>
<td>13 (5)</td>
</tr>
<tr>
<td>PG + TS + Ery + Cip + T</td>
<td>10 (4)</td>
</tr>
<tr>
<td>TS + Gen + Cip + C + T</td>
<td>3 (1)</td>
</tr>
<tr>
<td>TS + Ery + Cip + C + T</td>
<td>3 (1)</td>
</tr>
<tr>
<td>PG + TS + Ery + Cip</td>
<td>15 (6)</td>
</tr>
<tr>
<td>PG + TS + Gen + Cip</td>
<td>3 (1)</td>
</tr>
<tr>
<td>PG + TS</td>
<td>3 (1)</td>
</tr>
</tbody>
</table>

Abbreviations: PG, Penicillin; TS, Co-trimoxazole; Gen, Gentamicin; Ery, Erythromycin; CD, Clindamycin; Cip, Ciprofloxacin; C, Chloramphenicol; Te, Tetracycline

3.3 Determination of the Genotypes of the MRSA Strains

The genotypes of the MRSA strains namely Staphylococcus cassette chromosome mec types and spa types were determined in the study.
3.3.1 Staphylococcus Cassette Chromosome mec Types

The presence of the mecA gene and determination of the SCCmec types of the isolates was confirmed by running the multiplex PCR products on an agarose gel (Figure 3.3).

![SCCmec typing gel picture of controls and selected samples](image)

Figure 3.3: SCCmec typing gel picture of controls and selected samples, M: 50bp marker; Lane 1: Negative control; Lane 2: Positive Control (SCCmec type I); Lane 3: MS16; Lane 4: Negative control; Lane 5: Positive Control (SCCmec type II); Lane 6: Negative control; Lane 7: Positive Control (SCCmec type III); Lane 8: MS54; Lane 9: MS63; Lane 10: Negative control; Lane 11: Positive Control (SCCmec type IV); Lane 12: MS67; Lane 13: MS85 ; Lane 14: MS89 ; Lane 15: MS97 ; M: 50bp marker

The mecA gene was detected in 40 out of 41 isolates. The distributions of the SCCmec types determined in the study are shown in Figure 3.4. Three SCCmec types were found. The most predominant SCCmec type was SCCmec type IV (63.4% of MRSA isolates). SCCmec type III (14.6%) and SCCmec type I (2.4%) were also identified. SCCmec types that seemed to be a combination of more than one SCCmec types were also observed and were called complexes. The SCCmec type of 7.3% of the MRSA isolates were untypeable.
3.3.2 Staphylococcal protein A (Spa) Types

Seventy-eight percent (32/41) of the MRSA isolates were positive for spa gene after PCR (Fig 3.5). The PCR was repeated for spa negative samples to ensure they were truly negative. DNA sequences of the spa gene of representative isolates are shown in Figure 3.6.

Figure 3.5: spa typing gel picture of controls and selected samples. M: 50bp marker; Lane 1: Negative control; Lane 2: Positive control; Lane 3: MS01; Lane 4: MS02; Lane 5: MS04; Lane 6: MS05; Lane 7: MS06; Lane 8: MS09; Lane 9: MS10; Lane 10: MS11; Lane 11: MS18; Lane 12: MS20
Figure 3.6: DNA sequences of the spa gene of representative isolates: a) spa type t064: DNA sequence for isolate MS01, b) spa type t2104: DNA sequence for isolate MS02, c) spa type t355: DNA sequences for isolate MS09, d) spa type t1257: DNA sequence for isolate MS10 e) novel spa type: DNA sequence for isolate MS33
The MRSA strains were found to be of 5 spa types (Table 3.4). The most common spa type was t064 (40.6% of isolates). Notably one isolate had an unknown spa type.

<table>
<thead>
<tr>
<th>Table 3.4: Distribution of Spa Types among the MRSA Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of Isolates % (n)</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>40.6 (13)</td>
</tr>
<tr>
<td>31.3 (10)</td>
</tr>
<tr>
<td>3.1 (1)</td>
</tr>
<tr>
<td>21.9 (7)</td>
</tr>
<tr>
<td>3.1 (1)</td>
</tr>
</tbody>
</table>

Phylogenetic analysis of the MRSA strains based on the spa types showed that the strains cluster into two clusters (Figure 3.6). One of these two clusters then further divided into two other clusters.
Figure 3.7: Phylogenetic relationships of the MRSA strains based on the *spa* types.

