DETECTION OF ANTIMICROBIAL RESISTANCE AND VIRULENCE GENES IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CLINICAL SAMPLES AT THE UNIVERSITY TEACHING HOSPITALS, LUSAKA, ZAMBIA

BY

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DECLARATION

I, Milner Malambo Mwenda do hereby declare to the Senate of the University of Zambia, that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for the degree award in any other institution.

Signature_____Date_____

CERTIFICATE OF APPROVAL

This dissertation submitted by MILNER MALAMBO MWENDA has been approved as partial fulfillment for the award of the degree of MASTER OF SCIENCE IN ONE HEALTH ANALYTICAL EPIDEMIOLOGY at the University of Zambia.

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ABSTRACT

The pathogenicity of *Staphylococcus aureus* (*S. aureus*) is attributed to development of antimicrobial resistance and production of many virulence factors. Several genes are implicated in resistance to antimicrobials and virulence factors. This study was aimed at investigating the presence of selected antimicrobial resistance and virulence genes in *S. aureus* isolated from clinical samples at the University Teaching Hospitals in Lusaka, Zambia. This was a cross-sectional study involving forty-three (43) *S. aureus* isolates. Species identification gene *(nuc*), antimicrobial resistance genes (*erm*A, *erm*B, *erm*C, *mec*A, *tet*K *tet*L, *tet*M, and *tet*O) and virulence genes (*spa*, *pvl*, *sea*, *seb*, *sec*, *sed*, and *see*) were detected using Polymerase Chain Reaction. The *nuc* gene was detected in all the 43 isolates thereby confirming them as S. aureus. Of the 43 isolates, 14 (32.6%) were from blood samples while 29 (67.4%) were from skin and soft tissue samples. Five (11.6%) *S. aureus* isolates harboured the *mec*A gene (MRSA). Nine (20.9%) isolates tested positive for *erm*C gene while none were positive for neither *erm*A nor *erm*B. The t*et*K and *tet*L genes were detected in 3 (7.0%) and 1 (2.3%) isolates, respectively. None of the isolates tested positive for *tet*M, and *tet*O genes. One *spa* type t015 was detected in two isolates whereas the spa types for five isolates were unknown. Five (11.6%) isolates tested positive for *sec* enterotoxin gene. None of the 43 isolates possessed any of the *sea*, *seb*, *sed* and *see* enterotoxin genes as well as the *pvl* gene. Two (22.2%) of *erm*C positive isolates harboured enterotoxin *sec* gene. This study adds to existing knowledge by giving insight into the presence of *mec*A gene (MRSA) among *S. aureus* isolates as well as noting the presence of one MRSA strain that harboured both *tet* (*tet*K) and macrolide resistance (*erm*C) genes which poses a significant therapeutic challenge in disease management of staphylococcal infections in resource limited countries like Zambia. Furthermore, the carriage of staphylococcal enterotoxin *S. aureus* strains suggests a high potential of staphylococcal food poisoning at the largest referral hospital. This study has demonstrated the need to develop an efficient control programme to curb the transmission and spread of antimicrobial resistant particularly multi-antimicrobial resistant and food toxin producing *S. aureus* strains at the largest referral hospital.

DEDICATION

I dedicate this work to my dearest wife Maureen Mofu Mwenda and children, whose words of encouragement, love, and support allowed me to reach this far in my studies. I further dedicate this work to my beloved mother, Mrs Esther Mwenda for her words of wisdom, which inspired me to excel in my education. My late father, Mr Molosi Mwenda would have been the happiest man alive to see me reach this level in my academic endevours.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AMR	Antimicrobial resistance
Bp	Base Pair
CA-MRSA	Community-associated Methicillin Resistant *Staphylococcus aureus*
CC	Clonal Complex
CCR	Complex chromosome rearrangement
ccr	Cassette chromosome recombinases
Fc	Fragment Crystallizable
HA-MRSA	Hospital-associated Methicillin Resistant *Staphylococcus aureus*
Ig	Immunoglobulin
Luk	Leukocidin
MDR	Multi-Drug Resistance
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequencing Typing
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Susceptible *Staphylococcus aureus*
Nor	Nucleolus Organizer Region
PBP	Penicillin-binding Protein
PCR	Polymerase Chain Reaction
PVL	Panton-Valentine Leukocidin
QRDR	Quinolone-Resistance Determining Region
SCC	Staphylococcal Cassette Chromosome
SE	Staphylococcal Enterotoxin
SSTI	Skin and soft-tissue infection
ST	Sequence Type
UNZABREC	University of Zambia Biomedical and Research Ethics Committee
UTH	University Teaching Hospitals
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 Background

Staphylococcus aureus is a virulent and one of the major human pathogens associated with life threatening nosocomial, and community acquired infections (Ayeni et al., 2018, Monteiro et al., 2019). Irrespective of methicillin-resistant or methicillin-susceptible phenotype, *S. aureus* can cause numerous infections in humans, such as superficial lesions, deep seated infections, osteomyelitis, and infective endocarditis (Bhatta et al., 2016, Guo et al., 2020). *S. aureus* can acquire resistance to almost all the antibiotics that enter clinics to date, and the World Health Organisation (WHO) in 2016 defined it as a high-priority pathogen for research and development of new antimicrobials (Manara et al., 2018). Emergence of multidrug-resistant (MDR) *S. aureus* has become an increasing health concern worldwide (Yousefi et al., 2016). Methicillin resistance is one of the most important clinical resistance traits acquired by *S. aureus*. Methicillin-resistant *S. aureus* (MRSA) is resistant to most β-lactams due to the expression of an extra penicillin-binding protein 2a (PBP2a), encoded by the *mec*A gene located on a genomic island known as staphylococcal cassette chromosome *mec* (SCC*mec*) (Fergestad et al., 2020). Resistance to erythromycin in *S. aureus* is usually associated with resistance to other macrolides. Three genes, *erm*A, *erm*B, and *erm*C encoding methyltransferases are responsible for resistance to erythromycin (Zmantar et al., 2011). *S. aureus* has also developed mechanisms of resistance against tetracyclines which is mediated by plasmid encoded *tet*K and *tet*L genes and ribosomal protection that is encoded by chromosomal or transposonal *tet*M or *tet*O genes (Khoramrooz et al., 2017).

The pathogenicity of *S. aureus* is also related to a number of virulence factors that allow the organism to adhere, avoid the immune system and cause harmful effects to the host (Bhatta et al., 2016, Lee et al., 2018, Yunita et al., 2020). One of the important virulence factors produced by some strains of *S. aureus* is the Panton Valentine leukocidin (PVL), encoded by two genes, *lukS-PV* and *lukF-PV* (Bhatta et al., 2016). Colonisation with PVL expressing strains of *S. aureus* is characterised by recurrent skin and soft tissue infections (Hoppe et al., 2018). The Staphylococcal Protein A encoded by Staphylococcal Protein A

(*spa)* gene is a virulence factor of *S. aureus* that binds to the IgG molecules by their Fc portion and inhibits phagocytosis of the bacteria and thus contributes to the development of the disease (Choudhary et al., 2018). Staphylococcal Protein A genes are also a good choice to be able to identify and distinguish *S. aureus* strain variability (Yunita et al., 2020). Staphylococcal food poisoning is one of the most common food-borne diseases and results from the ingestion of staphylococcal enterotoxin (SE) preformed in food by enterotoxigenic strains of *S. aureus* (Hennekinne et al., 2010). The SE toxins' genes in *S. aureus* encode different virulence factors which if expressed, can produce the corresponding enterotoxins (Ayeni et al., 2018). Among the 23 SEs identified and reported in literature, only five SEs namely, *sea*, *seb*, *sec*, *sed* and *see* have been well characterized (Basanisi et al., 2017). Staphylococcal enterotoxin B, represents a powerful super antigenic toxin and is considered a bioweapon (Bae et al., 2020).

The number of comprehensive studies addressing clinical cohorts of *S. aureus* infections by simultaneously looking at the epidemiology, phylogenetic reconstruction, genomic characterisation, and transmission pathways of infective *S. aureus* clones in sub-Saharan African countries including Zambia is currently low, thus limiting surveillance and epidemiological monitoring. Therefore, this study was aimed at investigating the presence of antimicrobial resistance and virulence genes in *S. aureus* isolated from clinical samples at the University Teaching Hospitals (UTH) in Lusaka, Zambia.

