PREVALENCE AND ANTIMICROBIAL PROFILES OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM RAW BOVINE MILK IN DAIRY AND PASTORAL FARMS OF MOROGORO, TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

2020

ABSTRACT

Staphylococcus aureus is an economic significant bacterium in dairy industry that can be spread to humans through consumption of raw milk causing foodborne infections. Severity of *S. aureus* infections either in animals or humans is enhanced by acquisition of resistance to methicillin. A cross-sectional study was carried out to establish prevalence, antibiotic susceptibility patterns and molecular characteristics of *S. aureus* in raw bovine milk from dairy and pastoral farms in Mvomero and Morogoro Urban Districts, Tanzania. A total of 397 milk samples were randomly collected from various wards in the study area. The pure isolates were identified by their cultural, morphological and biochemical features. Kirby Bauer Disk Diffusion method was used for the susceptibility testing. Multiplex PCR was used for detection of Methicillin resistance and virulence genes. Analysis of results revealed a prevalence of 124/397 (31.2%) for coagulase positive *S. aureus* (COPS) and 29/397 (7.3%) for coagulase negative *Staphylococci* (CONS) isolates based on conventional identification. All Coagulase positive *S. aureus* isolates were susceptible to cefoxitin(30µg) and chloramphenicol (50µg) but had resistance to penicillin G (10 UI), tetracycline (30µg), amoxicillin-clavulanic (30µg), oxacillin (1µg), gentamicin (10µg), and trimethoprim-sulfamethoxazole (30µg) at 93.5%, 28.2%, 25%, 22.6%, 8.1% and 1.6% respectively. The results also revealed that CONS had resistance of 86.2%, 17.2%, 17.2%, 10.3%, and 3.4% to penicillin G (10 UI), tetracycline (30µg), oxacillin (1µg), amoxicillin-clavulanic (30µg) and trimethoprim-sulfamethoxazole (30µg) but were susceptible to chloramphenicol (50µg), cefoxitin (30µg) and gentamicin (10µg). Of the 124 *S. aureus* isolates, 80 (64.5%) had *spa* gene and 1/124 (0.8% *mecA gene. S. aureus* (31.2%) isolated from raw bovine milk at farm level constitutes a health hazard to

consumers hence, highlighting the importance of observing hygienic milking practices as well as educating livestock farmers on proper usage of antimicrobials.

DECLARATION

I Nancy Kalee Evans, do hereby declare to the 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 degree award in any other institution.

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Name: Nancy Kalee Evans Date

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The declaration is hereby confirmed by;

_____ _____

Dr. Abubakar S. Hoza Date

(Supervisor)

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ACKNOWLEDGEMENT

I sincerely thank Inter-University Council of East Africa-World Bank for funding this project and my Masters coursework at Sokoine University of Agriculture.

I also express my profound gratitude to my supervisor, Dr. Abubakar S. Hoza for his dedicated guidance and wise counsel towards the success and completion of my research project.

To the field assistant officer Mrs. Enesa Mlay, Livestock extension officers of Morogoro Urban and Mvomero districts, I am indebted to them for providing the needed support during sample collection at respective study sites. Many thanks are also extended to the farmers, not forgetting the workers and colleagues at the microbiology laboratory in Department of Microbiology, Parasitology and Biotechnology.

Above all I owe it all the Almighty GOD for His faithfulness.

DEDICATION

To my beloved mum, husband and daughter.

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
bp	Base pair
CA-MRSA	Community-associated Methicillin resistant *Staphylococcus aureus*
CLSI	Clinical and Laboratory Standards Institute
CONS	Coagulase-negative *Staphylococci*
COPS	Coagulase-positive *Staphylococci*
DNA	Deoxyribonucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GARP	Global Antibiotic Resistance Partnership
IU	International Unit
MRSA	Methicillin resistant *Staphylococcus aureus*
^{o}C	Degrees Celsius
SCC*mec*	Staphylococcal Cassette Chromosome *mec* elements
SFP	Staphylococcal food poisoning
WHO	World Health Organization
µg	Microgram
µl	Microliter

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Staphylococcus aureus is an opportunistic pathogen in humans and an economic important pathogen in dairy industry (Mekonnen *et al*., 2018). *S. aureus* can be spread to humans through consumption of animal products such as raw/unpasteurized milk. Milk contamination by *S. aureus* and other *Staphylococci* species is a major concern in public health and dairy industry (Ateba *et al*., 2020). It can either occur from a cow infected with mastitis or unhygienic handling of milk by the milk handlers such as use of unsanitary utensils or milking equipment and unclean water (Ngasala *et al*., 2015). Cows infected with mastitis normally sheds these organisms into milk making it unfit for public consumption (Boerhout *et al*., 2016).

Veterinary usage of antimicrobials for therapeutic purposes or growth promotion influences the occurrence of resistant bacteria in livestock and their spread to humans (Lathers, 2002). The spread of antimicrobial resistant *S. aureus* to humans can be through consumption of animal products such as raw milk or meat and also close and regular proximity of humans to animals especially livestock keepers and veterinary professionals. Dissemination of antimicrobial resistant bacteria from animals to humans through foods of animal origin together with associated economic and health impact is well documented. Infections resulting from antimicrobial resistant bacteria are associated with high healthcare costs, increased hospitalization, longer duration of illness infections, increased mortality and lost labour and wages (Angulo *et al.,* 2004).

The increased resistant bacteria strains in dairy industry is attributed to misuse and uncontrolled sale of veterinary drugs in developing countries for livestock production (Massawe *et al*., 2019). Cost burden to the local farmers can be immense because intramammary infections with resistant strains leads to increased treatment or other veterinary expenses and substantial reductions in milk production. Therefore, antimicrobial resistance is clearly a threat to livestock production and public health (Shiferaw *et al*., 2016) warranting close monitoring and control of antibiotic resistant *S. aureus.*

1.2 Problem Statement

Unhygienic milking practices by farmers might be factors that can contribute to the development and spread of antimicrobial-resistant *Staphylococcus* species (Acar and Moulin, 2006). Most (75%) of raw/unpasteurized milk in developing countries is sold through informal networks with no regards to regulations and milk quality control measures (Bertu *et al*., 2010). Local markets in Tanzania rely mostly on raw milk supplied by pastoral and dairy farms because of its affordability and accessibility compared to pasteurized milk from the industries (Gwandu *et al*., 2018). Some of these farms are characterized by poor milking practices which contaminates milk with *S. aureus*. A study in Tanzania highlighted some of these practices such as; use of cold water without detergent for cleaning hands, udder and milking utensils, dirty milking shades and storing milk under room temperature (Ngasala *et al*., 2015). In Pemba Island, Zanzibar (Gwandu *et al*., 2018) reported similar poor hygiene milking practices including; washing of hands and cows udder with cold water, use of dirty plastic containers for milking and wiping udder/teats with one piece of cloth for all cows during milking.

1.3 Justification

Antimicrobial resistant *S. aureus* isolates in animal products have attracted great public and scientific concern because of their negative impact on human and livestock treatment (Shiferaw *et al*., 2016). Information on prevalence of *S. aureus* isolates in raw milk in from dairy and pastoral farms in Morogoro, Tanzania is limited. There are two studies in Morogoro where one was carried out in three dairy farms belonging to Sokoine University of Agriculture (Kashoma *et al.*, 2015) another one was conducted at sales points in Morogoro municipal (Mohammed *et al*., 2018). This study is therefore, providing an insight to both Public Health and Veterinary authorities on the current status of *S. aureus* and their resistance profiles that can inform decisions of the control strategies.

1.4 Research Objectives

1.4.1 Overall objective

To determine the prevalence, antimicrobial profiles and molecular characteristics of *S. aureus* isolated from raw bovine milk in dairy and pastoral farms of, Morogoro, Tanzania.