A minimum spanning tree showing the relationship of the isolates in relation to the most frequent *spa* type determined (t064) is included in Figure 3.7.
Figure 3.8: Minimum spanning tree showing the relationship of the isolates in relation to the most frequent spa type determined (t064)
Chapter 4: Discussion

4.1 Discussion

Methicillin-resistance *Staphylococcus aureus* has been recognised as one of the major causes of nosocomial infections world-wide responsible for a wide range of infections (Gould *et al.*, 2012; Wang *et al.*, 2012). Since MRSA was first discovered in the UK in the early 1960s (Barber, 1961; Moellering, 2012), its prevalence has been steadily increasing overall in the world with geographical variations (Vindel *et al.*, 2009; Johnson, 2011; Moellering, 2012). The burden of MRSA infections has further been heightened by the emergence of CA-MRSA and LA-MRSA (Mammina *et al.*, 2010; van Cleef *et al.*, 2011). While the clonal relatedness of MRSA isolates from developed countries has been extensively analysed there is sparse information from developing countries (Breurec *et al.*, 2010). To the best of our knowledge this study was the first of its kind to use molecular tools to determine the clonal relatedness of MRSA in Zambia.

The detection and screening of MRSA among patients upon hospital admission has been attributed to low prevalence rates in some countries (Tiemersma *et al.*, 2004; van Rijen *et al.*, 2008; van Cleef *et al.*, 2010). Several methods have been employed in the detection of resistance to methicillin by *S. aureus*. In this study, the disc diffusion using oxacillin and cefoxitin discs was used to detect methicillin resistance. The results of this study show that 43% of the isolates were MRSA and the cefoxitin disc detected more (40%) isolates as MRSA compared to the oxacillin disc (28%) ($\chi^2$; p=0.0388). This finding is in agreement with studies that have shown that the cefoxitin disc performs better than the oxacillin disc in the detection of methicillin
resistance (Felten et al., 2002; Skov et al., 2003; Cauwelier et al., 2004). However, two isolates were resistant to oxacillin but susceptible to cefoxitin. A possible explanation for this might be that resistance to methicillin in these isolates was not due to the mecA gene since other modes of resistance to methicillin such as the presence or overexpression of β-lactamase enzymes and chromosomal mutations have been documented (Gal et al., 2001; Nadarajah et al., 2006; Banerjee et al., 2010; Ba et al., 2014). Currently, resistance to cefoxitin is taken as the surrogate marker for resistance due to the mecA gene (CLSI, 2012). The finding of this study, taken together with the findings of Mwenya (2003) and Kapatamoyo and others (2010), suggest that there is an increase in the rates of MRSA infections at the UTH in Lusaka. However, since all these studies have been conducted at a particular point in time with relatively small sample sizes, caution must be applied in the interpretation of the data. We recommend that more comprehensive studies be conducted over a longer period of time.

Determination of antimicrobial susceptibility of bacterial pathogens using classical phenotypic tests in clinical microbiology is important in the therapeutic treatment of patients which depends on assessing the susceptibilities of the bacteria (Fluit et al., 2001). Accumulated susceptibility testing data gathered at the local, regional, or national level form the basis for empiric treatment schemes for infectious diseases (Sundsfjord et al., 2004). In addition, careful detection of resistant bacteria provides a fundamental basis for infection control measures and antimicrobial surveillance systems (Sundsfjord et al., 2004). While comparison of antimicrobial susceptibilities are difficult between studies, our findings correspond more closely with reports from South Africa in which MRSA resistance rates to gentamicin (68% vs. 65.7%) and
erythromycin (78% vs. 78.6%) were observed (Marais et al., 2009). However, our study detected higher resistance rates to ciprofloxacin (95% vs. 69.7%) and co-trimoxazole (100% vs. 55%) than the South African study. All isolates in our study were susceptible to amikacin while the South African isolates were highly resistant (72%). Notably, some studies from Spain showed high (greater than 90%) resistance rates to ciprofloxacin as observed in our study (Cuevas et al., 2007; Vindel et al., 2009). Our findings are also very similar to those of a study done in Taiwan in which resistance rates of 94.9%, 71.8% and 78.2% to erythromycin, co-trimoxazole and gentamicin respectively were observed (Wang et al., 2012). However, resistance to clindamycin was higher in the Taiwanese study than in our study (86.5% vs. 2.5%). The differences in the antimicrobial resistance rates to the various drugs observed might reflect a difference in regional antimicrobial usage and subsequent epidemiology due to inappropriate use of antibiotics in some regions.

