THE PREVALENCE OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) ISOLATED FROM RAW BOVINE MILK IN THE MOROGORO MUNICIPALITY, TANZANIA

JIBRIL MOHAMMED

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ABSTRACT

Methicillin-Resistant *Staphylococcus aureus* (MRSA) have the ability to cause superficial skin infections and occasionally causes invasive and serious diseases. This study was performed in a cross sectional design to determine the prevalence and molecular characterization of MRSA in raw bovine milk in the Morogoro Municipality. Raw milk samples (117) were collected from 18 administrative wards and cultured on Baird-Parker agar to isolate *S. aureus*. Presumptive colonies were analyzed byslide microscopy and biochemically for catalase and coagulase production. PCR was used to determine*S. aureus* species,*mecA* and coagulase gene. Kirby-Bauer Disk Diffusion method was used for the susceptibility test and multiplex PCR for the SCC*mec* typing of the MRSA isolates. A total of 75 (64.10%) isolates were positive for catalase and coagulase reactions, and 42 (35.90%) were positive for catalase but negative for coagulase reactions.PCR test of the 75 isolates todetect species-specific gave 46*S. aureus* identified from the coagulase-positve and two from the coagulase-negative isolates. The susceptibility test for *S. aureus* on oxacillin (1µG), cefoxitin (30µg), clindamycin (2µg), vancomycin (30µg), trimethoprim-sulfamethoxazole (25µg), tetracycline (30µ) and penicillin G (10 IU) revealed a resistance of 6.52%, 4.35%, 23.91%, 2.17%, 30.43%, 41.30%, and 71.74% respectively to these antibiotics. The coagulase-negative staphylococci isolates also recorded the resistance to oxacillin and cefoxitin as 19.05% and 2.40% respectively. Multi-drug resistance was found in 12 (26.09%)*S. aureus* and none in coagulase-negative staphylococci isolates. PCR screening for methicillin-resistance in both coagulase-positive*S. aureus*and coagulase-negativestaphylococci isolates detected*mecA* gene in three isolates;one from coagulase-negative staphylococci and two from coagulase-negative *S. aureus*.The three isolates were *coa* gene negative and their SCC*mec* type could not be determined by multiplex PCR. This gives theMRSA

prevalences of 4.17% and 2.38% of coagulase-negative*S. aureus* and coagulase-negative staphylococci respectively in the raw milk samples. This study reports for the first time the presence of a presumptive coagulase-negative variant of MRSA and multi-drug resistant *S. aureus*in Morogoro, Tanzania.

DECLARATION

I, **Jibril Mohammed**, do hereby declare to Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted for a higher degree award in any other institution.

……..... ……..

Jibril Mohammed Date

(MSc. Molecular Biology and Biotechnology)

The declaration is hereby confirmed by:

…… …….

Dr. Huruma Nelwike Tuntufye Date

(Supervisor)

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DEDICATION

To Mona M. Abbas.

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LIST OF ABBREVIATIONS AND SYMBOLS

µl	microlitre
µm	micrometre
µM	micromole
%	percent
^{o}C	degrees celsius
µg	microgram
bp	base pair
CA-MRSA	Community-Associated MRSA
ccr	chromosome recombinase complex
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CPS	coagulase-positive Staphylococci
CNS	coagulase-negative Staphylococci
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EFSA	European Food Safety Authority
egc	enterotoxin gene cluster
g	gravitational force
HA-MRSA	Hospital-Associated MRSA
hr	hour
HVR	hypervariable region
IU	International Unit
k	kilo

KCl	potassium chloride
Kms	kilometres
LA-MRSA	Livestock-Associated MRSA
min	minute
MH	Mueller-Hinton
MLST	Multilocus Sequence Typing
mm	milimetre
M-PCR	Multiplex-PCR
MRCN	Methicillin resistant coagulase-negative
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
MSCRAMM	Surface Components Recognizing Adhesive Matrix Molecules
NaCl	sodium chloride
NC	negative control
ODD	oxacillin disk diffusion
PBP-2a	Penicillin Binding Protein-2a
PC	positive control
PCR	Polymerase Chain Reaction
pH	hydrogen ion concentration
PVL	Panton-Valentine leukocidin
QGIS	Quantum Geographic Information System
S. aureus	*Staphylococcus aureus*
SaPIs	pathogenicity islands
SCC	staphylococcal cassette chromosome

SCC*mec*	Staphylococcal Cassette Chromosome *mec*
sec	second
SEs	Staphylococcal Enterotoxins
SEIs	enterotoxin-like proteins
SFP	Staphylococcal Food Poisoning
SSTI	skin and soft tissue infection
ST	Sequence type

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Infection with methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious human health issue that is evolving from a problem once confined to hospitals to a much more general concern and certainly deserves attention. *Staphylococcus aureus*(*S. aureus*) is commonly found everywhere, particularly on the skin and mucous membranes of animals and humans(Friendship *et al.*, 2009). It is a commensal bacterium that colonizes the nares (its primary reservoir), axillae, vagina, pharynx, and/ or damaged skin surfaces (Boucher *et al*., 2010;Casewell, 1986). *Staphylococcus aureus* causes a wide spectrum of disease including skin and soft tissue infections (SSTI), pneumonia, bacteremea, endocarditis, and osteomyelitis (Sowash*et al*., 2014;Lowy, 1998).

Foodborne illness related to improper handling and storage of food and the production of heat stable enterotoxins is another health concern associated with certain strains of *S.aureus*(Friendship *et al*., 2009). Wound infections and food poisoning due to staphylococcal toxins in food have long been recognized. In spite of the constantly increasing need and the alarming epidemic of multi-drug resistant bacteria, antibiotic drug discovery and development seem to have greatly decelerated in recent years.This has forced clinicians to reintroduce forgotten antibiotics into their practice (Kalita *et al*., 2015).The problem of antibiotic resistance by*S. aureus* and the spreading of resistant strains of the bacteria outside the hospital setting is a new concern of public health workers (Friendship *et al.*, 2009). This fact has necessitated investigations directed at animals and animal products as a

potentialsource of human MRSA. Therefore, the monitoring of food processing such as milk production and storage is essential to avoid potential health problems.

1.2 Problem statementand justification

1.2.1 Problem statement

Many strains of *S. aureus* produce penicillinases, making them resistant to penicillin. Methicillin was developed as a substitute to penicillin to solve this problem and immediately became the antibiotic of choice to treat penicillin-resistant staphylococcal infections(Friendship *et al*., 2009). By 1961 reports of methicillin resistance began to appear (Barber, 1961) and afterwards MRSA emerged as a serious problem in most hospitals worldwide. Resistance to methicillin and β-lactam antibiotics in staphylococci is mediated by penicillin binding protein 2a (PBP2a) encoded by the methicillin resistance gene *mecA* (Kinnevey *et al.,* 2013). There is currently increased public and scientific concern regarding extensive use of antimicrobials for therapeutic purpose or as growth promoters in food animals, due to the emergence and dissemination of multiple antibiotic resistant zoonotic bacterial pathogens (Normanno *et al*., 2007;Hardy, 2002). Such antibiotic resistant bacteria do not respond to regular antibiotic treatments and prolong the duration of illness (Joshi *et al.*, 2014). There has also been another increasing concern that human infections in the community may be caused by the MRSA from contaminated foods and livestock products including bovine milk (Kwon *et al*., 2005;Lee, 2003). According to the Centers of Disease Control and Prevention (CDC), 240,000 illnesses with 1,000 hospitalizations and six deaths associated with staphylococcal food poisoning occur annually(Oguttu *et al*., 2014;Tallent *et al.,* 2013).In Tanzania, high level of resistance by MRSA was seen to first-line and inexpensive antimicrobial agents (Moyo *et al.,* 2010).

1.2.2 Justification

In recent years, MRSA has been detected in livestock animals, including pigs, veal calves and chickens(de Neeling *et al.,* 2007). Most animals may be colonized by*S. aureus*, but only recently were MRSA strains isolated from several food production animals (de Boer *et al*. 2009).

There is no previous report on MRSA isolation from raw bovine milk in Tanzania. This research is the first of its kind to isolate MRSA from bovine milk in the Morogoro Municipality, Tanzania, and would subsequently be the baseline for which future MRSA surveillance on animal based food products would be studied. Furthermore, the awareness of milk contamination with MRSA should be raised to help consumers to avoid using unpasteurized milk and to enforce healthy food handling practices to prevent contamination

1.3 Objectives of the study

1.3.1 Main objective

Determine the prevalence and SCC*mec* types (I-V) and subtypes IVa-IVe of Methicillin-Resistant *Staphylococcus aureus* in raw bovine milk.

1.3.2 Specific objectives

I. To determine the prevalence of *S. aureus* isolates in raw bovine milk.
II. To establish the antimicrobial resistance profile of *S. aureus* and other staphylococci isolated from raw bovine milk.
III. To compare the MRSA isolates from the raw bovine milk obtained from the different Wards in the municipality of Morogoro.

1.3.3 Research questions

I. What is the prevalence of *S. aureus* isolates in raw bovine milk?

II. What is the antimicrobial resistance pattern of *S. aureus* and other staphylococci isolated from the raw bovine milk?