1.4.2 Specific objectives

i. To determine the prevalence of *S. aureus* isolates from raw bovine milk in dairy and pastoral farms of Morogoro.
ii. To establish the antimicrobial profiles of *S. aureus* isolates from raw bovine milk in dairy and pastoral farms of Morogoro.

iii. To characterize Methicillin resistant Staphylococcus *aureus* isolates from raw bovine milk in dairy and pastoral farms of Morogoro using molecular methods.

iv. To find out if there is any association between risk factors and prevalence of *S. aureus* in raw bovine milk.

1.5 Research Questions

i. Is raw bovine milk from dairy and pastoral farms in Morogoro contaminated with *S. aureus*?

ii. What are the antimicrobial profiles of *S. aureus* isolates from raw bovine milk from dairy and pastoral farms in Morogoro?

iii. What are the molecular characteristics of the *S. aureus* isolates in raw bovine milk from dairy and pastoral farms in Morogoro?

iv. Is there any association between risk factors and prevalence of *S. aureus* in raw bovine milk?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 General Description of *S. aureus*

S. aureus is a gram-positive coccus in shape bacterium belonging to the family Staphylococaccaceae (Mohammed *et al*., 2018). Upon gram staining, they usually appear like a bunch of grapes when observed under light microscope. On general purpose medium like nutrient agar, it forms medium yellow or white colonies, in blood agar *S. aureus* forms a clear zone of beta hemolysis due to production of beta hemolysins while in selective mannitol salt agar it produces golden yellow colonies with yellow zone suggesting fermentation of mannitol. It is identified biochemically by coagulase tests because of its capacity to clot blood by producing coagulase enzyme, distinguishing it from *S. saprophyticus, S. epidermidis, S. schleiferi, S. lugdunensis and S. haemolyticus* that are unable to produce coagulase enzyme (Harris *et al*., 2002) .

2.2 Prevalence of *S. aureus* Isolates from Raw Bovine Milk

There are several studies reporting varying rates of *S. aureus* in raw bovine milk from various geographical region between 2010 to 2020. A study in Morogoro Municipality involving raw milk samples from retail shops in 2015 reported 41% *S. aureus* (Mohammed *et al*., 2018). Another study involving 3 dairy farms located within a similar climatic region in Tanzania reported a prevalence of 49% *S. aureus* in raw milk (Kashoma *et al*., 2015). Massawe *et al*. (2019) found 15% *S. aureus* in raw milk samples from farmers and retail markets in Mbozi and Mbeya rural Tanzania between March-June 2015. A study in Algeria reported a prevalence of 41.8% (Chaalal *et al*., 2014) in raw

bovine milk samples collected from five farms between November 2011 to June 2012. A higher prevalence of 51.2% *S. aureus* from subclinical mastitis cows was reported in Ethiopia (Abebe *et al*., 2016) where cross bred cattle were more infected with mastitis than local cattle. Among the reasons contributing to milk contamination by *S. aureus* mentioned in these studies include poor hygiene and farm management practices. Improper washing of milking utensils/containers and hands or using untreated borehole water for sanitation (Ateba *et al*., 2010) .

2.3 *S. aureus* and Food Poisoning

Consumption of food containing *S. aureus* enterotoxin causes Staphylococcal food poisoning (SFP), a common food borne disease of major health concern globally (Argudín *et al*., 2010). Under optimal environment for growth, enterotoxigenic *S. aureus* strains produces toxins that cause food poisoning (Kwon *et al*., 2005). Symptoms of SFP includes nausea, hypersalivation, vomiting, diarrhea and abdominal cramping (Lin *et al*., 2016). SFP can be severe in immunocompromised patients, though in normal cases it is usually mild resolving within one to two days (Reddy *et al*., 2017). Most SFP especially of animal-origin foods are result of unhygienic handling and processing of food and related products (Massawe *et al*., 2011). Several types of foods such as milk and dairy products provide favorable environment (medium) for the growth of micro-organisms (Le Loir *et al*., 2003). Street vendors usually do not adhere strictly to food safety measures and yet about 80% of the families in Tanzania purchase unpasteurized milk from them (Kurwijila *et al*., 2009). This exposes such families to the risk of Staphylococcal food poisoning.

2.4 Emergence and Evolution of MRSA: Penicillin and Methicillin Resistance

Over the years *S. aureus* has acquired resistance to virtually all antibiotics used to treat it. *S. aureus* resistance to penicillin emerged in 1942 only 2 years after the introduction of this antibiotic (Rammelkamp and Maxon, 1942). Resistant *S. aureus* strains produced a beta- lactamase enzyme encoded by plasmid which inactivates beta-lactam ring of penicillin (Lowy, 2003). As early as 1950s penicillin resistant *S. aureus* were frequently reported in hospitals and subsequently spread out into the community (Chambers, 2001). Methicillin was then introduced in 1959 as an alternative to penicillin but two years later *S. aureus* became resistant to methicillin through acquisition of *mecA* gene (Jevons, 1961). MRSA was first reported in hospitals but later on it also emerged in community and livestock settings becoming an important bacteria pathogen (Miragaia, 2018).

Beta-lactams antibiotics including penicillin's, carbapenems, cephalosporins, monobactams and carbapenems act on bacteria by targeting bacterial penicillin binding proteins (PBPs) (Miragaia. 2018). Penicillin binding proteins are enzymes that are involved in biosynthesis of peptidoglycan, an essential component of bacterial cell wall. Therefore, binding of beta-lactams to PBPs inhibits synthesis of peptidoglycan and hence the growth of bacteria. In MRSA genome, *mecA* gene codes for penicillin- binding protein 2a (PBP2a) whose expression causes resistance to virtually all beta lactam antibiotics (Monecke *et al*., 2013). PBP2a is resistant to inhibition by the available beta-lactam antibiotics (Fuda *et al.*, 2004); therefore, allowing cell wall biosynthesis and survival of bacteria.

MecA genes are located on mobile genetic elements termed as Staphylococcal Cassette Chromosome *mec* (SCC*mec*) elements in *S. aureus* and other staphylococcal species (Ito

et al., 2009). The novel *mecC* gene located on a novel SCCmec element was first reported amongst bovine population in England (García-Álvarez *et al*., 2011). Later on, *mecC* (0.7%) was also reported for the first time in Sweden among dairy cows (Unnerstad *et al*., 2013). Besides *mecA, mecC* (*mecA*$_{LGA251}$) has also been reported to be associated with resistance to beta lactams in *S. xylosus* and *S. aureus (Harrison et al., 2014*; *García-Álvarez et al., 2011)*.

2.5 Methicillin-resistant *S. aureus* (MRSA) in Food

Foods of animal origin are vital in dissemination of antimicrobial resistant bacteria to humans. This spread can be through ingestion of resistant strains or antibiotic residues in food (Pesavento *et al*., 2007). MRSA strains have been isolated from domestic animals such as chicken, cattle, pigs, goats and other animals (Wachtmeister, 2018; Neeling *et al*., 2007) and their related food products including fresh Indian mackerel fish, raw milk, raw chicken meat and beef (Ali *et al*., 2017; Aqib *et al*., 2017; Wu *et al*., 2019; Kitai *et al*., 2004).

2.6 *S. aureus* Virulence Factor

S. aureus protein A is an important virulence factor encoded by *spa* gene. Protein A contributes to the development of the diseases by evading phagocytosis as it binds to the IgG molecules. *S. aureus* pathogenicity and clinical manifestations in cattle mastitis is therefore associated with *spa gene (Yadav et al., 2015)*. There are variable number of small tandem repeats in X region of *spa* gene with a length of 24 nucleotides that are used in genotyping of *S. aureus (Yadav et al., 2015)*.

