DETECTION OF VIRULENCE GENES AND ANTIMICROBIAL RESISTANCE GENES IN *STAPHYLOCOCCI* ISOLATED AT MALAMULO HOSPITAL IN MALAWI

By

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A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science in One Health Laboratory Diagnostics Sciences

The University of Zambia

Declaration

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

Date……. Signature……...

Certificate of Approval

This dissertation of **Wilfred Taika** has been approved in partial fulfilment of the requirements for the degree of **Master of Science in One Health Laboratory Diagnostics Sciences** at University of Zambia.

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Abstract

Staphylococci associated morbidities and mortalities have been on the rise in Malawi due to paucity of molecular AMR surveillance data critical for improved patient care. This retrospective laboratory-based study was conducted at Malamulo Hospital, a sentinel AMR surveillance site in Malawi under Fleming fund. The study aimed at detecting selected virulence and AMR genes in 36 *Staphylococci* bacteria stocked between December 2021 and May 2022 for a molecular AMR surveillance. Sample source types included blood (16), urine (14), pus (three), skin wedge (one), pleural fluid (one), and ascitic fluid (one). Patients age categories recorded were 12 paediatrics, 19 young adults, two middle aged adults and three elderly adults**.** The *nuc*, *spa* and *coa* genes were used to detect and identify *S. aureus species*. Disc diffusion susceptibility test was used to determine antimicrobial susceptibility levels. PCR was performed to detect antimicrobial resistance genes (*mec*A**,** *erm*A, *erm*B, *erm*C**,** *tet*K, *tet*L, *tet*M and *tet*O) and virulence genes (PVL (*lukS-PV* and *lukF-PV*), *spl*A, *spl*B, *spl*C, *spl*D, *spl*E, and *spl*F) respectively. The *spa* gene was sequenced after which Spa typing was also done. Descriptive statistics were used for analysis. From the sample source types, the proportion of PCR confirmed *S. aureus* was 38.9% (14/36) and 61.1% (22/36) were other *Staphylococci*. Noteworthy disc diffusion antimicrobial resistance levels in the study were against trimethoprim/sulfathomexazole (50%) and MDR-MRSA (three *S. aureus* isolates). Antimicrobial resistance genes detected included *tet*K (57.1%), *mec*A (14.3%) and *tet*M (7.1%). The *spl*A, *spl*B, *spl*C, *spl*D, *spl*E and *spl*F genes were detected in 86% (12/14) of confirmed *S. aureus* isolates. PVL (*lukS-PV* and *lukF-PV*) gene was detected in four *S. aureus* isolates. Spa type t941 was common at 33.3% (3/9) and an unknown spa type was detected. These findings indicate probable life-threatening infections caused by virulent, multidrug and methicillin resistant *S. aureus* of several *spa* lineages among patients at Malamulo Hospital. Hence a call for molecular epidemiology and molecular characterization studies preferably using whole genome sequencing tools at Malamulo Hospital in Malawi for more insights into the *S. aureus* infections.

Dedication

I dedicate this dissertation to my late grandfather, Wilfred Taika the first.

Acknowledgements

I thank the Almighty God for the providence that has seen this work come to a successful completion. A project of this nature could not have been completed by one-person's effort. First, I acknowledge my supervisors, namely, Professor Bernard Mudenda Hang'ombe who has enriched the course of my career towards one health in laboratory diagnostics sciences; knowing him has been such a great honor. Many thanks to Professor Musso Munyeme and Ms. Rosie Panulo for their invaluable time. To Ms. Mulemba Tillika Samutela who helped me with stimulating suggestions, encouragement, proofreading and correcting my many mistakes throughout all the ups and downs of this research project both in the laboratory and during writing, thank you.

I would also like to acknowledge my colleagues at Malamulo Adventist Hospital in Malawi, Mr. Mphatso Mafunga, Mr. Odala Phiri, Mr. Lugard Lupapa, Mr. Zefaniah Katua, Mr. Jonathan Majamanda, Mr. Chimwemwe Phiri, Dr. Wilson Tomas, Ms. Lucy Mlendo and Dr. Moses Kasumbu Kagya for the invaluable support in the preliminary work of this research. My appreciation also extends to colleagues at The University of Zambia School of Veterinary Medicine research laboratories, Ms. Charlotte S. Mabhena, Ms. Lebang Matokwane Mr. Andrew Mukubesa, Mr. Joseph Ndebe, Mr. Ladslav Moonga, and Dr. Misheck Shawa for the much needed support during the molecular biology work of this project.

Last but not the least, I gratefully acknowledge the African Centre of Excellence for Infectious Diseases in Humans and Animals (ACEIDHA), The University of Zambia, School of Veterinary Medicine for the scholarship to study in this program including financial and technical support to conduct this research project.

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List of Abbreviations

AMR	Antimicrobial resistance
CA-MRSA	Community acquired methicillin resistant *Staphylococcus aureus*
CLSI	Clinical laboratory standards institute
DNA	Deoxyribonucleic acid
EUCAST	European committee on antimicrobial susceptibility testing
HA-MRSA	Hospital acquired methicillin resistant *Staphylococcus aureus*
LIS	Laboratory information system
MDR-MRSA	Multi-drug resistant, methicillin resistant *Staphylococcus aureus*
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MSSA	Methicillin susceptible *Staphylococcus aureus*
NHRSC	National Health research science committee
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PhLOPSA	Phenicols Lincosamides Oxazolidinones Pleuromutilins Streptogramin A resistant phenotype
UNZABREC	University of Zambia Biomedical research ethics committee
UV	Ultraviolet light
PVL	Panton valentine leukocidine
SNPs	Single nucleotide polymorphisms
WGS	Whole genome sequencing

CHAPTER ONE

INTRODUCTION

1.1 Background

Staphylococci species are a group of Gram-positive spherical bacteria that inhabit the environment, skin and nares of healthy people and animals as either normal flora, opportunistic pathogens and/or true pathogens depending on the virulence of the strain and host immunity (Bierowiec *et al*., 2019). Incidence of both hospital and community acquired *Staphylococcal* infections is linked with specific clonal types(Feng *et al.*, 2008). Increased mortality due to *S. aureus* infections has been prominent with COVID 19 pandemic (Vaillancourt and Jorth, 2020; Sharifipour *et al*., 2020). *Staphylococcus aureus* co-infection with the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is reported to increase disease fatality and mortality especially among patients with *staphylococcal* bacteremia and pneumonia (Adalbert *et al.*, 2021). Furthermore, among patients infected with the Human Immunodeficiency Virus-1 (HIV-1), there is increased development of antibiotic resistance, for instance, Methicillin Resistant *S. aureus* (MRSA) due to increased prophylactic use of antibiotics and consequently increased mortality to *Staphylococcus aureus* bacteremia, from deep soft-tissue infections, and pneumonia (Adesida *et al.*, 2017).

Staphylococcus aureus and other pathogenic *Staphylococci* species are the most isolated bacteria in patients with highest resistance to commonly used antibiotics in Malawi (Kumwenda *et al.*, 2021). Lack of capacity to detect virulence and antimicrobial resistance genes in antimicrobial resistance (AMR) surveillance laboratories in Malawi has led to paucity of data on virulence and AMR genes in *Staphylococcus* species affecting humans and animals (Gordon *et al.*, 2020).

The current global increase in prevalence of pathogenic *Staphylococcal* infections with significant regional strain variations, demands profiling of resistance and antimicrobial resistance genes of locally circulating *S. aureus* lineages (Seni *et al.*, 2013). These genes responsible for virulence and antimicrobial resistance can be potential candidates for targeted diagnosis and treatment methods to control infection burden in a particular locality (Haddad *et al.*, 2018).

1.2 Prevalence of *S. aureus* infections

It is possible that prevalence of human *S. aureus* infections in Africa is underestimated due to reasons that include neglected research in clinical *S. aureus* in many African countries leading to a scarcity of data (Egyir *et al.*, 2014).

Underutilization of health care facilities in rural populations compared to urban populations in African countries could also result in low numbers of hospital reported cases (Oladipo, 2014). Recent estimations have shown that prevalence of MRSA is increasing in African countries for example from 2002-2007 prevalence of MRSA in Tunisia increased from 16% to 41%; in Botswana MRSA prevalence fluctuated between 23-44% from 2000-2007; while Algeria and Egypt registered MRSA prevalence of 45% and 52% between 2003-2005, respectively (Falagas *et al.*, 2013). Increased isolation of PVL producing *S. aureus* and reports of fatal *S. aureus* pneumonia, skin and soft tissue infection in European travelers returning from Africa in two decades have led to considerations that Africa is a PVL-endemic region with high rates of PVL-positive MSSA isolates ranging from 17% to 74% (Schaumburg *et al.*, 2014).

1.3 Distribution of risk sources for *S. aureus* infections in hospitals and community

Pathogenic *Staphylococci* are ubiquitously distributed with preferred sites being skin and mucus membranes of humans and animals as well as the environment. Transmission cycles leading to human colonization in both hospital and community acquired infections are maintained by direct contact, inhalation of aerosols or dust, and fomites such as stethoscopes in the hospital (Russell *et al*., 2012).

Anterior nares of the nose are the most regular carriage site for *S. aureus* in human beings as compared to other body sites (Al-Humaidan *et al.*, 2015). Finger picking habits from source to the nose have been connected with nasal colonization with *S. aureus* strains in adults and considered the major factor (LaCour *et al.*, 2020).

Among hospital workers, nasal carriage of MRSA and MSSA rates are reported to be high in nurses, technicians, physicians and sanitary workers and very low among pharmacists, radiologists and administrative workers (Rongpharpi *et al*., 2013; Shibabaw *et al*., 2013). Nevertheless, in the

presence of robust infection prevention measures, it has been shown that nosocomial transmissions are kept at insignificant levels despite nasal carriage of *S. aureus* by hospital workers and/or patients (Price *et al.*, 2017). In the hospital environment, factors such as prolonged hospitalization, intensive care unit admission, antibiotic exposure, surgery, chronic wounds, hemodialysis, indwelling catheters and other invasive devices increases the risk of Hospital-Acquired MRSA (HA-MRSA) infections (Epstein *et al.*, 2016).

Among the risk factors considered to be catalysts of increased transmission and prevalence of CA-MRSA infections in Africa are HIV/AIDS, malnutrition, increased close contact with animals, crowded living conditions, high temperatures and humidity that increases *S. aureus* survival in the environment (Schaumburg *et al*., 2014).

Soft tissue community acquired methicillin resistant *S. aureus* (CA-MRSA) infections, in Human Immunodeficiency Virus (HIV) infected patients, are reported to be predominantly caused by PVL-positive *S. aureus* in Gabon, Tanzania and Mozambique (Ruffing *et al.*, 2017). Maternal carriage has been considered the main determinant for neonatal nasal carriage (Maayan-Metzger *et al.*, 2017). Community acquired MRSA (CA-MRSA) was first reported in 1960 and interaction of carrier household members with other households or at community sites such as schools is currently considered the basis for the ability of these strains to remain endemic in certain communities (Knox *et al.*, 2015).

There is evidence of interspecies transmission of human t148 spa-type MSSA in central Africa indicating the ability of *Staphylococcus aureus* to thrive, spread and cause infections in various animal and human hosts (Nagel *et al.*, 2013). Host switching result in host-specific mutations as the organism acclimatize to the new host population environment that lead to clonal expansion and development of antimicrobial resistance through gain or loss of mobile genetic elements (Haag *et al.*, 2019). Carriage rates of *S. aureus* among healthy human hosts from different geographical regions can be comparable (Ngoa *et al.*, 2012), but apomorphic genetic diversities of *S. aureus* are mostly region specific, for instance, hypervirulent community-acquired *Staphylococcus aureus* multilocus sequence type 8 (ST8) has at least 224 different clades globally disseminated across all continents that originally evolved from a European ancestor (Strauß *et al.*, 2017). Emergence of

novel spa types, such as the recent discovery of an unknown spa type in Zambia, implies that MRSA is developing more well adapted geographically unique clones in Africa (Samutela *et al.*, 2017).

Unlike CA-MRSA infections, HA-MRSA infections are the most common and exhibit high levels of antimicrobial resistance including MRSA especially among patients with other co-morbidities in the hospitals (Matta *et al.*, 2018). Inadequate previous use of antimicrobial therapy among patients is an important risk factor for multidrug resistance selection of *S. aureus* species during patient treatment for *S. aureus* clinical infections in hospitals (Cardoso *et al.*, 2012).MRSA is an important zoonotic public health threat because potent multiple drug resistance through clonal expansion result in infections that are difficult to treat (Algammal *et al.*, 2020).

1.4 Types of clinical specimen

Staphylococcus aureus can be isolated from a wide range of clinical specimens depending on the infected tissue, organ or system. These include blood (sepsis or bacteremia); bone tissue, sub periosteal fluid, muscle abscess, synovial tissue or fluid (musculoskeletal infections) (Miguel *et al.*, 2019).

Other clinical specimens include sputum, tracheal aspirate, paranasal sinuses, pleural fluid (respiratory infections), peritoneal fluid, urine, cerebrospinal fluid, skin and soft tissue, eye swabs, vaginal swabs, rectal swabs, skin tissue biopsies, ear drainage, lymph node tissue, esophageal brush specimens (Miguel *et al.*, 2019). Clinical microbiology laboratory manuals provide guidelines for standardized collection of clinical samples using a sterile technique and appropriate equipment (Miller *et al.*, 2018).

1.5 Diagnosis of *S. aureus* infections

Laboratory techniques used to diagnose clinical *S. aureus* infections can be grouped into phenotypic and molecular methods which are used for diagnosis according to preference (Song *et al*., 2013; Sabat *et al*., 2013). Phenotypic methods are used to affirm expressed traits such as resistance and/or virulence and gain wide routine usage in spite of accompanying disadvantages; poor reproducibility and prolonged turnaround time (Sanchini, 2022). Compared to phenotypic

methods molecular methods are superior in reproducibility but they vary in their discriminatory power. Moreover they do not detect actual expressed resistance and/or virulence therefore sometimes a resistance gene may be detected in a bacteria that is yet susceptible to the antibiotic (Williamson *et al*., 2015). This therefore warrants use of phenotypic and genotypic approaches as complementary methodologies for accurate and rapid identification of pathogens (Atshan and Shamsudin, 2011; Mistry *et al*., 2016).

1.6 Phenotypic tests for *Staphylococcus aureus*

These tests are the mainstream laboratory diagnosis methods in resource-limited settings (Kali *et al.*, 2014). Inorder to guarantee reliable results for the identification of *S. aureus* in resource limited settings, sequel testing of the isolates is proposed when these phenotypic tests are in use (Kateete *et al.*, 2010).

1.6.1 Gram's stain test

The Gram's stain is a technique based on the principle that bacteria whose cell wall has a thick layer of peptidoglycan and low lipid content, retain the primary stain when fixed by mordant (purple colored Crystal Violet-Iodine complex) after alcohol decolorization (Sizar and Unakal, 2022).Bacteria cell wall with thin layer of peptidoglycan and high lipid content gets its purple crystal Violet-Iodine complex washed off by alcohol decolorization eventually appearing red/or pink in color when stained by saffranin. The morphological appearance of *Staphylococcus aureus* is a gram-positive cocci in clusters when examined using light microscopy (Sizar and Unakal, 2022).

1.6.2 Biochemical identification of *S. aureus*

Involves phenotypic identification of *S. aureus* species on the basis of a variety of physiological or biochemical characteristics that are linked to its pathogenic capacity to produce exoproteins and toxins (Karmakar *et al.*, 2016).

1.6.2.1 Catalase test

This is a test that detects *Staphylococcus aureus* ability to produce the enzyme catalase which catalyses the release of O_2 by giving out bubbles when bacterial suspension in normal saline is mixed with two to three drops of 3% hydrogen peroxide (Duza, 2021).

1.6.2.2 Coagulase test

This test is used to detect the ability of *S. aureus* to produce the enzyme coagulase. The enzyme coagulase is capable of clotting the plasma (appearance of coagulation) after 24 hours incubation at 37°C (Marek *et al.*, 2021). A positive test shows that the bacterial strain present in the tube is a coagulase producer which is characteristic of *Staphylococcus aureus* strains (Rakotovao-Ravahatra *et al.*, 2019).

1.6.2.3 Deoxyribonuclease (DNase) test

This test is based on ability of *Staphylococcus aureus* to produce an enzyme DNase which hydrolyzes nucleic acid (media contains 0.2% deoxyribonucleic acid) in DNase agar. A positive reaction is seen as a colorless zone around the colonies after 18 hours incubation at 37°C (Pumipuntu *et al.*, 2017).

1.6.2.4 Mannitol fermentation

This test aims at demonstrating the ability of most strains of *S. aureus* to ferment mannitol sugar when inoculated on Mannitol Salt Agar producing an acid end product that results in phenol red indicator in the medium changing its color from red to yellow (Tigabu and Getaneh, 2021).

1.6.2.5 Gelatin hydrolysis

Gelatin hydrolysis test aims at testing the ability of bacteria to produce gelatinases which cause a liquefaction reaction that release carbon particles into the medium resulting in complete disintegration of charcoal disc in the medium after 24 hours of incubation at 37°C. This test is used to discriminate between gelatinase-positive pathogenic *Staphylococcus aureus* from nonpathogenic *Staphylococcus epidermidis* which are gelatinase negative (Chakraborty *et al.*, 2011).

1.6.2.6 Urea hydrolysis

This test is used to detect the ability of some *S. aureus* strains to produce urease which breaks down urea in the medium releasing ammonia which changes the pH of the medium to become more alkaline. A pH change is detected by the change of pH indicator (phenol red) color from yellow to red or pink after 24 hours incubation at 37°C (Ahmed, 2017).

1.6.2.7 Protease activity on milk agar medium

Test is used to detect *S. aureus* extracellular protease activity that is shown by clear zones surrounding supernatants (20 µL) of bacteria previously added through a hole in the skim milk agar plates and incubated at 37°C for 24 hours (Park *et al.*, 2012).

1.6.2.8 Hydrolysis of esculin

Test is based on the ability of some *S. aureus* strains to hydrolyze aesculin in the presence of bile results in a black precipitate formed from interaction of iron with 6, 7,dihydroxyxouramin in the medium (Habib *et al*., 2015).

1.6.2.9 Hemolytic activity on 5% sheep blood agar

Staphylococcus aureus produce cleared halo of hemolysis when plated on 5% sheep blood agar plates that can be categorized as strong (complete cleared zone) or weak due to β-hemolysin (Hlb) expression (Stulik *et al.*, 2014).

1.6.2.10 Antimicrobial Susceptibility Testing

Classical culture dependent phenotypic antimicrobial susceptibility testing (AST) examines the bacterial response to the presence of an antimicrobial agent as a disc in an agar plate or as diluted in broth (Gajic *et al.*, 2022). Information on local patterns of bacteria response to antimicrobial agents can be collected using AST, so that policies guiding the empiric choice of therapy can be based on current data on local resistance trends (van Belkum *et al.*, 2019).

1.6.2.10.1 Dilution method

Dilution was one of the earliest tools in microbiological practice, starting in the early 1870s, and it allows the growth and identification of bacterial populations in suspension. The two basic types

of dilution are micro dilution and macro dilution, wherein broth and agar are the most commonly used mediums (Khan *et al*., 2019).

1.6.2.10.1.1 Macro dilution technique

In standardized broth dilution method, consecutive two-fold dilutions of antimicrobial agents are made and dispensed into a series of equal volume micro-centrifuge tubes containing bacterial growth medium to make final volumes that are incubated overnight at 35°C. Clinical Laboratory Standards Institute (CLSI) guidelines which are standard laboratory protocols for best laboratory practice are followed (Khan *et al.,* 2019). Finally, the bacterial growth is examined based on turbidity of culture media to confirm minimum inhibitory concentration (MIC) and antimicrobial susceptibility tubes in which CLSI recommended guidelines are also used to set the breakpoints for interpretation (Khan *et al.*, 2019).

1.6.2.10.1.2 Micro dilution technique

This is a miniaturized prototype of the macro dilution method in which susceptibility testing is performed using 96-well micro titer plates wells of capacity ~0.1 mL. Mechanical dispensers may be used to minimize handling errors and after overnight incubation at 35°C, bacterial growth turbidity and MIC are determined by using optical instruments for instance specialized spectrophotometer (Khan *et al.*, 2019).

1.6.2.10.2 Disk diffusion method

Disk diffusion antimicrobial susceptibility tests are usually performed on Mueller Hinton (MH) agar plated with a standardized bacterial inoculum and standard antimicrobial imbedded paper disks placed in the medium according to susceptibility testing standards. After 16 to 20 hours' incubation at 37°C (with 5% CO_2 for *S. aureus*), zone diameters are measured, interpreted and considered for resistant, intermediate or susceptible strains. Internationally approved standards for instance the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and/or CLSI protocols for best laboratory practice are used for interpretation of antimicrobial susceptibility results (Hagstrand Aldman *et al.*, 2017).

1.6.2.10.3 Epsilometer testing (E – test)

This is a simple antimicrobial susceptibility testing technique in which plastic strips are coated with defined antibiotic concentrations, and the corresponding interpretive MIC ranges are marked on the surface and back of the strip, respectively. The procedure involves placing multiple strips on the surface of bacteria streaked agar plate (Khan *et al.*, 2019). After an overnight incubation at 35°C, elliptical inhibition zones appear around the strips, indicating the MIC at the intersection point between the inhibition zone and the strip edge that can be directly used for test interpretation (Khan *et al.*, 2019).

1.6.2.11 Matrix-assisted laser desorption ionization time of flight (MALD – TOF) mass spectrometry

This is a method that has been applied in the detection of *S. aureus* protein virulence factors for example phenol soluble modulin molecule (PSM) along with protein resistance markers such as PBP2a in MRSA encoded by *mecA* gene and therefore also has ability to discriminate between MSSA, MRSA, VRSA based on fragmented protein mass spectrum patterns in Daltons (Bryson *et al.*, 2019).