III. What is the comparison of the MRSA isolates obtained from the different Wards?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background information

Staphylococcus aureus is one of the leading causes of foodborne disease outbreaks due to its ability to produce staphylococcal enterotoxins (Tenhagen *et al*., 2014;Hennekinne *et al*., 2012). More recently, MRSA has been isolated from most animals and foods of animal origin. MRSA strains have been isolated from cows' or small ruminants' milk and various dairy products in many countries. The MRSA prevalence in milk and dairy products reported from different countries or even regions of the same country differs significantly (Pexara *et al.,* 2013).There are variations in MRSA prevalence in milk produced in most African countries, for instance it is as high as 60% in Ethiopia(Daka *et al*., 2012). In Nigeria, Suleiman *et al*. (2012) isolated 8% *S. aureus* habouring the *mecA* gene and in South Africa, Ateba *et al*. (2010) found a MRSA prevalence of 6% in cow's milk produced in commercial farms. The MRSA prevalence in Asian countries varies from high e.g. 28.3% in Iran to low (e.g. in Korea 1.5% and 1.10% in Japan) (Pexara *et al.*, 2013). In most European countries, the MRSA prevalence in milk and dairy products has been found to be low and in the U.S (1-2%) and Canada (0.06%) MRSA prevalence estimates have been reported (Pexara *et al.*, 2013).

2.2Microbiology of *Staphylococcus aureus*

2.2.1 Macromorphology of *Staphylococcus aureus*

Depending on growth conditions, the colony pigmentation varies from grey, grey-white with yellowish to orange shades and a typical β-haemolysis on the blood agar(Medveďová *et al.*, 2012). On Baird-Parker Egg Yolk Tellurite medium

(OXOID, Hampshire, England), colonies appear grey-black, shiny and convex measuring 1-1.15 mm diameter (18 hrs) up to 3 mm (48 hrs) with narrow white entire margin surrounded by zone of clearing of about 2-5 mm. Mannitol salt agar(Hardy Diagnostics, CA, USA) is another growth media, where typical *S. aureus* appear as yellow colonies with yellow zones in the medium at 35-37^{o}C after 24-48 hrs of growth. *Staphylococcus aureus* colonies will appear pink to mauve when cultured on CHROMagar (CHROMagar, Paris-France).

2.2.2 Micromorphology of *Staphylococcus aureus*

The staphylococci are Gram positive, facultative anaerobic, nonmotile cocci in clusters and are non spore formingmicroorganisms.

2.2.3 Biochemical characteristics of *Staphylococcus aureus*

Staphylococcus aureus is a chemoorganotroph with a respiratory and fermentative metabolism. Under aerobic conditions, acids are produced from glucose, lactose, maltose and mannitol while under microaerophilic conditions, acids are produced from many other sugars and alcoholic sugars (Baird-Parker, 2000). Most *S. aureus* strains hydrolyse native animal proteins (casein, gelatine, fibrin), lipids, phospholipoproteins and tween. They also coagulate animal plasma which is mediated by a coagulase and clumping factor (Medveďová*et al.,* 2012). A positive catalase reaction is also produced by this species, this enzyme allows the bacteria to better resist intra- and extra-cellular killing by hydrogen peroxide (Gruner *et al*., 2007).

2.3 Identification of MRSA bacteria

2.3.1 Conventional methods of identification

The use of a rapid and accurate detection of methicillin resistance in *S. aureus* is important to guide appropriate antimicrobial therapy for the control of infections spread of MRSA strains. The efficiency of conventional methods for detection of methicillin resistance in *S. aureus* such as the disk diffusion, agar dilution, oxacillin agar screen test, and the latex agglutination test and MRSA-Screen latex has shown that, MRSA-screen latex yielded 100% sensitivity and 100% specificity. It also has the advantage of giving a reliable relatively fasterresults (Soloaga *et al.*, 2004). Another study which compared two conventional phenotypic methods, that is oxacillin disk diffusion (ODD) and mannitol salt agar (MSA) resulted in the sensitivity and specificity of ODD and MSA to be 93.5% and 83.5%; 87.1% and 89.3% respectively. The time taken for diagnosing MRSA by these methods was 48-72 hrs, which is longer than PCR, which takes 18-24 hrs(Pillai *et al.*, 2012).Yamazumi *et al*. (2001), also reported 96.9 and 100%; 98 and 98%; 98 and 100%; 99 and 99% sensitivities and specificities for MRSA-Screen agar, Oxacillin-Screen agar, VITEK and microdilution tests. Cefoxitin was reported by several authors to give high quality results than Oxacillin (Broekema *et al*., 2009;Anand *et al*., 2009;Arya *et al*., 2005;Sharp *et al*., 2005). The sensitivity and specificity of cefoxitin disk diffusion test was found to be 97.3 and 100% in another study (Kali *et al*., 2014; Broekema *et al.,* 2009).

2.3.2 Molecular methods of identification

Use of different methods of DNA-based molecular typing (e.g. macro-restriction patterns, PCR-based typing) revealed considerable *S. aureus* host specificity or strain, although distinct ‘bovine types’ are often interspersed among the human

genotype clusters suggesting that human-adapted MRSA were the evolutionary ancestors of *S.aureus* adapted to cattle in modern agriculture (Cuny *etal*., 2010). Multilocus sequence typing (MLST) which is a powerful method for bacterial population analysis, has been widely applied for studying *S.aureus* from humans, in particular MRSA (Cuny *et al*., 2010), but to a less extent isolates from animals. This method is based on the sequences of ~450 bp the internal fragments of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*) (Enright *et al*., 2000).

Genotypic techniques to type MRSA must be particularly discriminatory, as MRSA strains probably originate from a single clone or at least a few strain types (Schmitz *et al.*, 1998). In comparison with other detection methods, such as Southern blotting and pulsed-field gel electrophoresis (PFGE) which involves creating large DNA fragments from intact bacterial chromosomes using rare cutting restriction endonucleases, PCR assays such as multiplex-PCR (M-PCR), real-time PCR, hypervariable region (HVR) and the amplification of protein A mediatory gene (*spa*) techniques can provide a rapid amplification, detection and typing tool for MRSA strains (Strande´n *et al.*, 2003). The *spa* method generates a staphylococcal strain-specific amplification pattern, which can be used to classify MRSA strains (Schmitz *et al.*, 1998). The HVR-typing method is however based on the amplification of the 40-bp repeat unit elements between the *IS431mec* and *mecA* genes, which are situated on the staphylococcal cassette chromosome (Senna *et al.*, 2002). Both*spa*- and HVR-typing have been reported to provide a rapid and cost effective method for the genotyping of MRSA strains(Strande´n *et al*., 2003). Therefore epidemiological conditions should determine the best typing method to be employed.

2.4The epidemiology of MRSA

The epidemiology of MRSA in hospitals and community settings in the developed world has been extensively studied but remains largely understudiedin lower and middle-income countries, and in Africa in particular(Abdulgader *et al.*, 2014). MRSA can be categorized according to where the infection was acquired thus hospital-associated MRSA (HA-MRSA), community-associated MRSA (CA-MRSA) and the recently discovered livestock-associated MRSA (LA-MRSA). MRSA was initially reported as a nosocomial pathogen in human hospitals and was isolated from patients with compromised immune systems. In the 1990s, a major change in the epidemiology of MRSA occured, with the appearance of cases affecting people with no epidemiological connection to hospitals. Strains that caused such infections were referred to as community-associated MRSA (EFSA, 2009). The CA-MRSA strains that cause non-hospital-associated infections are characteristically susceptible to many antibiotics, habour type IV SCC*mec*, which is smaller in size than other SCC*mec* elements, and contains no other antibiotic resistance genes except *mecA*(Kwon *et al*., 2005;Baba *et al.*, 2002;Fey *et al*., 2003). In contrast to CA-MRSA, the majority of HA-MRSA strains carry one of two types of SCC*mec*, type II or III, and show characteristics of multidrug resistance (Kwon *et al*., 2005;Sharp *et al*., 2005;Ito *et al.*, 2001). There is now increasing concern about the public health impact of MRSA associated livestock, because their resistant genes can spread to humans by direct contact or through the food chain (Kluytmans, 2010). Approximately 20% to 60% of humans are permanent or intermittent carriers of*S. aureus* and relevant sites include the anterior nares, axillae, perineum and vagina (Kluytmans, 2010). The prevalence of MRSA was lower than 50% in most of the African countries, although it appears to have increased since 2000 in many African countries, except for South Africa (Falagas *et al*., 2013). The prevalence of MRSA in

Tanzania has been increasing, for instance at the Muhimbili National Referral Hospital Dar es Salaam, the prevalence was 0.4% in 1999, 2% in 2004, and 23.3% in 2010 (Mshana *et al.*, 2013). At the Bugando Medical Center Mwanza, the prevalence of MRSA was 16.3% (Mshana *et al.*, 2009) and 18.8% in 2011 (Mawalla *et al.*, 2011). The new MRSA clone ST1797/ t7231 was isolated in Tanzania, thus emphasizing the diversity of MRSA clones in Africa (Moremi *et al.*, 2012). A recent study in Uganda concluded that, SCC*mec* types V and I are the most prevalent MRSA genotypes among patients with post surgical infection (Seni *et al.*, 2013). A similar study was carried out in Tanzania where 44% of *S. aureus* isolated from patients with wound infections at the Muhimbili National Hospital were MRSA(Manyahi *et al.*, 2014). In another study in Lusaka, *S.aureus*was isolated from 205 paper currency notes, where 2.92% of vancomycin resistant strains was found (Neel, 2013). A similar study was done by the same researcher in Tanzania where 28.125% of MRSA isolated from local currency notes were resistant to vancomycin and methicillin (Neel, 2012). A high prevalence of clindamycin resistance was observed among *S. aureus* and a significant association was reported between MRSA and inducible clindamycin resistance (Mshana *et al.,*2009). There is the potential public health threat of *S. aureus* from contamination of milk and milk products with pathogenic bacteria following unhygienic processing and handling(Thaker *et al.*, 2013).