Panton-Valentine leucocidin (PVL) is also a bacteriophage-encoded virulence factor which causes mild to severe infections such as necrotizing pneumonia and cutaneous infections to chronic osteomyelitis. (Shrivastava *et al*., 2018). *S. aureus* harboring PVL-encoding genes are common in human populations, so their occurrence in dairy industry is a risk to food safety and subsequently the health of consumers (Shrivastava *et al*., 2018).

2.7 Convectional Identification of *S. aureus*

Definitive identification of *S. aureus* is important for prevention and control of *S. aureus* infections in veterinary and human medicine. Conventional identification of *S. aureus* from cultured milk sample involves examination of colony morphological features, hemolysis on Blood agar, gram staining and biochemical tests such as catalase and coagulase (Muftah *et al*., 2011). Hemolytic characteristics can either be alpha, beta or gamma. *S. aureus* isolates usually show beta hemolysis on incubation for 18-24 hours under 37 ^{o}C.

Mannitol salt agar is a selective and differential media used to identify *Staphylococcus species*. Its high concentration of salts (7-9%) allows only members of *Staphylococcus* to grow since they can tolerate high saline levels (Ali *et al*., 2017). Its differential because of the presence of mannitol sugar and a pH indicator phenol red. *S. aureus* ferments mannitol sugar giving a characteristic golden yellow colony in mannitol salt agar, while other species are non-fermenters.

2.8 Detection of *S. aureus* and MRSA Using Polymerase Chain Reaction

Molecular tools are more sensitive and specific than conventional microbiological assays in detection of resistance and virulence genes in *S. aureus* strains from animal products (Corrente *et al*., 2007).

The *nuc* gene is specific to *S. aureus* strains hence used for PCR detection of *S. aureus* using specific primers (Barkstad *et al.,* 1992; Khan *et al.,* 2007). A novel *mecA* homologue, *mecC,* that is also associated with beta-lactamase resistance was discovered in *S. aureus* isolated from bulk milk of dairy cattle in England (García-Álvarez *et al*., 2011). Routine susceptibility testing detected methicillin resistance of the reported *mecC* gene but on *mecA* gene specific PCR it was negative. Because of its genomic composition difference from *mecA* (70% similar at the DNA level), *mecA* gene specific PCR cannot detect *mecC*-positive isolates (Ariza-miguel *et al*., 2014). Multiplex PCR was therefore adopted to co-detect and differentiate the two resistance genes and detect pathogenic *spa* and PVL-encoding genes *(Stegger et al., 2012)*.

2.9 Control of *S. aureus*

Prevention and control of *S. aureus* contaminations and spread at food production and distribution level is very crucial (Lin *et al*., 2016). Good hygienic milking practices are important in reducing microbial contaminations and intramammary infections. Washing udder/teat with clean warm water or sanitizing solution and drying with individual towels, preferably disposable towels are recommended for preventing milk contamination and the spread of infection between cows (Muftah *et al*., 2011). Creating awareness on Antimicrobial resistance and their consequences is important especially among village farmers and general public at large (Grema *et al*., 2015). Treatment of livestock infections

using antibiotics should always rely on the results of routine antimicrobial susceptibility testing rather than self-prescription by the farmers (Grema *et al*., 2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Design

A cross-sectional study was conducted from October, 2019 to June, 2020 in Morogoro urban and Mvomero district, Morogoro region. Morogoro region is situated in the eastern part of Tanzania, 196 kilometers west of Dar es Salaam, the country's largest city and commercial center, and 260 kilometers east of Dodoma, the country's capital city. The region lies between latitudes 5˚58’ and 10˚00’ South of the Equator and between longitudes 35˚25’ and 38˚30’ East of Greenwich. It is divided into six (6) districts namely; Mvomero, Morogoro Urban, Morogoro Rural, Kilombero, Kilosa and Ulanga. It covers an area of 70 624 square kilometers with a human population of 2 218 492 based on 2012 census (Tanzania National Bureau of Statistics, 2013).

Morogoro urban District was purposely selected because of the availability of dairy farms operated by institutions, large scale and small-scale farmers in the urban region of Morogoro who supplies milk to the local market. Morogoro urban district has 29 administrative wards from which seven study administrative wards were selected randomly. On the other hand, Mvomero district is dominated by agro-pastoralists whose livelihoods mostly relies on livestock through sales of live animals and milk (Fred *et al*., 2013). Out of 30 wards in Mvomero district, seven were randomly selected for the study.

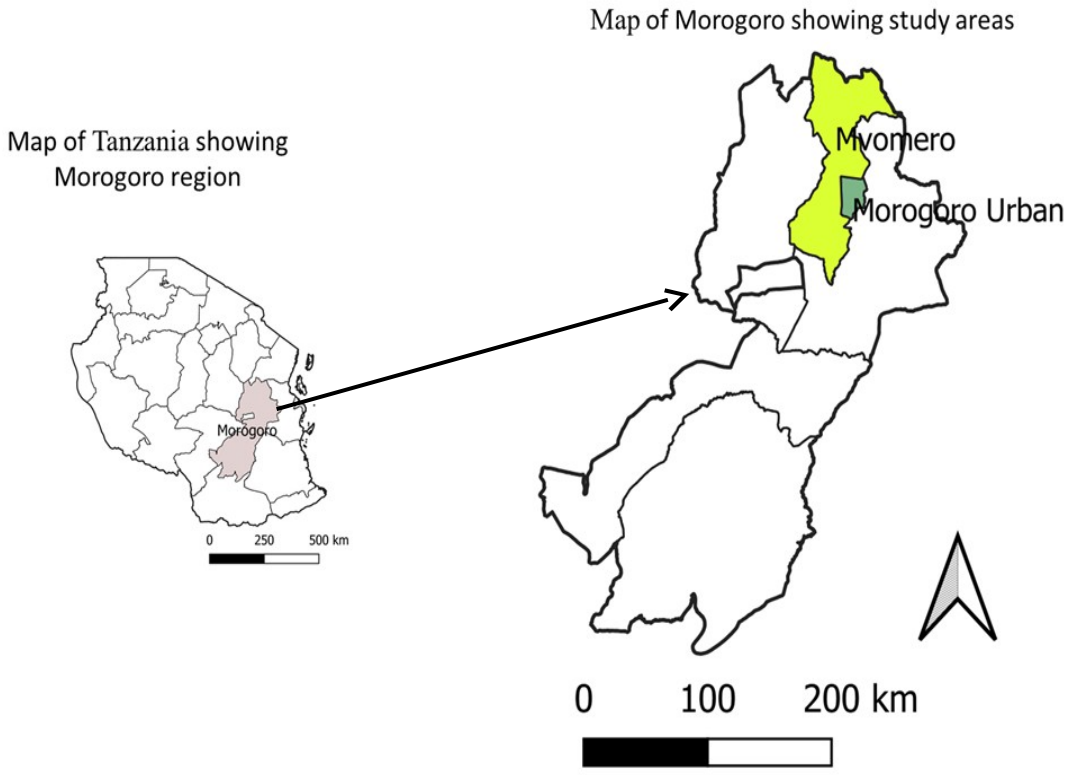

Figure 1: Map of Tanzania and study districts (constructed using QGIS Software version 3.14)

3.2 Study Population and Husbandry Practices

Animals included in this study were lactating cattle including dairy and indigenous cattle. Dairy cattle in dairy farms where managed with semi-intensive system where cattle are kept in cattle sheds for some time and fed with cut and carry pasture. The cattle shed floors were either stoned paved, concrete or earthen. The model of production of indigenous cattle in pastoral communities was free grazing of large herds of cattle in open grazing lands.

3.3 Sample Size

Assuming 49% prevalence (p) of *S. aureus* in raw bovine milk (Kashoma *et al*., 2015), error rate (e) of 5% at 95% confidence interval (z= 1.96), the formula below described by Thrusfield (2005) was used estimate the minimum sample size (n) required of milk samples.

$$n=\frac{Z^2 p(1-p)}{e^2}$$

n = 384, however, 397 raw bovine milk samples were collected for this study.