The choice of antibiotic for therapy of MRSA infections is usually complicated. Therefore, an important implication of the antimicrobial susceptibility results from the current study is their interpretation in relation to treatment of MRSA infections. For example, co-trimoxazole and a combination of co-trimoxazole and other drugs are among the currently available treatment options for MRSA, especially in the community (Grim, et al., 2005; Rybak, et al., 2013). However, where resistance to co-trimoxazole is high, this may not be viable. The high resistance to co-trimoxazole found in this study corroborate the findings of Kapatamoyo and others (2010) who found co-trimoxazole resistance rates (among Staphylococci isolates) at 86% and recommended that it should not be used for treatment of acute bacterial infections (Kapatamoyo et al., 2010). The high resistance to co-trimoxazole could be attributed
to its use in prophylactic treatment of *Pneumocystis jiroveci* infections in HIV positive patients (Alonso *et al*., 1997; Martin *et al*., 1999; McDonald *et al*., 2003; Chintu *et al*., 2004). Co-trimoxazole is currently the considered main drug for treatment and prophylaxis for *Pneumocystis jiroveci* pneumonia (PCP) in immunocompromised individuals including HIV patients (Centers for Disease Control and Prevention, 2000; Sepkowitz, 2002; Grim, *et al*., 2005). Co-trimoxazole is also used as a prophylactic drug in sickle cell patients (Donkor *et al*., 2013). However, a study in Ghana found no significant difference between the resistance patterns of *Staphylococcus aureus* isolates from sickle cell patients and non-sickle cell patients (Donkor *et al*., 2013).

Another potential antibiotic in the treatment of MRSA infections is ciprofloxacin. Ciprofloxacin and other quinolone antibiotics have been proposed as possible alternatives to parenteral vancomycin therapy on the basis of several *in vitro* and *in vivo* animal model data (Gade and Qazi, 2013). In this study the resistance rate to ciprofloxacin was very high (95%). Although this result differs from those of Kapatamoyo and others (2010), who found very low ciprofloxacin resistances rates, it is consistent with those of several other studies (Qureshi *et al*., 2004; Cuevas *et al*., 2006; Vindel *et al*., 2009; Sarma *et al*., 2010; Gade and Qazi, 2013). Such ciprofloxacin resistance rates in healthcare settings may render ciprofloxacin not to be useful as a first-line antibiotic (Gade and Qazi, 2013). Resistance to ciprofloxacin is usually due to spontaneous mutations in the gyrase and topoisomerase genes (Didier *et al*., 2011; Hashem *et al*., 2013). Moreover, it has been reported that ciprofloxacin resistant MRSA isolates tend to show increased resistance to other antibiotics, including aminoglycosides (Tsering *et al*., 2011). Further studies on the
susceptibility of MRSA to ciprofloxacin and other quinolones in our setting are recommended.

Clindamycin has also been shown to be useful in the treatment of MRSA infections, especially community acquired infections (Rybak, et al., 2013; Lall and Sahni, 2014). However, inducible resistance is an important factor to consider when treating a patient with the use of clindamycin (Lall and Sahni, 2014). In our study although resistance to clindamycin was very low (2.5%), inducible clindamycin resistance was relatively high (68.3%). A possible explanation for this is that isolates with inducible resistance appear sensitive to clindamycin on disc diffusion testing when tested singly. Staphylococcal strains with MLSBi are resistant to inducer macrolides but susceptible to non-inducer macrolides, lincosamides and streptogramin B (Schreckenberger et al., 2004; Lall and Sahni; 2014). Consequently, it would be recommendable to routinely screen for erythromycin-induced clindamycin resistance among S. aureus isolates in our setting. Notably, all isolates were sensitive to vancomycin, teicoplanin and amikacin. Currently, glycopeptides, vancomycin and teicoplanin are the mainstay of therapy for most MRSA infections (Gade and Qazi, 2013; Rybak, et al., 2013). Therefore, these antibiotics still remain a viable option treatment of MRSA infections in our setting.