1.6.2.12 Latex agglutination tests

These are manufacturer protocol tests that are available for rapid detection of coagulase production, catalase production and also identifying MRSA by detecting penicillin binding protein 2a (PBP2a) based on an immuno-agglutination reaction on a card (Pumipuntu *et al.*, 2017).

1.7 Molecular diagnostic and typing methods for *Staphylococcus aureus*

Molecular testing for *S. aureus* can greatly reduce laboratory turnaround time, and in some circumstances, may lead to improved clinical outcomes. In addition, advances in DNA sequencing technology and bioinformatics analysis have shed new lights on the molecular epidemiology and transmission dynamics of *S. aureus* (Williamson *et al*., 2015).

1.7.1 Xpert MRSA/SA blood culture assay

This is an automated DNA extraction and real-time PCR processing assay with a turnaround time of one hour that detects *Staphylococcal* protein A (*spa*) gene, *nuc* gene, *mecA* responsible for

methicillin resistance and the *Staphylococcal* cassette chromosome mec, SCCmec for rapid identification of methicillin resistant *S. aureus* (McHugh *et al*., 2020). The coagulase (*coa*) gene has also been used in multiplex with *spa* gene for accurate PCR identification of *S. aureus* with 100% specificity (Manukumar and Umesha, 2017).

1.7.2 Pulsed field gel electrophoresis (PFGE)

This is a standardized two days' molecular typing protocol that begins with culture and ends with a gel image that is used to subtype MRSA isolates in server-based systems for DNA fingerprint data collection so called BioNumerics. The protocol involves growth and lysis of bacteria, preparation of agarose gel, washing of sample, restriction enzyme digestion and electrophoresis of DNA fragments in a gel matrix electric field that periodically changes direction. Pulse field gel electrophoresis (PFGE) has a high discriminatory ability and is the documented gold standard for DNA fingerprinting among the various DNA sequence-based methods (Gökmen *et al.*, 2018).

1.7.3 Multilocus sequence typing (MLST)

This is a sequence-based typing method that relies on analysis of relatively conserved *Staphylococcus aureus* genes that encode essential proteins in order to provide a relatively detailed picture of the global dissemination of the pathogen. This is accomplished by using web-based bioinformatics tools that are maintained as a coherent global asset and assist users in the analysis of their data (Saunders and Holmes, 2014). In MLST, fragments of seven *Staphylococcus aureus* housekeeping genes namely; *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL* are amplified by PCR, sequenced, compared to known alleles at each locus and submitted to a central database which generates an allelic profile that is used to generate the *Staphylococcus aureus* sequence type, (ST) (Williamson *et al*., 2015).

1.7.4 *spa* typing

The *spa* gene of MRSA encodes protein A and consists of three distinct regions: Fc, X, and C (Goudarzi *et al.*, 2016). This molecular typing method relies on the number of tandem repeats in region X that are assigned an alpha-numerical code in order to deduce the *spa* type based on the order of specific repeats and the sequence variation in region X of the gene. The *spa* type distribution of clinically isolated MRSA strains are unique to different geographic locations so that

worldwide they exhibits a different pattern because the repeat region of MRSA *spa* gene is subject to spontaneous mutations that lead to loss or gain of repeats caused by different selection pressures (Goudarzi *et al.*, 2016).

1.7.5 Microarray-based typing

This is also another method used to type *S. aureus* that provides strain type as well as determine presence or absence of antimicrobial resistance and/or virulence genes. This method utilizes genomic *S. aureus* DNA in a multiplex linear PCR assay, in which the resulting biotin-labelled amplicons are hybridized to target sequences in the *S. aureus* genome, corresponding to known genes and their allelic variants. This method therefore allows for broader classification of *S. aureus* into clonal complexes (CCs), SCC*mec* complex, distinct allotypes and detection of genes associated with antimicrobial resistance and/or virulence (Williamson *et al*., 2015).

1.7.6 Whole genome sequencing (WGS)

This is also a molecular typing method that involves collation and comparison of single nucleotide polymorphisms (SNPs) in the *Staphylococcus aureus* core genome and is the most discriminatory method for typing and phylogenetic analysis of *S. aureus* strains. WGS has applications in the prevention, diagnosis and surveillance of *S. aureus* infections in terms of identification of antimicrobial resistance genes in the context of an outbreak (Williamson *et al*., 2015).

1.8 Treatment of *S. aureus* infections

The recommended oral agents for therapy of *S. aureus* systemic and non-systemic infections that are prescribed according to approved guidelines are; Oral antistaphylococcal β-lactams, clindamycin, trimethoprim (TMP)- sulfamethoxazole (SMX), doxycycline, minocycline and rifampin (David and Daum, 2017).

On the other hand, recommended intravascular antimicrobial agents are vancomycin, daptomycin, tigecycline, ceftaroline, dalbavancin, oritavancin, telavancin, and quinupristin/dalfopristin are (David and Daum, 2017). Antimicrobials linezolid and Tedizolid are available in both oral and intravascular formulations for treatment of *S. aureus* infections (Jame *et al.*, 2021). Ampicillin/penicillin (50,000 IU/kg intramuscular stat dose for children < 15 years and 2.4 mega

units' intramuscular stat dose for adults) with gentamicin (7.5mg/kg intramuscular stat dose for children < 15 years and 240mg stat dose for adults), or ceftriaxone (100mg/kg intravascular once daily for 7 days) is the standard antimicrobial treatment for *S. aureus* infections in Malawi (Tam *et al*., 2019). Following World Health organization guidelines, cotrimoxazole is prescribed 400mg twice daily in adults and cotrimoxazole 8mg/kg once daily in children as part of antiretroviral therapy in Malawi (Dixon *et al.*, 2021).

1.9 *Staphylococcus aureus* impact on health and economy

Antimicrobial resistance including methicillin resistance in *S. aureus* is a global public health problem responsible for approximately 111,000 deaths each year in the European Union and more than 23,000 annual deaths in the United States of America with approximately 2 million people affected. Especially CA-MRSA confers a greater economic burden than many other acute infectious diseases in the USA for example, the hospitalization cost per CA-MRSA infection ($7070–$20 489 per case) is two to five times that of an influenza case ($3000–$4000 per case), and over 17 times that of Lyme disease ($397–$923 per case) and CA-MRSA infections occur more frequently than many other infectious diseases (Antonanzas *et al.*, 2015). The impact of MRSA on health and economy is not well characterized in Africa (Wangai *et al.*, 2019). This therefore warrants more studies on MRSA in the African context.

Therefore, this study aimed at detecting the virulence and antimicrobial resistance genes in *Staphylococci* affecting patients at Malamulo hospital in Malawi in order to improve patient care.

1.10 Statement of the problem

The prevalence trends of MRSA infections in Malawi are reportedly rising as evidenced by an initial prevalence of 7.7% in 1998 that increased to 18.4% in 2016 (Musicha *et al.* 2017). *Staphylococcal* sepsis is among the top five causes of paediatric deaths in Malawian tertiary hospitals (Harris *et al*. 2019). Moreover, lack of regular AMR surveillance on *S. aureus* AMR, molecular types and virulence is negatively affecting control and treatment efforts in Malawi (Makoka *et al.* 2012). Since inception of Fleming's AMR surveillance programme in December 2021, there are 14 sentinel laboratory sites in Malawi that report quarterly disc diffusion and MIC antimicrobial susceptibility patterns of priority bacterial pathogens including *S. aureus* and

antibiotics use (Gordon *et al.*, 2020). Monthly monitoring and reporting of antimicrobial susceptibility levels, antibiotics usage, strain types including virulence and AMR genes could ensure timely availability of data throughout the year for more robust AMR surveillance (Acharya *et al.*, 2021).

1.11 Justification of the study

Molecular characterization of *Staphylococcus aureus* is important for surveillance, treatment and control of public health important strains responsible for infections in a particular locality (Zarazaga *et al*., 2018). Current knowledge of genetic drivers of virulence and resistance among locally circulating clinical *Staphylococcus* strains is a requirement for robust antimicrobial stewardship and surveillance.

Public health policies and guidelines used for surveillance, treatment, diagnosis, prevention and control of MRSA and (antimicrobial resistance) AMR can be realigned to meet the current resistance and virulence challenge. Insights into the lineages of *S. aureus* and their virulence including AMR traits gained from the current study will help influence the guidelines for diagnosis, treatment and control of *Staphylococcal* infections.

1.12 Research questions

1. What are the antimicrobial susceptibility levels of the *Staphylococci* isolates?
2. What are the antimicrobial resistance genes embodied in the *Staphylococci* isolates?
3. What are the virulence genes responsible for pathogenicity among the *Staphylococci* isolates?
4. What *spa* types of *S. aureus* are isolated at Malamulo Hospital laboratory?

1.13 Objectives

1.13.1 General objective

To determine antimicrobial susceptibility levels, antimicrobial resistance genes, virulence genes and spa types of *Staphylococci* isolated from patients at Malamulo Hospital laboratory in Malawi.

1.13.2 Specific objectives

1. To determine antimicrobial susceptibility levels among the *Staphylococci* isolates.
2. To detect selected antimicrobial resistance genes (*mec*A, *mec*C, *erm*A, *erm*B, *erm*C, *tet*L, *tet*O, *tet*M, *tet*K) in the *Staphylococci* isolates.
3. To detect selected virulence genes responsible for pathogenicity (*spl*A, *spl*B, *spl*C, *spl*D, *spl*E, *spl*F, and Panton Valentine Leukocidin (*lukS-PV* and *lukF-PV*) gene.
4. To determine the *Staphylococcal* Protein A (*spa*) types of *S. aureus* isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of *Staphylococcus aureus*

Staphylococcus aureus was first described by Sir Alexander Ogston in 1880 (Missiakas and Schneewind, 2013). *Staphylococcus aureus* belong to *Staphylococceae*, a large family that comprises 98 species grouped within nine genera. The genus *Staphylococcus* which is the most populated in the *Staphylococceae* family contains 55 species and 23 subspecies. *Staphylococcus aureus* has two subspecies which are *Staphylococcus aureus* subspecies *aureus* and its heterotypic synonym *Staphylococcus aureus anaerobius* (Schaumburg *et al.*, 2014)

2.2 Organization of *Staphylococcus aureus* genome

Staphylococcus aureus has an average genome size of ~2.8 Mbp comprising of a single circular chromosome and extrachromosomal genetic elements such as plasmids, prophages, genomic islands, pathogenicity islands, and the *Staphylococcal* chromosomal cassette *mec* (SCC*mec*) (Shukla *et al.*, 2012). The extrachromosomal elements (also called mobile genetic elements) represent 15 – 25% of *S. aureus* genome and are acquired by horizontal gene transfer (Alibayov *et al.*, 2014). The mobile genetic elements are key to the dissemination of antimicrobial resistance and virulence genes especially among *S. aureus* lineages through horizontal gene transfer (Lindsay, 2014). Recently allotypes of arginine catabolic mobile element (ACME) from coagulase negative *Staphylococcus epidermidis* have been detected in methicillin resistant *Staphylococcus aureus* (MRSA) and may provide further evolutionary advantage to an already successful pathogen (Shokrollahi *et al.*, 2022).

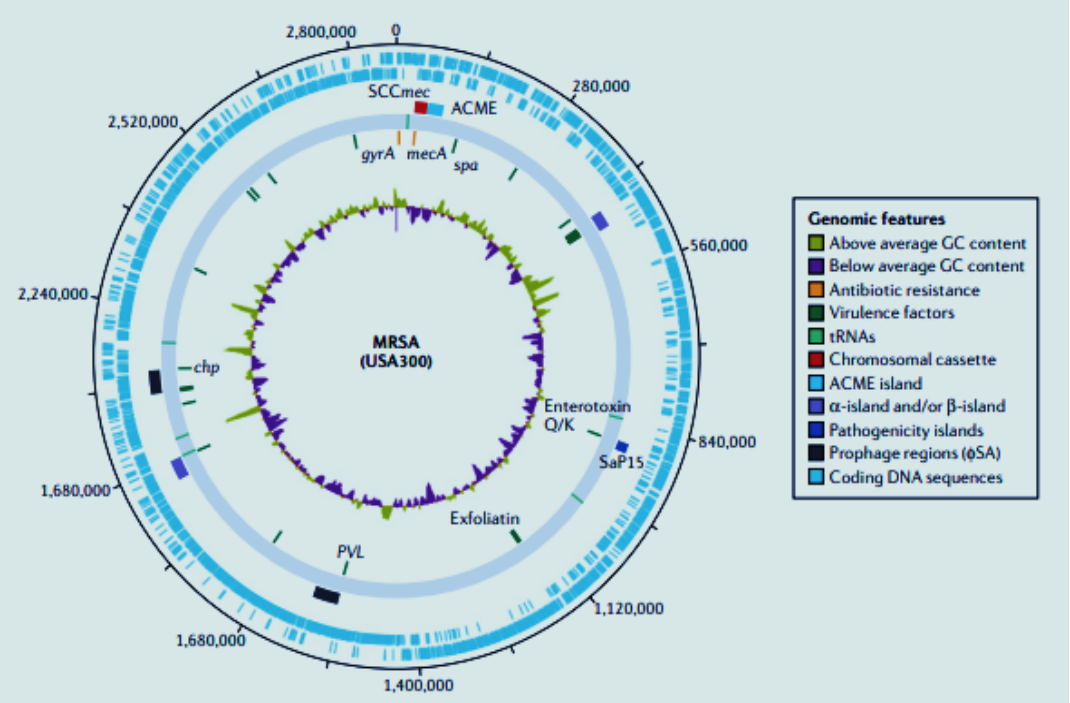


Figure 2.1: Organization of major genomic elements in methicillin resistant *S. aureus* (Turner *et al.*, 2019).

2.3 Pathogenesis of *S. aureus* in human clinical infections

In order to successfully cause infections *S. aureus* must overcome hosts' protective barriers such as the skin and as they find their way into the blood stream and other organs they must resist immune cells including antimicrobial agents to achieve systemic distribution (Cheung *et al.*, 2021). Therefore, during multiplication *S. aureus* produce damaging enzymes and toxins that facilitate *S. aureus* invasion into a human host and survival (Otto, 2014).

2.3.1 Adhesion and invasion of host cells

During infection *S. aureus* interact with the host initially by adhering to eukaryotic cells, extracellular matrix (ECM) components, and serum proteins by means of adhesins (Hammerschmidt *et al.*, 2019). *Staphylococcus aureus* adhesins are grouped into proteins that are cell wall peptidoglycans called microbial surface components recognizing adhesive matrix molecules, MSCRAMMs (Ghasemian *et al.*, 2015).

The second group consists of secreted proteins that rebind to the bacterial cell surface called secretable expanded repertoire adhesive molecules, SERAMs (Bur *et al.*, 2013). Exposure of *S. aureus* to mechanical stresses, for example hydrodynamic flow of urine and cell-surface contacts activate the adhesive function of *Staphylococcus aureus* MSCRAMMs so that the pathogen withstands high shear stress by expressing *S. aureus* adhesins clumping factor A (ClfA) and clumping factor B (ClfB) strong binding conformations (Geoghegan and Dufrêne, 2018). Another group of MSCRAMMs called fibronectin binding proteins (FnBPs), highly expressed by infecting strains of clinical *S. aureus*, mediate the process of host cell invasion by a binding process that signals the host cells to take up the bacteria enabling the pathogen to become intracellular hence evading the immune system (Bur *et al*., 2013).

2.3.2 Genetic basis for virulence

S. aureus strains responsible for most clinical infections have a tendency to cause infections in immunocompromised individuals whereas some strains show enhanced virulence and are capable of causing severe infections even in previously healthy people with no predisposing risk factors (Sahukhal and Elasri, 2014). This is so because of coordinated genetic regulation of more than 40 protein virulence factors used to establish and maintain infections that are produced by a vast majority of *S. aureus* strains that have enormous genomic plasticity (Costa *et al.*, 2013).

The most studied and characterized virulence factors include aureolysin (*aur*), the clumping factors A and B (*clfA, clfB*), the coagulase (*coa*), the extracellular adherence protein (*eap*), the extracellular matrix protein–binding protein (*emp*), the fibronectin-binding protein A (*fnbPA*), α-hemolysin (*hla*), lipase (*lip*), phenol-soluble modulins (*psms* genes, *hld*), protein A (*spa*), and von Willebrand factor–binding protein, *vWbp* (Bonar *et al.*, 2015). Amongst the most virulent strains, Panton-Valentin Leukocidin (PVL) is expressed in about 25% of *S. aureus* strains, and causes the destruction of leukocytes and also tissue necrosis (Raineri *et al.*, 2020).

In toxic shock syndrome; PVL exfoliative toxins A and B (ETA and ETB) function as “molecular scissors” and facilitate the invasion of the skin leading to atopic dermatitis and other dermal infections (Dunyach-Remy *et al.*, 2016). Furthermore, alpha, beta and delta hemolysins are cytotoxins that affect a wide range of human cells types including epithelial cells, endothelial cells,

T cells, monocytes and macrophages, especially *hla* (alpha hemolysins), is one of the main factors of pathogenicity of *S. aureus* (Silva *et al*., 2020).

Another group of virulence factors unique to *S. aureus* strains responsible for human clinical infections that are known to increase the lethality of *S. aureus* pneumonia but their specific mode of pathogenesis is unknown are six serine proteases (Spl proteases) that are encoded on the νSaβ pathogenicity island and expressed through lac operon regulation of six genes namely *spl* A, *spl* B, *spl* C, *spl* D, *spl* E, and *spl* F (Paharik *et al.*, 2016).

2.3.3 Basis of AMR in *Staphylococcus aureus*

Ability of to survive after in vivo and/or in vitro exposure to one or more antimicrobial drugs can arise when *S. aureus* acquire resistance genes encoded by mobile genetic elements through horizontal gene transfer or though mutations in chromosomal genes (Foster, 2017).

2.3.3.1 Drug resistance

Mechanisms by which *S. aureus* express drug resistance include; limiting uptake of the drug, modifying target of the drug, enzymatic inactivation of the drug, and active efflux pumping out of the drug (Yılmaz and Aslantaş, 2017). Conjugative transfer of mobile genetic element-encoded drug resistance genes and bacteriophage transduction are the main routes by which *S. aureus* gain novel drug resistance genes by horizontal gene transfer (Haaber *et al.*, 2017).

Horizontally transferred mobile genetic element-encoded drug resistance genes are integrated into the staphylococcal chromosome by site specific recombinase genes and this results in acquisition of drug resistance when *S. aureus* begins to synthesize novel proteins that enables the *S. aureus* to resist lethal effects of the drugs *(*Liu *et al*., 2016).

Multiplex PCR assays are currently useful molecular tools for rapid detection of AMR genes in *S. aureus* using a panel of primers targeting several resistance genes (Anjum *et al.*, 2017). Some of the common *Staphylococcus aureus* resistance genes and associated antimicrobial drugs classes and mechanisms of resistance are summarized in Table 2.1.

Table 2.1: *S. aureus* AMR genes, associated antimicrobial agent classes, and mechanism of resistance (Reygaert, 2013)

AMR Gene	**Antimicrobial class: Antimicrobial agents**	**Mechanism of resistance**
mecA, mecC *blasZ*	β-lactams: Penicillins, Cephalosporins, Monobactams,carbapenems	Altered drug target, Penicillin binding protein 2 a (PBP2a) Hydrolysis of β-lactam antibiotics by β-lactamase
tetK *tetM*	Tetracyclines: Tetracycline Minocycline Tigecycline	Active efflux pumping Ribosomal protection by competitive binding
Cat	Chloramphenicol	Inactivation by acetylation of the drug
ermA *ermB* *ermC*	Macrolides and Lincosamides: Erythromycin Clindamycin	Methylation of ribosome to decrease binding
rrn, cfr	Oxazolidinones: Linezolid	Mutation of ribosome Methylation of ribosome
ermA, ermB, ermC	Streptogramins: Quinupristin/Dalfopristin	Methylation of ribosome
gyrA *grlA* *norA*	Fluoroquinolones: Ciprofloxacin Norfloxacin Levofloxacin Gatifloxacin Moxifloxacin	Gyrase modified target Topoisomerase IV modified target Active efflux
dhfr/ dhps	Metabolic Pathway Inhibitors: Trimethoprim/Sulfamethoxazole	Target enzyme modification

2.3.3.2 Types of drug resistance

Staphylococcus aureus is among World Health Organization's list of priority pathogens due to emergence of multidrug resistant *S. aureus* phenotypes (Gatadi *et al.*, 2019). Multidrug resistance occurs when *S. aureus* acquires resistance to more than three classes of antimicrobial agents (Ge *et al*., 2017). The types of *S. aureus* drug resistances associated with multidrug resistant phenotypes that are well characterized are multidrug resistant methicillin resistant *S. aureus* (MDR - MRSA),and multidrug resistant vancomycin resistant *S. aureus* (MDR - VRSA) (Hiramatsu *et al.*, 2014).

Methicillin resistant *S. aureus* (MRSA) phenotype results from uptake of *mec* gene (*mec*A and/or *mec*C) by horizontal gene transfer of a mobile genetic element Staphylococcal cassette chromosome *mec*(SCC*mec*) (Lee *et al.*, 2018). Insertion of the 20 - 65 kb SCC*mec* element into the core genome enables *S. aureus* to express the *mec* gene (*mec*A and/or *mec*C) that encodes penicillin-binding protein 2a (PBP2a) (Turner *et al.*, 2019). PBP2a is a transpeptidase with a reduced affinity for β-lactam drugs that inhibit cell wall synthesis by transpeptidase-inhibition (Fergestad *et al.*, 2020). Consequently, MRSA strains continue cell wall synthesis and multiplication in the presence of nearly all β-lactam drugs (Fisher and Mobashery, 2021). Eleven SCC*mec* subtypes (Types I up to XI) of MRSA have been described (Shore and Coleman, 2013).