3.4 Sample Collection

A total of 397 raw bovine milk samples were collected from individual cattle. Udder was cleaned with warm clean water and gloved hands used to collect milk from the cow's four teats into a sterile universal bottle. The first streams of milk (foremilk) were discarded before collecting actual sample. In situations where farmers did not approve the use of gloves to milk their cows, cleaning hands with hand detergent and water after collecting from each cow was observed to minimize cross contamination. All collected samples were numbered accordingly and packed in a cool box with ice packs then transported to

Microbiology laboratory in the Department of Veterinary Microbiology, Parasitology and Biotechnology, Sokoine University of Agriculture within 2 hours of collection. A semi-structured questionnaire was used to collect data on milking practices and usage of antibiotics from each farm's herd (Appendix 1).

3.5 Isolation and Identification of *S. aureus*

A procedure described by Jahan *et al*. (2014) was used for isolation where briefly, 10µl of milk sample was inoculated on 5% horse blood agar (HiMedia, India) using sterile wire loop and incubated at 37 ºC for 24-48 hours. Colonies were gram stained and sub cultured on Mannitol salt agar (HiMedia, India) and incubated at 37ºC for 24-48 hours. Identification was based on morphological features of beta hemolysis patterns, mannitol fermentation, gram staining and biochemical tests including catalase and coagulase.

3.5.1 Catalase test

Hydrogen peroxide (3%) was prepared and tested with a positive control *S. aureus* ATCC 25923 before testing the isolated pure colonies. A small amount of bacterial colony from an overnight grown culture was transferred to a surface of a glass slide with a drop of normal saline (0.85%) using a sterile wire loop then emulsified to make a smooth suspension. A drop of 3% H_2O_2 was added over the test smear and observed for the appearance of gas bubbles.

3.5.2 Coagulase test

One to two colonies from an overnight grown culture were emulsified in a small drop of distilled water on a surface of a glass slide to make a suspension. A drop of rabbit plasma was added to the test suspension and mixed well by rocking as clumping was being observed. Some colonies which did not show clumps (agglutination) were further

identified by tube coagulase test. Rabbit plasma was diluted with sterile distilled water at a ratio of 1:10. Colonies of test isolate were resuspended in 2ml of diluted plasma in a sterile glass test tube. A positive control *S. aureus* ATCC 25923 and negative control which included diluted plasma with no culture were included. All the test tubes were incubated at 37 ^{o}C and observed after every hour for four hours. A positive result was indicated by a gel formation. If gel did not form after four hours the test tubes were kept at room temperature overnight and results observed. Isolates which did not form a gel after four hours and after overnight were then confirmed as negative. Isolates that were positive for coagulase testing were identified as coagulase positive *S. aureus* (COPS) and coagulase negative isolates as coagulase negative *Staphylococci* (CONS). Pure isolates were stored on a nutrient agar slants at -20 ^{o}C until further analysis.

3.6 Antimicrobial Susceptibility Testing

Confirmed isolates were tested for their susceptibility to eight antimicrobials using Kirby-Bauer disk diffusion method (Massawe *et al*., 2019) according the Clinical and Laboratory Standards Institute guidelines (CLSI, 2019). The following antimicrobials that are commonly used for treatment of mastitis and growth promotion in livestock production and as primary class of antibiotic for treatment of bacterial infections in human (GARP-Tanzania, 2015) were used; oxacillin (1 µg), cefoxitin (30 µg) amoxicillin/clavulanic acid (30 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (25 µg), tetracycline (30 µg), chloramphenicol (50 µg) and penicillin G (10 IU) manufactured by (Liofilchem, Italy). Briefly, three to four colonies were picked using a sterile inoculating loop and suspended into 4ml sterile normal saline. Turbidity of bacterial suspension was adjusted to 0.5 McFarland standard then streaked on to the surface of sterile Muller Hinton agar (Oxoid, UK) in a petri dish using sterile cotton

swabs and allowed to air dry. Antimicrobial disks were placed on the surface of the inoculated agar plates and incubated at 37 ºC for 18 hours. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used as quality control. Diameter of inhibition zones was measured using a ruler and the results interpreted according to the zone diameter interpretative standards (CLSI, 2019) for *Staphylococcus* species.

3.7 Molecular Characterization of *S. aureus*

3.7.1 DNA extraction and quantification

Quick-DNA fungal/bacterial Miniprep Kit (ZymoResearch Corp, USA) was used to extract bacterial genomic DNA of isolates grown overnight on nutrient agar according to the manufacturer's instructions. Briefly, three to four bacteria colonies were suspended in 200 µL of nuclease free water in a 1.5 µL microcentrifuge tube. Bacterial suspension was transferred into ZR BashingBead™ lysis tube (ZymoResearch Corp, USA) and 750 µL BashingBead™ buffer added, then processed in a Disruptor Genie™ (ZymoResearch Corp, USA) for 10minutes and centrifuged at 1000xg for 1minute, then transferred 400 µL of the supernatant to a Zymo-Spin™ IIC Column in a collection tube and centrifuged again. Genomic Lysis buffer (1200 µL) was added to the filtrate, mixed well then transferred to a Zymo-Spin™ IIC Column in a collection tube, centrifuged and flow through was discarded. A DNA pre-Wash Buffer was added and centrifuged. Finally, 70 µL of DNA elution was added to elute DNA from the Zymo-Spin™ IIC column into 1.5 µL microcentrifuge tube. The concentration and purity of the extracted genomic DNA were estimated using Nanovue plus™ spectrophotometer (Biochrom Ltd, UK) at 260 and 280 nm. The genomic DNA was then stored at -20º C until used for molecular analyses.

3.7.2 Polymerase chain reaction

A multiplex PCR was used for the detection of Methicillin resistance and virulence genes. Methicillin resistance was achieved by amplification of both *mecA* and *mec*C and virulence genes of *S. aureus* by amplification of the *spa* gene and Panton Valentin Leukocidin (PVL)-encoding genes following the protocol from (Stegger *et al.*, 2012) with modifications to suit our experiment using the primers described previously as shown in Table 1. PCR contained 12.5 µL of premix, 5.5 µL of nuclease free water, 0.5 µL of each of the four sets of primers with variable product size and 3 µL of bacterial genomic DNA. The thermal cycling conditions were; initial denaturation at 94 ºC for 5 minutes, then 35 cycles of 94 ºC for 30 seconds, 59 ºC for 1 minute and 72 ºC for 1 minute with a final extension at 72 ºC for 2 minutes. Agarose gel (2%) electrophoresis was used to separate PCR products in 1X Tris–acetate-EDTA (TAE) buffer with 10 µl E-Z Vision staining dye at 120 volts for 60 minutes using Midi plus 15 electrophoresis system (VWR, USA). Calculation of number of tandem repeats (N) in *spa* gene product to ascertain virulence was done using the formula below given by Frenay *et al*. (1996):

$$N = \frac{¿\,the\ amplified\ spa\ gene\ product - ¿\,primers\,(forward + reverse)}{24}$$

3.8 Risk Factors Associated with Milk Contamination by *S. aureus*

Presence of *S. aureus* in raw bovine milk is either from mastitis dairy cow, milk handlers or poor hygiene farm management practices (Fagundes *et al*., 2010). Under favorable growth condition *S. aureus* multiplies to high levels of contamination posing risk to public health (Shiferaw *et al*., 2016). Type of floor in the milking and cow's sleeping shades is very key in reducing the risk of milk contaminations. Due to low income with increased needs and demands, most dairy and pastoral farms do not put-up concrete floors

in the milking area or use detergent for cleaning hands during milk and they also use a single piece of cloth to wipe teats for all cows during milking.