It is generally expected that MRSA resistance rates to penicillin are very high (Chambers, 2001; Mollering, 2012). Consequently, penicillin and other β-lactam antibiotics are not used in the therapy of MRSA infections. The most important resistance mechanism to penicillin is production of the β-lactamase which inactivates penicillin by hydrolysing the β-lactam ring. In our study, we employed the penicillin
disc diffusion zone edge test to detect β-lactamase production and most of the MRSA isolates were positive for β-lactamase production. Susceptibility testing of other β-lactam antibiotics was not undertaken as per recommendation from the CLSI since MRSA strains tend to be resistant in vivo despite being susceptible in vitro (CLSI, 2012).

In our study, most of the MRSA strains were multi-drug resistant. These results are in agreement with global findings of MRSA strains being multi-drug resistant (Kesah et al., 2003; Peng et al., 2010; Seni et al., 2013; Al-haddad et al., 2014). In a study conducted in Ethiopia, 86% of the S. aureus strains isolated were multi-drug resistant (Godebo et al., 2013). In our study, a total of 13 resistance patterns were determined of which five of them accounted for 79% of the isolates. This finding correlates well with a study from Spain where 12 resistance patterns were determined (Vindel et al., 2009). However, a study from South Africa detected 61 resistance patterns and multi-drug resistance was very high (81.5%) (Marais et al., 2009). Notably, both of these studies analysed more isolates than our study.

In recent years several molecular detection techniques have been employed to detect and type MRSA. It has been shown that complete characterization of MRSA lineages requires not only the identification of the genetic background of the bacteria but also identification of the structural types of the large and heterologous mec element (SCCmec element), which carries methicillin resistance determinant mecA (Oliveira and de Lencastre, 2002; Turlej et al., 2011). A multiplex PCR strategy that detects the mecA gene as well as determines the SCCmec subtypes and variants was used in this study. The mecA gene was not detected in one of isolates detected as MRSA.
phenotypically. Despite repeating the PCR, the isolate was negative for the mecA gene. A possible explanation for this is that the observed resistance to methicillin in this particular isolate was due to mechanisms other than those due to the PBP2a encoded by the mecA gene referred to earlier (Gal et al., 2001; Nadarajah et al., 2006; Banerjee et al., 2010; Ba et al., 2014). Bignardi and colleagues (1996) described similar isolates and according to Swenson and colleagues (2001), this non-mecA mediated resistance is still clinically relevant since such strains are still MRSA (Bignardi et al., 1996; Swenson et al., 2001). To our knowledge this was the first study in Zambia to detect the mecA gene in S. aureus isolates.