2.5 The*Staphylococcus aureus* resistance

The methicillin resistance mechanism is well understood inMRSA strains (Lowy, 2003). It is caused by the production of a novel penicillin-binding protein, PBP-2a, with a decreased binding affinity for β-lactams(Hartman *et al.*, 1984). This process is encoded by the chromosomal gene *mecA* that is found in the *mec* region. The

sequence of *mecA* is conserved in all methicillin-resistant strains of *S. aureus*(Weller, 1999). Theβ-lactam antibiotics damage bacteria by inactivating penicillin-binding proteins, that are essential in the assembly of the bacterial cell wall(Pinho *et al.*, 2001). Treated bacteria become osmotically fragile and easily lysed as a result of the weakened cell wall. The presence of the *mecA* gene has until recently been the 'gold standard' for the detection of MRSA worldwide. However, a novel *mecA* homologue that also confers methicillin resistance was identified in *S. aureus* isolates from dairy cattle and humans (Pexara *et al.*, 2013). This gene has been designated *mecC*(García-Álvarez *et al.*, 2011). Some strains of *S.aureus* possess an alternative resistance mechanism, attributable to the hyper-production of the *S. aureus*β-lactamase enzyme, which inactivates the antibiotic agents by hydrolysing the β-lactam ring of penicillin and cephalosporin compounds (Brown *et al.*, 2005). However, some SCC*mec* types carry various additional genetic elements (e.g Tn*554*), which encodes resistance to macrolides, clindamycin and streptogramin B;*pT181*, which encodes resistance to tetracyclines. These genetic elements are especially common in HA-MRSA. Until recently, the only antibiotic available for treating MRSA infections was vancomycin, however, vancomycin-resistant MRSA strains, including CA-MRSA strains, have increasingly been reported(Pexara *et al*., 2013;Tenover *et al*., 2009).

2.6 Production of virulence factors

Due to the production of surface-associated factors like microbial surface components recognizing adhesive matrix molecules (MSCRAMM), protein A, polysaccharide A, peptidoglycan and a clumping factor, *S. aureus* is responsible for resistance to opsonophagocytosis, the formation of biofilm and adhesion to the host cell matrix (Garzoni *et al.*, 2009). The role of enzymes like coagulase, catalase, hyaluronidase, lipase, heat-resistant nuclease, staphylokinase and β-galactosidase is

to disrupt cell structure, degrade cell lipids and hyaluronic acid, and to convert fibrinogen to fibrin(Halpin-Dohnalek *et al*., 1989).It has since been demonstrated that coagulase is an important virulence factor during the infection process (Roodmajani *et al.*, 2014; Himabindu *et al*., 2009; Goh *et al*., 1992; Hookey *et al*., 1998).

Enterotoxins are short, extracellular proteins that are water-soluble(Schelin *et al.*, 2011). To date, 21 staphylococcal enterotoxins (SEs) or enterotoxin-like proteins (SEIs) have been identified and designated SEA to SEIV (Thomas *et al.*, 2006). The genes encoding the different enterotoxins are carried and disseminated by different mobile genetic elements, i.e., prophages, plasmids, pathogenicity islands (SaPIs), enterotoxin gene cluster (*egc*) and the staphylococcal cassette chromosome (SCC) (Shalita *et al.*, 1977).

2.7Disease transmission of MRSA

MRSA can be transmitted from person to person, as well as from animals to humans and *vice-versa*. Transmission usually occurs by direct contact, often via the hands, with colonized or infected people or animals (Lee, 2003). Food may easily be contaminated with MRSA; thus handling or eating contaminated food is also a potential means of transmitting these microorganisms to humans.

2.8 MRSA disease infection and symptoms

MRSA symptoms of infection depend on the part of the body that is infected. The disease can occur following skin and soft tissue infections or as invasive infections. During skin and soft tissue infection the bacteria often enter the skin through a cut, bruise or hair follicle and may develop into a boil or abscess. Cellulitis is another

skin tissue infection affecting the deeper layers of the skin, fat and soft tissues underneath. In a typical MRSA invasive infection, the bacteria penetrate the circulatory system causing blood poisoning (sepsis), urinary tract infection, pneumonia and osteomyelitis. *Staphylococcus aureus* has the ability to grow, and produce staphylococcal enterotoxins (SE), the causative agent of staphylococcal food poisoning (SFP), over an extensive range of temperature, pH, sodium chloride concentration and water activity (Schelin *et al.*, 2011). Staphylococcal food poisoning symptoms include copious vomiting, diarrhea, abdominal pain or nausea (Murray *et al.*, 2002).

2.9Treatment of MRSA bacterial desease

Resistance to beta-lactams and other agents has resulted in the increasing use of glycopeptides, such as vancomycin, as first-line therapy for the treatment of serious MRSA infections (Ruef, 2004). However, various forms of glycopeptides resistance have appeared in MRSA strains, including rare high-level resistance, homogenous and heterogenous intermediate resistance (French, 2006;May *et al*., 1998). In response to this challenge, a number of new antimicrobials have been developed. These include thestreptogramins (quinupristin/ dalfopristin), (Gurk-Turner, 2000), the oxazolidinones (linzolid), (Wilcox, 2005), and more recently daptomycin which are reliable in the treatment of complicated skin infections, infective right-sided endocarditis, and bacteremia caused by gram-positive agents. The unique mechanism of action of daptomycin and its low resistance profile, together with its rapid bactericidal action make it a favourable alternative to vancomycin in multi-drug resistant cocci (Beiras-Fernandez *et al.*, 2010).The current treatment options for MRSA infections are limited, however, linzolid has proven to be a valuable addition

to the antibiotic used in treatment against this common and dangerous pathogen (Watkins *et al*., 2012).

2.10 Controlmeasures of MRSA bacteria

The emergence of methicillin-resistance in previously sensitive strains of *S. aureus* appears to be relatively rare. Excessive use of antibiotics, however, promotes the spread of existing strains of MRSA through reduction in colonization resistance in patients and by giving resistance strains a survival advantage in the environment (Coia *et al.*, 2006). Therefore, antibiotic use and compliance with local guidelines needs to be audited. Inappropriate antibiotic use, such as underdosing, multiple or excessive duration of courses, and the use of broad-spectrum agents are major factors in the spread of antibiotic resistance (Coia *et al.*, 2006). Other preventive measures such as control of raw ingredients, proper handling and processing, adequate cleaning, and disinfection of equipment used in food processing and preparation should be deployed (Kadariya *et al*., 2014;Hennekinne *et al*., 2012;James *et al*., 2008). Strict implementation and adherence to the microbiological guidelines is imperative. These include Hazard Analysis and Critical Control Points (HACCP), Good Manufacturing Practices (GMPs), and Good Hygienic Practices (GHPs) developed by World Health Organization (Kadariya *et al*., 2014; Syne *et al*., 2013; Lammerding, 1997).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area and the study design

Morogoro Municipality was the chosen study area. The Municipality has 531.6 Km^2 of total land area and the population growth rate is 4.7% per annum and a projected total population of 316,603 (Region and District Projection Volume XII, 2011). About 33% of the population engage in subsistence farming and livestock keeping. The Municipal Council has one division, which is subdivided into 29 Administrative Wards. The current study was an experimental cross-sectional design, in which 18 Wards were randomly selected as sampling areas. The wards and their respective locations are as shown in Figure 1 below, and the permit document for the study is indicated in Appendix 5.