1.2 Statement of the Problem

S. aureus has become a major public health concern as a result of the steadily increasing incidence of antimicrobial resistance (Ding et al., 2008, Bennett and Thomsen, 2020). Studies conducted at UTH in 2012 and 2014 estimated the presence of MRSA isolated from clinical samples at 37% and 43% respectively (Mulipilwa, 2012 (Samutela et al., 2015). This suggests that the antimicrobial resistance is on the increase. Recent studies conducted at UTH also revealed that *S. aureus* isolates were highly resistant to commonly prescribed antibiotics for example trimethoprim/ sulfamethoxazole ciprofloxacin, penicillin G, erythromycin, tetracycline and gentamicin (Samutela et al., 2015, Chakolwa et al., 2019). The emergence of virulent and multidrug-resistant strains has heightened the morbidity rates and impeded effective treatment of *S. aureus* infections at the referral hospital (Chakolwa et al., 2019). Despite high phenotypic antimicrobial resistance shown using the Kirby-Bauer disc diffusion method in the *S. aureus* isolates at the referral

hospital, studies to detect respective antimicrobial resistance genes have not been carried out which is quite worrisome. In addition, the presence of virulence genes has also not been thoroughly studied. Little information is available regarding the detection of antimicrobial resistance and virulent factors genes of *S. aureus* isolates at the largest referral and teaching hospital in Zambia. It is well known that antimicrobial resistance, toxigenic equipment carriage and clonal analyses in *S. aureus* can provide useful insights into the virulence potential and nature of *S. aureus* populations. With limited studies there is concern that in the not-too-distant future, we may be faced with a growing number of potentially untreatable staphylococcal infections, if no action is taken there will be no cure tomorrow.

1.3 Justification of the Study

Identification of antimicrobial resistance genes is extremely important for understanding the underlying mechanisms and the epidemiology of antimicrobial resistance. Therefore, a deeper understanding of the molecular variability of *S. aureus* in clinical settings can lead to better comprehension of its pathogenic potential and improved strategies to counteract its virulence and antimicrobial resistance. To develop new anti-staphylococcal antimicrobials, it takes a more thorough understanding of the current role *S. aureus* virulence factors play in contributing to human disease. The accurate and rapid detection of antimicrobial resistance and virulence genes in *S. aureus* is extremely important in the treatment of staphylococcal infections and preventing the spread of infections. This study focused on antimicrobial resistance and virulent factor genes found in *S. aureus* isolates from clinical samples. Furthermore, the presence of virulence genes would increase understanding of virulence factors and their importance to *S. aureus* pathogenesis. To establish better staphylococcal infection control in Zambia, it is important to understand the local epidemiology and clonal lineages of *S. aureus* strains in circulation. The genetic diversity in relation to antimicrobial resistance and virulence factors of *S. aureus* in circulation is poorly understood in Zambia. The information from this study is useful by giving insight to policy that would regulate the use of antimicrobials and best management practices to prevent the emergence and spread of antimicrobial resistance.

1.4 Study Objectives

1.4.1 General Objective

To characterise *S. aureus* strains isolated from clinical samples at UTH in Lusaka, Zambia using molecular tools.

1.4.2 Specific Objectives

1.4.2.1 To detect the antimicrobial resistance genes using PCR in *S. aureus* isolates at UTH, Lusaka.

1.4.2.2 To detect virulence genes using PCR in the *S. aureus* isolates.

1.4.2.3 To determine co-occurrence of antimicrobial resistance and virulence genes.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mechanism of Antimicrobial Resistance in Bacteria

Antimicrobial resistance (AMR) occurs when bacteria, viruses, fungi and parasites change over time and no longer respond to antimicrobials making infections harder to treat and increasing the risk of disease spread, severe illness and death. Bacteria may be intrinsically resistant to one or more classes of antimicrobial agents, or may acquire resistance genes by de novo mutation or via the acquisition of antimicrobial resistance genes from other organisms (Tenover, 2006). Resistance can be caused by a variety of mechanisms: (i) the presence of an enzyme that inactivates the antimicrobial agent; (ii) the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent; (iii) a mutation in the antimicrobial agent's target, which reduces the binding of the antimicrobial agent; (iv) posttranscriptional or posttranslational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent; (v) reduced uptake of the antimicrobial agent; (vi) active efflux of the antimicrobial agent; (vii) overproduction of the target of the antimicrobial agent and (viii) lack of target, no cell wall; innately resistant to penicillin. In addition, resistance may be caused by a previously unrecognized mechanism (Fluit et al., 2001). Acquisition of new genetic material by antimicrobial-susceptible bacteria from resistant strains of bacteria may occur through conjugation, transformation, or transduction, with transposons often facilitating the incorporation of the multiple resistance genes into the host's genome or plasmids (Poulose et al., 2021).

2.2 *Staphylococcus aureus* as a Main Antimicrobial Resistant Bacterium in Human Settings

AMR is one of the greatest current threats in international public health (Waddington et al., 2022). *S. aureus* is one of the four antimicrobial resistant pathogens of global concern (Taylor and Unakal, 2021). Since the introduction of antimicrobials in medicine, *S. aureus* has shown a frequent and rapid development and spread of AMR and has developed resistance to almost all types of antimicrobials (Perovic et al., 2015). AMR contributes to antimicrobial treatment failure and increasing rates of mortality in various infectious

diseases. Misuse of specific antimicrobial and overuse of broad-spectrum antimicrobials are attributed to the emergence of AMR, which in some strains of *S. aureus* results in multidrug resistance (MDR). Despite warnings regarding overuse, antibiotics are overprescribed worldwide (Nature, 2013). According to Antimicrobial Resistance Global Report on Surveillance (2014) the following overall reported range of *S. aureus* AMR proportion percentages (%) were recorded across the globe; Region of the Americas 21–90%, Eastern Mediterranean Region 10–53%, European Region 0.3–60%, South-East Asia Region10–26%, Western Pacific Region 4–84% and African Region 12–80%.

The implication of high reported proportions of AMR is that treatment for suspected or verified *S. aureus* infections in many countries may require second-line antimicrobials. This may also be the case for prophylaxis in orthopaedic and many other surgical procedures. Second-line drugs needed to treat or prevent AMR infections are expensive. Information concerning the true extent of the problem of AMR in the African Region is limited because surveillance of AMR is carried out in only a few countries.

2.3 Drivers in *S. aureus* Antimicrobial Resistance

AMR in *S. aureus* occurs naturally as an adaptive mechanism of bacteria while global increases in antimicrobial use in humans, animals and agriculture, have increased the selective pressure that drives the development of resistance, drastically accelerating this natural phenomenon (Holmes et al., 2016). Although frequently overlooked, the emergence and dissemination of AMR is also affected by socioeconomic and environmental factors, including inadequate water, sanitation, and hygiene infrastructure, living conditions, waste management, education and awareness, economic activities, and other factors such as climate, health care quality, and migration (Allel et al., 2020).

2.4 Epidemiology of *S. aureus* and its Antimicrobial Resistance and Virulence Genes

The epidemiology of *S. aureus*, especially of MRSA, showed a rapid evolution in the last years. After presenting as a typical nosocomial multidrug-resistant pathogen, MRSA has recently emerged in the community and among farmed animals due to its ability to evolve and adapt to different settings (Monaco et al., 2017) Global surveillance has shown that

MRSA represents a problem in all continents and countries where studies have been carried out, determining an increase in mortality and the need to use last-resource expensive antibiotics (Monaco et al., 2017). Since the evolution of *S. aureus* will continue, it is important to maintain the attention on the epidemiology of *S. aureus* in the future with a global view.

2.5 Some Selected Antimicrobial Resistance Genes in *S. aureus*

Presence of AMR genes in *S. aureus* is usually associated with phenotypical AMR (Momtaz et al., 2013). Genes that confer AMR to commonly prescribed antimicrobials in *S. aureus* include *mec*A (methicillin), *erm* (erythromycin), *tet* (tetracycline), *van*A (Vancomycin), *bla*Z (penicillin), and *aac*A-*aph*D (gentamycin) (Akpaka et al., 2017). Detection of these antimicrobial resistance genes is required not only for therapy but also to monitor the spread of resistant genes in *S. aureus* throughout the hospital and community settings. Furthermore, the presence of a resistance gene is highly predictive for clinical outcome of antimicrobial therapy. The advent of PCR offers a cost-effective, user-friendly genetic method for the detection and characterization of AMR genes in *S. aureus.*

2.5.1 Methicillin Resistance Genes

Beta-lactam antimicrobials kill bacteria by inhibiting cell wall synthesis. Methicillin resistance of *S. aureus* to beta-lactam group is due to a protein mutant of penicillin-binding protein 2a (PBP2a) encoded by *mec*A gene (Nasution et al., 2018). Penicillin binding protein is a group of enzymes in the cell membrane of *S. aureus* that catalyzes the transpeptidation chain webbing (cross linkage) (Nasution et al., 2018). Affinity of PBP2a to antimicrobial beta-lactam group is so low that MRSA strains remain alive in high concentration of antimicrobial exposure (Boswihi and Udo, 2018). Detection of *mec*A gene is also important in classifying *S. aureus* into MRSA and methicillin susceptible *S. aureus* (MSSA).