Vancomycin resistant *S. aureus* (VRSA) strains result from an independent conjugation transfer of the *van* gene cluster from a donor (mostly vancomycin resistant *Enterococcus*) to a *S. aureus* recipient (Cong *et al.*, 2020). Minimum inhibitory concentration (MIC) method is the preferred technique for determining *S. aureus* susceptibility to vancomycin (Wu *et al.*, 2021). Variants of vancomycin resistant *S. aureus* phenotypes include heterogeneous vancomycin-intermediate *S. aureus* (hVISA) as defined by an MIC within the range ≤2 μg/ml and vancomycin-intermediate *S. aureus* with an MIC range ≥4 μg/ml (Zehra *et al.*, 2023).

2.3.3.3 Causes of drug resistance

widespread use of antibiotics is considered the main cause of emergence of drug-resistant bacterial strains and antibiotic resistance gene (Tang *et al.*, 2019).The earliest lineage of MRSA was

identified in 1960s in England and Denmark, the time when methicillin was introduced into clinical practice, but phylogenetic studies have traced the period of acquisition to be the mid-1940s, a period when β-lactamase-mediated penicillin resistance was becoming widespread due to usage of penicillin for the treatment of staphylococcal infections (Harkins *et al.*, 2017). Use of antibiotics may not be the only driving factor of antibiotic resistance in clinical settings as ineffective use of biocidal chemical disinfectants in a recent report has also been linked with proliferation of AMR *S. aureus* strains (Meade *et al.*, 2021). Long term infections leading to AMR selection when there is repeated antibiotic treatment, or when pathogen adapts to new host immune system, or through horizontal resistant gene transfer in the environment or during co-infection with other bacterial species has also been reported (Haim *et al.*, 2021).

2.4 *Staphylococcus aureus* molecular lineages

Based on molecular typing techniques such as multilocus sequence typing (MLST), distinct set of lineages, called clonal complexes of *S. aureus* are highly implicated in HA-MRSA infections especially the following; CC5, CC8, CC22, CC30, and CC45 (Abdulgader *et al.*, 2015). Molecular lineages CC1, CC8, CC30, and CC80, are geographically distinct lineages that are predominantly associated with community acquired MRSA infections (Di Gregorio *et al.*, 2021).

Clonal complexes CC8 and CC30 are pandemic lineages that are known to cause infections both in the hospital and community settings (Abdulgader *et al.*, 2015). In Africa six main CCs of MRSA identified are CC5, CC22, CC30, CC45, CC80, CC88, other lineages CC398, CC9, CC97, CC130, CC1, which have been reported in human, wild animals and livestock interface (Zarazaga *et al*., 2018).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

This was a laboratory based retrospective study of *S. aureus* isolates previously isolated at Malamulo Adventist Hospital laboratory in Malawi during the period December 2021 to May 2022.

3.2 Study Site This study was conducted at Malamulo Hospital, a 275-bed capacity rural referral hospital located in Thyolo District, Southern Region of Malawi. Malamulo Hospital has a referral bacteriology laboratory serving a catchment area of about 500, 000 people from Thyolo, Mulanje, Chikwawa and Blantyre Districts through16 mobile clinics and three health centers. Malamulo Hospital bacteriology laboratory was earmarked as a sentinel AMR surveillance site under United Kingdom (U.K) Fleming Fund and started its AMR surveillance project in December, 2021. Molecular work for this study was conducted at University of Zambia School of Veterinary Medicine, an African Centre of Excellence for Diseases of Humans and Animals.

3.3 Sampling Frame

The study involved a convenient selection of glycerol stocked samples. These were isolates preliminarily identified as *S. aureus* by routine phenotyping methods such as gram stain, catalase and slide coagulase tests between December 2021 to May 2022 at Malamulo Hospital laboratory in Malawi.

3.4 Sample Size

An initial expected sample size of 231 was estimated using the sample size calculation formula (Sharma *et al.*, 2019) in ausvet epitools (epitools.ausvet.com.au):

$$n = \frac{Z^2 \times Pq}{d^2}$$

Where: n = was the expected sample size

Z= was 1.96 using 95% Confidence level

P = was 18.4% prevalence of *S. aureus* (Musicha *et al.*, 2017)

q = was 1 –P or 100 – P for decimal or percentage is respectively

d = 5% margin of error

3.5 Resuscitation of frozen bacteria isolatesA total of 36 glycerol stocked isolates of *S. aureus* previously frozen at − 20°C were resuscitated by scraping some of the frozen bacteria on the surface of an opened tube using a sterile loop and directly streaking on nutrient agar plate. The plate was incubated for 24 hours at 37°C and observed for single colonies of *Staphylococcus aureus* that were either golden yellow or creamy greyish in color (Naorem *et al*., 2020)**.**

3.6 Antimicrobial susceptibility testing of the isolates Phenotypic expression of antimicrobial resistance to selected antimicrobial agents by the *S. aureus* isolates was determined using the Kirby-Bauer Disc Diffusion Susceptibility Test as previously described (Chakolwa *et al*., 2019). All 36 *S. aureus* isolates were subjected to BD BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs (Becton Dickinson. New Jersey, United States. Company) of Erythromycin E-15 µg, Penicillin P-10 µg, Ciprofloxacin CIP-5 µg, Sulfamethoxazole/Trimethoprim SXT-23.75/1.25 µg, Gentamicin GM-10 µg, Cefoxitin FOX-30 µg and MASTDISCS® Tetracycline TET 30µg (Mast Group Ltd. Liverpool, UK). Several isolated colonies of test organism grown on nutrient agar (Mast Group Ltd. Liverpool, UK) at 37°C for 24 hours were suspended in 2mL of 0.85% saline to make a heterogeneous mixture. Optical density (OD) of each tube was measured and paired with an estimated bacterial cell density McFarland 0.5 = 1.5×10^8 colony forming units (CFU)/mL using a DEN-1 McFarland Densitometer (Biosan SIA. Riga, Latvia).

A sterile cotton swab (HiMedia Laboratories Private Ltd. Mumbai, India) dipped in the *S. aureus* suspension, was used to swab over the entire surface of dry BD DIFCO™ Mueller Hinton Agar plate (Becton Dickinson. New Jersey, United States. Company) with depth standardized at 4mm. Sterile forceps were used to place BD BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs (Becton Dickinson. New Jersey, United States. Company) of Erythromycin E-15 µg, Penicillin P-10 µg, Ciprofloxacin CIP-5 µg, Sulfamethoxazole/Trimethoprim SXT-23.75/1.25 µg, Gentamicin GM-10 µg, Cefoxitin FOX-30 µg and MASTDISCS® Tetracycline TET 30µg (Mast Group Ltd. Liverpool, UK) on the streaked plates of Mueller Hinton agar. The plates were incubated at 35°C for 24 hours after which zones of inhibition were measured using a ruler in millimeters by placing "0" in the center of antibiotic disc and the reading taken at the edge of inhibition zone was multiplied by two in order to get the diameter length. The European Committee

on Antimicrobial Susceptibility Testing (EUCAST) clinical laboratory zone diameter breakpoints for *Staphylococcus aureus* (Appendix A) were used to define the measured zones of inhibition as susceptible, intermediate, or resistant. Multidrug resistance was considered wherever an isolate was resistant to at least 3 classes of antimicrobial agents (Ge *et al*., 2017). Phenotypic identification of MRSA was based on expressed cefoxitin (30µg) resistance and Methicillin susceptible ATCC 29213 *Staphylococcus aureus* as control organism (Soni *et al.*, 2015).

In resource limited settings where molecular detection of methicillin resistant *S. aureus* (MRSA) is not possible, use of cefoxitin disc diffusion method is recommended (Fri *et al.*, 2018). This is so because studies have shown that with reference to *mec*A gene PCR for MRSA detection, cefoxitin disk diffusion test produces more comparable results than oxacillin disc diffusion method (Umar *et al.*, 2023).

3.7 DNA extraction and purification

Boiling method was used to extract DNA from the *S. aureus* isolates as described by Dashti *et al*. (2009). Several bacteria colonies grown overnight on mannitol salt agar were suspended in 100µL of sterile nuclease free water in an Eppendorf tube using a sterile wire loop under sterile conditions. The mixture was homogenized at 1000 *xg* for 10 seconds on a vortex mixer (Vortex-Genie 2, Fisher Scientific Ltd. Leicestershire, UK) and afterwards heated at 95°C for 10 minutes in a water bath (Thermo Fisher Scientific Inc. Massachusetts, USA). After heating the Eppendorf tube was placed on ice for 2 minutes and centrifuged at 3000 *xg* for 3 minutes at 4°C using a high speed refrigerated micro centrifuge (Tomy MX-207, Tomy Digital Biology Co. Ltd. Tokyo, Japan). Supernatant containing the DNA was aliquoted under sterile conditions on ice by pipetting 50µL of supernatant into a sterile Eppendorf tube. NanoDrop-1000 spectrophotometer, (Thermo Fisher Scientific Inc. Massachusetts, USA) was used to assess DNA purity whereby absorbance 260/280 -ratio of DNA concentration of at least 1.8ng/µL was accepted as pure DNA. The pure DNA samples were stored at − 80°C pending use in all PCR reactions in this study.

3.8 Molecular confirmation of *Staphylococcus aureus* species

Conventional PCR was used to confirm *S. aureus* species among all 36 *Staphylococci* isolates in this study using previously described protocols and gene specific primers of three *S. aureus* specific genes namely *nuc*, *spa* and *coa* in Table 3.1.

Table 3.1: Primers for *S. aureus* identification

Gene	**Forward (F1) and reverse (R2) primer sequences**	**Product size (Base pairs)**	**Citation**
nuc	F1: 5' - GCG ATT GAT GGT GAT ACG GTT - 3'and R2: 5' - AGC CAA GCC TTG ACG AAC TAA AGC - 3'	279bp	Zhang *et al*.,2004
coa	F1: 5' - GTA GAT TGG GCA ATT ACA TTT TGG AGG - 3'and R2: 5' - CGC ATC AGC TTT GTT ATC CCA TGT A - 3'	117bp	Ibrahim *et al.*2019
spa	F1: 5' - AGACGATCCTTCGGTGAGC - 3'and R2: 5' - GCTTTTGCAATGTCATTTACTG - 3'	250 – 500bp	Ghaznavi-Rad and Ekrami, 2018

3.8.1 Molecular confirmation of *S. aureus* using spa gene

A conventional PCR assay was performed to amplify and detect *spa* gene using a primer pair in Table 3.1 as described by Ghaznavi-Rad and Ekrami, (2018). A final volume of 25µL PCR master mix was used so as to secure enough *spa* gene amplicons to be used for subsequent spa gene big dye terminator sequencing. PCR master mix contained 12.5µL OneTaq®Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs, Inc. Ipswich, United States), 0.5µL of 10µM concentration for each primer, 7 µL of nuclease free water and 5µL of DNA from the *S. aureus* isolates for PCR amplification. Nuclease free water was as used as negative control. Thermocycling conditions involved an initial denaturation step at 95°C for 4 minutes was followed by 30 cycles of 95°C denaturation for 30 seconds, 60°C annealing step for 30 seconds, 72°C extension for 45 seconds, and a final extension step at 72°C for 10 minutes after 30 cycles. Amplicons were run in parallel with a 100bp ladder molecular weight marker on 1.5% agarose gel

(Sigma Aldrich Ltd. Missouri, United States) pre-stained with ethidium bromide in an electrophoresis system (Mupid-exU, ADVANCE CO., Ltd. Tokyo, Japan). The electrophoresis system contained 1 × Tris – Acetate EDTA (TAE) and was run for 30 minutes at 100V. A picture was taken under ultraviolet (UV) light in an UV illuminator (Daihan Scientific Co., Ltd. Seoul City, South Korea).

3.8.2 Molecular confirmation of *S. aureus* using nuc gene

Amplification and detection of *nuc* gene using a primer pair in Table 3.1 as previously described by Zhang *et al*.(2004). PCR master mix was calculated to a final volume of 10µL by multiplying OneTaq®Quick-Load® 2X Master Mix reaction mixture for 25µL by a factor of 10/25 (0.4). A 10µL total volume was preferred as this protocol only aimed at *nuc* gene detection. The volume of 10µL PCR master mix contained 4.8µL OneTaq®Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs, Inc. Ipswich, United States), 0.2µL of 10µM concentration for each primer, 2.8 µL of nuclease free water and 2µL of DNA extracted from all 36 *S. aureus* isolates. for PCR amplification.

Nuclease free water and ATCC 29213 *Staphylococcus aureus* were used as negative control and positive control respectively. An initial denaturation step at 94°C for 5 min was followed by 30 cycles of 94°C denaturation for 1 minute, 50°C annealing step for 1 minute, 72°C extension for 2 minutes, and a final extension step at 72°C for 10 minutes after 30 cycles. After PCR, electrophoresis was run and picture taken as described in section 3.8.1.

3.8.3 Molecular confirmation of *S. aureus* using coa gene

PCR amplification and detection of coagulase (*coa*) gene using a primer pair listed in Table 3.1 was performed as described by Ibrahim *et al.* (2019). A final volume of 10µLwas calculated and a reaction mixture of PCR master mixture was prepared as in 3.8.2 above. Nuclease free water was used as negative control. An initial denaturation step at 94°C for 4 min was followed by 30 cycles of 94°C denaturation for 45 seconds, 50°C annealing step for 45 seconds, 72°C extension for 1 minute, and a final extension step at 72°C for 2 minutes after 30 cycles. Amplification products were also run and a picture was taken using apparatus and technique in section 3.8.1.

3.9 Molecular detection of selected antimicrobial resistance genes

Conventional PCR was performed according to previously described protocols using gene specific primers for detection of antimicrobial resistant genes as outlined in Table 3.2.

3.9.1 Molecular detection of *mecA* gene

A conventional PCR assay was performed to amplify and detect *mec*A gene according to a previously described protocol (Petinaki *et al.*, 2001). A primer pair as stated in Table 3.2 was used. OneTaq®Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs, Inc. Ipswich, United States) was used to prepare a final PCR master mix volume of 10µL as in section 3.8.2. Nuclease free water was used as negative control. An initial denaturation step at 94°C for 4 min was followed by 30 cycles of 94°C denaturation for 30 seconds, 53°C annealing step for 30 seconds, 72°C extension for 1 minute, and a final extension step at 72°C for 4 minutes after 30 cycles. Amplification products were run in a submarine electrophoresis system (Mupid-exU, ADVANCE CO., Ltd. Tokyo, Japan) containing 1 × Tris-Acetate EDTA. The amplicons were run in parallel with a 100bp ladder molecular weight marker on 1.5% molecular biology grade agarose gel (Sigma Aldrich Ltd. Missouri, United States) previously stained with ethidium bromide. After 30 minutes of electrophoresis at 100V, a picture was taken under ultraviolet (UV) light in a UV illuminator (Daihan Scientific Co., Ltd. Seoul City, South Korea).

Table 3.2: Primers for antimicrobial resistance gene amplification

Gene	**Forward (F1) and reverse (R2) primer sequences**	**Product size (Base pairs)**	**Citation**
*mec*A	F1: 5' - AAAATCGATGGTAAAGGTTGGC - 3'and R2: 5' - AGTTCTGCAGTACCGGATTTGC - 3'	533bp	Petinaki *et al*., 2001
*erm*A	F1: 5' - TCTAAAAAGCATGTAAAAGAA - 3'and R2: 5' - CTTCGATAGTTTATTAATATTAG - 3'	645bp	Matsuoka *et al*., 2002
*erm*B	F1: 5' - GAAAAGTACTCAACCAAATA - 3'and R2: 5' - AGTAACGGTACTTAAATTGTTTA - 3'	639bp	Sutcliffe *et al*.,1996
*erm*C	F1: 5' - TCAAAACATAATATAGATAAA - 3'and R2: 5' - GCTAATATTGTTTAAATCGTCAAT - 3'	642bp	Sutcliffe *et al*.,1996
*tet*K	F1: 5' - TTAGGTGAAGGGTTAGGTCC - 3'and R2: 5' - GCAAACTCATTCCAGAAGCA - 3'	616bp	Aarestrup *et al*., 2000
*tet*L	F1: 5' - CATTTGGTCTTATTGGATCG - 3'and R2: 5' - ATTACACTTCCGATTTCGG - 3'	456bp	Aarestrup *et al*., 2000
*tet*M	F1: 5' - GTTAAATAGTGTTCTTGGAG - 3'and R2: 5' - CTAAGATATGGCTCTAACAA - 3'	576bp	Aarestrup *et al*., 2000
*tet*O	F1: 5' - GATGGCATACAGGCACAGAC - 3'and R2: 5' - CAATATCACCAGAGCAGGCT - 3'	515bp	Aarestrup *et al*., 2000

3.9.2 Molecular detection of *erm*A, *erm*B and *erm*C genes

Uniplex conventional PCR assays were performed to individually amplify and detect *erm*A , *erm*B and *erm*C genes according to previously described protocol (Matsuoka *et al*., 2002; Sutcliffe *et al*.,1996). Forward and reverse primers used were as outlined in Table 3.2. A final volume of 10µL PCR master mix based on OneTaq®Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs, Inc. Ipswich, United States) was prepared as in section 3.8.2. Nuclease free water was used as negative control. Amplification cycles were an initial denaturation step at 93°C for 3 minutes which was followed by 30 cycles of 93°C denaturation for 1 minute, a 52°C annealing step for 1 minute, a 72°C extension for 1 minute, and a final extension step at 72°C for 5 minutes after 30 cycles. Amplification products were run in a submarine electrophoresis and pictures were taken as in section 3.9.1.

3.9.3 Molecular detection of *tet*K, *tet*L, *tet*M and tetO genes

The detection employed a conventional PCR that was used to amplify *tet*K, *tet*L, *tet*M and *tet*O genes as described by Aarestrup *et al*. (2000).The primer pairs that were used are listed in Table 3.2. A 10µL total volume of PCR master mix was prepared as in section 3.8.2. Nuclease free water was used as negative control. PCR thermocycling conditions were an initial denaturation step at 93°C for 3 minutes that was followed by 30 cycles of 93°C denaturation for 1 minute, 52°C annealing step for 1 minute, 72°C extension for 1 minute, and a final holding step at 72°C for 5 minutes after 30 cycles. The PCR products were run in a submarine electrophoresis system and picture was taken as in section 3.8.2.

3.9.10 Molecular detection of selected genes responsible for virulence

Gene specific primers were used for conventional PCR amplification and detection of virulence genes as in previously described protocols in Table 3.3.

Table 3.3: Primers for virulence genes amplification

Gene	**Forward (F1) and reverse (R2) primer sequences**	**Product size (base pairs)**	**Citation**
PVL (*luk*S/*luk* F-PV)	F1: 5’ - GCTGGACAAAACTTCTTGGAATAT - 3’and R2: 5’ - GATAGGACACCAATAAATTCTGGATTG - 3’	83bp	Stegger *et al*., (2012)
*spl*A	F1: 5’ - CATTCAATTGCCGGATCCGAAAAGAATGTC - 3’and R2: 5’ - CACGAATGAATTGACTCGAGTTATTTTTCAATAT - 3’	641bp	Zdzalik *et al*., 2012
*spl*B	F1: 5’ - CAACAAACTGCCGGATCCGAAAATAATGTC - 3’and R2: 5’ - GCTCGTTTAAAGTCACTCGAGTTATTTATCTATG - 3’	654bp	Zdzalik *et al*., 2012
*spl*C	F1: 5’ - GGATCCGAGAAGAATGTTACGCAAGTTAAAG - 3’and R2: 5’ - CTCGAGTTATTGTTCAATGTGCTTTTGAATAAAATC - 3’	621bp	Zdzalik *et al*., 2012
*spl*D	F1: 5’ - GGATCCGAAAATAGTGTGAAATTAATTACCAACACG - 3’and R2: 5’ - CTCGAGTTATTTATCTAAATTATCTGCAATAAATTTC - 3’	635pb	Zdzalik *et al*., 2012
*spl*E	F1: 5’ - GGATCCGAACATAATGTGAAACTAATCAAAAATAC- 3’and R2: 5’ - CTCGAGTTATTTATCTGTGTTATCTGCAATGAATTTC - 3’	633bp	Zdzalik *et al*., 2012
*spl*F	F1: 5’ - CAACAAACAGCCGGATCCGAAAATACTGTTAAAC - 3’and R2: 5’ - GTCTAAGCTCGTGTTATTTATCTAAATTATC - 3’	621bp	Zdzalik *et al*., 2012

3.9.10.1 Molecular detection of PVL (*lukS-PV* and *lukF-PV*) gene

A conventional PCR assay was performed to amplify and detect PVL (*lukS-PV* and *lukF-PV*) gene using a primer pair in Table 3.3 as previously described by Stegger *et al*. (2012). A final volume of PCR master mix contained 4.8µL OneTaq®Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs, Inc. Ipswich, United States). Then 0.2µL of 10µM concentration for each primer, 2.8 µL of nuclease free water and 2µL of bacterial DNA for PCR amplification were added make a total volume of 10µL. Nuclease free water and ATCC 29213 *S. aureus* were used as negative control and positive control respectively. PCR thermocycling conditions involved an initial denaturation step at 94°C for 5 minutes which was followed by 30 cycles of 94°C denaturation for 30 seconds, 59°C annealing step for 1 minute, 72°C extension for 1 minute, and a final extension (holding) step at 72°C for 10 minutes after 30 cycles. Amplification products were run in a submarine electrophoresis system (Mupid-exU, ADVANCE CO., Ltd. Tokyo, Japan) containing 1 × Tris – Acetate EDTA (TAE) in parallel with a 100bp ladder molecular weight marker on ethidium bromide stained 1.5% molecular biology grade agarose gel (Sigma Aldrich Ltd. Missouri, United States). The electrophoresis was run for 30 minutes at 100V. A picture was taken under ultraviolet (UV) light in a UV illuminator (Daihan Scientific Co., Ltd. Seoul City, South Korea). DNA extracted from all 36 *Staphylococci* isolates was subjected to these PCR conditions.