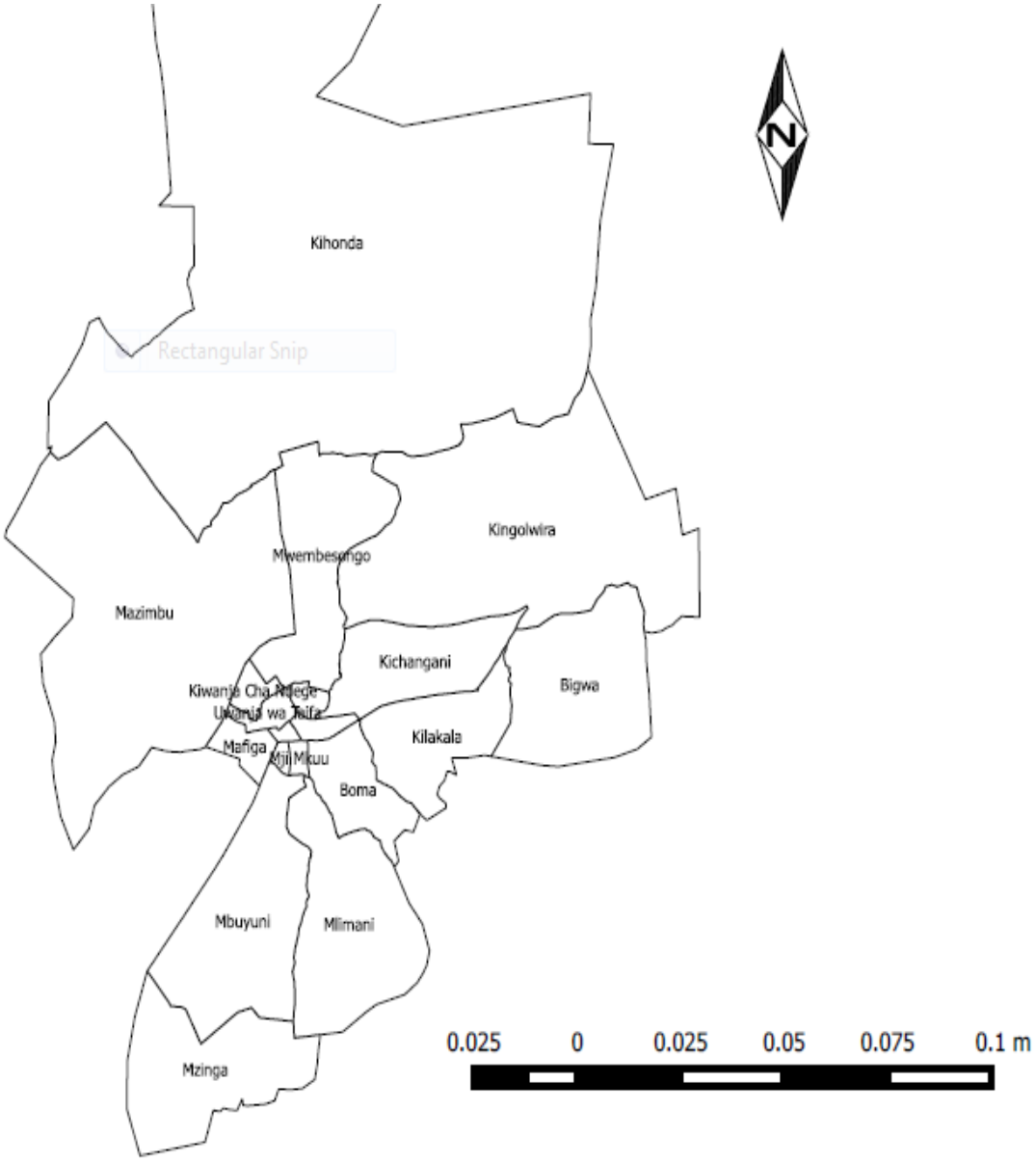

Figure 1: The map of Morogoro Municipality wards (map constructed using QGIS Software).

3.2 Sampling area

The sampling area comprised 18 Wards in the Morogoro Municipality, selected randomly by a lottery in which each ward was assigned a unique number (1-12 and 14-19). Sales points and local shops where fresh and unboiled cow milk is sold were randomly selected.

3.3 Sample size

The sample size was estimated with a confidence level (Zα) of 95% and maximum tolerable error (e) of 5%. Using the prevalence rate of 7.6% (Suleiman *et al*., 2012). Required sample size was calculated using the formula (Charan *et al.*, 2013) as shown below:

$n=Z\alpha^2 * P(1-P)/e^2$

$n=1.96^2 * 0.076\ (1-0.076)/0.05^2$

$n=107.909$

Therefore, 108 samples would be required for the study.

3.4 Sampling

For 6 samples from each Ward (with ±2 samples); a maximum of eight and a minimum of four samples were collected. A total of 117 milk samples of at least 10 ml each were collected in appropriately labelled sterile Universal Bottles and transported immediately to the laboratory for further processing.

3.5 Isolation of Staphylococcus species

The fresh milk samples collected from the sales point were transported to the laboratory and immediatedly cultured on Baird-Parker media (OXOID, Hampshire, England). According to the manufacturer's instructions, the selective Baird-Parker agar enriched with Egg Yolk Tellurite Emulsion (OXOID, Hampshire, England) was prepared by adding 50ml ofthe supplement to one litre of the agar. Each media plate was appropriately labelled and cultured by streaking three loopful of the milk. The culture plates were incubated at 37^{o}C for 24 hrs after which first readings were taken and recorded. Cultures were reincubated for 48 hrs and thereafter, read and recorded.

Colonies characterized by small(1 to 1.5 mm) shiny black with surrounding clear zones or of small/ medium size with opaque zone/ halo were selected and stored in nutrient broth (Nutrient Broth No. 2, OXOID, Hampshire, England) and refridgerated at 4^{o}C for further analysis.

3.6 Biochemical testsfor*Staphylococcusaureus*

3.6.1 Tube coagulase test

The colonies were subcultured in Nutrient Broth and incubated at 37^{o}C for 18-24 hrs. Rabbit plasma was diluted withsterile distilled water at 1:10 ratio. The 117 tubes were appropriately labelled according to each sample and one ml of the diluted plasma was aliquoted into each tubeand extra tube was labelled as control. Approximately 200 µlof each sample broth was added to the tubes and incubated at 37^{o}C. Tubes were observed after every hour of incubation for four hrs for coagulation. Where no reaction occured tubes were further incubated overnight(Katz, 2013). A positive reaction was noted by a thick clot or jelly formation that did not flow readily when tube was inverted. A negative reaction was where the mixture flowed when tube was inverted. Tubes were not agitatedto avoid breaking the clots.

3.6.2 Catalase test

Samples from the nutrient culture broth were grownon nutrient agar and incubated at 37^{o}C for 18-24 hrs. This was followed by preparing 3% hydrogen peroxide in a tube. A loopful of sterile normal saline (0.85% NaCl) solution was put on a sterile grease free microscope slide. A loopful of the culture was added and homogenized in the normal saline. Then a drop (0.2 ml) of hydrogen peroxide was added to

thehomogenate. A positive reaction was indicated by the formation of bubbles from the culture(Reiner, 2013).

3.7 DNA extraction

The *S. aureus* genomic DNA was extracted by boiling. Frozen cultures stored at -45^{o}C in 15% glycerol nutrient broth were thawed and homogenized in nutrient broth and subcultured on nutrient agar for 24 hrs at 37^{o}C. The DNA extractionwas done following the protocol by Zhang *et al*. (2004) with some modification on amount of nuclease-free water, centrifugation force and volume of supernatant. Three to five bacterial colonies were added into 1.5 ml eppendorf tubes containing 200 µl of nuclease-free water. The tubes' caps were sealed with clean masking tapes to prevent opening during heating, then they were placed in a boiling water bath at 99^{o}C for 10 mins. After centrifugation at 30,000 x g for 1 min, 3 µl of the supernatant was used as template DNA PCR mixture.

3.8 PCR detection of *S. aureus*

The detection of *S. aureus* was performed using primers which were species specific for *S. aureus*(Table 1).The primers were obtained from Martineau *et al*. (1998) and were manufuctured by Macrogen Inc. (Seoul, South Korea). The PCR mixture comprised of an aliquot of 3 µl of bacterial DNA template, primers and distilled water to a total of 20 µl into AccuPower® PCR PreMix tubes (BioneerInco., South Korea) which contained 1 U *Taq* DNA polymerase, 250 µM each of dATP, dCTP, dGTP, dTTP, 10 mM Tris-HCL (pH 9.0), 30 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye. The concentration of each primer was 0.4 µM. They were derived from a chromosomal DNA specific for *S. aureus* that amplifies a 108 bp product, and codes for the enzyme glutamate synthase (*gltB*). The PCR mixtures were incubated

in a TAKARA PCR Thermal CyclerDice Gradient TP600 (Takara Bio, Tokyo, Japan) under thermal cycling conditions of initial denaturation step at 95^{o}C for 5 min, and then 35 cycles of amplification at 95^{o}C for 30 sec, with annealing at 55^{o}C for 30 sec, extension at 72^{o}C for 30 sec and final extension step at 72^{o}C for 5 min and a hold at 4^{o}C.

3.9 Antimicrobial susceptibility test

The Kirby-Bauer Disk Diffusion Susceptibility test was used to obtain the antimicrobial resistance profile of the isolates. This was done following the protocol by Hudzicki (2013). Samples were cultured in nutrient broth at 37^{o}C for 18-24 hrs. The *S. aureus* isolates were tested against the antibiotics including oxacillin (1µg) and cefoxitin (30µg) for the phenotypic detection of MRSA, whereas, clindamycin (2µg), vancomycin (30µg), trimethoprim-sulfamethoxazole (25µg), tetracycline (30µ) and penicillin G (10 IU) for the routine drug susceptibility test. The coagulase-negative staphylococci (CNS) were also tested to oxacillin and cefoxitin. Methicillin was not included as a test antibiotic because it is no longer produced, and oxacillin remains a second option, though it was demonstrated that cefoxitin was more reliable than oxacillin for detecting MRSA (Zurita *et al.*, 2010;Cauwelier *et al*., 2004;Skov *et al*., 2003;Felten *et al*., 2002;Mougeot *et al.,* 2001). All antibiotic discs were manufactured by Liofilchem®Italy. Mueller-Hinton (MH) agar(Liofilchem s.r.l., Italy) supplemented with 2% NaCl for oxacillin, to improve the detection of heteroresistant MRSA, was prepared according to the manufacturer's instructions. The*S. aureus* andCNSwere subcultured on Nutrient agar at 37^{o}C for 18-24 hrs. Using a sterile inoculating loop, three to four colonies were picked and suspended in 3 ml sterile saline solution.Turbidity of the bacteria suspension was adjusted to 0.5 Standard McFarland. The dried surface of the media was inoculated with 200µl ofthe

bacteria suspension and allowed to dry. The antimicrobial-impregnated disks were placed on the surface of the agar plates and incubated at 37^{o}C except oxacillin which was at 35^{o}C for 24 hrs. Following incubation, zone sizes were measured and the results interpreted according to the zone diameter interpretative standards (CLSI, 2014) for *Staphylococcus* species (Appendix1). However,further routine susceptibility test was performed on all oxacillin-resistant CNS to clindamycin, vancomycin, trimethoprim-sulfamethoxazole, tetracycline and penicillin G.