In Saudi Arabia 13 of 15 *S. aureus* isolates resistant to methicillin expressed *mec*A gene, while the remaining two showed the resistance to methicillin without the expression of *mec*A gene (Al-Ruaily and Khalil, 2011). In Iran among 162 *S. aureus* isolates, 55 (34%)

were MRSA (*mec*A positive) and 107 (66%) were methicillin-susceptible *S. aureus* (Sedaghat et al., 2017).

Research in Africa has demonstrated that *S. aureus* has been largely neglected in the past, despite the cultural and geographical diversity in Africa, which has a significant impact on the epidemiology of this pathogen. The polarity between developed urban societies and remote rural populations, combined with close contact with animals (e.g. livestock and domestic animals, and wildlife), makes the epidemiology of *S. aureus* on the African continent unique and fascinating (Schaumburg et al., 2014). In Sub Saharan Africa numerous studies exist describing various *S. aureus* strain collections with phenotypic and genotypic methods, yet, they have been collected from retrospective strain collection, lack accompanying clinical data, are not controlled for hospital acquisition of the isolate, and have not been performed strictly comparing the genotype. In other words cross sectional molecular epidemiologic studies on both MSSA and MRSA are largely lacking in Sub Sahara Africa (Ruffing et al., 2017). In Nigeria among the 194 strains, 20.6% were MRSA using 10-µg methicillin disc. PCR analysis showed that *mec*A gene was present only in 22.2% of 194 *S. aureus* isolates (Alli et al., 2011). A study involving five major African towns reported an overall MRSA prevalence rate of 15% in clinical samples (Breurec et al., 2011). A study in South Africa, methicillin resistance was detected in 46% isolates with the minimum inhibitory concentration method and confirmed with *mec*A in 43% isolate (Perovic et al., 2015). In a study by Samutela et al, (2017) at a referral hospital in Zambia, the *mec*A gene was detected in all the MRSA (100%) isolates (Samutela et al., 2017).

The prevalence of MRSA has increased in many parts of the world causing serious infections in hospitals that pose a serious burden in terms of medical and socio-economic costs and cause significant morbidity and mortality (Sajith Khan et al., 2012). According to this literature data the prevalence of MRSA is on the increase worldwide. This observation is of clinical significance because MRSA always shows a multidrug-resistant pattern, not only for penicillin but also for various antimicrobial classes including macrolides, fluoroquinolones, aminoglycosides, tetracyclines, and lincosamides.

2.5.1.1 Staphylococcus Cassette Chromosome *mec* (SCC*mec*) Typing

Emergency of MRSA is associated with acquiring of the SCC*mec* element, which provides the bacterium with two essential components, the *ccr* gene complex (ccr) and the *mec* gene complex (*mec*) harboring the *mec*A gene. SCC*mec* typing classifies SCC*mec* elements on the basis of their structural differences (Katayama et al., 2000). There are five major types of SCC*mec* elements (I–V) (Deurenberg et al., 2007). Studies have indicated that majority hospital-associated MRSA (HA-MRSA) strains carry SCC*mec* type I, II or III (Oliveira et al., 2001) whereas well-defined community-associated MRSA (CA-MRSA) strains carry SCC*mec* type IV or V (Turnidge et al., 2000, Ma et al., 2002, Ito et al., 2004. SCC*mec* types IV and V are relatively small in size; for SCC*mec* type IV, this appears to have resulted in its increased mobility and therefore greater potential for horizontal spread to diverse *S. aureus* genetic backgrounds, compared with other SCC*mec* types (Cooper et al., 2004). SCC*mec* typing is a suitable method for detection of strains associated with nosocomial infection. SCC*mec* typing is also applied in epidemiological studies to distinguish MRSA strains or to define an MRSA clone in combination with the genotype of MSSA strain in which an SCC*mec* element has integrated.

In Pakistan a study at a tertiary hospital, MRSA, SCC*mec* typing revealed that 3% were type I; 9% were type II; 47% were type III, and 29% were type IV (Asghar et al., 2014). In Zambia a study by Samutela et al (2017) at UTH showed that MRSA strains were of SCCmec types I, II, and III.

2.5.2 Erythromycin Resistance Genes

Erythromycin is a metabolic product of *Streptomyces erythreus* and consists of a 14-member lactone ring to which are attached two deoxy-sugars, desosamine and cladinose. The macrocyclic lactone ring is the source of the class name, macrolide. Erythromycin, like most macrolides, appears to act by binding in the ribosomal tunnel through which the nascent peptide moves and thus can be considered a peptidyl transferase inhibitor. The most well-known mechanism of resistance to erythromycin is the decreasing affinity of antibiotics for ribosomes due to ribosomal binding site alteration (Jelić and Antolović,

2016). Ribosomal methylase production, coded by *erm* genes (*erm*A, *erm*B, *erm*C) is involved in this modification.

In a study conducted in Iran, the most common erythromycin-resistant genes in *S. aureus* isolates were *erm*C (35.2%) and *erm*A (20.4%) (Sedaghat et al., 2017). A study in Turkey revealed that *erm*C gene was the most frequent gene detected in 91.9% of erythromycin resistant isolates and was found in 35 isolates, in 20 isolates with *erm*A and in two isolates with the *erm*B gene (Yılmaz and Aslantaş, 2017). The *erm*A gene was reported as the most common genotype among both erythromycin resistant MSSA and MRSA isolates in Turkey (Yılmaz and Aslantaş, 2017). In India, a study done by Abulkasim et al (2017) the most prevalent erythromycin resistance gene in *S. aureus* was *erm*A (61.7%), followed by *erm*C (23.40%) and *erm*B which occurred in (14.89%) of the erythromycin resistant *S. aureus* isolates tested (Abulkasim et al., 2017).

2.5.3 Tetracycline Resistance Genes

Protein synthesis is an essential requirement of any cell. It involves the use of ribosomes, which translate an mRNA code into functioning proteins. Tetracyclines specifically inhibit the 30S ribosomal subunit, hindering the binding of the aminoacyl-tRNA to the acceptor site on the mRNA-ribosome complex. When this process halts, a cell can no longer maintain proper functioning and will be unable to grow or further replicate (Shutter and Akhondi, 2020). Two main mechanisms of resistance to tetracycline have been described in *S. aureus* that's active efflux pumps, resulting from the acquisition of the plasmid-located *tet*K and *tet*L genes and ribosomal protection by elongation factor-like proteins that are encoded by chromosomal or transposonal *tet*M or *tet*O determinants (Emaneini et al., 2013). A study in Lao revealed that tetracycline resistance was common (50%) and mainly associated with *tet*K (90%), with *tet*M found in three isolates (Yeap et al., 2017). In Iran, the rate of tetracycline resistance genes associated with *mec*A was 61%. All the tetracycline resistant isolates, excluding 11 isolates, gave positive results for *tet* genes. Forty-nine isolates (32.4%) contained *tet*M, 17.2% possessed *tet*K and 13.9% included both *tet*M and *tet*K genes. Four isolates were sensitive to tetracycline but positive for the *tet* genes (two isolates contained both *tet*M and *tet*K and two isolates contained only *tet*K). None of the isolates were positive for *tet*L and *tet*O in the PCR assay (Emaneini et al.,

2013). The *tet*K and *tet*M genes were detected in 82.75% and 56.9% of 58 tetracycline resistant isolates, respectively, in which 39.65% isolates harboured *tet*K and *tet*M simultaneously. None of the isolates were positive for *tet*L and *tet*O genes (Khoramrooz et al., 2017). In Pakistan, the *tet*K gene was found in 58 isolates and *tet*L in one isolate. No *tet*M and *tet*O were detected (Ullah et al., 2012). A study done in Nigeria revealed that isolates resistant to tetracycline carried either one of the resistance genes *tet*K or *tet*M, which mediate resistance through active drug efflux or ribosomal protection mechanisms, respectively (Shittu et al., 2011).