3.9.10.2 Molecular detections of *spl*A, *spl*B, *spl*C, *spl*D, *spl*E, and *spl*F genes

A uniplex PCR for detection and amplification of each gene was performed using primers listed in Table 3.3 according to a protocol previously described by Zdzalik *et al*.(2012). A final volume of 10µL PCR master mix was prepared as in section 3.9.10.1. Nuclease free water was used as negative control. The amplification cycles involved an initial denaturation step at 95°C for 2 minutes which was followed by 30 cycles of 95°C denaturation for 30 seconds, 48°C annealing step for 30 seconds, 72°C extension for 5 minutes, and a final extension step at 72°C for 5 after 30 cycles. Amplification products were run in an electrophoresis system and were visualized as in section 3.9.10.1.

3.11 Determining the *spa* types of the *S. aureus* isolates

Big dye terminator sequencing method was performed on *spa* gene positive PCR products as previously described by Shopsin *et al.* (1999). A total of nine *spa* gene DNA amplicons were

purified by DNA clean & concentrator-5 kit (Zymo Research Biotechnology company. California, USA). About 100µL of DNA binding buffer were added to 20µL of *spa* gene amplicons (ratio 5:1). The mixture was homogenized on a vortex mixer (Vortex-Genie 2, Fisher Scientific Ltd. Leicestershire, UK) for 5 seconds and transferred to a Zymo-Spin™ Column in a collection tube then followed by centrifugation at 14000 *xg* for 30 seconds in a refrigerated centrifuge at 4°C. The flow through was discarded. Two wash steps followed that utilized a new collection tube to add 200µL of DNA wash buffer (previously prepared by adding 24mL of 100% ethanol to 6mL of DNA wash buffer concentrate) to the column, centrifugation at 14000 *xg* in a refrigerated centrifuge at 4°C for 30 seconds and flow through discarded. 10µL of DNA elution buffer containing 10mM Tris-HCl at pH 8.5 and 0.1mM EDTA (Ethylene Diamine Tetra acetic acid) was added directly to the column matrix and incubated at room temperature for 1 minute. Finally, the column was transferred to a clean sterile Eppendorf tube and centrifuged for 30 seconds at 14000 × g in a refrigerated centrifuge at 4°C to elute ultra-pure DNA that was verified by an A260/A280 -ratio of at least 1.8ng/µL on NanoDrop-1000 spectrophotometer, (Thermo Fisher Scientific Inc. Massachusetts, USA). Big dye master mix PCR reaction for sequencing was set up and contained 1µL of Big dye terminator reaction mix (Applied Bio systems, Thermo Fisher Scientific Inc. Massachusetts, USA), 3.75µL of 5 × sequencing buffer, 0.35µL of 10µM primer, (forward and reverse primers were separated in two PCR reaction tubes representing one sample) 2µL of sample DNA template, and 12.92µL of nuclease free water. The PCR thermocycling conditions were an initial denaturation at 96°C for 1 minute, followed by 28 cycles of 96°C for 10 seconds, 50°C for 5seconds and 60°C for 2 minutes and holding at 4°C after the cycles on a Verit 96 well thermo cycler (Applied Bio systems, Thermo Fisher Scientific Inc. Massachusetts, USA).

The Big dye PCR products were precipitated prior to sequencing by alcohol precipitation method. Firstly, 2µL of 3M sodium acetate and 2µL of 125M EDTA were added to the reaction tubes at the same time followed by 90µL of 100% ethanol. The mixture was then incubated for 10 minutes in a dark place at 4°C. After incubation, the mixture was centrifuged at 15000 *xg* for 20 minutes at 4°C in a refrigerated centrifuge. The supernatant was discarded after centrifugation and 200µL of 70% alcohol was added to the remaining substance followed by centrifugation at 15000 *xg* for 10 minutes at 4°C in a refrigerated centrifuge. During these centrifugation steps the supernatant was discarded by pipetting at the slant (down side) direction that the tubes made during

centrifugation to avoid discarding DNA. Therefore, the tubes were held on the side where DNA was leaning. The supernatant was pipetted directly on the opposite side and discarded. The tube was then wrapped in an aluminium foil paper and The DNA was dried in a centrifugal evaporator (Tomy mini vacuum MV-100, Tomy MX-207, Tomy Digital Biology Co. Ltd. Tokyo, Japan) for 8 minutes with heating at 55°C for 2 minutes in order to dry the DNA sample. After drying 20µL of highly deionized formamide was added to the dry sample that was then homogenized by vortex mixing for 1 minute and spun down.

Finally, the sample was denatured at 95°C for 10 minutes in a Verit 96 well thermocycler and loaded in a sequencer, Seq Studio Genetic Analyzer (Applied Bio systems, Thermo Fisher Scientific Inc. Massachusetts, USA) for DNA sequencing. The sequences were assembled and aligned to generate consensus sequences in FASTA format using Genetyx ATGC software (www.genetyx.co.jp). FASTA format consensus sequences were aligned using basic local alignment search tool (BLAST) in NCBI (www.ncbi.nlm.nih.gov) to check for highly similar sequences (megablast). Two online spa typing tools namely spaTypeFinder (spatyper.fortinbras.us) and spaTyper, center for genomic epidemiology (genomicepidemiology.org) were used to assign spa stypes to the FASTA format consensus sequences and to match the spa typing results.

3.12 Data Collection and Analysis

Social demographic information from Laboratory information system (LIS) that included age, sex, clinical specimen, and medical laboratory test performed (e.g., blood culture, urine culture) was manually double recorded using a designed form (Appendix B) and entered in a Microsoft Excel sheet and backed up in terabox cloud storage (www.terabox.com). Antimicrobial susceptibility testing results were manually double recorded on a designed form (Appendix C) and also entered in a Microsoft Excel sheet and backed up in terabox cloud storage. Conventional PCR test results on *nuc* gene, *coa* gene, *spa* gene, selected antimicrobial resistance genes, and virulence genes were also manually double recorded on a designed form (Appendix D) and also entered in a Microsoft Excel sheet and backed up in cloud storage. Images of DNA bands on an ultraviolet illuminator were captured by an internal camera and images were exported into a new flash drive and also backed up in terabox cloud storage. Sequencing data of the *Staphylococcus aureus* isolates *spa*

gene types was exported from the Sequencer into a new flash drive and also backed up in terabox cloud storage.

Descriptive statistics were used to analyze all categorical data using Stata/IC software version 14.2 (www.stata.com). All charts were constructed in Microsoft excel 2016 and Tables were constructed in Microsoft word 2016.

3.13 Ethical Considerations

Ethics approval was obtained from National Health Sciences Research Committee (NHSRC) in Malawi, protocol # 22/08/2966 (Appendix E and Appendix F). Furthermore, the permission to conduct the study was obtained from Malamulo Adventist Hospital Management. The material transfer agreement process was done prior to ethical clearance as required by the National Health Sciences Research Committee (NHSRC) in Malawi.

A material transfer agreement was signed by all involved parties i.e., investigator, Malamulo Hospital and School of Veterinary Medicine, The University of Zambia. The material transfer agreement was used to obtain an export permit and import permit for transfer of biological samples from Malawi to Zambia respectively. Copies of material transfer documents were submitted and authenticated by the National Health Sciences Research Committee (NHSRC) in Malawi.

The samples were triple packaged and transported according to World Organization for Animal Health (OIE) and the World Health Organization (WHO) regulations for international transfer of biological materials. Anonymity was maintained by use of study's sample identification numbers and not patient names nor laboratory accession numbers in the laboratory information system at Malamulo Hospital.

CHAPTER FOUR

RESULTS

4.1 Number of *Staphylococci* isolates collected

A total of 36 glycerol stocked *Staphylococci* were conveniently selected as they were available *Staphylococci* isolated during the study period which was lower that the calculated expected sample size of 231 *Staphylococci* isolates.

4.2 Sample types and social demographic details of participants

Confirmed *Staphylococcus aureus* isolates representing 4.4% (14 from a total of 321 glycerol stocked isolates) were included in this study. Other *Staphylococci* species included in this study were 6.9% (22 from a total of 321 glycerol stocked isolates). The *Staphylococcus aureus* isolates were from a variety of human clinical specimens that were examined during the period December, 2021 to May 2022 at Malamulo Hospital. The clinical samples included 14 urine samples (38.9%), three pus swabs (8.3%), 16 blood culture samples (44.4%), one skin wedge sample (2.8%), one pleural fluid sample (2.8%) and one ascitic fluid sample (2.8%). Patients were 14 males (38.9%), and 22 females (61.1%). The ages of patients were categorized according to risk stratification. Among the patients, 12 (33.3%) belonged to paediatric group (ages 0 – 14), 19 (52.8%) belonged to the young group (ages 15 – 47), two (5.6%) belonged to the middle age group (48 – 63) and three (8.3%) belonged to the elderly group (ages ≥ 64).

4.3 Detection and identification of *Staphylococcus aureus*

All isolates in this study had previously been identified as *Staphylococcus aureus* using routine methods namely gram's stain, catalase test and slide coagulase test at Malamulo Adventist Hospital laboratory. Molecular detection of the *nuc* gene, *coa* gene and *spa* gene confirmed 38.9% (14/36) isolates as *S. aureus* species representing 4.4% (14/321) total confirmed *S. aureus* during the study period.

Among the PCR confirmed *S. aureus*, 2.8% (1/36) were *coa* gene positive, 5.6% (2/36) were *spa* gene positive, 5.6% (2/36) were *nuc* gene positive, 5.6% (2/36) were both *coa* and *spa* gene

positive, 13.9% (5/36) isolates were both *nuc* and *spa* gene positive, and 5.6% (2/36) were positive to all three genes. The *nuc* gene was the most detected and confirmed in 71.4% (10/14) isolates as *Staphylococcus aureus* species. A total of 64% (9/14) were confirmed by *spa* gene and 35.7 % (5/14) by *coa* gene.

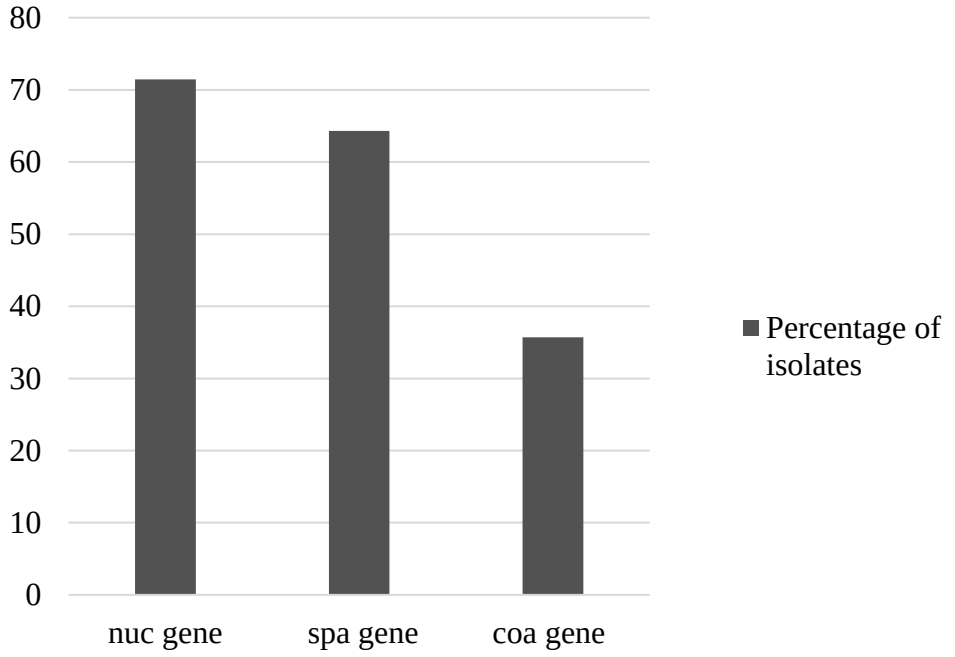


Figure 4.1: Molecular confirmation of *S. aureus* species

4.4 Antimicrobial susceptibility and resistance genes profile

4.4.1 Phenotypic antimicrobial susceptibility profile and ranking

Overall, 38.9% (14/36) of the isolates were multidrug resistant. Among the multidrug resistant isolates, 57.1% (8/14) were PCR confirmed *S. aureus* species whereas 42.9% (6/14) were other Staphylococci species. Moreover, among the PCR confirmed multidrug resistant *S. aureus* isolates 37.5% (3/8) were multidrug resistant-methicillin resistant *S. aureus* (MDR-MRSA). The multidrug resistant *Staphylococci* species group (unconfirmed species) had 33.3% (2/6) multidrug resistant-methicillin resistant *Staphylococci* isolates (Table 4.1).

Table 4.1: Multidrug antimicrobial susceptibility pattern of isolates

Antimicrobial drugs resistance pattern	**Proportion of isolates**	
	S. aureus group % (n)	Staphylococci spp. group % (n)
Trimethoprim/sulfamethoxazole + Penicillin + Ciprofloxacin	7.1 (1)	-
Trimethoprim/sulfamethoxazole + Penicillin + Tetracycline	7.1 (1)	-
Trimethoprim/sulfamethoxazole + Penicillin + Erythromycin	-	4.6 (1)
Trimethoprim/sulfamethoxazole + Tetracycline + Erythromycin + Ciprofloxacin	-	4.6 (1)
Trimethoprim/sulfamethoxazole + Tetracycline + Penicillin + Cefoxitin	7.1 (1)	4.6 (1)
Trimethoprim/sulfamethoxazole + Tetracycline + Penicillin + Erythromycin	7.1 (1)	-
Trimethoprim/sulfamethoxazole + Tetracycline + Erythromycin+ Gentamicin	7.1 (1)	-
Tetracycline + Penicillin + Erythromycin + Cefoxitin	7.1 (1)	-
Trimethoprim/sulfamethoxazole + Tetracycline + Erythromycin + Ciprofloxacin + Gentamicin	-	4.56 (1)
Trimethoprim/sulfamethoxazole + Tetracycline + Penicillin + Erythromycin + Ciprofloxacin	-	4.56 (1)
Trimethoprim/sulfamethoxazole + Tetracycline + Penicillin + Erythromycin + Gentamicin	7.1 (1)	-
Trimethoprim/sulfamethoxazole + Tetracycline + Penicillin + Erythromycin + Ciprofloxacin + Cefoxitin + Gentamicin	7.1 (1)	4.6 (1)

The highest resistance observed amongst all the isolates was against Sulfamethoxazole/trimethoprim recorded at 50% and the least resistance recorded was against gentamicin at 13.9%. Resistance to penicillin and tetracycline was at 38.9% while resistances to erythromycin, ciprofloxacin, cefoxitin were recorded at 30.6%, 19.4%, and 16.7%, respectively (Figure 4.2).

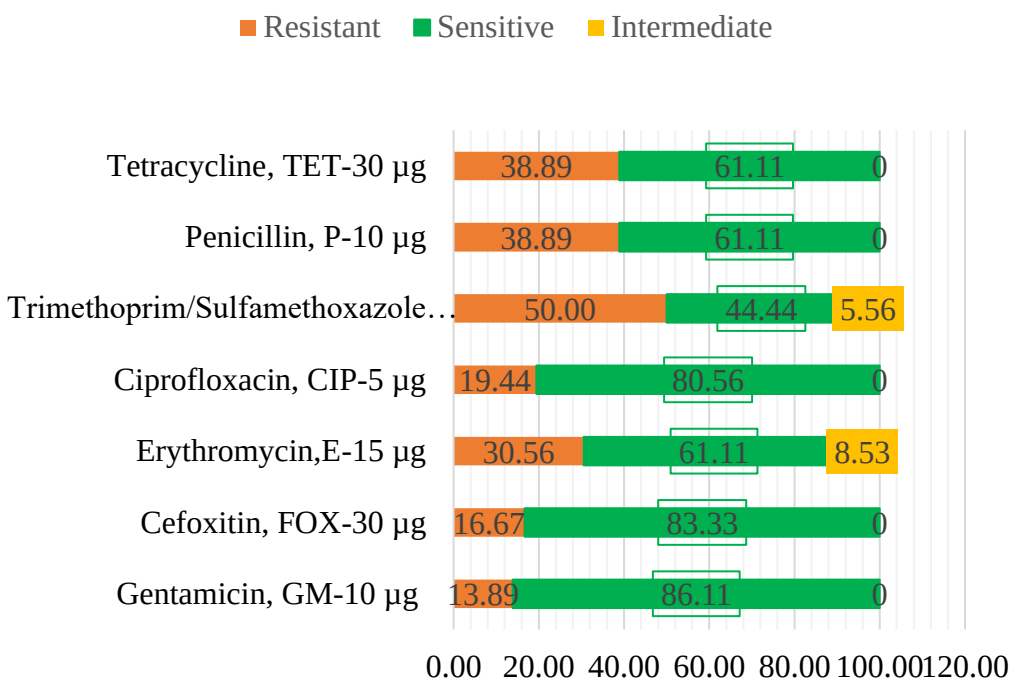


Figure 4.2: Overall antimicrobial susceptibility levels of isolates to antimicrobial agents ranking. Figure was generated in Microsoft Excel 2016.

The clustering of all multidrug resistant *Staphylococci* isolates in this study (n = 14) based on patients' age categories and sample source types showed that most of the multidrug resistant *Staphylococci* were isolates from blood and urine sample sources. The clustering of multidrug resistant *Staphylococci* in blood sample source was as follows; two isolates from paediatric age group (age: 0 – 14), two isolates from young age group (age: 15 – 47), and one isolate from elderly age group (age: ≥ 64). In urine sample source, multidrug resistant *Staphylococci* clustered as follows; four isolates from middle age groups (age: 15 - 47) and one isolate from the elderly group (age: ≥ 64).

One multidrug resistant *Staphylococcus* was from each pus sample source of young (age: 15 – 47) and middle (age: 48 – 63) age groups respectively. One multidrug resistant *Staphylococcus* was also from each pleural fluid and skin wedge sample source of paediatric (age: 0 – 14) and young (age: 15 – 47) age groups respectively (Table 4.2).

Table 4.2: Multidrug resistant *Staphylococci* stratified by sample source types and patients age categories.

Multidrug resistant Staphylococci isolates %(n)				
Patients' age category				
	Age: 0 – 14	Age: 14 – 47	Age: 48 – 63	Age ≥ 64
Blood	14.3% (2)	14.3% (2)	0	7.1% (1)
Pus	7.1% (1)	0	7.1% (1)	0
Urine	0	28.3% (4)	0	7.1% (1)
Pleural Fluid	0	7.1% (1)	0	0
Skin wedge	7.1% (1)	0	0	0

4.4.2 Antimicrobial resistance genes detected

Antimicrobial resistance genes were detected in 30.6% (11/36) of both the *S. aureus* and *Staphylococci* species group of isolates. The detection rates were as follows, *tet*K 22.2% (8/36), *mec*A 8.3% (3/36), *tet*L 5.6% (2/36), *tet*M 5.6% (2/36), and *erm*A 2.8% (1/36). Among the *S. aureus* group 57.1% (8/14) isolates had *tet*K detected, 14.3% (2/14) had *mec*A gene detected. The AMR genes *erm*A, B and C genes were not detected among *S. aureus* isolates. In contrast, among the *Staphylococci* species group all antimicrobial resistance genes *mec*A, *tet*K, *tet*L, *tet*M and *erm*A were detected in 27.3% (6/22) of the isolates (Table 4.3).

Table 4.3: Comparison of AMR genes detection between PCR confirmed *S. aureus* isolates and other *Staphylococci* species

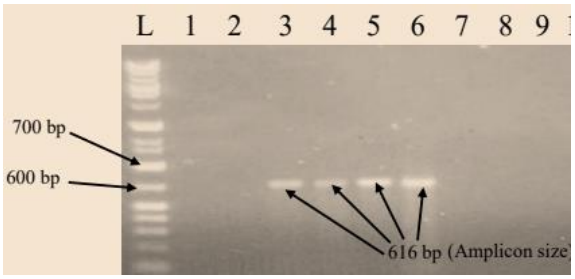
	Proportion of isolates %(n)	
AMR gene	*S. aureus* group (Total number = 14),	*Staphylococci* species group (Total number = 22)
tet K	57.1% (8)	4.6% (1)
tet L	0	9.1% (2)
tet M	7.1% (1)	4.6% (1)
mec A	14.3 % (2)	4.6% (1)
erm A	0	4.6% (1)

The highest detection of the antimicrobial resistance genes occurred in blood specimens across all age categories (Table 4.4).

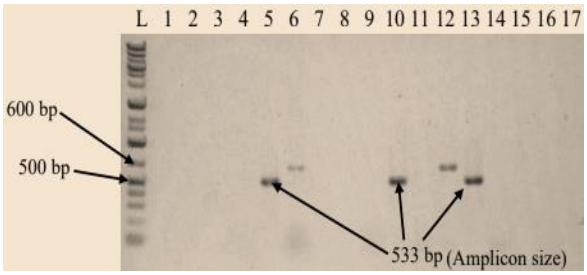
Table 4.4: Detection of AMR genes in *Staphylococci* isolates stratified by sample source types and patient age categories.