Misuse of antimicrobials by the farmers for therapeutic purposes or growth promotion also accelerates the occurrence of antimicrobial resistant *S. aureus* in foods of animal origin (Massawe *et al*., 2019). Easy access to antimicrobials from unauthorized drug sellers and lack of knowledge on antimicrobial use and resistance among livestock keepers complicates the situation (Kimera *et al*., 2020).

Table 1: Primers used in this study

Primer name	Gene	Oligonucleotide sequence (5' - 3')	Amplicon size (bp)	Reference
spa-1113F	*Spa*	TAAAGACGATCCTTCGGTGAGC	Variable (180-600)	Stegger *et al*., 2012
spa-1514R		CAGCAGTAGTGCCGTTTGCTT		
mecA P4F	*mecA*	TCCAGATTACAACTTCACCAGG	162	Stegger *et al*., 2012
mecA P7R		CCACTTCATATCTTGTAACG		
pvl-F	*lukF-PV*	GCTGGACAAAACTTCTTGGAATAT	85	Stegger *et al*., 2012
pvl-R		GATAGGACACCAATAAATTCTGGATTG		
$mecA_{LGA251}$ MultiFP	$MecA_{LGA251}$	GAAAAAAAGGCTTAGAACGCCTC	138	Stegger *et al*., 2012
$mecA_{LGA251}$ MultiRP		GAAGATCTTTTCCGTTTTCAGC		

3.9 Data Analysis

Collected data was entered into Microsoft excel spread sheet and subjected to descriptive statistics such as frequencies and proportions. Chi-squared (χ^2) test (Epi info Version 7) was used to determine the prevalence and variables on milking practices and antimicrobial usage. All results at $p < 0.05$ were considered statistically significant.

3.10 Ethical Clearance

This study was approved by Ethical Committee, Director of Postgraduate Studies, Research Technology Transfer and Consultancy, Sokoine University of Agriculture (Appendix 2). Executive directors of Mvomero and Morogoro Urban Districts granted the permission to collect data in the respective wards of their jurisdiction. The objective and importance of the study was well explained to farmers for their consent before sample collection.

CHAPTER FOUR

4.0 RESULTS

4.1 Study population

In this study, 190 dairy and 207 indigenous cows were used for sample collection. The study dairy farms were located in seven different wards in Morogoro Urban which included; Magadu, Mazimbu, Kichagani, Mindu, Kingolwira, Bigwa and Mlimani as shown in table 2. Majority of the cows sampled from the dairy farms were crossbreeds including Friesian, Ayrshire and Jersey.

In Mvomero district, the 207 indigenous cows were from 26 homesteads in seven wards of Mvomero, Melela, Mlali, Mangae, Dakawa, Mzumbe and Lubungo. Cattle in pastoral communities were indigenous Tanzanian short horn zebu (TSHZ) and their crosses of Friesian, Ayrshire and Jersey.

Table 2: Distribution of samples collected from respective districts and wards

Morogoro Urban District		**Mvomero District**		**Sample size**
Ward	**No. of cows**	**Ward**	**No. of cows**	
Mindu	53	Lubungo	38	
Magadu	39	Dakawa	37	
Mlimani	29	Mlali	37	
Kingolwira	26	Mvomero	33	
Mazimbu	19	Melela	31	
Kichagani	14	Mangae	17	
Bigwa	10	Mzumbe	14	
Total	**190**		**207**	**397**

4.2 Isolation and Identification of *S. aureus*

4.2.1 Macromorphological and micromorphological characteristics

A total of 153/397 (38.5%) isolates had a typical characteristic of *Staphylococcus* species colonies. Of the 153 isolates, 124 (81.1%) showed zones of clear beta hemolysis on Blood agar and characteristic golden yellow colonies on mannitol salt agar, suggesting fermentation of mannitol (Figure 2). While 29/153 (19.0%) *Staphylococci* isolates showed gamma hemolysis in B and no fermentation observed in mannitol. However, they were all gram positive arranged in clusters (Figure 2).

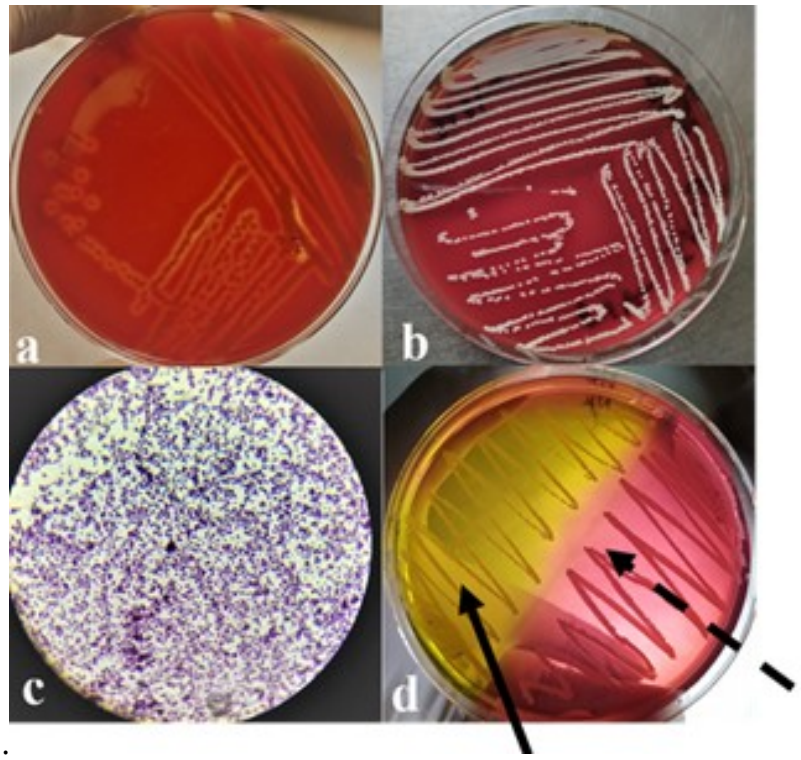

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Figure 2: Bacteria culture results indicating *Staphylococci* isolates: (a) beta hemolytic colonies on Blood Agar, (b) gamma hemolytic colonies on blood agar (c) Microscopic appearance of gram-positive cocci in clusters and (d) Mannitol fermenter (see solid arrow) and non-fermenter (see broken arrow) colonies on Mannitol salt agar.

4.2.2 Biochemical characteristics of the isolates

Of the 153 *Staphylococci* isolate suspects, 124 (81.1%) were coagulase positive *S. aureus* and 29 (19.0%) coagulase negative *Staphylococci* (Table 3).

Table 3: Distribution of isolates showing a typical characteristic of *Staphylococci* in dairy and pastoral farms

Source of samples	Total milk samples	No. of MSA fermenter isolates	Gram + cocci, grape like clusters	Catalase test (Positive)	β- hemolytic and coagulase (Positive)
Dairy farms	190	78	90	90	78
Pastoral farms	207	46	63	63	46
Total	**397**	**124**	**153**	**153**	**124**

MSA, Mannitol salt agar; +, positive

The overall prevalence of *S. aureus* isolates in this study was 124/397 (31.2%). Notably, the dairy farms had higher prevalence of 78/190 (41.1%) than pastoral farms 46/207 (22.2%) Figure 3.