2.6 Virulent Factors associated with *S. aureus* Pathogenicity

AMR is not the only weapon in the arsenal of *S. aureus*. In contrast to many other bacterial pathogens, which often rely on only one or a few toxins to promote disease, *S. aureus* produces an astounding array of virulence factors that promote pathogenicity of this organism (Sause et al., 2019). These include a plethora of toxins and immune evasion factors, and a vast array of protein and non-protein factors that enable host colonization during infection**.** Among the virulence factors that have an important role in pathogenicity of *S. aureus* are adhesins and surface proteins, such as protein A, and, in the particular case of toxins, enterotoxins and *β*-Hemolysin (Adame-Gómez et al., 2020).

2.7 Some Selected Virulence genes in *S. aureus*

While there has always been great interest in *S. aureus* virulence ever since this bacterium was first recognized as an important pathogen at the end of the 19th century, recent developments have increased research efforts into unraveling *S. aureus* virulence mechanisms (Cheung et al., 2021). Simplex and multiplex PCR assays can be used as rapid and sensitive diagnostic tools to detect the presence of *S. aureus* and characterize its virulence factors that help in detection of severity of infection, distribution and stating preventive and control strategies (Elsayed et al., 2015).

2.7.1 Panton Valentine Leukocidin Virulence Factor

PVL is a virulence factor produced by some strains of *S. aureus* that causes leukocyte lysis and tissue necrosis (Darboe et al., 2019). PVL-associated *S. aureus* predominantly causes skin and soft-tissue infections (Darboe et al., 2019). The PVL is encoded by the two genes *lukS-PV* and *lukF-PV* and thought to be associated with increased disease

severity (Dekker et al., 2016). PVL was named after Sir Philip Noel Panton and Francis Valentine who associated it with soft tissue infections in 1932 (Bhatta et al., 2016, Hoppe et al., 2018). It is a member of the synergohymenotropic toxin family that induces pores in the membranes of cells (Bhatta et al., 2016). PVL is frequently associated with severe and recurrent skin and soft-tissue infections (SSTIs) and has previously been found in *S. aureus* isolates from various complexes (Shittu et al., 2011). In particular, PVL-producing MSSA affiliated to CC121 are known to be common in many countries on all continents (Shittu et al., 2011). In German a major difference was observed with leucocidins: the genes encoding for the PVL *lukF-PV* and *lukS-PV* were recognized in almost one half of African clinical strains and were virtually absent in German isolates (Ruffing et al., 2017). A study conducted in the United Kingdom revealed that thirty-three (34%) patients were noted to have abscesses, of which 85% had isolates which encoded PVL genes (Yeap et al., 2017). In a study carried out in Indonesia, *S. aureus* isolated from skin and soft tissue infections carried genes encoding PVL were detected in 21.8% of methicillin susceptible *S. aureus* and PVL positive MRSA was not detected (Santosaningsih et al., 2018). In Brazil *S. aureus* isolated from blood culture showed only one isolate carried the PVL gene (Monteiro et al., 2019). In Iran, 7 and 10 PVL genes were detected in MSSA and MRSA respectively (Sedaghat et al., 2018).

In Africa several studies have been carried out to detect PVL genes in *S aureus*. A study conducted in Gabon by Schaumburg et al (2011) detected a significant association of PVL-encoding genes with isolates derived from abscesses. In Ghana, the prevalence rate of PVL-positive isolates among all *S. aureus* was 75% (Dekker et al., 2016). In Gambia, *S. aureus* PVL positive strains accounted for 61.4% of *S. aureus* isolates (Darboe et al., 2019). A study conducted in West and Central Africa found that the PVL genes encoding PVL, present in 130 MSSA isolates overall (57%), were highly prevalent in isolates from Cameroon, Niger, and Senegal. This finding is of major concern with regards to both as a source of severe infections and a potential reservoir for PVL genes. This over representation of PVL in MSSA could lead to the emergence and spread of successful, highly virulent PVL-positive MRSA clones, a phenomenon that has already started in Africa (Breurec et al., 2011). In a study done in Nigeria the proportion of PVL-positive isolates among MSSA isolates was high (40%). Most of the PVL-positive MSSA isolates were obtained from wound infections and associated with clonal complexes CC1, CC30,

CC121 and with sequence type ST152 (Shittu et al., 2011). In a study done in Zambia, PVL genes were detected in 1.9% isolates (Samutela et al., 2017). In another study from Zambia, *lukS-PV* gene was detected in 10% of the *S. aureus* isolates (Phiri et al., 2022). These findings demonstrate that, the prevalence of PVL gene in West and Central Africa is on the increase.

2.7.2 *S. aureus* Staphylococcal Protein A

The Staphylococcal Protein A, encoded by *spa* gene is a virulence factor of *S. aureus* that binds to the IgG molecules by their Fc portion and inhibits phagocytosis of bacteria and thus contributes to the development of the disease (Choudhary et al., 2018).

2.7.2.1 Spa Typing

The spa typing technique uses the sequence of a polymorphic variable number of tandem repeats (VNTR) in the 3' coding region of the *S. aureus* specific staphylococcal protein A. Each new base composition of the polymorphic repeat found in a strain is assigned a unique repeat code. The repeat succession for a given strain determines its spa type. The spa typing of *S. aureus* strains provides information that can group isolates in clonal lineages. Clonal analyses can also provide useful insights into the virulence potentials and nature of *S. aureus* populations. This is important for the detection of transmission routes and monitoring of bacterial strains circulation (Kolawole et al., 2013).

In Ghana, twenty-five different spa types were identified in the *S. aureus* isolates. The most prevalent were t355 (11/19.6%) followed by t314 (8/14.3%), t084 (8/14.3%) and t311 (5/8.9%). The most frequent sequence types (ST) were ST152 (17/32.1%), followed by ST121 (14/26.4%) and ST15 (9/17.0%) (Dekker et al., 2016). In South Africa, spa typing of 569 of the isolates revealed 47 different spa types, nine of which were novel and had not yet been assigned. The five most common spa types were t037 (n =274), t1257 (n =120), t045 (n =42), t064 (n =34) and t012 (n =22), which accounted for 87% of the isolates tested. Spa types t037 and t1257 were related to hospital- and community-associated infections respectively (Perovic et al., 2015). In Zambia a study carried out at UTH, the MRSA strains were found to be of 5 spa types namely t064 (40.6%), t2104 (31.3%), t355 (3.1%), t1257 (21.9%) and unknown spa type (3.1%) (Samutela et al.,

2017). Phylogenetic analysis of the MRSA strains based on the spa types showed that isolate with spa type t355 was most distantly related when compared with the rest of the strains characterised (Samutela et al., 2017). In a similar study at UTH 26% of the *S. aureus* isolates were positive for the *spa* gene on PCR. One isolate positive for the *spa* gene could not be sequenced. Two spa types namely t015 (42.8%) and t069 (14.3%) were identified, whereas 43% were of unknown spa type (Chakolwa et al., 2019). These data appear to indicate that the most common spa types associated with hospital infections are spa type t064 and spa type t1257, which were also found at UTH. This study aims at determining the spa types found in *S. aureus* strains isolated from clinical samples at the referral hospital.

2.7.3 *S. aureus* Enterotoxins

The *S. aureus* enterotoxins are a superfamily of secreted virulence factors that share structural and functional similarities and possess potent super antigenic activity causing disruptions in adaptive immunity (Fisher et al., 2018). The classical antigen-based classification of Staphylococcal Enterotoxins (SEs) includes five classical types: *sea*, *seb*, *sec*, *sed*, and *see.* In latest years, new types of SEs (*seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *ser*, and *seu*) have been reported (Riva et al., 2015). The enterotoxins, *sea*, *seb*, and *sec* have been widely studied for their involvement in food poisoning disease.

In South Korea, among the 70 *S. aureus* isolates obtained from blood, 50 of the isolates (71.4%) harboured at least one enterotoxin gene (Perk et al., 2009). The *see* and PVL genes were not detected in any of the *S. aureus* isolates obtained from blood. MRSA and MSSA isolates from blood showed different prevalence of enterotoxin genes (Perk et al., 2009). With regard solely to the *sea* gene, the MSSA isolates showed significantly higher prevalence than was observed in the MRSA isolates (Perk et al., 2009). Whereas the majority of MRSA isolates from blood contained *sec* (66.7%), *seg* (86.1%), *sei* (83.3%), and less than 30% of the MSSA isolates from blood were positive for those genes (Perk et al., 2009). In Mexico a study found that the *sea* gene coding for enterotoxin A was amplified in 53.1%, and *sec* with 6.2% and with less frequency the *sed*, *seb*, and *see* genes with 4.6%, 3.1%, and 1.5%, respectively (Adame-Gómez et al., 2020).