The multiplex PCR strategy used in this study essentially detects four SCCmec types and their variants namely SCCmec type I, II, III, IV and Variants IA and IIIE. The method was used for its simplicity and ease of performance. The most prevalent SCCmec type found in the study was SCCmec type IV (63.4%). A similar study conducted in Pakistan also found the SCCmec type IV as one of most predominant SCCmec types with a prevalence of 38.1% (Zafar et al., 2011). However, compared to our study, their prevalence rate was relatively low. The SCCmec type IV has been associated with CA-MRSA strains which are generally susceptible to more drug classes compared to HA-MRSA (Boyle-Vavra and Daum, 2007; Afroz et al., 2008; Wu et al., 2010). However, the SCCmec type IV has been increasing found in HA-MRSA in the recent past. Studies conducted in India and Malaysia showed that SCCmec type IV had emerged in the HA-MRSA strains (Lim et al., 2013; Dhawan et al., 2014). Moreover, the HA-MRSA strains with SCCmec type IV were multidrug resistance unlike their counterpart CA-MRSA strains which had SCCmec type IV (Dhawan et al., 2014).
In our study, the second predominant SCC\textit{mec} type found was SCC\textit{mec} type III (14.6%). This finding is similar to that of a study conducted in South Africa in which the SCC\textit{mec} type III was found to be the second most common (14%) SCC\textit{mec} type (Makgotlho \textit{et al.}, 2009). While only 2.4% of the isolates in our study harboured SCC\textit{mec} type I, SCC\textit{mec} type I was the second most predominant (16.7%) SCC\textit{mec} type found among isolates in a study from Uganda (Seni \textit{et al.}, 2013). SCC\textit{mec} types I and III are normally harboured by HA-MRSA (Boyle-Vavra and Daum, 2007; Afroz \textit{et al.}, 2008; Wu \textit{et al.}, 2010). HA-MRSA strains containing SCC\textit{mec} type III tend to be multi-drug resistant since this SCC\textit{mec} type is known to harbour plasmids and transposons that encode resistance to tetracycline, cadmium, erythromycin and spectinomycin (Ito \textit{et al.}, 2001; Ito \textit{et al.}, 2003; Deurenberg \textit{et al.}, 2007; Chen \textit{et al.}, 2014). The 7.3% of isolates detected in this study that were SCC\textit{mec} non-typeable is comparable to the 8% from the South African study (Makgotlho \textit{et al.}, 2009) but slightly higher (4%) compared to those from a study in Belgium (Denis \textit{et al.}, 2005). In 12.2% of the isolates investigated in this study, the SCC\textit{mec} type could not be easily deduced as they seemed to contain more than one SCC\textit{mec} type namely SCC\textit{mec} type III and type IV. This finding is not unusual since the SCC\textit{mec} type IV has been shown to be mobile and has been found in MRSA of several different genetic backgrounds, which had other SCC\textit{mec} types (Oliveira \textit{et al.}, 2001; Oliveira and de Lencastre, 2002).

It is now generally agreed that a combination of genotyping methods such MLST, \textit{spa} typing and/or PFGE with SCC\textit{mec} analysis be used to define MRSA strains (Friedrich \textit{et al.}, 2008; Otter and French, 2012; Stefani \textit{et al.}, 2012; Dhawan \textit{et al.},
In this study we employed 

spa
typing, a PCR and DNA sequencing-based technique, in addition to SCCmec typing. Its advantages include high speed, ease of use and interpretation of data, simplicity of large database creation and effortless information exchange between laboratories. The most prevalent 

spa
type determined in the study was t064 and was found in 40.6% of the isolates. 

spa
type t064 has been identified in the USA where it was designated ST 8 ORSA I and was also associated with persons living with AIDS (McDougal et al., 2003; Gordon et al., 2005). A study in Nigeria also found 

spa
type t064 as the most predominant 

spa
type in HIV positive 

S. aureus carriers (Olalekan et al., 2012). This 

spa
type was also found among the most common 

spa
types identified in South Africa although it was not linked to any particular clinical condition (Moodley et al., 2010). However, in a study from Uganda, 

spa
type t064 was found in only 4% of the isolates and was associated with the obstetrics and gynaecology wards (Seni et al., 2013). 

spa
type t064 is associated with the MLST sequence type 8 (ST-8) (McDougal et al., 2003; Gordon et al., 2005; Oosthuysen, 2007; Olalekan et al., 2012). Other strains associated with ST-8 include the Archaic/Iberian clones, USA500, ORSA IV, USA500 ORSA II, ORSA IV and ORSA III. The Archaic/Iberian clones are widely spread HA-MRSA isolates (McDougal et al., 2003; Gordon et al., 2005; Oosthuysen, 2007).


spa
types t2104 (31.3%) and t1257 (21.9%) were the second and third most common 

spa
types identified in our study respectively. We could not find other studies documenting 

spa
type t2104 among MRSA isolates despite the relatively high frequency of t2104 in our study. However, according to the 

spa
t sever (www.spa.ridom.de) this 

spa
type has been reported in association with MRSA in the United States, Sweden and Japan (Spa Sever, 2014). Notably, this 