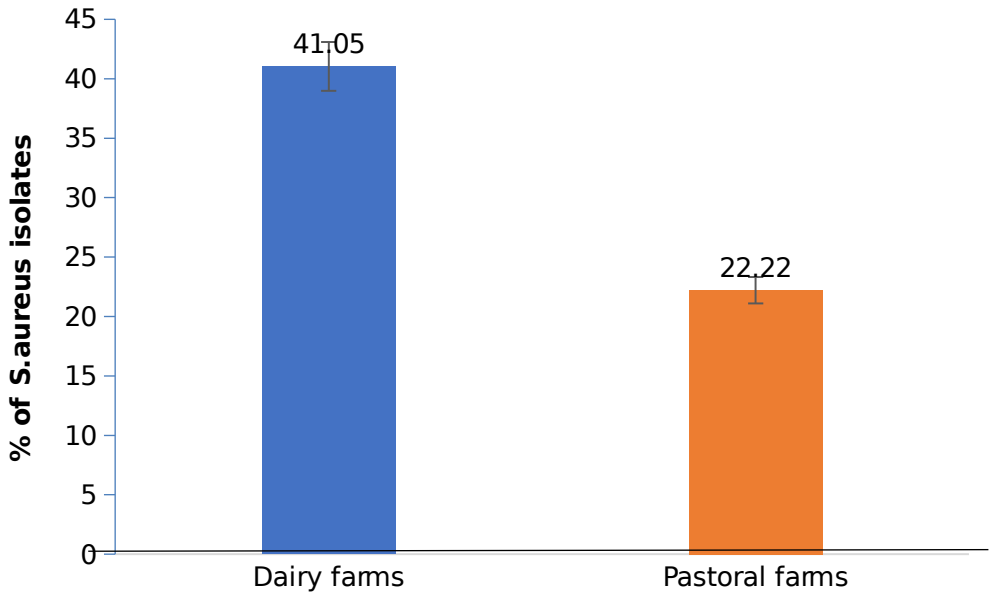

Figure 3: Prevalence of *S. aureus* in dairy and pastoral farms.

4.3 Antimicrobial Susceptibility Testing Results

Antimicrobial susceptibility profiles of 124/397(31.2%) coagulase positive *S. aureus* isolates from dairy and pastoral farms are shown in Table 4. They were susceptible to chloramphenicol and cefoxitin but had resistance to penicillin, tetracycline, amoxicillin-clavulanic acid, oxacillin, gentamicin, and trimethoprim-sulfamethoxazole at 93.5%, 28.2%, 25%, 22.6%, 8.1% and 1.6% respectively.

Table 4: Antimicrobial profiles of *S. aureus* isolates from dairy and pastoral farms

Antimicrobial	% Susceptible		% Intermediate		% Resistant	
	Dairy farms (n=78)	Pastoral farms (n=46)	Dairy farms (n=78)	Pastoral farms (n=46)	Dairy farms (n=78)	Pastoral farms (n=46)
Penicillin G (10 UI)	5.1	8.7	0	0	94.9	91.3
Tetracycline (30µg)	57.7	65.2	16.7	2.2	25.6	32.6
Oxacillin (1µg)	60.3	50	16.7	28.3	23.1	21.7
Amoxicillin-clavulanic (30µg)	66.7	89.1	0	0	33.3	10.9
Gentamicin (10µg)	91.0	86.9	2.6	2.2	6.4	6.4
Trimethoprim-sulfamethoxazole (30µg)	100	95.7	0	4.3	0	4.4
Chloramphenicol (50µg)	100	100	0	0	0	0
Cefoxitin(30µg)	100	100	0	0	0	0

Of the 124 COPS isolates, 8 (6.5%) were susceptible to all tested antimicrobials, 48 (38.7%) had single resistance, 53 (42.7%) double resistance, 5 (4.03%) multiple resistance and 10/124 (8.1%) intermediate profile. Figure 4 below shows a representative image of drug susceptibility testing results of *S. aureus* isolate showing respective antibiotic profiles on Muller Hinton agar according to CLSI, 2019 guidelines.

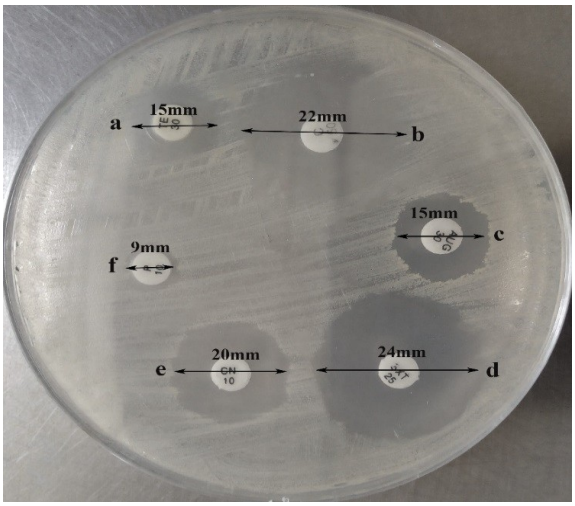

Figure 4: Drug susceptibility testing results showing *S. aureus* isolate susceptible (b, d, e) Intermediate (a) and Resistant (c, f) to different antibiotic discs on Muller Hinton agar according to CLSI, 2019 guidelines.

Results revealed that 29/397 (7.3%) CONS had resistance of 86.2%, 17.2%, 17.2%, 10.3%, and 3.4% to penicillin G (10 UI), tetracycline (30 µg), oxacillin (1 µg), amoxicillin-clavulanic acid (30 µg) and trimethoprim-sulfamethoxazole (30 µg) respectively however, they were susceptible to chloramphenicol (50 µg), cefoxitin (30 µg) and gentamicin (10 µg). Of 29 CONS isolates, 2 (6.9%) were susceptible to the eight antimicrobials, 15 (51.7%) had single resistance, 6 (20.7%) double resistance, 4 (13.8%) multiple resistance and 2 (6.9%) intermediate profile.

4.4 Molecular Detection of MRSA and Genotyping of *S. aureus*

4.4.1 Genotyping of *S. aureus* isolates from raw milk

Of 124 *S. aureus* isolates, 80 (64.5%) produced amplicons of *spa* gene (Figure 5) with eight different product size amplified at 180, 200, 300, 340, 370, 390, 400 and 600 bp with 6, 6, 11, 12, 13, 14, 15 and 24 number of tandem repeats respectively. In terms, of pathogenicity, 78/124(62.9%) were pathogenic possessing more than 7 tandem repeats

(Table 6). The most frequent tandem repeat observed was 14 in 71/80 (88.8%) of the isolates. On the other hand, 79/80 (98.8%) isolates produced only single *spa* amplicon whereas 1/80 (1.2%) isolate produced two *spa* amplicons of 390 and 600bp. There was no S. *aureus* isolate harboring PVL-encoding gene that was amplified at 85bp.

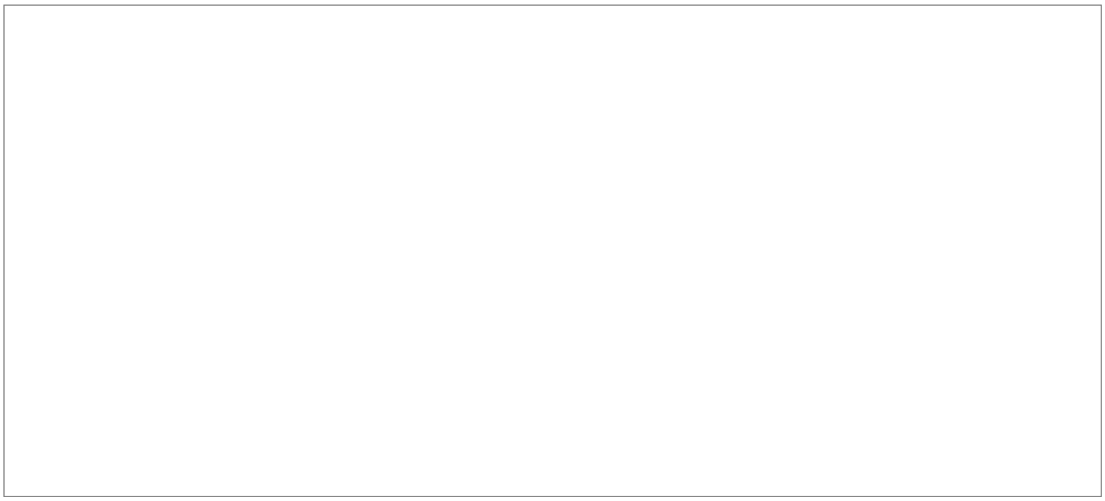

Figure 5: PCR amplification of *spa* gene in *S. aureus* isolates; *spa* gene positive isolates (Lane 1, 2, 3, 4, 5, 7, 8, 9, 10, 11 and 12), *spa* gene negative isolate (Lane 6), PC; positive control, NC; negative control, M; DNA ladder marking from 100bp to 1kb.