In Nigeria, the study revealed that *seb* gene was the most prevalent followed by *sea* gene and *sec* gene. The *sed* gene was detected in two isolates, while *see* gene was not observed

(Kolawole et al., 2013). From these data it was observed that *sea*, *seb*, and *sec* were the most frequently detected enterotoxin associated with food poisoning. Regrettably, there is no published evidence on the presence of enterotoxins genes in *S. aureus* isolates in Zambia.

2.8 Relationship Between Antimicrobial Resistance and Virulence in *S. aureus*

There is an overly complex relationship between AMR and virulence in bacteria (Beceiro et al., 2013). Multiple factors associated with bacteria and their environment affect the evolution of antibiotic resistance and virulence. Both virulence factors and ability to resist antimicrobials in *S. aureus* contribute to successful host-microbe colonisation (Pérez et al., 2020). Virulence mechanism are necessary to overcome host defense systems, and the development of AMR is essential to enable pathogenic bacteria to overcome antimicrobial therapies and to adapt to and survive in competitive and demanding environments (Beceiro et al., 2013). Increased resistance is associated in most cases, either directly or indirectly, with decreased virulence and fitness (Beceiro et al., 2013). However, evidence also shows the opposite, and it is increasingly evident that the relationship is often of greater benefit to the pathogen, resulting in a growing public health problem (Beceiro et al., 2013). In *S. aureus* accessory gene regulator (*agr*) gene is a dominant regulator of pathogenesis and represents a clear example of the link between antimicrobial resistance and virulence (Shopsin et al., 2003). It can control the expression of virulence factors and antimicrobial resistance (Derakhshan et al., 2021).

In a study in West Iran, among MRSA isolates, 92.0% harboured enterotoxins genes and the most frequent enterotoxin was *sea* (45.0%), followed by *sec* (39.0%). Among 100 MSSA isolates, 89 (89.0%) harbored enterotoxins genes and the most prevalent genes were *sea* (42.0%), *sek* (38.0%), and *sec* (35.0%,) (Arabestani et al., 2018). In a study done in Poland 90.9% of the MRSA harboured virulence genes (*sea, seb sec, sed, seg*, *sei,* and PVL) (Budzyńska et al., 2021). In a study in China 56 MRSA isolates harboured *sea* (54%), *seb* (47%), *sec* (16%) and PVL (39%) virulence genes (Luo et al., 2018) The correlation between virulence factors and antimicrobial resistance is a serious public health threat that calls for a concerted global action to reduce the impact of *S. aureus* infections (Perez et al., 2020)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The *S. aureus* isolates were collected from UTH. UTH is the largest and main referral hospital in Zambia. It is located in the capital city Lusaka approximately 4 km east from the city centre. It has an approximately bed capacity of 2000 and its catchment population is estimated at about 2 million people. The laboratory analysis of the isolates was done at the University of Zambia, School of Veterinary Medicine in the Microbiology and Virology laboratories in the Department of Para clinical studies and Department of Disease control respectively.

3.2 Study Design

This was a retrospective laboratory based cross-sectional study.

3.3 Sampling Techniques

Retrospectively collected consecutive *S. aureus* isolates from clinical samples taken from 2018 to 2019 and processed (isolated) at the Microbiology Laboratory at the University Teaching Hospitals were used in this study.

3.4 Sample Size

A convenient sample size of 43 stored clinical isolates of *S. aureus* isolated from blood, skin and soft tissue infections, were used for the study. All the stored isolates were included in the study.

3.5 Isolates Subculture

The 43 stored *S. aureus* isolates from clinical samples were cultured on nutrient agar and incubated overnight at 37 °C. Briefly a wire loop was used to inoculate stored isolates

onto nutrient agar and incubated at 37 °C for 24 hours. Pure colonies were selected for DNA extraction.

3.6 DNA Extraction

The boiling method technique described by Dashti et al (2009) was used to extract the DNA from 24 hours old colonies. Briefly, 200 µl of sterilized distilled water was used to emulsify a loop full of pure colonies from nutrient agar. The mixture was then vortexed and heated at 95°C for 15 minutes on a heating block. This was followed by centrifugation at 1,954 xg for 5 minutes. The supernatant containing the DNA was transferred to another tube and stored at -20°C until needed for further analysis.

3.7 Primers Preparation

Commercially prepared primers were used. Reconstitution was done following manufacturer instruction from stock primer solution and read to use primers aliquoted in cryo vials and stored at -20°C.

3.8 *S. aureus* Strain Identification and Detection of Methicillin Resistance

S. aureus identification and resistance to methicillin of the 43 isolates was confirmed by PCR amplification using species-specific (*nuc*) gene and *mec*A gene primers, respectively (Table 3.1). Briefly, for the *nuc* gene, PCR was performed in a final volume of 25µl and the reaction compositions included 0.5µl (5ng/ µl) of each Nuc1 and Nuc2 primers (10 Pmol), 10µl of deionized water, 12.5µl of reaction buffer of One*Taq* Quick-load Master Mix (BioLabs, New England) and 1.5µl of the template DNA extracted. The amplification was performed on a Veriti thermal cycler (Thermo Scientific, Hanover, MD, USA), using the following PCR conditions: an initial denaturation step at 94°C for 5 min; 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The PCR products were electrophoresed in 1.5% agarose gels with 1X Tris-acetate-EDTA (Baba et al., 2008) buffer at 100V for 30 min. The gels were pre-stained with ethidium bromide (Cinnagen Co., Tehran, Iran) to visualise the amplified DNA fragments (279 bp) under UV light box by comparison with a molecular size marker (100 bp ladders, eurobio, UK). Positive and negative controls were *S. aureus* ATCC29213 and molecular grade water, respectively. To detect the *mec*A, PCR reaction was carried out in

a 25µl reaction volume containing 0.5µl of each primer forward and reverse primers, 10µl of deionized water, 12.5µl of reaction buffer of One*Taq* Quick-load Master Mix (BioLabs, New England) and 1.5µl of the template DNA extracted. Thermocycling conditions in Veriti thermal cycler (Thermo Scientific, Hanover, MD, USA), was as follows: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 52 °C for 30 sec, and 72 °C for 30 sec, with a final extension at 72 °C for 5 min. The PCR products were electrophoresed in 1.5% agarose gels with 1X Tris-acetate-EDTA buffer at 100V for 30 min and the gels were pre-stained with ethidium bromide (Cinnagen Co., Tehran, Iran) to see the amplified DNA fragments (1339 bp) under UV light box by comparison with a molecular size marker (100 bp ladders, eurobio, UK). Methicillin resistant *S. aureus* clinical isolate containing *mec*A gene and molecular grade water was used as positive and negative controls, respectively.

Table 3.1: Primer Sets to Identify S. aureus and Detection of mecA Gene

Primer name	Target gene	Primer Seq (5'-3')	Amplicon size (bp)	Reference
Nuc1	*Nuc*	GCG ATT GAT GGT GAT ACG GTT	279	(Zhang et al., 2004)
Nuc2		AGC CAA GCC TTG ACG AAC TAA AGC		
mecA F	*mec*A	GTA GAA ATG ACT GAA CGT CCG ATA A	1339	(Sogut et al., 2020)
mecA R		CCA ATT CCA CAT TGT TTC GGT CTA A		

3.8.1 Staphylococcus Cassette Chromosome *mec* Typing

SCC*mec* types of *mec*A positive (MRSA) isolates were determined using the method described by (Nasution et al., 2018). PCR reactions for *SCCmec* typing primers listed in Table 3.2 were performed on a Veriti thermal cycler (Thermo Scientific, Hanover, MD, USA). A final volume of 25µl was used, containing 2µl bacterial DNA template, primer and 2X PCR Master Mix (Thermo Scientific, Hanover, MD, USA). The cycle conditions used were as follows: an initial denaturation at 95°C for 4 min, followed by 30 cycles of 95°C for 30 seconds (denaturation), 53°C for 30 seconds (annealing) and 72°C for 1 min (extension), 7 min at 72°C as the final extension time. The PCR products were

electrophoresed in 1.5% agarose gels with 1X Tris-acetate-EDTA buffer at 100V for 30 min and the gels were pre-stained with ethidium bromide (Cinnagen Co., Tehran, Iran) to see the amplified DNA fragments under UV light box by comparison with a molecular size marker (100 bp ladders, eurobio, UK).