3.10Detection of *mecA* gene by PCR

The detection of *mecA* gene was carried out as a single target PCR amplification using the primer pairs listed in Table 1. All *S. aureus* and CNS isolates were screened for the detection of *mecA* gene for the genotypic identification of MRSA. The primer and PCR conditions were obtained from Zhang *et al.* (2005) withsome modifications. The initial primer concentration was 0.046 µM and amplicon size147 bp. The PCR was run in 20 µl of AccuPower® PCR PreMix tubes (Bioneer Inco., South Korea) containing 3 µl of template DNA, with cycling parameters beginning with an initial denaturation step at 95^{o}C for 5 min followed by 35 cycles of 95^{o}C for 30 sec, 52^{o}C for 45 sec, and 72^{o}C for 30 sec, ending with a final extension step at 72^{o}C for 7 min and a hold at 4^{o}C.

3.11 PCR amplification of the coagulase (*coa*) gene

The PCR amplification of*coa* gene on *mecA* positive of the *S. aureus* and the CNS isolateswas performed using the primer pairs(Table 1) which were obtained from Akineden *et al*. (2008). This was to genotypically confirm the coagulase producing status of the isolates.The PCR mixture contained 20 µl of AccuPower® PCR PreMixwith 3 µl of bacterial DNA and concentration of 1 µl each of the two primers

with variable product size (bp). The thermal cycling condition was performed according to Hookey *et al*.(1998) as follows: initial denaturation at 94^{o}C for 45sec. The cycling preceeded for 30 cycles of 94^{o}C for 20sec, 57^{o}C for 15sec, and 70^{o}C for 15sec with a final step at 72^{o}C for 2 min; and a hold at 4^{o}C.

3.12The Multiplex PCR typing of staphylococcal cassette chromosome(SCC*mec*) Types I-V and subtypes IVa-IVe in MRSA

The primer sequences used for SCC*mec* typing of the MRSA isolates were obtained from Zhang *et al.* (2012). The multiplex PCR mixture comprises an aliquot of 3 µl of bacterial DNA template, primers and distilled water to a total volume of 20 µl into AccuPower® PCR PreMix.Thermocycling conditions as decribed by Zhang *et al*.(2012) were set at 94^{o}C for 5 min, followed by 10 cycles of 94^{o}C 45 sec, 65^{o}C 45 sec, 72^{o}C 1.5 min. A further 25 cycles of 94^{o}C 45 sec, 52^{o}C 45 sec, 72^{o}C 2 min were followed by a 10 min incubation at 72^{o}C and a hold at 4^{o}C. The primers used in the multiplex PCR reaction are listed in Table 1.

3.13 Gel electrophoresis and visualization of PCR products

Ten µl of each PCR product were run on a 1.5 % agarose gel electrophoresis in 0.5x TAE buffer with 3 µl gel red (Excellgen, Rockville, MD, USA) at 80 volts for 30 mins using Mupid-One Electrophoresis System (Advance, Tokyo, Japan), with 100bp DNA ladder (BioLabs, New England, USA) as molecular marker. Since the PCR PreMix contained a dye, DNA loading dye was not required. The agarose gel was visualized under UV light in Gel Doc EZ Imager machine (Bio Rad, California, USA).

Table 1: The list of primers used in this study

Primer	Oligonucleotide sequence (5′-3′)	Conc.(μM)	Ampliconsize (bp)	Specificity	References
Type I-F	GCTTTAAAGAGTGTCGTTACAGG	0.1	613	SCC*mec* I	Zhang *et al*., 2005
Type I-R	GTTCTCTCATAGTATGACGTCC				
Type II-F	CGTTGAAGATGATGAAGCG	0.1	398	SCC*mec* II	Zhang *et al*., 2005
Type II-R	CGAAATCAATGGTTAATGGACC				
Type III-F5	TTCTCATTGATGCTGAAGCC	0.16	257	SCC*mec* III	Zhang *et al*., 2012
Type III-R6	GTGTAATTTCTTTTGAAAGATATGG				
Type Iva-F	GCCTTATTCGAAGAAACCG	0.1	776	SCC*mec* Iva	Zhang *et al*., 2005
Type Iva-R	CTACTCTTCTGAAAAGCGTCG				
Type Ivb-F	TCTGGAATTACTTCAGCTGC	0.28	493	SCC*mec* Ivb	Zhang *et al*., 2005
Type Ivb-R	AAACAATATTGCTCTCCCTC				
Type Ivc-F2	CCTGAATCTAAAGAGATACACCG	0.1	200	SCC*mec* Ivc	Zhang *et al*., 2012
Type Ivc-R2	GGTTATTTTCATAGTGAATCGC				
Type Ivd-F5	CTCAAAATACGGACCCCAATACA	0.72	881	SCC*mec* Ivd	Zhang *et al*., 2005
Type Ivd-R6	TGCTCCAGTAATTGCTAAAG				
Type IVE-F3	CAGATTCATCATTTCAAAGGC	0.3	175	SCC*mec* IVE	Zhang *et al*., 2012
Type IVE-R4	AACAACTATTAGATAATTTCCG				
Type V-F	GAACATTGTTACTTAAATGAGCG	0.132	325	SCC*mec* V	Zhang *et al*., 2005
Type V-R	TGAAAGTTGTACCCTTGACACC				
MecA147-F	GTG AAG ATA TAC CAA GTG ATT	0.046	147		Zhang *et al*., 2005
MecA147-R	ATG CGC TAT AGA TTG AAA GGA				
Sa442-1	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG	0.4	108	Species-specific target	Martineau *et al.*, 1998
Sa442-2	CGT AAT GAG ATT TCA GTA GAT AATACA ACA				
Coa-1	ATAGAGATGCTGGTACAGG	1	Variable		Akineden *et al*., 2008
Coa-2	GCTTCCGATTGTTCGATGC				

CHAPTER FOUR

4.0 RESULTS

4.1 Bacterial isolation

All 117 raw milk samples collected were immediately cultured on Baird Parker agar for observations in 24 and 48 hrs. Colonies showing the typical *S. aureus* colonial characteristics were selected and Gram staining performed to observe the gram-positive reactions as shown in the Fig. 2:

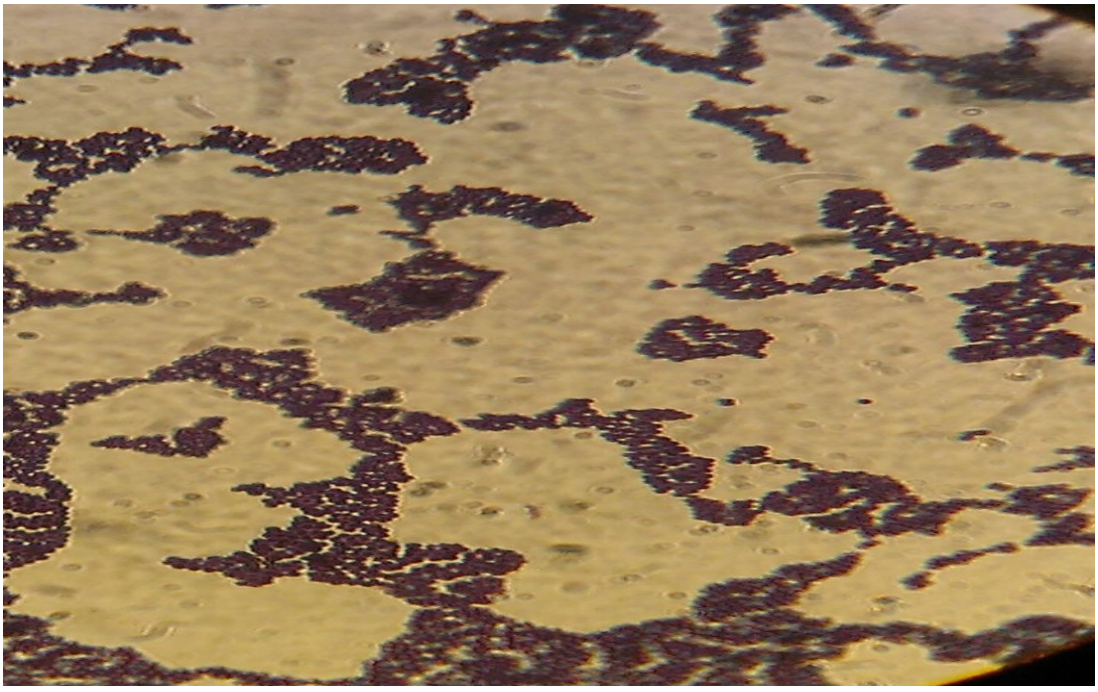

Figure 2: A picture showing Gram-positive cocci in clusters

4.2 Phenotypic identification of coagulase-positive and coagulase-negative staphylococci

Suspected colonies where subjected to catalase and tube coagulase tests. Out of117 raw milk samples, 75 (64.10 %) tested positive for both catalase and coagulase reactions, while 42 (35.90%) tested positive for catalase but negative for coagulase.