Table 5: Polymorphic protein A (*spa*) gene in *S. aureus* isolates from raw milk

Serial no.	No. of isolates	*Spa* gene amplicon size (bp)	No. of tandem repeats
1	1	180	6
2	3	200	6
3	1	300	11
4	1	340	12
5	2	370	13
6	69	390	14
7	1	390,600	14,24
8	2	400	15

4.4.2 PCR Detection of *mecA* and *mecC* Gene Amongst *S. aureus* Isolates

Of 124 *S. aureus* isolates, 1 (0.8%) had *mecA* resistance gene. The amplification of *mecA* gene was detected at 162bp as shown in Figure 6 below. There were no positive isolates in detection of *mecC* to confirm *S. aureus* resistance gene at 138bp.

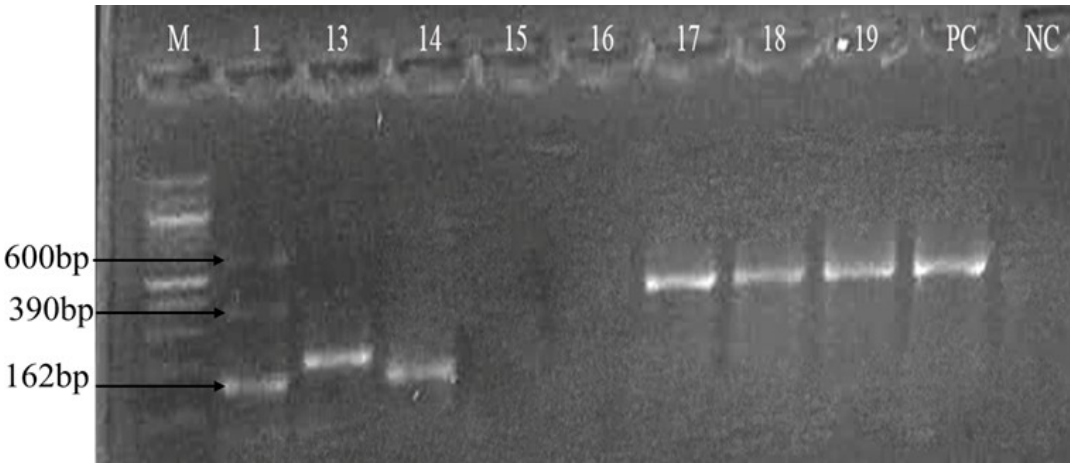

Figure 6: Multiplex PCR detection of *mecA* and *mecC* gene; *S. aureus mecA* (162bp) positive isolate (Lane 1), *S. aureus spa* gene positive isolates (Lane 1,13,14,17,18,19); *S. aureus spa* negative isolates (Lane 15,16); PC, positive control; NC, negative control; M, DNA ladder.

4.5 Risk Factors Associated with Prevalence of *S. aureus*

Prevalence of *S. aureus* isolates in raw bovine milk was significantly associated with hygiene status of the farm (*p* value=0.009; 95% confidence interval (1.313-14.687), hand washing without detergent (*p* value=0.0008; 95% confidence interval, 1.9006-104.316) and hand washing after milking every cow (*p* value=0.0008; 95% confidence interval, 1.9006-104.316) (Table 4).

Table 6: Association between farm hygiene, usage of antimicrobials and prevalence of *S. aureus* in dairy and pastoral farms

Variables	Category	Frequency (n=32)	χ^2 value	*p*-value	CI-95%
Hygiene status of the farm	Good	3	6.79	0.009	1.313-14.687
	Poor	29			
Floor type	Concrete	5	3.4312	0.064	0.923-6.519
	Soil	27			
Cleaning quality of milking utensils	Good	7	1.223	0.269	0.683-3.851
	Poor	25			
Method of milking	Hands	30	50.295	0	0.007-0.129
	Machine	2			
Hand pre washing	With detergent	1	11.332	0.0008	1.9006-104.316
	Without detergent	31			
Hand washing after milking every cow	Yes	1	11.332	0.0008	1.9006-104.316
	No	31			
Udder/teat washing before milking	Yes	32	60.514	0	0
	No	0			
Veterinary usage of antibiotics	Yes	32	60.514	0	0
	No	0			
Observing withdrawal periods	Yes	29	45.524	0	0.014-0.157
	No	3			
AMR awareness	Yes	10	0	0	0.459-2.174
	No	22			

CHAPTER FIVE

5.0 DISCUSSION

S. aureus is one of the economically significant bacteria in dairy industry (Kummel *et al*., 2016) and can be spread to humans through consumption of raw milk and related products. The observed overall prevalence of *S. aureus* (31.2%) is an indication that raw bovine milk is not safe for consumption by people in Morogoro urban and Mvomero districts, Tanzania. This study found that milk samples analyzed from the dairy farms were more contaminated with *S. aureus* than milk samples from pastoral communities which largely keeps indigenous cattle. The higher prevalence observed in dairy cows could probably be due to their high milk production rate which is directly proportional to udder infection (Saifudeen *et al*., 2018) and reduced period the calves are allowed to suckle after milking (Shem *et al*., 2002).

Milk contamination by *S. aureus* can occur through shedding of the organism into milk from cows with clinical or subclinical mastitis (Mcmillan *et al*., 2016), but high proportion of *S. aureus* contamination in this study often relates to poor hygienic milking practices (Reta *et al*., 2016). Milking environment and practices significantly contributes to contamination of raw milk by microbes (Gwandu *et al*., 2018). Notably, milking areas in the study farms were poorly maintained in terms of hygiene where majority of the floors were dirty or muddy favoring *S. aureus* proliferation and spread. Hands of milkers can be a primary source of microbial transmission during milking (Zadoks *et al*., 2011). Therefore, washing hands with a detergent or soap is essential for infection control. Cold water without detergent was used to wash hands before milking in most of the farms (96.9%). In addition, washing of hands after milking each cow was not observed as well.

Comparable to our results, previous studies in Ethiopia and Pakistan reported frequencies of 39.2% and 34.2% *S. aureus* respectively *(Elemo et al., 2017;* Maalik *et al*., 2019). In contrast to our study, the findings of Bitew *et al.* (2010) and Zeryehun *et al*. (2017) in Ethiopia and Hamid *et al*. (2017) in India found a prevalence of 20.3 % , 24.1% and 22.5% *S. aureus* respectively in raw bovine milk. Similar studies have reported comparable prevalence in raw bovine milk from the pastoral communities including Asiimwe *et al*. (2017) in Uganda and Girma *et al*. (2012) Ethiopia at the rate of 20.3% and 24.2% respectively. Proper milk pasteurization minimizes the risks of infections to the public, unfortunately, most of the milk in low middle income countries is sold through informal channels as raw/unpasteurized (Bertu *et al*., 2010). Under optimal environment for growth, enterotoxigenic *S. aureus* isolates present in food produces enterotoxins that causes food poisoning (Kwon *et al*., 2005). Staphylococcal food poisoning can be severe in immunocompromised patients, though in normal cases it resolves within one to two days of onset (Reddy *et al*., 2017).