Table 3.1: Primers Sequences used for SCC*mec* Typing

Primer	Sequence (5'-3')	Amplicon size (bp)	Specificity
RIF5 F10	TTCTTAAGTACACGCTGAATCG	414	III
RIF5 R13	GTCACAGTAATTCCATCAATGC		
KDP F1	AATCATCTGCCATTGGTGATGC	284	II
KDP R1	CGAATGAAGTGAAAGAAAGTGG		
DCS F2	CATCCTATGATAGCTTGGTC	342	I, II. IV
DCS R1	CTAAATCATAGCCATGACCG		
CIF2 F2	TTCGAGTTGCTGATGAAGAAGA G	495	I
CIF2 R2	ATTTACCACAAGGACTACCAGC		
RIF4 F3	GTGATTGTTCGAGATATGTGG	243	II
RIF4 R9	CGCTTTATCTGTATCTATCGC		
MECI P2	ATCAAGACTTGCATTCAGGC	209	1I, III
MECI P3	GCGGTTTCAATTCACTTGTC		
IS431 P4	CAGGTCTCTTCAGATCTACG	303	IIIA
pT181 R1	GAAGAATGGGGAAAGCTTCAC		
IS431 P4	CAGGTCTCTTCAGATCTACG	381	IA
pUB110 R1	GAGCCATAAACACCAATAGCC		

NB: Primer sequences were those previously reported by (Hanssen and Ericson Sollid, 2006)

3.9 Determination of other Antimicrobial Resistance Genes

The presence of antimicrobial resistant genes including the genes responsible for erythromycin-resistant (*erm*A, *erm*B, and *erm*C) and tetracycline-resistant (*tet*M, *tet*K, *tet*L, and *tet*O), were determined using PCR with gene-specific primers shown in Table 3.3. The PCR reaction mixtures and conditions to detect the antimicrobial resistance genes are briefly described below. To detect erythromycin-resistant, tetracycline-resistant genes, amplification reactions were performed in a total volume of 25µl containing 12.5µl of reaction buffer of One*Taq* Quick-load Master Mix (BioLabs, New England), 0.5µl of each primer forward and reverse primers, 10µl of deionized water, and 1.5µl of the template

DNA extracted. The DNA was denatured at 95°C for 4 min and then annealed at 52°C for 60 sec. The amplification cycles consisted of elongation at 72°C for 1.5 min, denaturation at 95°C for 30 sec, and annealing at 52°C for 45 sec. After 35 amplification cycles, the last elongation step was performed at 72°C for 7 min. Amplification was performed by using a Veriti thermal cycler (Applied Biosystems, Foster City, California). The PCR amplicons were visualized by using a UV light box after electrophoresis at 100V voltages for 30 min on a 1.5% agarose gel containing 0.5µg of ethidium bromide/ml.

Table 3.2: Primer Sets for Determining Antimicrobial Resistance Genes of *S. aureus* Isolates

Primer name	Target gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
ermA-1	*erm*A	TCTAAAAAGCATGTAAAAGAA	645	(Sutcliffe et al., 1996)
ermA-2		CTTCGATAGTTTATTAATATTAG		
ermB-1	*erm*B	GAAAAGTACTCAACCAAATA	639	(Sutcliffe et al., 1996)
ermB-2		AGTAACGGTACTTAAATTGTTTA		
ermC-1	*erm*C	TCAAAACATAATATAGATAAA	642	(Sutcliffe et al., 1996)
ermC-2		GCTAATATTGTTTAAATCGTCAAT		
tetK-1	*tet*K	TTAGGTGAAGGGTTAGGTCC	697	(Aarestrup et al., 2000)
tetK-2		GCAAACTCATTCCAGAAGCA		
tetM-1	*tet*M	GTTAAATAGTGTTCTTGGAG	576	(Aarestrup et al., 2000)
tetM-2		CTAAGATATGGCTCTAACAA		
tetL-1	*tet*L	CATTTGGTCTTATTGGATCG	456	(Aarestrup et al., 2000)
tetL-2		ATTACACTTCCGATTTCGG		
tetO-1	*tet*O	GATGGCATACAGGCACAGAC	615	(Aarestrup et al., 2000)
tetO-2		CAATATCACCAGAGCAGGCT		

3.10 Determination of Virulence Genes

Uniplex PCR was performed on all 43 *S. aureus* isolates to detect genes encoding virulence factors including, *spa, lukS-PV* and *lukF-PV* genes encoding staphylococcal protein A and PVL respectively. Genes coding for SEs: *sea*, *seb*, *sec*, *sed*, and *see* were

also detected using the primers sets listed in Table 3.4. To detect each virulence genes, a reaction mixture was prepared in a final volume of 25µl reaction mixture comprising of 0.5µl of each gene specific forward and reverse primers, 10µl of deionized water, 12.5µl of reaction buffer of One*Taq* Quick-load Master Mix (BioLabs, New England), and 1.5µl of the template DNA extracted. To detect *spa* gene the Uniplex PCR cycle conditions was as follows: an initial denaturation at 95°C for 4 min, followed by 30 cycles of 95°C for 30 sec (denaturation), 60°C for 30 sec (annealing) and 72°C for 45 sec (extension), 10 min at 72°C was be used as the final extension time. To detect PVL gene the PCR cycle conditions was as follows: an initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 1 min at 59°C, extension for 1 min at 72°C, and final extension for 10 min at 72°C. The Uniplex PCR conditions for detecting the SEs genes was an initial denaturation at 95°C for 15 min, then 35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 57°C, then extension for 90 sec at 72°C and final extension for 10 min at 72°C. Amplification for all the genes was performed by using a Veriti thermal cycler (Applied Biosystems, Foster City, California). The PCR amplicons were visualized by using a UV light box after electrophoresis at 100V voltages for 30 minutes on a 1.5% agarose gel containing 0.5 µg of ethidium bromide/ml. All isolates with bands that correspond to the expected amplicon sizes were taken as positive for virulence genes.

Table 3.3: Primer Sets for Determining the Virulence of *S. aureus* Isolates

Primer name	Target gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
Luk-PV-1	*LukS/F-PV* (PVL)	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	(Moussa et al., 2012)
Luk-PV-2		GCATCAAGTGTATTGGATAGCAAAAGC		
1095F	*spa*	AGACGATCCTTCGGTGAGC	~230	(Shopsin et al., 1999)
1095R		GCTTTTGCAATGTCATTTACTG		
SEA-3	*sea*	CCTTTGGAAACGGTTAAAACG	127	(Becker et al., 1998)
SEA-4		TCTGAACCTTCCCATCAAAAAC		
SEB-1	*seb*	TCGCATCAAACTGACAAACG	477	(Becker et al., 1998)
SEB-4		GCAGGTACTCTATAAGTGCCTGC		
SEC-3	*sec*	CTCAAGAACTAGACATAAAAGCTAGG	271	(Becker et al., 1998)
SEC-4		TCAAAATCGGATTAACATTATCC		
SED-3	*sed*	CTAGTTTGGTAATATCTCCTTTAAACG	319	(Becker et al., 1998)
SED-4		TTAATGCTATATCTTATAGGGTAAACATC		
SEE-3	*see*	CAGTACCTATAGATAAAGTTAAAACAAGC	178	(Becker et al., 1998)
SEE-2		TAACTTACCGTGGACCCTTC		

3.10.1 Spa Typing

The spa typing was performed to seven selected isolates *spa* positive isolates, which harboured either antimicrobial resistance gene or virulence factor gene. The spa types

were determined with the database accessible via https://www.genomicepidemiology.org using spaTyper software. Briefly, the *spa* gene was amplified using the aforementioned PCR reactions using primers listed in Table 3.4. The PCR reactions were performed on a Veriti thermal cycler (Thermo Scientific, Hanover, MD, 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 concentration of 3mM. Prior to sequencing PCR products were purified using Zymo Research DNA purification kit (Irvin, California, USA). The amplicons were sequenced using Brilliant Dye terminator. DNA sequences were obtained with AB 3500 Genetic Analyzer sequencer (Applied Biosystem, UK). Editing of the DNA sequences was done using ATCG software.

3.11 Data Analysis

The data was stored and established in excel. No statistical analysis was done due to small sample size (43).