spa
type was
reported among MSSA isolates in a study to determine the prevalence and molecular epidemiology of *S. aureus* among rural Iowans, including individuals with livestock contact (Wardyn *et al.*, 2012). *Spa* type t1257 has been reported in South Africa and it accounted for about 9.7% of the isolates (Moodley *et al.*, 2010). This *spa* type has been associated with HA-MRSA strains. The only singleton *spa* type found in this present study was *spa* type t355. Although this *spa* type is rarely reported, it was the most prevalent *spa* type identified among MRSA isolates in a study from Ghana (Egyir *et al.*, 2014). It has also been documented in Nigeria and Uganda (Shittu *et al.*, 2011; Seni *et al.*, 2013). Only one novel *spa* sequence was determined in our study. It has not been reported previously from any other country and its name could not be determined by the *Spa* Sever. Although this novel *spa* type seems to be closely related to *spa* type t2104, its repeat sequence contains two repeats r25 end and is shorter than the repeat sequence for t2104. This finding possibly denotes mutations in the *S.aureus* genome.

*Spa* typing also allows for the grouping of isolates into groups called *spa*-clonal complex (Ruppitsch *et al.*, 2006). When examining the *spa* types belonging to a specific *spa*-CC, there is usually a repeat or several repeats that all the *spa* types have in common (Ruppitsch *et al.*, 2006). The general hypothesis regarding the evolution of the *spa* types is either through; insertions or deletions of an individual repeat unit or a group of repeat units; or point mutations within a specific repeat unit, thus changing it to a different repeat unit (Frenay *et al.*, 1996; Shopsin *et al.*, 1999). It is generally accepted that MRSA strains are related, i.e. belong to the same *spa*-Clonal Complex (*spa*-CC), if the *spa* type repeat motif is related (Ruppitsch *et al.*, 2006). From literature, we could deduce that most of our isolates belong to the *spa*-CC 064.
This is because alignment of the repeat patterns of spa types t064, t1257 and t2104 showed the presence of both motifs 11-19-, (start); followed by motif 05-17-34-; followed by motif 24-34-22; and finally repeat r25 (end). Although spa type t2104 has relatively fewer repeats since it lacks the motifs 05-17-. In addition the repeat sequence of the novel spa type is also very similar to that of spa type t2104. The spa-CC 064 is associated with HA-MRSA (McDougal et al., 2003; Gordon et al., 2005; Oosthuysen, 2007). Consequently, we may deduce that most of the strains studied were hospital acquired and clonally disseminated.

The clinical significance of MRSA is that it presents treatment challenges with regard to antimicrobial resistance. This is due to few options for suitable drugs. Further, the genotypes identified in the study have been shown to cause multi-drug resistance in MRSA (Dhawan et al., 2014). In addition, a growing problem associated with multi-drug MRSA is the socioeconomic implications. This includes: longer hospital stays, inadequate or delayed antibiotic use and the use of newer, more expensive antimicrobial agents needed to treat patients with MRSA appropriately. Consequently, there is an overall increase in resource utilization associated with MRSA (Schorr, 2006). However, all of the isolates in our study were still susceptible to glycopeptides which are the main stay drugs for the treatment of MRSA.

4.2 Conclusion
The study shows a possibility of an increase in the prevalence of MRSA at the UTH. Furthermore, multi-drug resistance was extremely high amongst the MRSA isolates, with some of the isolates being resistant to as many as seven antibiotics. This could
be due to the isolates harbouring genotypes SCCmec type IV and SCCmec type III, as detected in the study. However, all the isolates were susceptible to the glycopeptides and amikacin, implying that these antibiotics are still viable as treatment options of MRSA infections. Five spa types were determined, four of which clustered into one group, the spa-clonal complex 064. To the best of our knowledge this was the first study to report on the molecular characteristics of MRSA isolates from UTH and indeed Zambia.

### 4.3 Limitations of the Study

One major limitation of this study was the lack of clinical data. This data could have been useful in correlating the laboratory findings to the clinical outcomes of the patients. Unfortunately, these data were not available due to lack of access to patient records. Another limitation of the study was that only available archived isolates were studied and all these isolates came from UTH alone. Therefore, further work is warranted to determine the distribution of MRSA strains isolated from other parts of Zambia. This will give an accurate picture of the MRSA strains circulating in the country.

### 4.4 Future Directions

Based on the findings of this study, we would like to recommend that the infection prevention and control strategies at UTH should be strengthened since most of the MRSA isolates were hospital acquired and multi-drug resistant. Adopting infection prevention and control strategies such as the screening of patients at admission and isolation of those with MRSA could help reduce the transmission of MRSA. In addition, we recommend that treatment of MRSA infections should be guided by
antimicrobial susceptibility test results. Notably, routine screening for inducible clindamycin resistance and the cautious use of co-trimoxazole in acute infections is recommended.