Number of Staphylococci isolates (n), actual AMR genes detected.				
Participant age category				
	Age: 0 – 14	Age: 14 – 47	Age: 48 – 63	Age ≥ 64
Blood	(2), *tet* K, *erm*A	(2), *tet*K, *tet*L, *tet* M, *mec* A	(1), *tet* K	(1), *tet*K, *tet*M, *mec*A
Urine	0	(2), *tet*K, *tet*L	0	(1), *mec* A
Skin wedge	(1/12), *tet*K	0	0	0
Ascitic Fluid	0	(1), *tet* K	0	0

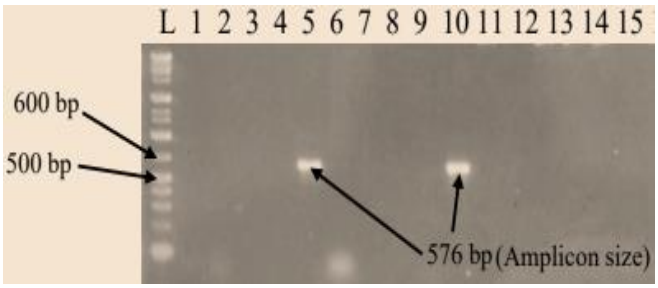
Representative agarose gel pictures of the detected antimicrobial resistance genes are shown in Figure 4.3A to E.



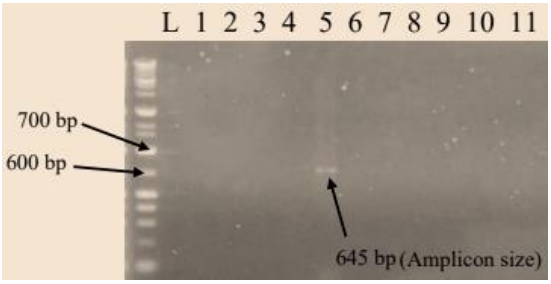
A.



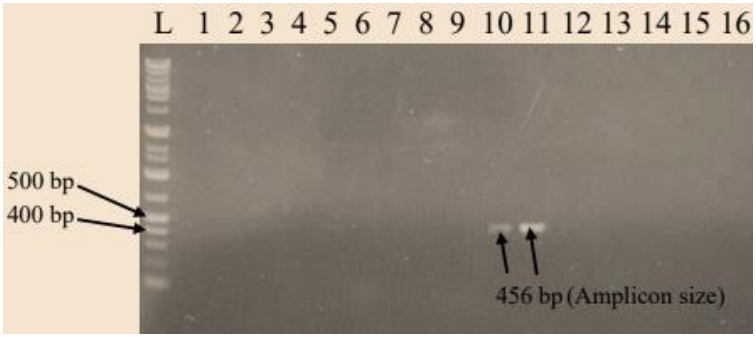
B.



C.



D.



E.

Figure 4.3A to E: Lane L: 100bp molecular marker, Lane 1: Negative control, A Lanes 3, 4, 5, and 6: *tet*K gene; B Lanes 5, 10, and 13: *mec* A gene; C Lanes 5 and 10; *tet*M; D Lane 6: *erm* A; E Lanes 10 and 11; *tet*L detection by ethidium bromide stained 1.5% agarose gel electrophoresis at 100V.

4.5 Virulence genes detected

4.5.1 PVL (*lukS-PV* and *lukF-PV*) gene

The PVL (*lukS-PV* and *lukF-PV*) gene was detected in four confirmed *S. aureus* isolates. None of the isolates from the *Staphylococci species* group were positive for PVL (*lukS-PV* and *lukF-PV*) gene in this study. Two of the *S. aureus* isolates with PVL (*lukS-PV* and *lukF-PV*) gene detected were from pus sample sources of young age group patients (age: 15 – 47). Other two *S. aureus* isolates with PVL (*lukS-PV* and *lukF-PV*) gene detected were each from blood sample sources of a paediatric patient and in ascitic fluid of young age group patients respectively (Table 4.5).

Table 4.5: PVL (*lukS-PV* and *lukF-PV*) gene detection stratified by sample source types and patients' age categories (n = 4)

(n) = number of *S. aureus* isolates with PVL (*lukS-PV* and *lukF-PV*) gene detected				
	Age: 0 – 14	Age: 14 – 47	Age: 48 – 63	Age ≥ 64
Blood	(1)	0	0	0
Pus	0	(1)	(1)	0
Ascitic Fluid	0	(1)	0	0

Figure 4.4 is a representative agarose gel picture for PVL (*lukS-PV* and *lukF-PV*) gene PCR detection.

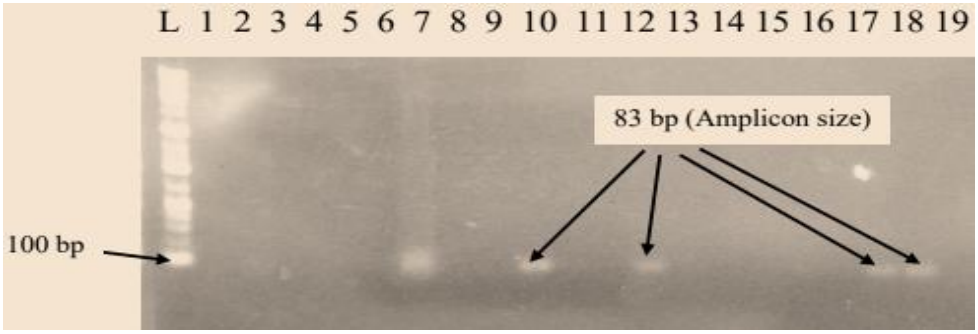


Figure 4.4: Lane L: 100bp molecular weight marker; Lane 1: Negative control; Lane 10, 12,17 and 18: PVL (*lukS-PV* and *lukF-PV*) gene detection by ethidium bromide stained 1.5% agarose gel electrophoresis at 100V.

4.5.2 *spl* genes

The *spl* genes were detected in 85.7% (12/14) exclusively *S. aureus* isolates in this study. The most detected *spl* genes were *spl*A 42.9% (6/14) and *spl*D 42.9% (6/14) whereas *spl*B 35.7% (5/14), *spl*C and *spl*F were each detected in 21.4% (3/14), while *spl*E was detected in 14.3% (2/14) *S. aureus* isolates. The highest number (n) of *spl* genes detected clustered amongst the young age category whereby all six *spl* genes were detected in four different isolates from blood specimens (Table 4.6).

Table 4.6: Distribution of *spl* genes detection stratified by sample source types and patients' age categories

Number of *S. aureus* isolates with *spl* genes detected (n), actual *spl* genes detected				
	Patients' age categories			
	Age: 0 – 14	Age: 14 – 47	Age: 48 – 63	Age ≥ 64
Blood	(2), *spl*A, *spl*D	(4), *splA*, *spl*B, *spl*C, *spl*D, *spl*E, *spl*F	(1), *spl*F	(1), *spl*A
Urine	0	0	0	(1), *spl*C
Ascitic fluid	0	(1), *spl*A, *spl*B	0	0
Skin wedge	(1), *spl*A, *spl*D	0	0	0
Pus	0	(1), *spl*D	0	0

Figure 4.5 is a representative agarose gel picture for *spl* genes (*spl*A, *spl*B, *spl*C, *spl*D, *spl*E and *spl*F) PCR detection.

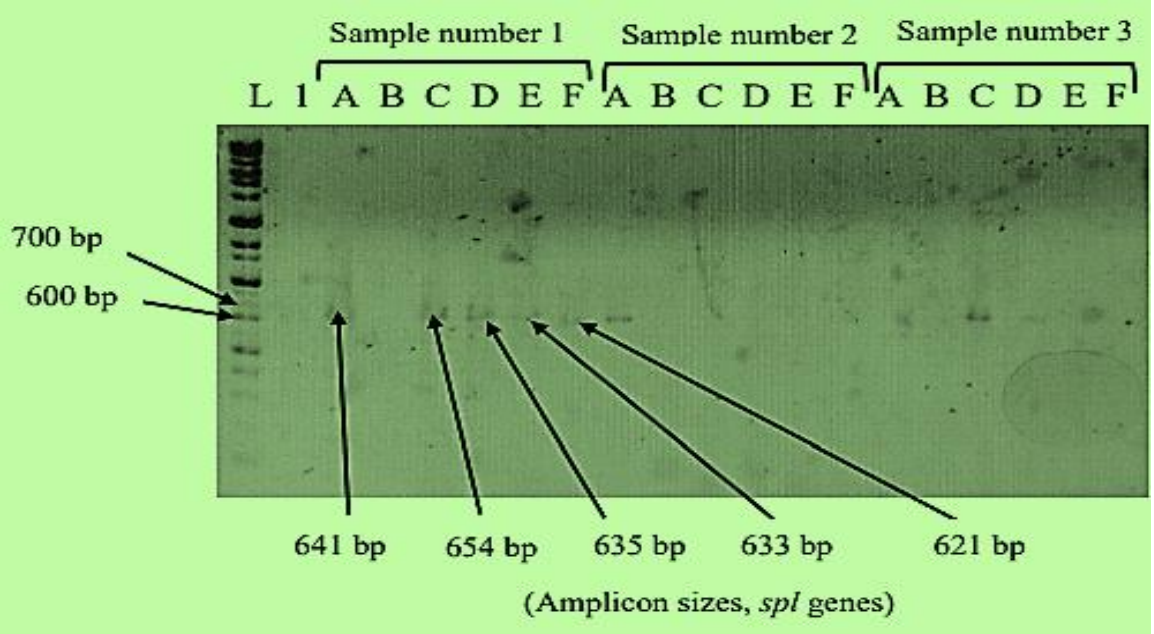


Figure 4.5: Lane L: 100bp molecular marker, Lane 1: Negative control, Sample 1: Lane A (*spl* A), Lane C (*spl* C), Lane D (*spl* D), Lane E (*spl* E), Lane F (*spl* F), Sample 2: Lane A (*spl*A), Sample 3: Lane C (*spl*C) detection by ethidium bromide stained 1.5% agarose gel electrophoresis at 100V.

4.6 *spa* typing

The most abundant *spa* type among the *spa* gene positive *S. aureus* isolates was t941 at 33.3% (3/9). Other spa types detected in this study were spa types t6140 at 11.1% (1/9), t1130 at 11.1% (1/9), t021 at 11.1% (1/9), t002 at 11.1% (1/9); t064 at 11.1% (1/9) and an unknown spa type at 11.1% (1/9) was also detected (Table 4.7).

Table 4.7: Distribution of *spa* types among *S. aureus* isolates from Malamulo Hospital in Malawi.

Proportion % (n)	spa type	Repeat succession
33.3 (3)	t941	04-21-12-17-20-17-12-12-17-16
11.1 (1)	t6140	07-23-12-13-12-12-23-02-12-23
11.1 (1)	t1130	15-12-16-16-16-02-16-02-25-17-24
11.1 (1)	t021	15-12-16-02-16-02-25-17-24
11.1 (1)	t002	26-23-17-34-17-20-17-12-17-16
11.1 (1)	t064	11-19-12-05-17-34-24-34-22-25
11.1 (1)	Unknown	11-19-12-05-17-34-24-34-22

The *spa* gene sequences of our isolates had 92.8% identity similarity to *S. aureus* subspecies *aureus* (NCTC 8325), in NCBI basic local alignment search tool (BLAST). Figure 4.6 is a representative NCBI hits table for the unknown *spa* type consensus sequence with identity similarity 92.8%.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Staphylococcus aureus subsp. aureus NCTC 8325 chromoso...	Staphyloc...	560	849	100%	4e-156	92.80%	2821361	NC_007795.1
Staphylococcus roterodami isolate Staphylococcus, whole ge...	Staphyloc...	425	2230	100%	2e-115	90.21%	479600	NZ_CAJGUT010000002.1
Staphylococcus schweitzeri strain NCTC13712 chromosome ...	Staphyloc...	409	1441	86%	2e-110	90.00%	2784939	NZ_LR134304.1
Staphylococcus argenteus, complete sequence	Staphyloc...	398	2563	99%	4e-107	88.69%	2762785	NC_016941.1
Staphylococcus singaporensis strain SS87 contig00002, whol...	Staphyloc...	298	1072	100%	4e-77	90.04%	480563	NZ_JABWHE010000002.1

Figure 4.6: Representative NCBI BLAST hits for unknown spa type

The distribution of *spa* types stratified by specimen types and age categories showed that most *spa* types were *S. aureus* from blood sample source type (Table 4.8).

Table 4.8: Distribution of *spa* types stratified by specimen types and age categories

Number of *S. aureus spa* types detected (n), actual *spa* types detected				
Patients' age categories				
	Age: 0 – 14	Age: 14 – 47	Age: 48 – 63	Age ≥ 64
Blood	(2), t021, t064	(2), Unknown spa type, t0941	0	(1), t6140
Pus	0	(1), t1130	0	0
Urine	0	0	0	(1), t002
Ascitic fluid		(1), t941		
Skin wedge	(1), t941			

CHAPTER FIVE:

DISCUSSION

5.1 Discussion

The proportion and number of confirmed *S. aureus* infections during the study period at Malamulo Hospital was low at 4.4% (n = 14) as compared to previous findings in Malawi. An overall 6.6% (n = 1925) *S. aureus* bacteremia infections was reported at Queen Elizabeth Central Hospital in Blantyre, Malawi from January 1998 to December 2016 (Musicha *et al.*, 2017). In another study an overall 34.7% (n = 783) *S. aureus* isolated in various clinical specimens that included pus, urine, blood, ear swabs, cerebrospinal fluid and other body fluids was reported by Kumwenda *et al*. (2021) at Mzuzu Central Hospital in Mzuzu, Malawi, from 2002 to 2014 and 26% (n = 86) *S. aureus* isolated in pus, blood, urine and cerebrospinal fluid reported by Choonara *et al*.(2022) at Kamuzu Central Hospital in Lilongwe, Malawi from June to December 2017.

This difference in *S. aureus* infections observed could be due to the small sample (n = 36) size and shorter time frame (6 months) for sample collection that was used in this study as compared to the previous studies. Furthermore, this study was conducted at a secondary rural referral facility that offer bacteriology referral services to a relatively smaller population of 500,000 as compared to the tertiary and larger bed capacity referral facilities where previous studies were conducted. For instance, Queen Elizabeth Central Hospital (QECH) in Blantyre, has a catchment population of about six million people and is a tertiary referral hospital for 12 public Districts Hospitals in the Southern Region of Malawi (Prin *et al.*, 2016).

A high proportion of *Staphylococci* reported in this current study were from blood sample source type. Previous research attests that *S. aureus* is the most isolated bacterial pathogen in blood culture specimens in febrile illnesses in Malawi (Makoka *et al.*, 2012).

S. aureus bacteremia has been associated with poor clinical outcomes with increased mortality worldwide if the infection occurs in the paediatric and older age categories or sickly patients with low immunity when diagnosis and treatment is delayed (Tong *et al.*, 2015). Timely laboratory

diagnosis and treatment is key to reduce mortality associated with *S. aureus* bacteremia (Yilmaz *et al.*, 2016). However antimicrobial resistance including methicillin resistance, and emergence of variant strains consequently make *S. aureus* bacteremia infections difficult to diagnose and treat on time and therefore *S. aureus* bacteremia continues to be a major public health threat (Hassoun *et al.*, 2017). *S aureus* bacteremia can result from various manifestations of *S. aureus* infection such as soft tissue infections, *S. aureus* urinary tract infection, endocarditis leading to *S. aureus* bacteremia infection through metastasis and upregulation of virulence factors involved in invasion such as *hla* (Horino and Hori, 2020). This study also explored other sample source types associated with *S. aureus* clinical infections that can lead to *S. aureus* bacteremia which included urine from *S. aureus* urinary tract infections, pus and skin wedge samples from *S. aureus* soft tissue infections.

Routine biochemical identification of all the *Staphylococci* isolates in this study consisted of gram-positive cocci grown overnight on 5% blood agar plate that were both catalase and slide coagulase test positive were considered as *S. aureus*. The low yield of *S. aureus* species by molecular testing observed in this study could be due to the fact that only three biochemical tests namely gram stain, catalase and coagulase tests were used to screen for *S. aureus* during routine testing.
The use of more sequel biochemical tests for routine screening of *S. aureus* in an ordered manner has been proven to reliably identify *S. aureus* species biochemically in resource limited countries.

In Nigeria for example, sequel testing using gram stain, hemolysis on blood agar, growth on mannitol salt agar, clear colonies on DNase agar, catalase test, tube coagulase test and Microgen™ STAPH-ID test has been proven to reliably identify *S. aureus* species (Confidence *et al.*, 2013). Inadequate identification of bacterial isolates can lead to a wrong prescription and potentially contribute to emergence of antimicrobial resistance (Saha and Sarkar, 2021). Use of catalase and coagulase tests alone can potentially lead to misidentification of *S. aureus* species. Misidentification of *S. aureus* can result because there are also other catalase and coagulase positive *Staphylococcus* species that are animal and human pathogens for instance *Staphylococcus intermedius* (Foster *et al*.,1997).

In this present study, the *nuc* gene was the most detected specific gene for *S. aureus* species as compared to *spa* gene and *coa* gene. The *nuc* gene is considered as a potential candidate gene for

rapid PCR identification of *S. aureus* species (Sahebnasagh *et al*., 2014; Brakstad *et al*., 1992). In spite of that, it has been shown that *nuc* gene can also be detected in *S. pseudintermedius, and S. schleiferi* besides *S. aureus* and therefore this warrants the coupled use of PCR along with sequencing to ascertain molecular species of *S. aureus* (González-Domínguez *et al.*, 2020). Alternatively, the use of several *S. aureus* specific genes for instance use of multiplex PCR has potential for adequate speciation and diagnosis (Montazeri *et al.*, 2015).

The highest phenotypic antimicrobial resistance observed in this study was against trimethoprim/sulfathomexazole, followed by tetracycline and penicillin resistance. High resistance to trimethoprim/sulfathomexazole up to 92% has recently been reported in *Enterobacteriaceae* (Onduru *et al.*, 2021), and *Pneumococci* up to 96% (Cornick *et al.*, 2014) in Malawi.
Previous findings signify that trimethoprim/sulfathomexazole resistance is gaining ground in both gram negative and gram-positive bacterial pathogens in Malawi.

Daily intake of trimethoprim/sulfamethoxazole as an antibacterial prophylactic drug among patients on anti-retroviral therapy may be an important trimethoprim/sulfathomexazole resistance selection pressure (Laurens *et al.*, 2021). Furthermore, according to Sambakusi, (2019), trimethoprim/sulfathomexazole is amongst home stocked antimicrobial agents used for self-medication against coughs, fever and malaria in Malawi. Evidence of circulating *S. aureus* strains with trimethoprim/sulfathomexazole resistance gene (*dfrG* gene) in sub-Saharan Africa has been previously reported by Nurjadi *et al*.(2014) and the trimethoprim/sulfathomexazole resistance gene (*dfrG* gene) is transferrable in *Staphylococci* species (López *et al.*, 2012). Tetracycline and penicillin resistance was also reported in the current study. Oxytetracycline is reportedly amongst the most frequently used veterinary antibiotics in Blantyre, Malawi according to Mankhomwa *et al*.(2022). Sustained high resistance to tetracycline and penicillin in human infections have resulted in declined use of these antimicrobial agents in Malawi which were previously used to empirically treat *N. gonorrhea*e infections (Brown *et al.*, 2010).

Multidrug resistance in this study was considered if an isolate showed phenotypic resistance to at least three different classes of antimicrobial agents of the seven antimicrobial classes used to treat *S. aureus* infections (Ge *et al*., 2017). The representative antimicrobial agents and classes were as

follows penicillin (beta lactam), cefoxitin (cephalosporins), tetracycline (tetracyclines), erythromycin (macrolides and lincosamides), ciprofloxacin (Quinolones), gentamicin (aminoglycosides) and trimethoprim/sulfathomexazole.

S. aureus species predominated *Staphylococci* species isolates in terms of multi-drug and/or methicillin resistance. However, at least one strain from each group was resistant to all seven representative antimicrobial classes.

Previous studies in Malawi have reported occurrence of methicillin resistant *S. aureus* in human clinical specimens but multidrug resistance has largely been reported in enterobacteriaceae (Gallaher *et al.,* 2018). In this study therefore, we report occurrence of methicillin and multidrug resistant *S. aureus* (MDR-MRSA) strains at Malamulo Hospital in Malawi. MDR-MRSA strains are a serious threat to public health and extensively contribute to high mortality rates among patients especially in developing countries as the infections are usually difficult to treat (Lim *et al.,* 2016).

Compared to phenotypic resistance pattern in this study, *tet*K was detected only in four isolates that were also phenotypically resistant to tetracycline whereas other four isolates had *tet*K gene detected but without corresponding tetracycline phenotypic resistance. The tetracycline resistance genes *tet*K and *tet*L encode energy dependent membrane-associated proteins that efflux pump out tetracycline from the bacterial cell as a mechanism of antimicrobial resistance (Ullah *et al.*, 2012). The tetracycline resistance genes *tet*M and *tet*O on the other hand code for ribosomal protection proteins, which function biochemically to reduce the affinity of tetracycline to the bacterial ribosome (Schmitz *et al.*, 2001). Surprisingly in this study some *S. aureus* strain that had *tet*K and *tet*L did not exhibit phenotypic tetracycline resistance whereas all isolates with *tet*M demonstrated phenotypic tetracycline resistance. The strain that lacked phenotypic expression of tetracycline resistance could possibly be an inducible tetracycline resistance strain that require pre-incubation with sub inhibitory tetracycline concentrations in order to express tetracycline resistance and can lead to false identification of susceptibility testing (Trzcinski *et al.,* 2000).

Unfortunately, inducible tetracycline resistance was not investigated in this protocol because minimum inhibition concentration (MIC) breakpoint method was not used in this study rather we used the disc diffusion method.