4.3 Detection of *Staphylococcus aureus*

PCR amplification to detect *S. aureus*was carried out on all 75 coagulase-positive and(CPS) catalase-positive strains and 46 isolates were genotypically confirmed to be*S.aureus*(Fig. 3):

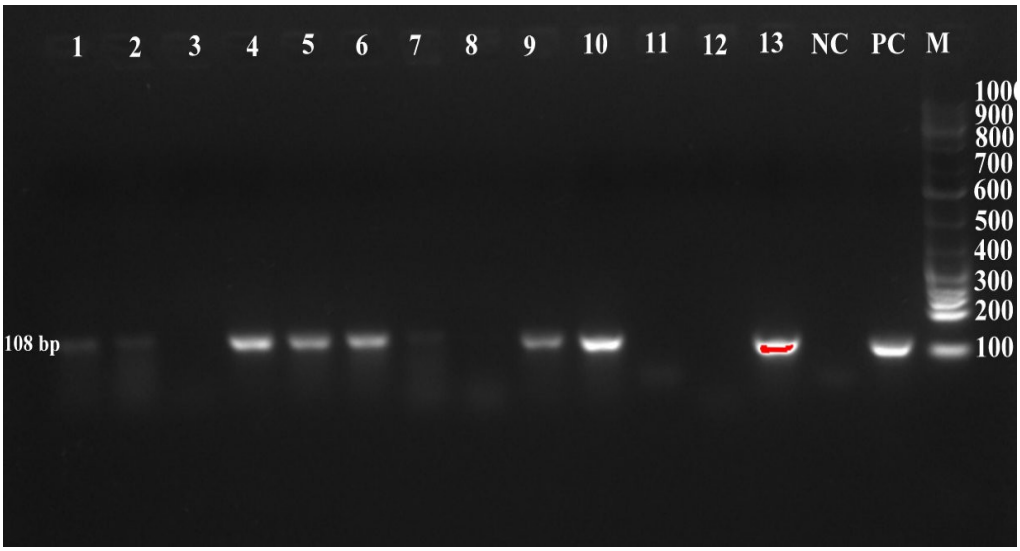

Figure 3: PCR detection for*S. aureus*isolates:PCR products visualized under UV transilluminator. Lanes: 3, 8, 11 and 12 are negative; while lanes: 1, 2, 4, 5, 6, 7, 9, 10 and 13 are positive for *S. aureus* specific gene (*gltB*)at 108 bp product. NC and PC are negative and positive controls respectively; and M: DNA ladder marking from 100 to 1k bp.

Table 2: The*S. aureus* isolated from milk in the Morogoro Municipality

Wards (Codes)	Samples analyzed	*S. aureus* isolated	% of *S. aureus*isolated
Boma (1)	7	1	2.1
Mazimbu (2)	8	3	6.3
Mwebesongo (3)	8	6	12.5
Msamvu (4)	8	8	16.7
Kihonda (5)	8	1	2.1
Kichangani (6)	4	2	4.2
Kilakala (7)	6	4	8.3
Mafiga (8)	6	2	4.2
Kiwanja Ndege (9)	8	3	6.3
Sabasaba (10)	5	3	6.3
Chamwino (11)	7	3	6.3
Mafisa (12)	6	3	6.3
Mbuyuni (14)	5	2	4.2
Mji Mpya (15)	6	2	4.2
Kingolwira (16)	4	1	2.1
Tungi (17)	9	1	2.1
Mkundi (18)	4	2	4.2
Magadu (19)	8	1	2.1

Total number of *S. aureus* isolated from Municipality (n= 48) and the proportion of isolates from each Ward (Ward code number 13 was not added because it did not yield enough samples for the study, therefore, was replaced by Ward code 19).

4.4 The antimicrobial susceptibility test

The five classes of antibiotics (beta-lactams, lincosamide, glycopeptide, sulphonamide and tetracycline) results are shown inAppendix 2 and 3. The *S.aureus*routine susceptibility test result is indicated in Appendix 2 and CNS susceptibility in Appendix 3. The eight (8) oxacillin-resistant CNS isolates and their resistance to clindamycin, vancomycin, sulfamethoxazole-trimethoprim, tetracycline and penicillin Gare shown in Appendix 3. Strains that showed resistance to three or more classes of antibiotics were tittled as multi-drug resistant (MDR) and are illustrated in Tables 5 and 6.

Table 3: The routine antimicrobial resistance profile

Isolates	CD/2µg	VA/30 µg	SXT/ 25 µg	TET/ µg 30	P/IU
S. aureus	23.91% (n=11)	2.17% (n=1)	30.43% (n=14)	41.30% (n=19)	71.74% (n=33)
OR-CNS	37.5% (n=3)	0	0	25% (n=2)	87.5% (n=7)

CD: clindamycin; VA: vancomycin; SXT: sulfamethoxazole-trimethoprim; TET: tetracycline; P: penicillin; OR-CNS: Oxacillin-resistant coagulase-negative staphylococci; n: number of isolates

Table 4: Phenotypic identification of methicillin-resistant isolates

Isolates	OX/1 µg	FOX/30 µg
S. aureus	6.52% (n=3)	4.35% (n=2)
CNS	19.05% (n=8)	2.38% (n=1)

OX: oxacillin; FOX: cefoxitin; n: number of isolates; CNS: coagulase-negative staphylococci

Table 5: The multi-drug resistance pattern of*S. aureus* isolated from raw milk

Resistant *S. aureus* isolates (n = 46)		
Number of antibiotic agent(s)	Number	Percent
0	9	19.57
1	12	26.09
2	13	28.26
3	9	19.57
4	2	4.35
5	1	2.17

The Table shows 12 isolates of *S. aureus*(MDR) resistant to three or more different classes of antibiotics

Table 6: The antibiotic resistance pattern ofCNS isolated from raw milk

Resistant CNS isolates (N = 42)		
Number of antibiotic agent(s)	Number	Percent
0	33	78.57
1	5	4.76
2	4	11.90

The Table aboveshows CNS isolates resistant to a maximum of two different classes of antibiotics

4.5 PCR detection of *mecA* gene

All coagulase-positive*S.aureus* screened for the presence of*mecA* gene were negative; while three of the isolates of CNS were reported to be positive for *mecA* gene (Kisanga *et al*., unpublished). The PCR gel result is shown in the Fig. 4 below:

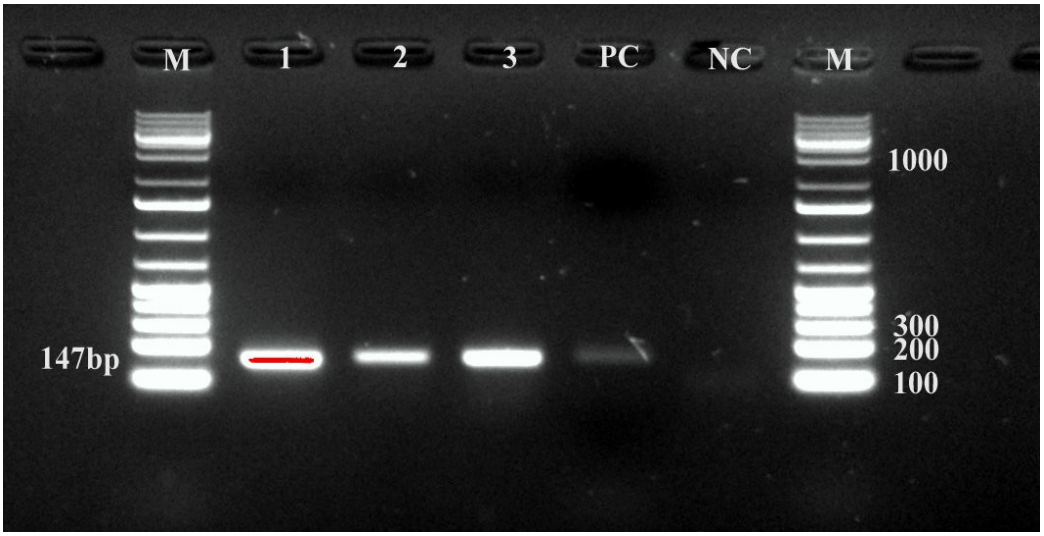

Figure 4: **PCR detection of *mecA* gene from coagulase-negative *S. aureus* and coagulase-negative staphylococciisolates:** PCR products showing *mecA* gene at 147-bp. Lane 1: Coagulase-negative Staphylococci *mecA* positive; Lane 2 and 3: Coagulase-negative *S. aureus mecA* positive; PC: positive control; NC: negative control and M: DNA marker.