3.12 Ethical Considerations

Ethics approval for this study was sought from the University of Zambia Biomedical and Research Ethics Committee (UNZABREC) and National Health Research Authority (NHRA) with the approval numbers 2149-2021 and Ref. No NHRA000003/15/01/2022**,** respectively. Permission to use archived bacterial isolates was obtained from the management at UTH. Study numbers were used to identify the bacterial isolates. The patient demographic data was not used in the study.

CHAPTER FOUR

RESULTS

4.1 *S. aureus* Strain Identification and Methicillin Resistance

All Forty-three *S. aureus* isolates tested positive for *S. aureus* species-specific *nuc* gene. Figure 4.1. shows part of *nuc* gene results. Five (11.6%) of the *S. aureus* isolates were identified as MRSA (*mec*A gene positive).

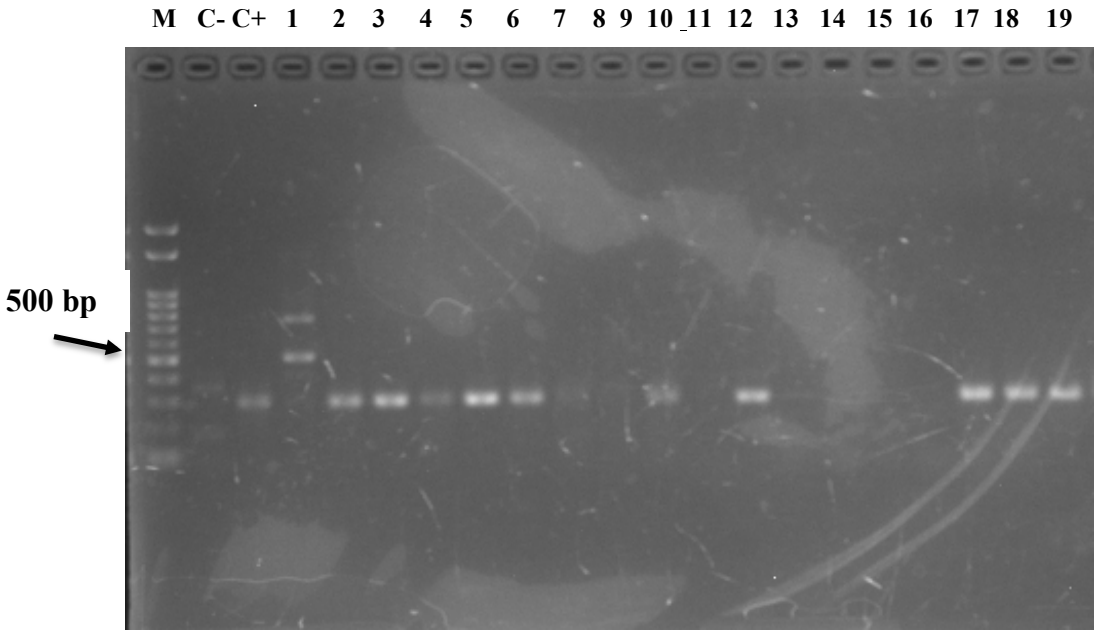

Figure 4.1: Agarose gel of electrophoresis of PCR products for *nuc* gene (279 bp), lane M is 100 bp DNA ladder,Lane C-Negative control,Lane,1-19 shows *S. aureus* samples.

4.1.1 Staphylococcus Cassette Chromosome *mec* Typing

The five MRSA were of three SCC*mec* types namely; type I, II, and III.

4.2 Determination of Antimicrobial Resistance Genes

Nine (20.9%) isolates had *erm*C gene amplified as shown in Figure 4.2, while none of the isolates were positive for neither *erm*A nor *erm*B gene. Three (6.9%) isolates tested positive for *tet*K gene and *tet*L gene was detected in one (2.3%) isolate. None of the

isolates tested positive for *tet*M, and *tet*O genes. The presence of tetracycline resistance genes among MRSA isolates was seen in 20% (1/5) of the isolates shown in Table 4.1.

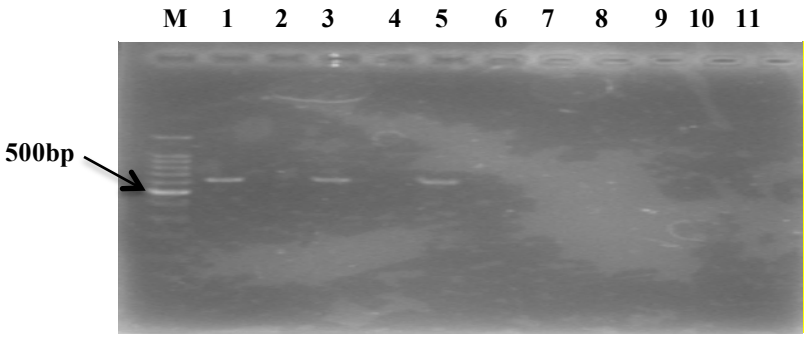

Figure 4.2: **Agarose gel electrophoresis of PCR product for *erm*C amplified gene (642 bp) Lane M is 100 bp DNA ladder, Lane C+ Positive control Lane C- Negative control, Lane 1-11 indicate the *S. aureus* samples.**

Table 4.1: Presence of Antimicrobial Resistance Genes among MRSA Isolates

Strain **ID**	**Source**	***mec*A**	***tet*K**	***erm*C**
1319	Blood	+	+	+
1241	Pus	+	_	+
1238	Pus	+	_	_
1293	Pus	+	_	_
1308	Blood	+	_	_

Key: + = Positive, _ = Negative

4.3 Determination of Virulence Genes

Of 43 isolates, 19 tested positive for *spa* gene. None of the isolates were positive for the PVL gene. Five (11.6%) isolates were found to be positive for *sec* gene shown in Figure 4.3. The *sea*, *seb*, *sed* and *see* enterotoxin genes were not detected in any of the isolates.

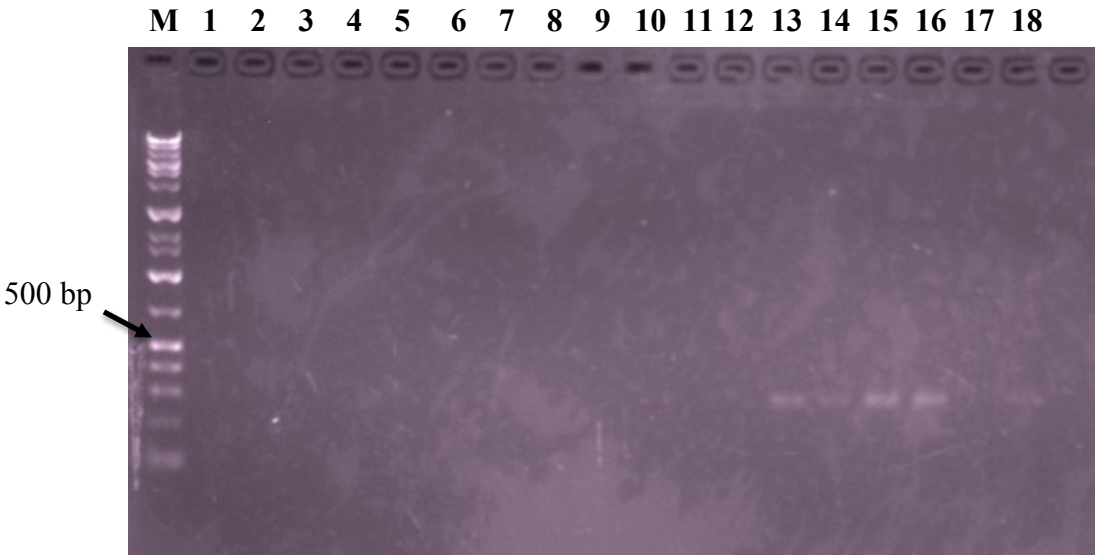

Figure 4.3: **Agarose gel electrophoresis of PCR product for *sec* amplified gene (271 bp) Lane, M is 1kb DNA ladder, Lane 13,14,15,16 and 18 show samples that tested positive for *sec* gene.**

4.3.1 Spa Typing

One spa type was identified among the seven selected *spa* positive isolates, which harboured antimicrobial or virulence genes as shown in Table 4.2. The spa type t015 was detected in two (28.6%) isolates whereas five (71.4%) isolates were of unknown spa types.