All isolates that had the *mec*A gene detected in this study had corresponding expression of phenotypic methicillin resistance. However, three isolates that had phenotypic expression of methicillin resistance tested negative for the *mec*A gene. MRSA isolates exhibit β-lactam resistance due to the acquisition of penicillin binding protein 2A (PBP2A), encoded by *mec*A (Panchal *et al.*, 2020). A variant of the *mec*A gene, *mec*C gene that also confer methicillin resistance to *S. aureus* through expression of a variant PBP2a has been reported (Laurent *et al.*, 2012). The *mec*C carrying MRSA has been associated with human (Lozano *et al.*, 2020), livestock (Aklilu and Chia, 2020) as well as wild animal infections (Gómez *et al.*, 2014). The use of cefoxitin as was done in this study is considered the most reliable method for phenotypic detection of both mecA and *mec*C carrying MRSA (Skov *et al.*, 2014). Therefore, it is possible that that the three isolates that had phenotypic expression of methicillin resistance but without *mec*A gene detected were likely β-lactamase hyper producers (Argudín *et al.*, 2018).

The erythromycin resistance gene *erm*A was detected in one *Staphylococci* species isolate in this study. We could not ascertain the species of this isolate in this study but literature evidence shows that a number of antimicrobial resistance genes including *erm*A, *tet*K, *tet*L, *tet*M and *mec*A have been detected in pathogens related to *S. aureus* such as *S.pseudintermedius* which are important pathogens of both humans and animals (Kadlec and Schwarz, 2012). The resistance genes that were detected in *S. aureus* isolates in this study namely *tet*K, *tet*M and *mec*A are all horizontally transferrable genes that can be passed from a resistant to a susceptible *S. aureus* strain (Bitrus *et al*., 2017; Baumgardner *et al*., 2021).

Pathogenicity of *S. aureus* highly relies on expression of virulence factors and in this study, we also investigated the presence of the *spl* genes and the PVL (*lukS-PV* and *lukF-PV*) gene among the isolates.

Staphylococcus aureus agr locus also a quorum-sensing gene cluster modulates expression of over 70 genes, 23 of which are virulence genes including genes that encode proteases investigated in this study *spl*A, *spl*B, *spl*C, *spl*D, *spl*E and *spl*F (Singh and Phukan, 2019). The PVL (*lukS-PV* and *lukF-PV*) gene of the *egc* operon (enterotoxin gene cluster) in *S. aureus* that encode Panton-Valentine leucocidin (PVL) responsible for invasive staphylococcal disease worldwide (Shallcross *et al.*, 2013).

Overall, the *spl* genes were detected in the highest proportion among the *S. aureus* isolates in this study as compared to PVL (*lukS-PV* and *lukF-PV*) gene (33.33%). Occurrence of *spl* genes in this study was highest in blood culture *S. aureus* isolates. Notably *spl*D was the most highly detected and none of the isolates had all six *spl* genes in this study. There is growing evidence that *spl* positive strains only contain a particular set of *spl* genes and not all six proteases which was also evidenced in this study. The *spl* genes are highly expressed in life-threatening *S. aureus* conditions, such as pneumonia, endocarditis, osteomyelitis, and sepsis according to the pathophysiology of the strain in question (Zdzalik *et al*., 2012). Biochemical structural studies have suggested that Spl proteases exhibit cooperative hydrolytic activities during an *S. aureus* infection but uniquely SplD selects threonine, isoleucine, leucine, valine, alanine, and serine at substrate's residue position 1 to perform an elastase-like activity (Zdzalik *et al.*, 2013).

The spa types t941 (33.33%) and t002 (11.1%) of which the former were the most abundant in this study were previously reported among European travelers from Tanzania. (Nurjadi *et al.*, 2014). Interestingly, they were trimethoprim/sulfathomexazole resistant in the previous study as well as in this present study (Nurjadi *et al*., 2014). Importantly, in the current study, the spa type t002 (11.1%) isolate was a MDR-MRSA. Further molecular epidemiology and phylogenetic studies in this case can help to establish if the Tanzanian and this present Malawian spa type t002 are evolutionary related strains and eventually help to limit their anticipated spread.

The spa type t064 has been reported in Uganda, Nigeria, Ethiopia, Zambia and Namibia in association with both hospital and community acquired as well as zoonotic infections (Kateete *et al*., 2019; Stastkova *et al*., 2009; Nurjadi *et al*., 2014; Samutela *et al*., 2017).

The spa type t021 (11.1%) has also been reported worldwide both in human and animal infection (Ishihara *et al*., 2014; Goudarzi *et al*., 2018). In this study the spa type t021 (11.1%) was a methicillin susceptible multidrug resistant *S. aureus* (MDR-MSSA) strain.

The other spa types t1130 (11.1%) and t6140 (11.1%) have not been widely reported and the unknown spa type (11.1%) reported in this study had a repeat succession similar to spa type t064 (11.1%). Whole genome sequence analysis can help to investigate if this novel spa type (11.1%) resulted from genetic evolution of spa type t064 (11.1%) as both strains showed similar phenotypic resistance pattern but only exhibited virulence *spl* genes differently.

5.2 Limitations of the study

One limitation of the study is that a small sample size of *S. aureus* isolates (n = 14) and other *Staphylococci* species (n = 22) was which implies that these results cannot be generalized. Only 36 *Staphylococci* isolates were stocked since the inception of the Fleming fund AMR surveillance program which was directly linked to the study period.

Another limitation of the study is that the conventional PCR and partial sequencing of the *spa* gene was only done on a representative number of isolates as such we could not ascertain the species of 22 *Staphylococci* isolates that were not confirmed as *S. aureus* during molecular detection of *S. aureus* species using *nuc*, *spa*, and *coa* genes.

Further, we employed the disc diffusion method for antimicrobial susceptibility testing which does not determine inducible resistance strains that require MIC method.

Furthermore, due to lack of clinical data on other underlying clinical conditions including HIV/AIDS status, treatment and length of hospital stay for the participants in this study, we could not link striking phenotypic resistances to the treatment strategies that were used on the patients.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

1. This study affirmed that *S. aureus* bacteremia may be a common presenting form of *S. aureus* infection at Malamulo Hospital.

2. MDR - MRSA and other multidrug-methicillin resistant *Staphylococci* species are a possible public health threat with noteworthy trimethoprim/sulfathomexazole, erythromycin, and tetracycline resistance at Malamulo Hospital.

3. The spl genes (*spl*A, *spl*B, *splC*, *spl*D, *spl*E and *spl*F) could be among the predominant genetic drivers of virulence in *S. aureus* bacteremia infections whereas PVL (*luk*S and *luk*F-*PV*) gene could be a predominant virulence determinant in skin and/or soft tissue at Malamulo Hospital.

4. The predominant spa type in this study of t941 along with t002, t021 and t064 are well documented and endemic in sub – Saharan Africa.

5. The unknown spa type reported in this study could be evolutionary closely related to spa type t064 as they had a similar repeat succession, a similar phenotypic resistance pattern but differed in the detection profile of virulence spl genes.

6.2 Recommendations and future directions

The MDR-MRSA and other multidrug methicillin resistant *Staphylococci* species observed in this study along with striking trimethoprim/sulfathomexazole resistance reported in this study can potentially frustrate treatment efforts in an already limited resource public health system.

1. This molecular study of clinical *S. aureus* isolates at Malamulo Hospital has provided some revealing insights that can be used to improve routine diagnosis, treatment of clinical *S. aureus*.

2. There is need for more molecular epidemiology and characterization studies on *S. aureus* and other pathogenic *Staphylococci* species preferably using whole genome sequence tools at Malamulo Hospital. This can provide more insights on how different pathogenic *Staphylococci* strains are gaining resistance to common antibiotics at alarming rates as with trimethoprim/sulfathomexazole as well as emergence of methicillin and multidrug resistant pathogens.

3. Based on the findings of this study several directions can be explored. Firstly, whole genome sequencing of *Staphylococci* species isolates to determine occurrence of other *Staphylococcal* pathogens that may be confused as *S. aureus* as well as determining their genotypic virulence and genotypic drug resistance profile in all the isolates. Whole genome sequencing of *S. aureus* isolates in this study can also help determine other molecular traits such as multilocus sequence types (MLST) and Staphylococcal cassette chromosome mec (SCCmec) types.

List of references

Aarestrup, F.M., Agerso, Y., Gerner–Smidt, P., Madsen, M., Jensen, L.B. (2000). Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagnostic Microbiology and Infectious Disease*, **37**: 127–137.

Abdulgader, S.M., Shittu, A.O., Nicol, M.P., Kaba, M. (2015). Molecular epidemiology of Methicillin-resistant *Staphylococcus aureus* in Africa: a systematic review. *Frontiers in Microbiology*, **0**.

Acharya, J., Zolfo, M., Enbiale, W., Kyaw, K.W.Y., Bhattachan, M., Rijal, N., Shrestha, A., Shrestha, B., Madhup, S.K., Raghubanshi, B.R., Kattel, H.P., Rajbhandari, P., Bhandari, P., Thakur, S., Sharma, S., Singh, D.R., Jha, R. (2021). Quality Assessment of an Antimicrobial Resistance Surveillance System in a Province of Nepal. *Tropical Medicine and Infectious Disease*, **6**: 60.

Adalbert, J.R., Varshney, K., Tobin, R., Pajaro, R. (2021). Clinical outcomes in patients co-infected with COVID-19 and *Staphylococcus aureus*: a scoping review. *BMC Infectious Diseases*, **21**: 985.

Adesida, S.A., Abioye, O.A., Bamiro, B.S., Amisu, K.O., Badaru, S.O., Coker, A.O. (2017). Staphylococcal bacteraemia among human immunodeficiency virus positive patients at a screening center in Lagos, Nigeria. *Beni-Suef University Journal of Basic and Applied Sciences*, **6**: 112–117.

Ahmed, K.S.H. (2017). In Vitro Susceptibility Patterns of Aqueous Extract of Green Tea on Bacteria Isolated from Pregnant Women with Urinary Tract Infection Attending Shendi Hospital. *SAR Journal of Pathology and Microbiology* , **2**: 21-27 .

Aklilu, E., Chia, H.Y. (2020). First mecC and mecA Positive Livestock-Associated Methicillin Resistant *Staphylococcus aureus* (mecC MRSA/LA-MRSA) from Dairy Cattle in Malaysia. *Microorganisms*, **8**: 147.

Algammal, A.M., Hetta, H.F., Elkelish, A., Alkhalifah, D.H.H., Hozzein, W.N., Batiha, G.E.-S., El Nahhas, N., Mabrok, M.A. (2020). Methicillin-Resistant *Staphylococcus aureus* (MRSA): One Health Perspective Approach to the Bacterium Epidemiology, Virulence Factors, Antibiotic-Resistance, and Zoonotic Impact. *Infection and Drug Resistance*, **13**: 3255–3265.

Al-Humaidan, O.S., El-Kersh, T.A., Al-Akeel, R.A. (2015). Risk factors of nasal carriage of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* among health care staff in a teaching hospital in central Saudi Arabia. *Saudi Medical Journal*, **36**: 1084–1090.

Alibayov, B., Baba-Moussa, L., Sina, H., Zdeňková, K., Demnerová, K. (2014). *Staphylococcus aureus* mobile genetic elements. *Molecular Biology Reports*, **41**: 5005–5018.

Anjum, M.F., Zankari, E., Hasman, H. (2017). Molecular Methods for Detection of Antimicrobial Resistance. *Microbiology Spectrum*, **5**: 5.6.02.

Antonanzas, F., Lozano, C., Torres, C. (2015). Economic Features of Antibiotic Resistance: The Case of Methicillin-Resistant *Staphylococcus aureus*. *PharmacoEconomics*, **33**: 285–325.

Argudín, M.A., Roisin, S., Nienhaus, L., Dodémont, M., de Mendonça, R., Nonhoff, C., Deplano, A., Denis, O. (2018). Genetic Diversity among *Staphylococcus aureus* Isolates Showing Oxacillin and/or Cefoxitin Resistance Not Linked to the Presence of mec Genes. *Antimicrobial Agents and Chemotherapy*, **62**: e00091-18.

Ngoa, U., Schaumburg, F., Adegnika, A.A., Kösters, K., Möller, T., Fernandes, J.F., Alabi, A., Issifou, S., Becker, K., Grobusch, M.P., Kremsner, P.G., Lell, B. (2012). Epidemiology and population structure of *Staphylococcus aureus* in various population groups from a rural and semi urban area in Gabon, Central Africa. *Acta Tropica*, **124**: 42–47.

Atshan, S.S., Shamsudin, M.N. (2011). Evaluation of phenotypic and genotypic detection methods for biofilm-forming methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* clinical isolates. *Annals of Microbiology*, **61**: 825–831.

Baumgardner, R.M., Berreta, A., Kopper, J.J. (2021). Evaluation of commercial probiotics for antimicrobial resistance genes. *The Canadian Veterinary Journal*, **62**: 379–383.

van Belkum, A., Bachmann, T.T., Lüdke, G., Lisby, J.G., Kahlmeter, G., Mohess, A., Becker, K., Hays, J.P., Woodford, N., Mitsakakis, K., Moran-Gilad, J., Vila, J., Peter, H., Rex, J.H., Dunne, W.M. (2019). Developmental roadmap for antimicrobial susceptibility testing systems. *Nature Reviews Microbiology*, **17**: 51–62.

Bierowiec, K., Korzeniowska-Kowal, A., Wzorek, A., Rypuła, K., Gamian, A. (2019). Prevalence of *Staphylococcus* Species Colonization in Healthy and Sick Cats. *BioMed Research International*, **2019**: e4360525.

Bitrus, A.A., Zunita, Z., Bejo, S.K., Othman, S., Nadzir, N.A.A. (2017). In vitro transfer of methicillin resistance determinants mecA from methicillin resistant *Staphylococcus aureus* (MRSA) to methicillin susceptible *Staphylococcus aureus* (MSSA). *BMC Microbiology*, **17**: 83.

Brakstad, O.G., Aasbakk, K., Maeland, J.A. (1992). Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. *Journal of Clinical Microbiology*, **30**: 1654–1660.

Brown, L.B., Krysiak, R., Kamanga, G., Mapanje, C., Kanyamula, H., Banda, B., Mhango, C., Hoffman, M., Kamwendo, D., Hobbs, M., Hosseinipour, M.C., Martinson, F., Cohen, M.S., Hoffman, I.F. (2010). Neisseria gonorrhoeae Antimicrobial Susceptibility in Lilongwe, Malawi, 2007. *Sexually Transmitted Diseases*, **37**: 169.

Bryson, A.L., Hill, E.M., Doern, C.D. (2019). Matrix-Assisted Laser Desorption/Ionization Time-of-Flight: The Revolution in Progress. *Clinics in Laboratory Medicine*, **39**: 391–404.

Bur, S., Preissner, K.T., Herrmann, M., Bischoff, M. (2013). The *Staphylococcus aureus* Extracellular Adherence Protein Promotes Bacterial Internalization by Keratinocytes Independent of Fibronectin-Binding Proteins. *Journal of Investigative Dermatology*, **133**: 2004–2012.

Cardoso, T., Ribeiro, O., Aragão, I.C., Costa-Pereira, A., Sarmento, A.E. (2012). Additional risk factors for infection by multidrug-resistant pathogens in healthcare-associated infection: a large cohort study. *BMC Infectious Diseases*, **12**: 375.

Dunyach-Remy, Ngba Essebe, C., Sotto, A., Lavigne, J.P. (2016). *Staphylococcus aureus* Toxins and Diabetic Foot Ulcers: Role in Pathogenesis and Interest in Diagnosis. *Toxins*, **8**: 209.

Chakolwa, G., Samutela, M.T., Kwenda, G., Mulundu, G., Mwansa, J., Hang'ombe, B.M., Simulundu, E., Lukwesa, C. (2019). Carriage rate and antimicrobial resistance profiles of *Staphylococcus aureus* among healthcare workers at a large tertiary referral hospital in Lusaka, Zambia. *Scientific African*, **5**: e00105.

Chakraborty, S.P., Mahapatra, S.K., Roy, S. (2011). Biochemical characters and antibiotic susceptibility of *Staphylococcus aureus* isolates. *Asian Pacific Journal of Tropical Biomedicine*, **1**: 212–216.

Cheung, G.Y.C., Bae, J.S., Otto, M. (2021). Pathogenicity and virulence of *Staphylococcus aureus*. *Virulence*, **12**: 547–569.

Choonara, F.E., Haldorsen, B.C., Ndhlovu, I., Saulosi, O., Maida, T., Lampiao, F., Simonsen, G.S., Essack, S.Y., Sundsfjord, A. (2022). Antimicrobial susceptibility profiles of clinically important bacterial pathogens at the Kamuzu Central Hospital in Lilongwe, Malawi. *Malawi Medical Journal*, **34**: 9–16.

Confidence, N., Anyanwu, J., John, W. (2013). Conventional and rapid methods for identification of *Staphylococcus aureus* from clinical specimens. *American Journal of Biomedical and Life Sciences*, **3**: 41–43.

Cong, Y., Yang, S., Rao, X. (2020). Vancomycin resistant *Staphylococcus aureus* infections: A review of case updating and clinical features. *Journal of Advanced Research*, **21**: 169–176.

Cornick, J.E., Harris, S.R., Parry, C.M., Moore, M.J., Jassi, C., Kamng'ona, A., Kulohoma, B., Heyderman, R.S., Bentley, S.D., Everett, D.B. (2014). Genomic identification of a novel co-trimoxazole resistance genotype and its prevalence amongst *Streptococcus pneumoniae* in Malawi. *Journal of Antimicrobial Chemotherapy*, **69**: 368–374.

Costa, A.R., Batistão, D.W.F., Ribas, R.M., Sousa, A.M., Pereira, M.O., Botelho, C.M. (2013). *Staphylococcus aureus* Virulence Factors and Disease. *Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education*, **1**: 702-710.

Dashti, A., Jadaon, M., Abdulsamad, A., Dashti, H. (2009). Heat Treatment of Bacteria: A Simple Method of DNA Extraction for Molecular Techniques. *Kuwait Medical Journal*, **41**.

David, M.Z., Daum, R.S. (2017). Treatment of *Staphylococcus aureus* Infections. In: Bagnoli, F., Rappuoli, R., & Grandi, G. (eds) *Staphylococcus aureus: Microbiology, Pathology, Immunology, Therapy and Prophylaxis*. Current Topics in Microbiology and Immunology. Springer International Publishing, Cham, pp. 325–383.

Di Gregorio, S., Haim, M.S., Vielma Vallenilla, J., Cohen, V., Rago, L., Gulone, L., Aanensen, D.M., Argimón, S., Mollerach, M. (2021). Genomic Epidemiology of CC30 Methicillin-Resistant *Staphylococcus aureus* Strains from Argentina Reveals Four Major Clades with Distinctive Genetic Features. *mSphere*, **6**: e01297-20.

Dixon, J., MacPherson, E.E., Nayiga, S., Manyau, S., Nabirye, C., Kayendeke, M., Sanudi, E., Nkaombe, A., Mareke, P., Sitole, K., Hutchison, C. de L., Bradley, J., Yeung, S., Ferrand, R.A., Lal, S., Roberts, C., Green, E., Willis, L.D., Staedke, S.G., Chandler, C.I.R. (2021). Antibiotic stories: a mixed-methods, multi-country analysis of household antibiotic use in Malawi, Uganda and Zimbabwe. *BMJ Global Health*, **6**: e006920.

Duza, S.S. (2021). Evaluation of Virulence Properties of *Staphylococcus aureus* Isolated from A Tertiary Level Hospital of Dhaka City. Thesis, University of Dhaka.

Egyir, B., Guardabassi, L., Sørum, M., Nielsen, S.S., Kolekang, A., Frimpong, E., Addo, K.K., Newman, M.J., Larsen, A.R. (2014). Molecular Epidemiology and Antimicrobial Susceptibility of Clinical *Staphylococcus aureus* from Healthcare Institutions in Ghana. *PloS One*, **9**: e89716.

Epstein, L., Mu, Y., Belflower, R., Scott, J., Ray, S., Dumyati, G., Felsen, C., Petit, S., Yousey-Hindes, K., Nadle, J., Pasutti, L., Lynfield, R., Warnke, L., Schaffner, W., Leib, K., Kallen, A.J., Fridkin, S.K., Lessa, F.C. (2016). Risk Factors for Invasive Methicillin-Resistant *Staphylococcus aureus* Infection After Recent Discharge From an Acute-Care Hospitalization, 2011–2013. *Clinical Infectious Diseases*, **62**: 45–52.

Falagas, M.E., Karageorgopoulos, D.E., Leptidis, J., Korbila, I.P. (2013). MRSA in Africa: filling the global map of antimicrobial resistance. *PloS One*, **8**: e68024.

Feng, Y., Chen, C.-J., Su, L.-H., Hu, S., Yu, J., Chiu, C.-H. (2008). Evolution and pathogenesis of *Staphylococcus aureus*: lessons learned from genotyping and comparative genomics. *FEMS Microbiology Reviews*, **32**: 23–37.

Fergestad, M.E., Stamsås, G.A., Morales Angeles, D., Salehian, Z., Wasteson, Y., Kjos, M. (2020). Penicillin-binding protein PBP2a provides variable levels of protection toward different β-lactams in *Staphylococcus aureus* RN4220. *Microbiology Open*, **9**: e1057.

Fisher, J.F., Mobashery, S. (2021). β-Lactams against the Fortress of the Gram-Positive *Staphylococcus aureus* Bacterium. *Chemical Reviews*, **121**: 3412–3463.

Foster, G., Ross, H.M., Hutson, R.A., Collins, M.D.Y. 1997. Staphylococcus lutrae sp. nov., a New Coagulase-Positive Species Isolated from Otters. *International Journal of Systematic and Evolutionary Microbiology*, **47**: 724–726.

Foster, T.J. (2017). Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. *FEMS Microbiology Reviews*, **41**: 430–449.