Table 7: **Comparison between phenotypic and genotypic tests for the identification of isolates**

Isolates	Phenotypic test (n)	Genotypic test (n)
S. aureus	75	48
MRSA	3	2
CNS	42	NA
MRCNS	8	1

MRSA: methicillin-resistant*S. aureus*; CNS: coagulase-negative staphylococci; MRCNS:methicillin-resistant CNS; NA: not applicable; n: number of isolates

4.6 PCR detection of coagulase (*coa*) gene among the three *mecA* positive isolates

The PCR *coa* analysis for the three *mecA* gene positive isolates showed that no isolate contained the coagulase gene. Therefore, they were coagulase-negative *S.aureus* and coagulase-negative staphylococci.

4.7 The staphylococcal cassette chromosome*mec* (SCC*mec*) type I-V and Subtypes IVa-IVe

This study was not able to determine the SCC*mec* types for any of the three*mecA* gene positiveisolates.

CHAPTER FIVE

5.0 DISCUSSION

This study was aimed at determining the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA)in fresh bovine milk and to determine the SCC*mec* of the isolates by molecular techniques. To the best of our knowledge this study has not been conducted in the Morogoro Municipality previously. The prevalence of *S. aureus* (Table 2)in raw milk was found to be 48 (41.03%) out of the total isolates examined (n=117).In addition, the prevalence of *S. aureus* was 46 (61.33%) from 75 coagulase-positive and 2 (4.76%) previously thought to be CNS were from the 42 coagulase-negative isolates. Therefore, 41.03% of the milk samples collected from the Wards were found to be contaminated by*S. aureus*. Samples from Msamvu and Mwebesongo were the most contaminated at the rate of 16.7% and 12.5% respectively. The Wards that recorded the lowest *S. aureus* contamination were Tungi (2.1%), Magadu (2.1%), Kihonda (2.1%) and Boma (2.1%). The result demonstrates the presence of *S. aureus* in all milk samples.Similar studies conducted in Morocco, Brazil, Ethiopia and Kenya reported prevalences of *S. aureus* to be 40%, 68%, 48.75% and 30.6% respectively (Bendahou *et al*., 2008), (de Oliveira *et al.,* 2011), (Daka *et al*., 2012), (Shitandi *et al.*, 2004).Similar to this study, the high prevalence were from milk samples collected from sale points and markets. However, the difference in prevalence may be due to the fact that our samples were collected froma smaller geographical area within a shorter period compared to those reported elsewhere. The high levels of*S. aureus* in milk relates to poor hygiene practices and also the health status of the animals.Milk collected directly from farms

or in the markets are prone to contamination under poor sanitary conditions. Bacterial contamination of milk usually occurs during the milking process and this depends on the sanitary condition of the environment, utensils used for milking and the milking personnel.Therefore, public awareness regarding safe food handling would help to prevent cross-contamination (Weese *et al*., 2010) as well as potential colonization of handlers from contaminated food products. Other public health interventions, such as personalized and tailored food safety and education programs targeting diverse sociodemographic population could be a cornerstone in preventing the outbreak of staphylococcal food poisoning(Kadariya *et al*., 2014; Bredbenner *et al*., 2013).

The antimicrobial susceptibilitytest for *S. aureus*revealed a relatively low resistance to oxacillin (6.52%), cefoxitin (4.35%), and vancomycin (2.17%) as shown in Tables 4 and 3. Few strains were resistant to clindamycin (23.91%), sulfamethoxazole-trimethoprim (30.43%) and tetracycline (41.30%), while the highest resistance was to penicillin (71.74%). The resistance profile forCNS to oxacillin and cefoxitin was 19.05% and 2.40% respectively (Table 4). Meanwhile, the Oxacillin-resistant CNS had shown no resistance to vancomycin and only intermediate resistance to sulfamethoxazole-trimethoprim (12.5%).Few strains were resistant to tetracycline (25%) and clindamycin (37.5%), but penicillin Grecorded the highest resistance at 87.5%. Therefore, the study was able to phenotypically identify three *S. aureus* and eight CNS that were methicillin-resistant (Table 4). The multi-drug resistance (MDR) patternfor *S. aureus*(Table 5) indicates that12 (26.09%) isolates were MDR while no MDR was observed among CNS (Table 6). Ateba *et al.* (2010) reported that

a larger proportion of the *S. aureus* isolates abtained from milk in different farms were resistant to three or more antibiotics. It is well known that antibiotic use in livestock production for the treatment and prevention of *S. aureus* infection is on the rise. Unfortunately, inappropriate use of these antibiotics is weakening their efficacy. The use of low doses of antibiotics in the livestock industry is responsible for the emergingdrug-resistant bacteria on farms, which reach the population through human or animal carriers, and through the consumption of food of animal origin. In general, the highest resistance was to penicillin G by*S. aureus*(71.74%) and Oxacillin-resistant CNS (87.5%) isolates. Different sources have reported different rates of penicillin resistance for *S. aureus* and CNS. Benhassen *et al*.(2003) reported that 64% and 22.6% of the *S. aureus* and CNS strains respectively from goat mastitis were resistant to penicillin G. Similar report byMessadi *et al*.(1991)presented 64% and 18.6%.The relatively high resistance to penicillin in *S. aureus* and CNS could be due to the production of beta-lactamases.El-Ghodban *etal*. (2006)found that 75% of penicillin resistant*S. aureus* strains originating from food in Libya were positive for beta-lactamase.The second highest resistance in *S. aureus* was observed to tetracycline (41.30%), followed by sulfamethoxazole-trimethoprim (30.43%), andclindamycin (23.91%). The Oxacillin-resistant CNSwere resistant to clindamycin (37.5%) and tetracycline (25%) but susceptible to vancomycin and sulfamethoxazole-trimethoprim.In Ethiopia, Mekuria *et al*. (2013)has reported resistance of *S. aureus* to tetracycline (66.7%), sulfamethoxazole-trimethoprim (21.6%) and clindamycin (17.6%). These organisms were isolated from milk and nasal swabs of farm workers. It is likely that these bacteria have the capacity to change their resistance behaviour to the antimicrobials they are exposed to(Mekuria

et al., 2013) giving the variations in the rate of resistance to the antibiotics. Little resistance was seen to oxacillin (6.52%), cefoxitin (4.35%) and vancomycin (2.17%) in the *S. aureus* strains, while no resistance was observed to vancomycin among the Oxacillin-resistant CNS. The low resistance of *S. aureus*to vancomycin in our study is in agreement withAteba *et al*. (2010),who reported 4.7% resistance of isolates from communal farms in South Africa. However,Daka *et al*. (2012)reported higher resistance in isolates from cow's milk to oxacillin (60.3%) and vancomycin (38.5%). This difference may be attributed to the fact that, vancomycin was the drug of choice for treating MRSA infections and used in treating infections on dairy animals, suggesting that the frequent use may have lead to the development of resistant strains. There may also have been a cross-contamination of the milk, either by the sellers or the animals, since *S. aureus* resides on healthy human and animal skin.Moreover,*S. aureus* has developed multidrug resistance in many regions of the world (World Health Organization, 2000) and the usage of antibiotics correlates with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains (Shitandi *et al*., 2004).

PCR was conducted to detect *mecA* gene in *S. aureus*. None of the isolates (*S. aureus*) contained *mecA* gene. But a report by Kisanga*et al*. (unpublished) detected threeisolates habouring the *mecA* gene from the CNS. Since the study's estimation of methicillin resistance was also based on the detection of *mecA* gene:further analyses for coagulase (*coa*) gene and*S. aureus* species-specific have shown that none of them had *coa* gene but species-specific detected twocoagulase-negative variance of *S. aureus* (CN-MRSA) and one coagulase-negative staphylococci (MRCNS) (Table 7).

This highlights the importance of accurate diagnosis of coagulase-negative variants of*S. aureus* because not all *S. aureus* are coagulase positive.

Diagnosis based on coagulase test should be revised since a strain can be coagulase-negative and yet it is *S. aureus*. Therefore, PCR to detect *S. aureus* is very important. Occurrence of coagulase-negative *S. aureus* in bovine milk is extremely rare (Akineden *et al.,*2011), and coagulase-negative variants of *S. aureus* strains were first isolated from milk samples derived from subclinical mastitis cases in dairy cattle (Fox *et al*., 1996; Laevens *et al*., 1996; Matthews *et al*., 1997; Malinowski *et al*., 2009; Akineden *etal.*, 2011). This study revealed theprevalence for CN-MRSA andMRCNS to be 4.17% and 2.38%respectively, and it is in accordance with 4.8% MRSA reported by Umaru *et al*. (2013) from raw and fermented milk in Nigeria.Kateete *et al*. (2013)from Uganda, upon susceptibility testing, also reported MRCNS prevalence of 57% in cows and 64% in humans, however, MRSA was not detected (Kateete *et al*., 2013). Also, Lim *et al*. (2013)detected seven MRCNS in milk samples and samples from a farmer's hand and nose in South Korea.