Future studies should focus on determining the antimicrobial resistance mechanisms of potential anti-MRSA drugs such as the quinolones (ciprofloxacin) and indeed other antibiotics. Furthermore, studies to determine the distribution of MRSA strains isolated from other parts of Zambia would give an accurate picture of the MRSA strains circulating in the country. This can be achieved by conducting national surveys involving isolates from several hospitals which would allow more systematic collection of epidemiological data. Lastly but not the least, to gain further insight into the molecular epidemiology and clonality of MRSA strains, studies using other molecular typing techniques such as Pulsed Field Electrophoresis, whole genome sequencing and single nucleotide polymorphism should be undertaken.
References


Appendices

Appendix A: Ethics Clearance Letter

THE UNIVERSITY OF ZAMBIA
BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telex: UNZU, LUSAKA
Telex: UNZALU ZA 44370
Fax: +260-1-250753
E-mail: unzeeco@unza.zm
Assurance No. FWA00000338
IRB00001131 of IORG0000774

12th October, 2012.

Your Ref: 014-09-12.

Ms Mulumba Tillika Samutela,
School of Medicine,
Department of Biomedical Sciences,
PO Box 50110,
Lusaka.

Dear Ms Samutela,

RE: SUBMITTED RESEARCH PROPOSAL: “MOLECULAR CHARACTERIZATION OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS ISOLATED AT THE UNIVERSITY TEACHING HOSPITAL, ZAMBIA”

Your application for a waiver of ethics review for the protocol “Molecular Characterization of Methicillin-Resistant Staphylococcus Aureus Isolated at the University Teaching Hospital, Zambia” was reviewed. The waiver is hereby granted. The approval was conducted in line with the University of Zambia Biomedical Research Ethics Committee guidelines on granting waiver of ethics review.

CONDITIONS:

- The waiver is based strictly on your submitted proposal. Should there be need for you to modify or make changes to the proposal you will need to seek clearance from the University of Zambia Biomedical Research Ethics Committee.
- This waiver does not release you from any other applicable obligations in ensuring confidentiality.
- If you need any clarifications please consult this office.
- Ensure that a final copy of the results is submitted to this Committee.

Yours sincerely,

Dr. J.C. Munthali
CHAIRPERSON

Date of approval: 12 October, 2012     Date of expiry: 11 October, 2013
Appendix B: Media and Reagent Preparation

A. Media

1.5% Agarose Gel
1.5g agarose powder
Up to 100ml 1x TBE buffer

5% Blood Agar (Mast Diagnostics, Mast Group Ltd, Merseyside, UK)
40g Columbia agar powder
50ml Sheep blood
Up to 1000ml distilled water, autoclave
Store at 4°C

DNase Agar (Mast Diagnostics, Mast Group Ltd, Merseyside, UK)
39g DNA agar powder
Up to 1000ml distilled water, autoclave
Store at 4°C

Mueller-Hinton Agar (Mast Diagnostics, Mast Group Ltd, Merseyside, UK)
38g Mueller-Hinton agar powder
Up to 100ml distilled water, autoclave
Store at 4°C

STTG Storage Media
2g Skimmed milk powder
3g Tryptone soy broth powder
0.5g Glucose
10ml Glycerol
Up to 100ml distilled water, autoclave

B. Reagents

Ethidium bromide solution
10mg/ml in distilled water
Stored in a dark bottle at 4°C

TBE Buffer (10x)
108g Tris
55g Boric acid
9.3g EDTA
Up to 100ml distilled water, autoclave and store at room temperature

TBE Buffer (1x)
1 volume of 10x TBE buffer
9 volumes distilled water

0.5 McFarland BaSO4 Standard
0.5 ml of 0.048M BaCl2 (1.175% W/V BaCl2.H2O)
99.5ml of 0.36N H2SO4
Appendix C: List of Publications and Presentations
The following manuscripts, based on this dissertation, have been prepared for publication


Part of this work has been presented at the following scientific meetings