Fri, J., Ndip, R.N., Njom, H.A., Clarke, A.M. (2018). First report of methicillin-resistant *Staphylococcus aureus* in tank cultured dusky kob (*Argyrosomus japonicus*), and evaluation of three phenotypic methods in the detection of MRSA. *Journal of Food Safety*, **38**: e12411.

Gajic, I., Kabic, J., Kekic, D., Jovicevic, M., Milenkovic, M., Mitic Culafic, D., Trudic, A., Ranin, L., Opavski, N. (2022). Antimicrobial Susceptibility Testing: A Comprehensive Review of Currently Used Methods. *Antibiotics*, **11**: 427.

Gallaher, J.R., Banda, W., Lachiewicz, A.M., Krysiak, R., Cairns, B.A., Charles, A.G. (2018). Colonization with Multidrug-Resistant Enterobacteriaceae is Associated with Increased Mortality Following Burn Injury in Sub-Saharan Africa. *World Journal of Surgery*, **42**: 3089–3096.

Gatadi, S., Madhavi, Y.V., Chopra, S., Nanduri, S. (2019). Promising antibacterial agents against multidrug resistant *Staphylococcus aureus*. *Bioorganic Chemistry*, **92**: 103252.

Ge, B., Mukherjee, S., Hsu, C.-H., Davis, J.A., Tran, T.T.T., Yang, Q., Abbott, J.W., Ayers, S.L., Young, S.R., Crarey, E.T., Womack, N.A., Zhao, S., McDermott, P.F. (2017). MRSA

and multidrug-resistant *Staphylococcus aureus* in U.S. retail meats, 2010–2011. *Food Microbiology*, **62**: 289–297.

Geoghegan, J.A., Dufrêne, Y.F. (2018). Mechanomicrobiology: How Mechanical Forces Activate *Staphylococcus aureus* Adhesion. *Trends in Microbiology*, **26**: 645–648.

Ghasemian, A., Najar Peerayeh, S., Bakhshi, B., Mirzaee, M. (2015). The Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) Genes among Clinical Isolates of *Staphylococcus aureus* from Hospitalized Children. *Iranian Journal of Pathology*, **10**: 258–264.

Ghaznavi-Rad, E., Ekrami, A. (2018). Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* Isolates, Isolated from a Burn Hospital in Southwest Iran in 2006 and 2014. *International Journal of Microbiology*, **2018**: e1423939.

Gómez, P., González-Barrio, D., Benito, D., García, J.T., Viñuela, J., Zarazaga, M., Ruiz-Fons, F., Torres, C. (2014). Detection of methicillin-resistant *Staphylococcus aureus* (MRSA) carrying the mecC gene in wild small mammals in Spain. *Journal of Antimicrobial Chemotherapy*, **69**: 2061–2064.

González-Domínguez, M.S., Carvajal, H.D., Calle-Echeverri, D.A., Chinchilla-Cárdenas, D. (2020). Molecular Detection and Characterization of the mecA and nuc Genes From Staphylococcus Species (*S. aureus, S. pseudintermedius*, and *S. schleiferi*) Isolated From Dogs Suffering Superficial Pyoderma and Their Antimicrobial Resistance Profiles. *Frontiers in Veterinary Science*, **7**.

Gordon, N., Amos, B., Azore, E., Bastiaens, G., Buhler, C., Issah, S., Jorakate, P., Raghu, S., Leslie, T. (2020). The UK Fleming Fund: Developing microbiology laboratory capacity for AMR surveillance. *International Journal of Infectious Diseases*, **101**: 86.

Goudarzi, M., Abiri, P., Nasirian, S., Afshari, S.G. (2018). SCCmec and spa Typing of *Staphylococcus aureus* Strains Isolated from Patients with Urinary Tract Infection: Emergence of spa Types t426 and t021 in Iran. *Jundishapur Journal of Microbiology*, **11**.

Goudarzi, M., Fazeli, M., Goudarzi, H., Azad, M., Seyedjavadi, S.S. (2016). Spa Typing of *Staphylococcus aureus* Strains Isolated From Clinical Specimens of Patients With Nosocomial Infections in Tehran, Iran. *Jundishapur Journal of Microbiology*, **9**: e35685.

Gökmen, T., Kalayci, Y., Yaman, A., Köksal, F. (2018). Molecular characterization of methicillin-resistant *Staphylococcus aureus* strains by spa typing and pulsed field gel electrophoresis methods. *BMC Microbiology*, **18**: 155.

Haaber, J., Penadés, J.R., Ingmer, H. (2017). Transfer of Antibiotic Resistance in *Staphylococcus aureus*. *Trends in Microbiology*, **25**: 893–905.

Haag, A.F., Fitzgerald, J.R., Penadés, J.R. (2019). *Staphylococcus aureus* in Animals. *Microbiology Spectrum*, **7**: 7.3.11.

Habib, F., Rind, R., Durani, N., Bhutto, A.L., Buriro, R.S., Tunio, A., Aijaz, N., Lakho, S.A., Bugti, A.G., Shoaib, M. (2015). Morphological and Cultural Characterization of *Staphylococcus aureus* Isolated from Different Animal Species. *Journal of Applied Environmental and Biological Sciences*,***5***: 15-26

Haddad, O., Merghni, A., Elargoubi, A., Rhim, H., Kadri, Y., Mastouri, M. (2018). Comparative study of virulence factors among methicillin resistant *Staphylococcus aureus* clinical isolates. *BMC Infectious Diseases*, **18**: 560.

Hagstrand Aldman, M., Skovby, A., I. Påhlman, L. (2017). Penicillin-susceptible *Staphylococcus aureus*: susceptibility testing, resistance rates and outcome of infection. *Infectious Diseases*, **49**: 454–460.

Haim, M.S., Zaheer, R., Bharat, A., Gregorio, S.D., Conza, J.D., Galanternik, L., Lubovich, S., Golding, G.R., Graham, M.R., Domselaar, G.V., Cardona, S.T., Mollerach, M. (2021). Comparative genomics of ST5 and ST30 methicillin-resistant *Staphylococcus aureus* sequential isolates recovered from paediatric patients with cystic fibrosis. *Microbial Genomics*, **7**.

Hammerschmidt, S., Rohde, M., Preissner, K.T. (2019). Extracellular Matrix Interactions with Gram-Positive Pathogens. *Microbiology Spectrum*, **7**: 7.2.36.

Harkins, C.P., Pichon, B., Doumith, M., Parkhill, J., Westh, H., Tomasz, A., de Lencastre, H., Bentley, S.D., Kearns, A.M., Holden, M.T.G. (2017). Methicillin-resistant *Staphylococcus aureus* emerged long before the introduction of methicillin into clinical practice. *Genome Biology*, **18**: 130.

Harris, C., Mills, R., Seager, E., Blackstock, S., Hiwa, T., Pumphrey, J., Langton, J., Kennedy, N. (2019). Paediatric deaths in a tertiary government hospital setting, Malawi. *Paediatrics and International Child Health*, **39**: 240–248.

Hassoun, A., Linden, P.K., Friedman, B. (2017). Incidence, prevalence, and management of MRSA bacteremia across patient populations—a review of recent developments in MRSA management and treatment. *Critical Care*, **21**: 211.

Hiramatsu, K., Katayama, Y., Matsuo, M., Sasaki, T., Morimoto, Y., Sekiguchi, A., Baba, T. (2014). Multi-drug-resistant *Staphylococcus aureus* and future chemotherapy. *Journal of Infection and Chemotherapy*, **20**: 593–601.

Horino, T., Hori, S. (2020). Metastatic infection during *Staphylococcus aureus* bacteremia. *Journal of Infection and Chemotherapy*, **26**: 162–169.

Ibrahim, O.M.A., Bilal, N.E., Azoz, M.E.H., Eltahir, H.B. (2019). Coagulase gene polymorphisms of *Staphylococcus aureus* isolates from patients at Kosti Teaching Hospital, Sudan. *Access Microbiology*, **1**: e000026.

Tam, P.-Y., Musicha, P., Kawaza, K., Cornick, J., Denis, B., Freyne, B., Everett, D., Dube, Q., French, N., Feasey, N., Heyderman, R. (2019). Emerging Resistance to Empiric Antimicrobial Regimens for Pediatric Bloodstream Infections in Malawi (1998–2017). *Clinical Infectious Diseases*, **69**: 61–68.

Ishihara, K., Saito, M., Shimokubo, N., Muramatsu, Y., Maetani, S., Tamura, Y. (2014). Methicillin-resistant *Staphylococcus aureus* carriage among veterinary staff and dogs in private veterinary clinics in Hokkaido, Japan. *Microbiology and Immunology*, **58**: 149–154.

Jame, W., Basgut, B., Abdi, A. (2021). Efficacy and safety of novel glycopeptides versus vancomycin for the treatment of gram-positive bacterial infections including methicillin resistant *Staphylococcus aureus*: A systematic review and meta-analysis. *PLOS ONE*, **16**: e0260539.

Kadlec, K., Schwarz, S. (2012). Antimicrobial resistance of *Staphylococcus pseudintermedius*. *Veterinary Dermatology*, **23**: 276-e55.

Kali, A., Stephen, S., Umadevi, S. (2014). Laboratory evaluation of phenotypic detection methods of methicillin-resistant *Staphylococcus aureus*. *Biomedical Journal*, **37**: 411–414.

Karmakar, A., Dua, P., Ghosh, C. (2016). Biochemical and Molecular Analysis of *Staphylococcus aureus* Clinical Isolates from Hospitalized Patients. *Canadian Journal of Infectious Diseases and Medical Microbiology*, **2016**: e9041636.

Kateete, D.P., Bwanga, F., Seni, J., Mayanja, R., Kigozi, E., Mujuni, B., Ashaba, F.K., Baluku, H., Najjuka, C.F., Källander, K., Rutebemberwa, E., Asiimwe, B.B., Joloba, M.L. (2019). CA-MRSA and HA-MRSA coexist in community and hospital settings in Uganda. *Antimicrobial Resistance & Infection Control*, **8**: 94.

Kateete, D.P., Kimani, C.N., Katabazi, F.A., Okeng, A., Okee, M.S., Nanteza, A., Joloba, M.L., Najjuka, F.C. (2010). Identification of *Staphylococcus aureus*: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. *Annals of Clinical Microbiology and Antimicrobials*, **9**: 23.

Khan, Z.A., Siddiqui, M.F., Park, S. (2019). Current and Emerging Methods of Antibiotic Susceptibility Testing. *Diagnostics*, **9**: 49.

Knox, J., Uhlemann, A.-C., Lowy, F.D. (2015). *Staphylococcus aureus* infections: transmission within households and the community. *Trends in Microbiology*, **23**: 437–444.

Kumwenda, P., Adukwu, E.C., Tabe, E.S., Ujor, Victor.C., Kamudumuli, P.S., Ngwira, M., Wu, J.T.S., Chisale, M.R.O. (2021). Prevalence, distribution and antimicrobial susceptibility pattern of bacterial isolates from a tertiary Hospital in Malawi. *BMC Infectious Diseases*, **21**: 34.

LaCour, M., Gleghorn, K., Wilson, J. (2020). The Dermatologist Nose Best: Correlation of Nose-Picking Habits and *Staphylococcus aureus*–Related Dermatologic Disease. *Cutis*, **106**.

Laurens, M.B., Mungwira, R.G., Nampota, N., Nyirenda, O.M., Divala, T.H., Kanjala, M., Mkandawire, F.A., Galileya, L.T., Nyangulu, W., Mwinjiwa, E., Downs, M., Tillman, A., Taylor, T.E., Mallewa, J., Plowe, C.V., van Oosterhout, J.J., Laufer, M.K. (2021). Revisiting Co-trimoxazole Prophylaxis for African Adults in the Era of Antiretroviral Therapy: A Randomized Controlled Clinical Trial. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, **73**: 1058–1065.

Laurent, F., Chardon, H., Haenni, M., Bes, M., Reverdy, M.-E., Madec, J.-Y., Lagier, E., Vandenesch, F., Tristan, A. (2012). MRSA Harboring mecA Variant Gene mecC, France. *Emerging Infectious Diseases*, **18**: 1465–1467.

Lee, A.S., de Lencastre, H., Garau, J., Kluytmans, J., Malhotra-Kumar, S., Peschel, A., Harbarth, S. (2018). Methicillin-resistant *Staphylococcus aureus*. *Nature Reviews Disease Primers*, **4**: 1–23.

Lim, C., Takahashi, E., Hongsuwan, M., Wuthiekanun, V., Thamlikitkul, V., Hinjoy, S., Day, N.P., Peacock, S.J., Limmathurotsakul, D. (2016). Epidemiology and burden of multidrug-resistant bacterial infection in a developing country. Abdool Karim, Q. (ed). *eLife*, **5**: e18082.

Lindsay, J.A. (2014). *Staphylococcus aureus* genomics and the impact of horizontal gene transfer. *International Journal of Medical Microbiology*, **304**: 103–109.

Liu, J., Chen, D., Peters, B.M., Li, L., Li, B., Xu, Z., Shirliff, M.E. (2016). Staphylococcal chromosomal cassettes mec (SCCmec): A mobile genetic element in methicillin-resistant *Staphylococcus aureus*. *Microbial Pathogenesis*, **101**: 56–67.

López, M., Kadlec, K., Schwarz, S., Torres, C. (2012). First Detection of the Staphylococcal Trimethoprim Resistance Gene dfrK and the dfrK-Carrying Transposon Tn559 in Enterococci. *Microbial Drug Resistance*, **18**: 13–18.

Lozano, C., Fernández-Fernández, R., Ruiz-Ripa, L., Gómez, P., Zarazaga, M., Torres, C. (2020). Human mecC-Carrying MRSA: Clinical Implications and Risk Factors. *Microorganisms*, **8**: 1615.

Maayan-Metzger, A., Strauss, T., Rubin, C., Jaber, H., Dulitzky, M., Reiss-Mandel, A., Leshem, E., Rahav, G., Regev-Yochay, G. (2017). Clinical evaluation of early acquisition of *Staphylococcus aureus* carriage by newborns. *International Journal of Infectious Diseases*, **64**: 9–14.

Makoka, M.H., Miller, W.C., Hoffman, I.F., Cholera, R., Gilligan, P.H., Kamwendo, D., Malunga, G., Joaki, G., Martinson, F., Hosseinipour, M.C. (2012). Bacterial infections in Lilongwe, Malawi: aetiology and antibiotic resistance. *BMC Infectious Diseases*, **12**: 67.

Mankhomwa, J., Tolhurst, R., M'biya, E., Chikowe, I., Banda, P., Mussa, J., Mwasikakata, H., Simpson, V., Feasey, N., MacPherson, E.E. (2022). A Qualitative Study of Antibiotic Use Practices in Intensive Small-Scale Farming in Urban and Peri-Urban Blantyre, Malawi: Implications for Antimicrobial Resistance. *Frontiers in Veterinary Science*, **9**: 876513.

Manukumar, H.M., Umesha, S. (2017). MALDI-TOF-MS based identification and molecular characterization of food associated methicillin-resistant *Staphylococcus aureus*. *Scientific Reports*, **7**: 11414.

Marek, A., Pyzik, E., Stępień-Pyśniak, D., Dec, M., Jarosz, Ł.S., Nowaczek, A., Sulikowska, M. (2021). Biofilm-Formation Ability and the Presence of Adhesion Genes in Coagulase-Negative Staphylococci Isolates from Chicken Broilers. *Animals*, **11**: 728.

Matsuoka, M., Inoue, M., Nakajima, Y., Endo, Y. (2002). New erm Gene in *Staphylococcus aureus* Clinical Isolates. *Antimicrobial Agents and Chemotherapy*, **46**: 211–215.

Matta, R., Hallit, S., Hallit, R., Bawab, W., Rogues, A.-M., Salameh, P. (2018). Epidemiology and microbiological profile comparison between community and hospital acquired infections: A multicenter retrospective study in Lebanon. *Journal of Infection and Public Health*, **11**: 405–411.

McHugh, M.P., Parcell, B.J., MacKenzie, F.M., Templeton, K.E., Scottish Microbiology and Virology Network (SMVN) Molecular Diagnostics Evaluation GroupY .(2020). Rapid

molecular testing for *Staphylococcus aureus* bacteraemia improves clinical management. *Journal of Medical Microbiology*, **69**: 552–557.

Meade, E., Slattery, M.A., Garvey, M. (2021). Biocidal Resistance in Clinically Relevant Microbial Species: A Major Public Health Risk. *Pathogens*, **10**: 598.

Miguel, C.P.V., Mejias, A., Leber, A., Sanchez, P.J. (2019). A decade of antimicrobial resistance in *Staphylococcus aureus*: A single center experience. *PLOS ONE*, **14**: e0212029.

Miller, J.M., Binnicker, M.J., Campbell, S., Carroll, K.C., Chapin, K.C., Gilligan, P.H., Gonzalez, M.D., Jerris, R.C., Kehl, S.C., Patel, R., Pritt, B.S., Richter, S.S., Robinson-Dunn, B., Schwartzman, J.D., Snyder, J.W., Telford, S., III, Theel, E.S., Thomson, R.B., Jr, Weinstein, M.P., Yao, J.D. (2018). A Guide to Utilization of the Microbiology Laboratory for Diagnosis of Infectious Diseases: 2018 Update by the Infectious Diseases Society of America and the American Society for Microbiologya. *Clinical Infectious Diseases*, **67**: e1–e94.

Missiakas, D.M., Schneewind, O. (2013). Growth and Laboratory Maintenance of *Staphylococcus aureus*. *Current Protocols in Microbiology*, **28**: 9C.1.1-9C.1.9.

Mistry, H., Sharma, P., Mahato, S., Saravanan, R., Kumar, P.A., Bhandari, V. (2016). Prevalence and Characterization of Oxacillin Susceptible mecA-Positive Clinical Isolates of *Staphylococcus aureus* Causing Bovine Mastitis in India. *PLOS ONE*, **11**: e0162256.

Montazeri, E.A., Khosravi, A.D., Jolodar, A., Ghaderpanah, M., Azarpira, S. (2015). Identification of methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from burn patients by multiplex PCR. *Burns*, **41**: 590–594.

Musicha, P., Cornick, J.E., Bar-Zeev, N., French, N., Masesa, C., Denis, B., Kennedy, N., Mallewa, J., Gordon, M.A., Msefula, C.L., Heyderman, R.S., Everett, D.B., Feasey, N.A. (2017). Trends in antimicrobial resistance in bloodstream infection isolates at a large urban hospital in Malawi (1998–2016): a surveillance study. *The Lancet Infectious Diseases*, **17**: 1042–1052.

Nagel, M., Dischinger, J., Türck, M., Verrier, D., Oedenkoven, M., Ngoubangoye, B., Le Flohic, G., Drexler, J.F., Bierbaum, G., Gonzalez, J.-P. (2013). Human-associated

Staphylococcus aureus strains within great ape populations in Central Africa (Gabon). *Clinical Microbiology and Infection*, **19**: 1072–1077.

Naorem, R.S., Urban, P., Goswami, G., Fekete, C. (2020). Characterization of methicillin-resistant *Staphylococcus aureus* through genomics approach. *3 Biotech*, **10**: 401.

Nurjadi, D., Olalekan, A.O., Layer, F., Shittu, A.O., Alabi, A., Ghebremedhin, B., Schaumburg, F., Hofmann-Eifler, J., Van Genderen, P.J.J., Caumes, E., Fleck, R., Mockenhaupt, F.P., Herrmann, M., Kern, W.V., Abdulla, S., Grobusch, M.P., Kremsner, P.G., Wolz, C., Zanger, P. (2014). Emergence of trimethoprim resistance gene dfrG in *Staphylococcus aureus* causing human infection and colonization in sub-Saharan Africa and its import to Europe. *Journal of Antimicrobial Chemotherapy*, **69**: 2361–2368.

Oladipo, J.A. (2014). Utilization of health care services in rural and urban areas: A determinant factor in planning and managing health care delivery systems. *African Health Sciences*, **14**: 322–333.

Onduru, O.G., Aboud, S., Nyirenda, T.S., Rumisha, S.F., Mkakosya, R.S. (2021). Antimicrobial susceptibility testing profiles of ESBL-producing Enterobacterales isolated from hospital and community adult patients in Blantyre, Malawi. *IJID Regions*, **1**: 47–52.

Otto, M. (2014). *Staphylococcus aureus* toxins. *Current Opinion in Microbiology*, **17**: 32–37.

Paharik, A.E., Salgado-Pabon, W., Meyerholz, D.K., White, M.J., Schlievert, P.M., Horswill, A.R. (2016). The Spl Serine Proteases Modulate *Staphylococcus aureus* Protein Production and Virulence in a Rabbit Model of Pneumonia. *mSphere*, **1**: e00208-16.

Panchal, V.V., Griffiths, C., Mosaei, H., Bilyk, B., Sutton, J.A.F., Carnell, O.T., Hornby, D.P., Green, J., Hobbs, J.K., Kelley, W.L., Zenkin, N., Foster, S.J. (2020). Evolving MRSA: High-level β-lactam resistance in *Staphylococcus aureus* is associated with RNA Polymerase alterations and fine tuning of gene expression. *PLOS Pathogens*, **16**: e1008672.

Park, J.-H., Lee, J.-H., Cho, M.H., Herzberg, M., Lee, J. (2012). Acceleration of protease effect on *Staphylococcus aureus* biofilm dispersal. *FEMS Microbiology Letters*, **335**: 31–38.

Petinaki, E., Arvaniti, A., Dimitracopoulos, G., Spiliopoulou, I. (2001). Detection of mecA, mecR1 and mecI genes among clinical isolates of methicillin-resistant staphylococci by combined polymerase chain reactions. *The Journal of Antimicrobial Chemotherapy*, **47**: 297–304.