The three*mecA* positive isolates in this study came from different Wards (Mafisa, Tungiand Mkundi), however, it was not possible to characterize these isolates for staphylococcal cassette chromosome *mec* (SCC*mec*) types I-V and the subtypes IVa-IVe when multiplex PCR was used. To date, eleven (I-XI) SCC*mec*types have been fully identified by the determination of *mec* (A, B, C1, C2 and D) and chromosome recombinase (*ccr*) complex (*ccr*AB1 to *ccr*AB5 and *ccr*C) complexes(Mkrtchyan *etal*., 2015;Kondo *et al*., 2007;Matsuhashi *et al*., 1986). However, the study was limited to test for types (I-V) due to minimum available funds. Other typing methods

such as MLST, PFGE and spa-typing were not done due to availability of funds as well.Among the three *mecA* positive isolates only the MRCNS was seen to be multi-drugresistant but theother two CN-MRSA showed resistance to only one class of antimicrobial. The*mecA* gene confers resistance to most currently available beta-lactam antibiotics (Berger-Bachi *et al*., 2002). But not all *mecA* positive clones are resistant to methicillin, and overall resistance levels in a population of MRSA depend on efficient production of PBP 2a, which is modulated by a variety of chromosomal and extrachromosomal factors(Appelbaum, 2007). This explains why MRSA resistance levels range from phenotypically susceptible to highly resistant (Berger-Bachi *et al*., 2002). According to Tavares (2000), the resistance to antibiotics, is explained not only by the presence of resistance genes, but also by expression of these genes, which is influenced by the environment. The*S. aureus* may be pathogenic or non-pathogenic with the pathogenic strains usually exhibiting coagulase-positivity and cause disease in their hosts (Jahan *et al.*, 2015).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The detection of *Staphylococcus aureus* in the milk samples indicates that the product is unwholesome for human consumption, and the population of Morogoro Municipality is at risk to staphylococcal infection or food poisoning.

The MDR *S. aureus* strains in the milk samples are a serious concern as these strains habour several antimicrobial resistance genes which may be transferred to antimicrobial susceptible strains, therefore increasing the population of microorganisms resistant to antimicrobials.

This study was undertaken mainly to estimate the prevalence of MRSA in the raw milk and to characterize the strains. The prevalence of CN-MRSA (4.17%) and MRCNS (2.38%) demonstrated by this study suggest the need for strict implementation and appropriate hygienic handling and processing techniques of milk and milk products from the farm to the sales point in order to prevent contamination. This study also report for the first time the presence of presumptive coagulase-negative variant of MRSA and MRCNS among multi-drug resistant *S. aureus* isolates from raw milk in Morogoro, Tanzania.

6.2 Recommendations

Based on the results of the current study, it would be pertinent to characterize further the CN-MRSA and MRCNS isolates so as to decide whether the isolates are clonally related. Also, the *S. aureus*, MDR strains and the CNS isolates must be screened for detection of the novel *mecA* homologue*mecC* gene and other antibiotic resistance genes. Furthermore, a range of sociological data by way of questionnaires or interviews should be applied to obtain general information from milk sellers/ distributorsregarding milk handling practices. Similar studies should also be conducted over a wider geographical area.

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APPENDICES

Appendix 1: The antibiotic susceptibility chart (CLSI, 2014)

Antibiotic Disk	Code	R / ≤ mm	I/mm	S/ ≥ mm
Cefoxitin	FOX	21		22
Clindamycin	CD	14	15-20	21
Oxacillin	OX	10	11-12	13
Penicillin	P	28		29
Tetracycline	TE	14	15-18	19
Vancomycin	VA	9	10-11	12
Sulfamethoxazole-trimethoprim	SXT	10	11-15	16

R : resistant ; I :intermediate ; S : susceptible

Appendix 2: The antibiotic resistance pattern of the coagulase positive*S. aureus* isolated from raw milk

Sample ID	OX/ 1µG	FOX/ 30µG	CD/ 2µg	VA/ 30 µg	SXT/ 25 µg	TET/ 30 µg	P/ 10 IU
1B	S	S	R	S	S	R	R
2C	I	S	S	S	R	S	R
2D	S	S	I	S	S	S	R
2G	S	S	I	S	S	R	R
3A	I	S	S	S	R	S	R
3C	S	S	R	I	S	R	R
3D	R	S	I	S	R	S	R
3E	S	S	I	S	R	R	R
3F	S	S	I	S	R	R	R
3H	S	S	I	S	R	I	R
4A	S	S	R	I	R	R	R
4B	S	S	R	S	S	R	R
4C	S	S	I	S	S	R	R
4D	S	S	I	I	R	R	R
4E	S	S	R	S	R	R	R
4F	S	S	I	S	S	S	S
4G	S	S	S	S	S	S	S
4H	S	S	S	S	S	S	S
5H	S	S	I	S	R	S	R
6B	S	S	S	S	S	S	S
6D	S	S	S	S	S	S	S
7A	S	S	I	S	S	S	S
7B	S	S	R	R	R	R	R
7C	S	S	S	S	S	S	S
7D	S	S	R	S	R	I	S
8A	S	S	R	S	S	R	R
8E	S	S	R	S	S	R	R
9C	S	S	S	S	S	R	R
9E	S	S	R	S	S	R	R
9H	S	S	I	S	S	R	R
10C	S	S	S	S	S	S	R
10D	R	R	I	S	S	S	R
10E	R	R	R	S	S	S	R
11A	S	S	S	S	S	S	S
11B	S	S	S	S	S	S	R
11C	S	S	S	S	S	R	S
12A	S	S	S	S	R	S	S
12E	S	S	S	S	S	R	R
12F	S	S	S	S	S	R	R

14D	S	S	S	S	S	S	R
14E	S	S	S	S	S	S	R
15B	S	S	S	S	S	S	R
15F	S	S	S	S	S	I	R
16A	S	S	S	S	S	S	R
18D	S	S	S	S	S	S	S
19A	S	S	S	S	R	S	S

S :susceptible; I :intermediate;R :resistance

Appendix 3: The antibiotic resistance pattern of coagulase negative staphylococci isolated from raw milk

sample ID	OX/ 1µG	FOX/ 30µG	CD/ 2µg	VA/ 30 µg	SXT/ 25 µg	TET/ 30 µg	P/ 10 IU
1A	S	S					
1C	S	S					
1E	R	S	S	S	I	R	
1G	R	S					
2A	R	S	R	S	S	S	R
2B	S	S					
2E	S	S					
3B	S	S					
5F	S	S					
7E	S	S					
7F	S	S					
8B	S	S					
8D	S	S					
9A	S	S					
9B	S	S					
9D	S	S					
9F	S	S					
9G	S	S					
10F	R	S	I	S	S	S	R
12B	S	S					
12C	R	R	S	S	S	R	R
12D	S	S					
14A	S	S					
14B	S	S					
15A	S	S					
15C	R	S	I	S	S	S	R
15D	S	S					
15E	S	S					
16B	S	S					
17B	I	S	R	S	S	S	R
17C	S	S					
17D	S	S					
17E	S	S					
17F	S	S					
17G	S	S					
17I	S	S					
18B	S	S					
18C	S	S					
19C	S	S					

19E	R	S	S	S	S	S	R
19F	R	S	R	S	S	S	R
19G	S	S					

Appendix 4: Research permit

CLEARANCE PERMIT FOR CONDUCTING RESEARCH IN TANZANIA

SOKOINE UNIVERSITY OF AGRICULTURE
OFFICE OF THE VICE-CHANCELLOR
P.O. Box 3000, MOROGORO, TANZANIA
Phone:023-2604523/2603511-4; Fax:023-2604651

Our Ref. SUA/ADM/R.1/8/ **Date: 10th October 2014**

The Municipal Director
Morogoro Municipal Council
P.O. Box 166
MOROGORO

Re: UNIVERSITY STAFF, STUDENTS AND RESEARCHERS CLEARANCE

The Sokoine University of Agriculture was established by Universities Act No.7 of 2005 and SUA Charter of 2007 which became operational on 1st January 2007 repealing Act No.6 of 1984. One of the mission objectives of the University is to generate and apply knowledge through research. For this reason the staff, students and researchers undertake research activities from time to time.

To facilitate the research function, the Vice-Chancellor of the Sokoine University of Agriculture (SUA) is empowered under the provisions of SUA Charter to issue research clearance to both, staff, students and researchers of SUA.

The purpose of this letter is to introduce to you **Mr. Mohamed Jibril** a bonafide **MSc. (Molec. Biol. & Biote.)** student with registration number **HD/X/SUA/VET/77/2013** of SUA. By this letter **Mr. Mohamed** has been granted clearance to conduct research in the country. The title of the research in question is **"Prevalence of Methicillin-Resistance Staphylococcus Aureus in Bovine Milk in Morogoro Municipality of Tanzania"**

The period for which this permission has been granted is from **October 2014 to May 2015.** The research will be conducted in **Morogoro Municipality.**

Should some of these areas/institutions/offices be restricted, you are requested to kindly advice the researcher(s) on alternative areas/institutions/offices which could be visited. In case you may require further information on the researcher please contact me.

We thank you in advance for your cooperation and facilitation of this research activity.

Yours sincerely,

Prof. Gerald C. Monela
VICE-CHANCELLOR

Copy to: Student – **Mr. Mohamed Jibril**

VICE CHANCELLOR
SOKOINE UNIVERSITY OF AGRICULTURE
P. O. Box 3000
MOROGORO, TANZANIA