Table 4.2: Distribution of spa Types among Selected *spa* Positive Isolates

Strain ID	Source	Gene(s) present	spa type	Repeat succession	Position
1561	Blood	*erm*C, *sec*	t015	08-16-02-16-34-13-17-34-16-34	48-325
1385	Pus	*erm*C, s*ec*	t015	08-16-02-16-34-13-17-34-16-34	33-310
1241	Pus	*erm*C, *mec*A	Unknown type		
1356	Pus	*Sec*	Unknown type		
1167	Pus	*erm*C	Unknown type		
1238	Pus	*mec*A	Unknown type		
1168	Blood	*Sec*	Unknown type		

4.4 Co-occurrence of Antimicrobial Resistance Genes and Virulence genes

Of nine *S. aureus* isolates that tested positive for erythromycin resistant gene (*erm*C), two (22%) harboured enterotoxin (*sec*) gene.

CHAPTER FIVE

DISCUSSION

The emergence and spread of antimicrobial resistance, and virulence factors of *S. aureus* is an important public health concern making molecular characterisation of AMR and virulence genes profiles of these strains of vital importance. The present study reports the molecular characteristics of *S. aureus* isolates from clinical samples at the largest referral and teaching hospital in Zambia from 2018 to 2019. Of these isolates, 11.6% were MRSA. MRSA strains are particularly of clinical importance because they carry mobile genetic elements, SCC*mec* that has a number of genes where the most important is *mec*A and genes that regulate the expression of *mec*A gene (Ito et al., 2001). The *mec*A gene confers resistance to methicillin and other β-lactam antimicrobials in *S. aureus* and encodes a 78-kDa PBP2a (Khan et al., 2020). MRSA is particularly important because it is one of the leading causes of nosocomial infections worldwide and has also emerged as a major cause of community acquired infections (Romero and de Souza, 2021). In the present study, the presence of *mec*A gene (11.6%) in the isolates was lower compared to 43% in South Africa but similar to a study involving five major African towns which reported 15% MRSA in clinical samples (Breurec et al., 2011). These MRSA strains were of SCCmec types I, II, and III, which is in agreement with the report by Samutela et al (2017) at the same hospital. SCCmec types I, II, and III are associated with hospital-associated methicillin resistant *Staphylococcus aureus* (HA-MRSA) (Romero and de Souza, 2021). These results confirm the idea that the HA-MRSA is more present than community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA) in hospital environments as SCCmec types IV and V which are carried by typical isolates of CA-MRSA, were not detected.

In this study, the co-expression of *tet*K gene among *mec*A positive isolates was at 20% (1/5), lower than 61% reported from Iran (Emaneini et al., 2013). Remarkably, one MRSA strain harboured both *tet* (*tet*K) and macrolide resistance (*erm*C) genes. These genes might be located on the same or associated genetic element. The co-occurrence of these AMR genes is of concern because it might give rise to resistance to different classes of antimicrobials. In the current study, tetracycline resistance genes, *tet*K and *tet*L genes

were detected. These genes are implicated in active efflux of tetracycline resistance, compared to chromosomal mediated *tet*M and *tet*O, which were not detected in any of the isolates (Schmitz et al., 2001). This is similar to the results of Ullah et al (2012) in which they found *tet*K in 58 isolates and *tet*L in one isolate and no *tet*M and *tet*O were detected. In our study the tetracycline resistance genes detected are plasmid mediated which is an indication of acquired AMR from other bacteria.

In the present study, erythromycin resistance gene *erm*C was detected. The findings of our study demonstrate that *erm*C (20%) gene is predominant, compared to the *erm*A (0%) and *erm*B (0%) genes which is similar to studies by Sedaghat et al (2017) and Yilmaz and Aslantas (2017) who reported *erm*C as predominant erythromycin resistance gene with prevalence rate of 35.2% and 91.9% respectively. These results have shown that *erm*C gene is responsible for majority of resistance to erythromycin in *S.aureus* isolates at the referal hospital.

The virulence factors of *S. aureus* play an important role during pathogenesis. In our study we did not find any isolate carrying the PVL gene despite similar studies reporting presence of PVL gene in low rates of 9.4%, 10% (Samutela et al., 2017, Phiri et al., 2022) and high rates of 61.5% (Darboe et al., 2019). It has been observed that prevalence of enterotoxin genes differs greatly depending on the geographic affiliation and the population structure tested (Kolawole et al., 2013). In our findings *sec* gene was the only enterotoxin gene detected among the most charcterised classical SEs in five *S. aureus* isolates. Results of the present study also gave new worrisome information regarding the presence of food toxin producing *S. aureus* strains at the largest referral hospital. The main sources of food contamination caused by enterotoxin producing *S. aureus* are food handlers through manual contact via noses and hands (Denayer et al., 2017). This could also apply to people staying closely together as in hospital settings.

The spa typing is an important tool that is used in the epidemiological study of *S. aureus* (Strommenger et al., 2008). The purpose of epidemiological typing is to determine the relationship between lines that were isolated from specific places and times for example during an outbreak. In our study, 41.8% *S. aureus* were positive for the *spa* gene. One spa type t015 (28.6%) was identified and five (71.4%) were unknown. Our observation is in

line with a previous study on clinical isolates from the same referral hospital which reported spa type t015 as most prevalent (42.8%) spa type among *S. aureus* isolates (Chakolwa et al., 2019). The spa type t015 is also of interest because it is associated with clinical cases in hospital environment. The spa type t015 was detected in Austria where it was associated with graft infections in patients (Konstantiniuk et al., 2016). In Germany, spa type t015 was also detected in patients with cystic fibrosis (Garbacz et al., 2018). A study at a Polish neonatal Intensive Care Unit, spa type t015 detected was associated with infections in newborns (Romaniszyn et al., 2015). In this study spa type t015 detection among food poisoning enterotoxin gene positive isolates was in line with findings of another study which also detected spa type t015 among staphylococcal food poisoning isolates (Wattinger et al., 2012). The five unknown spa types in this study could not be compared because the software that was used did not provide repeat succession to determine the spa type. This finding is similar to previous studies conducted at the same referral hospital where unknown spa types were reported (Samutela et al., 2017, Chakolwa et al., 2019).

Virulence factors and antimicrobial resistance are necessary to overcome the host's immune response and enhance the ability of bacteria to survive under adverse condition (Beceiro et al., 2013). In the present study we found the co-occurrence of AMR genes and virulence genes. Two *erm*C positive isolates also harboured *sec* gene. By grouping the strains according to methicillin resistance, it was observed that strains containing *sec* gene were more frequent in the group of MSSA (13.2%) compared to MRSA) (0%). This finding is inconsistent with those reported previously, where *sec* gene was more frequent in MRSA (39%) than in MSSA (35%) (Momtaz and Hafezi, 2014). However, our result was consistent with a study conducted in China where *sec* gene had higher percentage in MSSA (46) than in MRSA (16) strains (Luo et al., 2018). Most researchers focus on the presence of antimicrobial resistance and virulence genes in MRSA and MSSA, while the co-expression of virulence factors and antimicrobial resistance is less frequently studied. This could be attributed to reports from other Studies that *S. aureus* carrying antibiotic resistance genes may reduce the expression of virulence factors (Collins et al., 2010, Otto, 2010).

Although this study provides empirical evidence of antimicrobial resistance and virulence genes of *S. aureus* isolated from clinical samples at the referral hospital, this study also features some limitations;

1) The sample size of forty-three (43) was relatively small to generalize the prevalence of antimicrobial resistance and virulence genes of *S. aureus* at the referral hospital
2) Few number of antimicrobial and virulence genes used limited the study.

CHAPTER SIX

CONCLUSION AND RECOMENDATIONS

6.1 CONCLUSION

1) Of 43 isolates 5 (11.6 %) isolates harboured *mec*A gene (MRSA)
2) The presence of other antimicrobial resistance genes *erm*C, and tetracycline resistance genes (*tet*K, and *tet*L) indicated an important role these genes play in resistance to macrolides and tetracyclines respectively in *S. aureus* strains at the referral hospital
3) Notably one MRSA strain harboured both *erm*C and *tet*K genes
4) The detection of *sec* gene in *S. aureus* strains has given insight into the potential staphylococcal food poisoning threat at the University Teaching Hospitals

6.2 RECOMMENDATIONS

Based on the findings of this study it is recommended that;

1) Routine surveillance, prudent antibiotic use and optimised infection control practices are some of the strategies advocated for the containment of MRSA and MDR MRSA in healthcare system experiencing an ever-increasing incidence and prevalence of antimicrobial resistant and virulent strains of *S. aureus.*
2) More studies are needed for more detailed information about what drives antimicrobial resistance and virulence factors in *S. aureus* infection

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