Price, J.R., Cole, K., Bexley, A., Kostiou, V., Eyre, D.W., Golubchik, T., Wilson, D.J., Crook, D.W., Walker, A.S., Peto, T.E.A., Llewelyn, M.J., Paul, J. (2017). Transmission of *Staphylococcus aureus* between health-care workers, the environment, and patients in an intensive care unit: a longitudinal cohort study based on whole-genome sequencing. *The Lancet Infectious Diseases*, **17**: 207–214.

Prin, M., Itaye, T., Clark, S., Fernando, R.J., Namboya, F., Pollach, G., Mkandawire, N., Sobol, J. (2016). Critical Care in a Tertiary Hospital in Malawi. *World Journal of Surgery*, **40**: 2635–2642.

Pumipuntu, N., Kulpeanprasit, S., Santajit, S., Tunyong, W., Kong-ngoen, T., Hinthong, W., Indrawattana, N. (2017). Screening method for *Staphylococcus aureus* identification in subclinical bovine mastitis from dairy farms. *Veterinary World*, **10**: 721–726.

Raineri, E.J.M., Yedavally, H., Salvati, A., van Dijl, J.M. (2020). Time-resolved analysis of *Staphylococcus aureus* invading the endothelial barrier. *Virulence*, **11**: 1623–1639.

Rakotovao-Ravahatra, Z.D., Randriatsarafara, F.M., Milasoanjara, R.N., Ranaivosoa, M.K., Rakotovao, A.L., Rasamindrakotroka, A. (2019). Assessment of the Coagulase Test in the Identification of *Staphylococcus aureus* Strains. *Journal of Biotechnology and Biomedicine*, **2**: 105–111.

Reygaert, W.C. (2013). Antimicrobial resistance mechanisms of *Staphylococcus aureus*. *Microbial pathogens and strategies for combating them: science, technology and education*, **1**: 297-305.

Rongpharpi, S.R., Hazarika, N.K., Kalita, H. (2013). The Prevalence of Nasal Carriage of *Staphylococcus aureus* Among Healthcare Workers at a Tertiary Care Hospital in Assam with Special Reference to MRSA. *Journal of Clinical and Diagnostic Research: JCDR*, **7**: 257–260.

Ruffing, U., Alabi, A., Kazimoto, T., Vubil, D.C., Akulenko, R., Abdulla, S., Alonso, P., Bischoff, M., Germann, A., Grobusch, M.P., Helms, V., Hoffmann, J., Kern, W.V., Kremsner, P.G., Mandomando, I., Mellmann, A., Peters, G., Schaumburg, F., Schubert, S., Strauß, L., Tanner, M., Briesen, H. von, Wende, L., Müller, L. von, Herrmann, M. (2017). Community-Associated *Staphylococcus aureus* from Sub-Saharan Africa and Germany: A Cross-Sectional Geographic Correlation Study. *Scientific Reports*, **7**: 154.

Russell, A., Secrest, J., Schreeder, C. (2012). Stethoscopes as a Source of Hospital-Acquired Methicillin-Resistant *Staphylococcus aureus*. *Journal of PeriAnesthesia Nursing*, **27**: 82–87.

Sabat, A.J., Budimir, A., Nashev, D., Sá-Leão, R., Dijl, J.M. van, Laurent, F., Grundmann, H., Friedrich, A.W., Markers (ESGEM), on behalf of the E.S.G. of E. (2013). Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Eurosurveillance*, **18**: 20380.

Saha, M., Sarkar, A. (2021). Review on Multiple Facets of Drug Resistance: A Rising Challenge in the 21st Century. *Journal of Xenobiotics*, **11**: 197–214.

Sahebnasagh, R., Saderi, H., Owlia, P. (2014). The Prevalence of Resistance to Methicillin in *Staphylococcus aureus* Strains Isolated from Patients by PCR Method for Detec-tion of mecA and nuc Genes. *Iranian Journal of Public Health*, **43**: 84–92.

Sahukhal, G.S., Elasri, M.O. (2014). Identification and characterization of an operon, msaABCR, that controls virulence and biofilm development in *Staphylococcus aureus*. *BMC Microbiology*, **14**: 154.

Sambakusi, C.S. (2019). Knowledge, attitudes and practices related to self-medication with antimicrobials in Lilongwe, Malawi. *Malawi Medical Journal*, **31**: 225–232.

Samutela, M.T., Kalonda, A., Mwansa, J., Lukwesa-Musyani, C., Mwaba, J., Mumbula, E.M., Mwenya, D., Simulundu, E., Kwenda, G. (2017). Molecular characterisation of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated at a large referral hospital in Zambia. *The Pan African Medical Journal*, **26**: 108.

Sanchini, A. (2022). Recent Developments in Phenotypic and Molecular Diagnostic Methods for Antimicrobial Resistance Detection in *Staphylococcus aureus*: A Narrative Review. *Diagnostics*, **12**: 208.

Saunders, N.A., Holmes, A. (2014). Multilocus Sequence Typing (MLST) of *Staphylococcus aureus*. In: Ji, Y. (ed) *Methicillin-Resistant Staphylococcus aureus (MRSA) Protocols*. Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 113–130.

Schaumburg, F., Alabi, A.S., Peters, G., Becker, K. (2014). New epidemiology of *Staphylococcus aureus* infection in Africa. *Clinical Microbiology and Infection*, **20**: 589–596.

Schmitz, F.-J., Krey, A., Sadurski, R., Verhoef, J., Milatovic, D., Fluit, A.C., for the European SENTRY participants. (2001). Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. *Journal of Antimicrobial Chemotherapy*, **47**: 239–240.

Seni, J., Bwanga, F., Najjuka, C.F., Makobore, P., Okee, M., Mshana, S.E., Kidenya, B.R., Joloba, M.L., Kateete, D.P. (2013). Molecular Characterization of *Staphylococcus aureus* from Patients with Surgical Site Infections at Mulago Hospital in Kampala, Uganda. *PloS One*, **8**: e66153.

Shallcross, L.J., Fragaszy, E., Johnson, A.M., Hayward, A.C. (2013). The role of the Panton-Valentine leucocidin toxin in staphylococcal disease: a systematic review and meta-analysis. *The Lancet Infectious Diseases*, **13**: 43–54.

Sharifipour, E., Shams, S., Esmkhani, M., Khodadadi, J., Fotouhi-Ardakani, R., Koohpaei, A., Doosti, Z., EJ Golzari, S. (2020). Evaluation of bacterial co-infections of the respiratory tract in COVID-19 patients admitted to ICU. *BMC Infectious Diseases*, **20**: 646.

Sharma, S.K., Mudgal, S.K., Thakur, K., Gaur, R. (2019). How to calculate sample size for observational and experimental nursing research studies? *National Journal of Physiology, Pharmacy and Pharmacology*, **10**: 1–1.

Shibabaw, A., Abebe, T., Mihret, A. (2013). Nasal carriage rate of methicillin resistant *Staphylococcus aureus* among Dessie Referral Hospital Health Care Workers; Dessie, Northeast Ethiopia. *Antimicrobial Resistance and Infection Control*, **2**: 25.

Shokrollahi, P., Hasani, Alka, Aghazadeh, M., Memar, M.Y., Hasani, Akbar, Zaree, M., Rezaee, M.A., Sadeghi, J. (2022). Contribution of Arginine Catabolic Mobile Element and Copper and Mercury Resistance Element in Methicillin-Resistant *Staphylococcus aureus*: A Vantage Point. *Canadian Journal of Infectious Diseases and Medical Microbiology*, **2022**: e9916255.

Shopsin, B., Gomez, M., Montgomery, S.O., Smith, D.H., Waddington, M., Dodge, D.E., Bost, D.A., Riehman, M., Naidich, S., Kreiswirth, B.N. (1999). Evaluation of Protein A Gene Polymorphic Region DNA Sequencing for Typing of *Staphylococcus aureus* Strains. *Journal of Clinical Microbiology*, **37**: 3556–3563.

Shore, A.C., Coleman, D.C. (2013). Staphylococcal cassette chromosome mec: Recent advances and new insights. *International Journal of Medical Microbiology*, **303**: 350–359.

Shukla, S.K., Pantrang, M., Stahl, B., Briska, A.M., Stemper, M.E., Wagner, T.K., Zentz, E.B., Callister, S.M., Lovrich, S.D., Henkhaus, J.K., Dykes, C.W. (2012). Comparative Whole-Genome Mapping To Determine *Staphylococcus aureus* Genome Size, Virulence Motifs, and Clonality. *Journal of Clinical Microbiology*, **50**: 3526–3533.

Silva, V., Capelo, J.L., Igrejas, G., Poeta, P. (2020). Molecular Epidemiology of *Staphylococcus aureus* Lineages in Wild Animals in Europe: A Review. *Antibiotics*, **9**: 122.

Singh, V., Phukan, U.J. (2019). Interaction of host and *Staphylococcus aureus* protease-system regulates virulence and pathogenicity. *Medical Microbiology and Immunology*, **208**: 585–607.).

Sizar, O. and Unakal, C.G., 2022. Gram Positive Bacteria. 2021 Feb 1. *Stat Pearls [ncbi.nlm.nih.gov/books/NBK470553]*. *Treasure Island (FL): Stat Pearls Publishing*. Accessed 20 February,2022.

Skov, R., Larsen, A.R., Kearns, A., Holmes, M., Teale, C., Edwards, G., Hill, R. (2014). Phenotypic detection of mecC-MRSA: cefoxitin is more reliable than oxacillin. *Journal of Antimicrobial Chemotherapy*, **69**: 133–135.

Song, Y., Du, X., Li, T., Zhu, Y., Li, M. 2013. (2013). Phenotypic and molecular characterization of *Staphylococcus aureus* recovered from different clinical specimens of inpatients at a teaching hospital in Shanghai between 2005 and 2010. *Journal of Medical Microbiology*, **62**: 274–282.

Soni, I., Chakrapani, H., Chopra, S. (2015). Draft Genome Sequence of Methicillin-Sensitive *Staphylococcus aureus* ATCC 29213. *Genome Announcements*, **3**: e01095-15.

Stastkova, Z., Karpiskova, S., Karpiskova, R. (2009). Occurrence of methicillin-resistant strains of *Staphylococcus aureus* at a goat breeding farm. *Veterinární medicína*, **54 (2009)**: 419–426.

Stegger, M., Andersen, P.S., Kearns, A., Pichon, B., Holmes, M.A., Edwards, G., Laurent, F., Teale, C., Skov, R., Larsen, A.R. (2012). Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either mecA or the new mecA homologue mecA (LGA251). *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, **18**: 395–400.

Strauß, L., Stegger, M., Akpaka, P.E., Alabi, A., Breurec, S., Coombs, G., Egyir, B., Larsen, A.R., Laurent, F., Monecke, S., Peters, G., Skov, R., Strommenger, B., Vandenesch, F., Schaumburg, F., Mellmann, A. (2017). Origin, evolution, and global transmission of community-acquired *Staphylococcus aureus* ST8. *Proceedings of the National Academy of Sciences*, **114**: E10596–E10604.

Stulik, L., Malafa, S., Hudcova, J., Rouha, H., Henics, B.Z., Craven, D.E., Sonnevend, A.M., Nagy, E. (2014). α-Hemolysin Activity of Methicillin-Susceptible *Staphylococcus aureus* Predicts Ventilator-associated Pneumonia. *American Journal of Respiratory and Critical Care Medicine*, **190**: 1139–1148.

Sutcliffe, J., Grebe, T., Tait-Kamradt, A., Wondrack, L. (1996). Detection of erythromycin-resistant determinants by PCR. *Antimicrobial Agents and Chemotherapy*, **40**: 2562–2566.

Tang, C., Liu, C., Han, Y., Guo, Q., Ouyang, W., Feng, H., Wang, M., Xu, F. (2019). Nontoxic Carbon Quantum Dots/g-C3N4 for Efficient Photocatalytic Inactivation of *Staphylococcus aureus* under Visible Light. *Advanced Healthcare Materials*, **8**: 1801534.

Tigabu, A., Getaneh, A. (2021). *Staphylococcus aureus*, ESKAPE Bacteria Challenging Current Health Care and Community Settings: a Literature Review. *Clinical Laboratory*, **67**.

Tong, S.Y.C., Davis, J.S., Eichenberger, E., Holland, T.L., Fowler, V.G. (2015). *Staphylococcus aureus* Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. *Clinical Microbiology Reviews*, **28**: 603–661.

Trzcinski, K., Cooper, B.S., Hryniewicz, W., Dowson, C.G. (2000). Expression of resistance to tetracyclines in strains of methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, **45**: 763–770.

Turner, N.A., Sharma-Kuinkel, B.K., Maskarinec, S.A., Eichenberger, E.M., Shah, P.P., Carugati, M., Holland, T.L., Fowler, V.G. (2019). Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. *Nature Reviews Microbiology*, **17**: 203–218.

Ullah, F., Malik, S.A., Ahmed, J., Ullah, F., Shah, S.M., Ayaz, M., Hussain, S., Khatoon, L. (2012). Investigation of the Genetic Basis of Tetracycline Resistance in *Staphylococcus aureus* from Pakistan. *Tropical Journal of Pharmaceutical Research*, **11**: 925–931.

Umar, A.I., Garba, I., Ganau, A.M. (2023). Evaluation of Cefoxitin Disc Diffusion and Chromogenic Agar in the Detection of Methicillin Resistant *Staphylococcus aureus*. *South Asian Journal of Research in Microbiology*, **15**: 20–26.

Vaillancourt, M., Jorth, P. (2020). The Unrecognized Threat of Secondary Bacterial Infections with COVID-19. *American Society of Microbiology*, *mBio*, **11**: e01806-20.

Wangai, F.K., Masika, M.M., Maritim, M.C., Seaton, R.A. (2019). Methicillin-resistant *Staphylococcus aureus* (MRSA) in East Africa: red alert or red herring? *BMC Infectious Diseases*, **19**: 596.

Williamson, D.A., Heffernan, H., Nimmo, G. (2015). Contemporary genomic approaches in the diagnosis and typing of *Staphylococcus aureus*. *Pathology*, **47**: 270–275.

Wu, Q., Sabokroo, N., Wang, Y., Hashemian, M., Karamollahi, S., Kouhsari, E. (2021). Systematic review and meta-analysis of the epidemiology of vancomycin-resistance *Staphylococcus aureus* isolates. *Antimicrobial Resistance & Infection Control*, **10**: 101.

Yilmaz, M., Elaldi, N., Balkan, İ.İ., Arslan, F., Batırel, A.A., Bakıcı, M.Z., Gozel, M.G., Alkan, S., Çelik, A.D., Yetkin, M.A., Bodur, H., Sınırtaş, M., Akalın, H., Altay, F.A., Şencan, İ., Azak, E., Gündeş, S., Ceylan, B., Öztürk, R., Leblebicioglu, H., Vahaboglu, H., Mert, A. (2016). Mortality predictors of *Staphylococcus aureus* bacteremia: a prospective multicenter study. *Annals of Clinical Microbiology and Antimicrobials*, **15**: 7.

Yılmaz, E.Ş., Aslantaş, Ö. (2017). Antimicrobial resistance and underlying mechanisms in *Staphylococcus aureus* isolates. *Asian Pacific Journal of Tropical Medicine*, **10**: 1059–1064.

Zarazaga, M., Gómez, P., Ceballos, S., Torres, C. (2018). Chapter 10 - Molecular Epidemiology of *Staphylococcus aureus* Lineages in the Animal–Human Interface. In: Fetsch, A. (ed) *Staphylococcus aureus*. Academic Press, pp. 189–214.

Zdzalik, M., Kalinska, M., Wysocka, M., Stec-Niemczyk, J., Cichon, P., Stach, N., Gruba, N., Stennicke, H.R., Jabaiah, A., Markiewicz, M., Kedracka-Krok, S., Wladyka, B., Daugherty, P.S., Lesner, A., Rolka, K., Dubin, A., Potempa, J., Dubin, G. (2013). Biochemical and Structural Characterization of SplD Protease from *Staphylococcus aureus*. *PloS One*, **8**: e76812.

Zdzalik, M., Karim, A.Y., Wolski, K., Buda, P., Wojcik, K., Brueggemann, S., Wojciechowski, P., Eick, S., Calander, A.-M., Jonsson, I.-M., Kubica, M., Polakowska, K., Miedzobrodzki, J., Wladyka, B., Potempa, J., Dubin, G. (2012). Prevalence of genes encoding extracellular proteases in *Staphylococcus aureus* — important targets triggering immune response in vivo. *FEMS Immunology & Medical Microbiology*, **66**: 220–229.

Zehra, A., Singh, R., Gk, M. (2023). Vancomycin MIC variability among the *Staphylococcus aureus* isolates from the different sample types in Punjab. *The Pharma Innovation Journal*,**12**: 4456-4461

Zhang, K., Sparling, J., Chow, B.L., Elsayed, S., Hussain, Z., Church, D.L., Gregson, D.B., Louie, T., Conly, J.M. (2004). New quadriplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of *Staphylococcus aureus* from coagulase-negative staphylococci. *Journal of Clinical Microbiology*, **42**: 4947–4955.

Appendices

Appendix A: EUCAST susceptibility testing breakpoints for Staphylococcus species (www.eucast.org)

EUCAST

European Committee on Antimicrobial Susceptibility Testing

Staphylococcus species				
Antibiotic	**Disc content**	**Sensitive (mm)**	**Resistant (mm)**	**ATU(mm)**
Penicillins				
Penicillin	10µg	≥ 18	≤ 18	-
Cephalosporins				
Cefoxitin	30µg	≥ 22	≤ 22	-
Fluoroquinolones				
Ciprofloxacin (S. aureus)	5µg	≥ 21	< 21	-
Ciprofloxacin (CNS)	5µg	≥ 24	< 24	-
Aminoglycosides				
Gentamicin (S. aureus)	10µg	≥ 18	< 18	-
Gentamicin (CNS)	10µg	≥ 22	< 22	-
Macrolides				
Erythromycin	15µg	≥ 21	< 18	-
Tetracycline				
Tetracycline	30µg	≥ 19	< 19	-
Miscellaneous				
Trimethoprim/sulfamethoxazole	23.75µg/1.25	≥ 17	< 14	-

Appendix B: Social demographic data collection form.

Sampling tool Malamulo Hospital (LIS) information (English)

Isolate Number	Specimen Type	Anatomical Site	Patient Age	Patient Sex	Facility

Appendix C: Antimicrobial susceptibility results report form

Antimicrobial Susceptibility testing data collection tool (university of Zambia, School of Veterinary Medicine Laboratory)

Isolate Number	Antimicrobial agent dose tested, zone diameter and result (EUCAST guidelines)						
	FOX 30µg	GM 10µg	CIP 5µg	E 15µg	SXT 23.75µg/1.25µg	P 30µg	TE 30µg

Appendix D: PCR results report form

PCR *S. aureus* specific and antimicrobial resistance genes detection, data collection tool (University of Zambia, School of Veterinary Medicine Laboratory)

Isolate Number	Gene detected (insert 'pos' to indicate gene detected and 'neg' to indicate not detected																					
	nuc	*coa*	*spa*	*mecA*	*ermA*	*ermB*	*ermC*	*tetK*	*tetL*	*tetM*	*tetO*	PVL	*splA*	*splB*	*splC*	*splD*	*splE*	*splF*

Appendix E: Ethical clearance letter

Telephone: +265 1 789 400
Facsimile: +265 1 789 431
E-mail: researchlib@moh.gov.mw
All Communications should be addressed to: The Secretary for Health

In reply please quote No. MED/4/36c
Ministry of Health
P.O. Box 30377
Lilongwe 3
Malawi

4th November 2022

Wilfred Taika

University of Zambia

Dear Sir/Madam

RE: Protocol # 22/08/2966: Molecular Characterization of Clinical Staphylococcus Aureus Strains from Malamulo Hospital in Malawi

Thank you for the above titled proposal that researcher submitted to the National Health Sciences Research Committee (NHSRC) for review. Please be advised that the NHSRC has reviewed and approved the above named study.

- **APPROVAL NUMBER** :2966
- The above details should be used on all correspondences, consent forms and documents as appropriate.
- **APPROVAL DATE** :04/11/2022
- **EXPIRATION DATE** :03/11/2023
  This approval expires on 03/11/2023. After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the NHSRC Secretariat should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING:** All serious problems having to do with subject safety must be reported to the NHSRC within 10 working days using standard forms obtainable from the NHSRC Secretariat.
- **MODIFICATIONS**: Prior NHSRC approval using forms obtainable from the NHSRC Secretariat is required before implementing any changes in the protocol (including changes in the consent documents). You may not use any other consent documents besides those approved by the NHSRC.
- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the NHSRC using standard forms obtainable from the NHSRC Secretariat.
- **QUESTIONS**: Please contact the NHSRC on phone number *+265 999397913* or by email on mohdocscentre@gmail.com.
- **OTHER**: Please be reminded to send in copies of your final research results for our records (Health Research Database).

Kind regards from the NHSRC Secretariat.

National Health Sciences Research Committee
2022 -11- 04
APPROVED

.....

CHAIRPERSON, NATIONAL HEALTH SCIENCES RESEARCH COMMITTEE
Promoting Ethical Conduct of Research

Appendix F: Ethical clearance certificate

CERTIFICATE OF ETHICS APPROVAL

This is to certify that the National Health Sciences Research Committee has reviewed and approved the study titled:

Study Title: Protocol # 22/08/2966: Molecular Characterization of Clinical Staphylococcus Aureus Strains from Malamulo Hospital in Malawi

Investigator: Wilfred Taika

StartDate: 04/11/2022 End Date: 03/11/2023

Date of issue: 04/11/2022

Dr Martias Joshua
Chairperson-NHSRC

Mr Billy Nyambalo
NHSRC Administrator

National Health Sciences Research Committee
2022 -11- 04
APPROVED