Emergence and dissemination of resistant *S. aureus* in livestock production has been attributed mainly to the misuse of antimicrobials in either treatment or growth promotion (Kalayu *et al*., 2020). These bacteria can be transmitted to humans , negatively affecting management of the associated infections in humans as well as in animals (WHO, 2017). Therefore, it was of paramount importance to characterize antimicrobial resistant profiles of *S. aureus* isolates from dairy and pastoral farms. The *S. aureus* isolates exhibited high resistance to penicillin G (>90%) similar to a study in Addis Ababa (Mekuria *et al*., 2013) which reported (92.2%) resistance to penicillin. They also reported (33.3%) resistance to oxacillin which is close to the present study. Furthermore, *S. aureus* isolates with (100%) resistance to Penicillin G were reported from different farm settings in South Africa

(Ateba *et al*., 2010) . On contrary, a lower resistance of (15.8%) to penicillin G has been reported in India from bovine mastitis milk samples (Res and Samples, 2017). Results of this study for *S. aureus* were also comparable to Matallah *et al*. (2019) for single and multi-drug resistance (47.4% and 3.2%) respectively, nevertheless, they were different for double resistance (4.2%). This indicates that the proportion of multi drug isolates in the two study sites is still low.

CONS antimicrobial resistant profiles were also determined because they have been reported to have enterotoxins (Lourdes *et al*., 2007) and antimicrobial resistance genes. It is also an economic important bacterium in dairy industry that causes bovine mastitis (Gizaw *et al*., 2020). CONS can therefore, introduce toxins to food causing Staphylococcal food poisoning or be spread to human population as a resistant strain through consumption of unpasteurized milk. Similarly, to the present investigation, a high resistance (71.4%) to penicillin G was reported in Tunisia among CONS isolates from raw bovine milk (Klibi *et al*., 2018). Studies show that penicillin is commonly used in Tanzania for livestock production (Kashoma *et al*., 2015; Katakweba *et al*., 2012) because it is a primary choice of antibiotic for treatment. Resistant *S. aureus* strains produces a beta- lactamase enzyme encoded by plasmid genes which are transferred very easily within or between species through conjugation (Reta *et al*., 2016). Beta-lactamase enzymes inactivates penicillin's and its derivates such as oxacillin and amoxicillin-clavulanic acid.

This study observed wide degree of polymorphism confirming the pathogenicity potential of *S. aureus* isolates in dairy cows. The *spa* types in the present study are comparable with other studies such as that of Bhati *et al*. (2016) who found nine different size *spa* amplicons ranging from 140 to 280 bp, Isrina *et al*. (2004) who obtained nine different

sized spa gene bands of 100bp to 340 bp and Yadav *et al*. (2015) who reported seven diverse sized *spa* gene bands ranging from 120bp to 330bp. Furthermore, Bhati *et al*. (2016) also found one *S. aureus* isolate that produced two bands of *spa* amplicon similar to our study.

PVL encoding virulent genes are mostly associated with human population their presence in milk could be an indication of transfer from milkers during milking (Shrivastava et al., 2018). Importantly, this study did not detect any S. aureus isolate carrying PVL-encoding genes. Several studies have reported that PVL-encoding gene is not common in raw milk but is mostly associated with CA-MRSA strains (Fluit, 2012). A similar study in India did not amplify any PVL-encoding gene isolates from mastitis cattle (Prashanth *et al.*, 2011). On contrarily, Mitra *et al*. (2013) detected PVL-encoding gene positive *S. aureus* (41.6%) from raw bovine milk. Shrivastava *et al*. (2018) suggested that PVL-carrying *S. aureus* isolates can be transferred to raw bovine milk by the milkers during milking because it is rare in bovine population.

MecA mediated methicillin resistance in *S. aureus* remains a major bacteria pathogen due to the development and dissemination of pathogens with reduced susceptibility to the available classes of antibiotics (Monecke *et al*., 2013), constituting an important health challenge due to the limited treatment options (Klibi *et al*., 2012). Several studies have detected *S. aureus* strains possessing *mecA* gene in bovine milk and related products (Normanno *et al*., 2005) , posing risk of transmission of resistant strains to human. In this study *mecA* gene was amplified at 162bp but on susceptibility testing the *S. aureus* isolate had an inhibition zone of 27mm around cefoxitin disc, which is not considered as MRSA by CLSI 2019 guidelines. Failure to detect *mecA* gene phenotypically could be due to inconsistences of antimicrobial susceptibility tests efficiency or lack of *mecA* gene

expression (Cabrera-Contreras *et al*., 2019) by the specific isolate. Similar to this study, (Asiimwe *et al*., 2017) detected 23 MRSA isolates with *mecA* gene specific PCR, but on cefoxitin screening only two MRSA isolates were identified according to EUCAST guidelines. A study on the methods for detecting MRSA reported six MRSA strains that were not detected by phenotypic cefoxitin screening method but were identified by *mec*A gene specific PCR (Corrente *et al*., 2007). Based on these findings, they concluded that molecular tools are more sensitive and specific than conventional microbiological assays in detection of *mecA* gene in *S. aureus* strains from animal products. Comparatively, Kalayu *et al*. (2020) in Ethiopia did not find any *S. aureus* isolate from raw milk harboring *mecA*, but found 1 (4.5%) *S. aureus* isolates having *mecA* gene from the nares of a dairy worker.

This study did not detect *mecC* resistance gene at 138bp among the 124 screened *S. aureus* isolates. However, since its discovery in 2011 several studies in Europe have reported *mecC* genes from isolates of food origin. For instance a study Ariza-miguel *et al*. (2014) in Spain reported 1/601 (0.2%) *mecC* gene from milk tank upon sequencing. The current status of MRSA is not that much worse based on our findings but serious measures ought to be taken to prevent its spread.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

The prevalence of *S. aureus* at the rate of 31.2% in raw bovine milk at farm level in Morogoro Urban and Mvomero districts constitutes a major concern to the farmers and consumers of raw/ unpasteurized milk. High resistance to the tested antimicrobials was observed in beta lactams antibiotics which is an indication that they are plausibly the most commonly used antibiotics in the area of study for livestock production. Although the use of antimicrobials for growth promotion provides some economic benefits to producers and consumers at large, it contributes majorly to the current antimicrobial resistance crisis.

Based on the above results, effective hygiene practises are therefore very key at farm level especially during milking process to minimise infections with emphasis that food safety begins with a healthy animal. There is also a need for strict implementation of strategies that will control and monitor the development of MRSA or its dissemination through contaminated raw milk as well as creating awareness on AMR and proper usage of antimicrobials to the local farmers.

Sequencing techniques are higher in terms of sensitivity in detecting low frequency variants. This study did not use sequencing techniques to confirm the presence of resistant genes in *S. aureus* due to limitation of funds. However, Multiplex PCR was able to detect low prevalence of MRSA in milk providing baseline for future research to also consider confirming resistance genes using sequencing techniques. It would also be important to

sequence *spa* gene fragments in *S. aureus* isolates from dairy cows in Tanzania for comparing the differences in virulent phenotypes and epidemiological mapping of source of infection by establishing their genetic diversity. A similar study can also be conducted with inclusion of nasal or hand swab samples of the milkers among Masaai pastoralists in Tanzania.

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APPENDICES

Appendix 1: Study questionnaire

A questionnaire on farm management practices and usage of antibiotics in Dairy Farms and Pastoral communities of Morogoro, Tanzania.

Farm name……Location……Date…….

NB. Information provided will not be used for external purpose. Check with √ in the given box and write answers in the space provided appropriately.

A. Respondent's demographic data

Name …… Age……...

Gender……. Number of people in the household……...

B. General information

1. For what purpose do you keep cows?.....
2. How many dairy cows do you have?.....
3. What is the age of the cow and how many times has it given birth?
4. Which breed of cows are you keeping? …….
5. Hygienic condition of the farm

   Good ☐ Poor ☐

C. Milking practices

6. How do you milk the cows? Hands ☐

   Machine ☐
7. How often do you milk a cow in a day? …….
8. For what purpose do you milk?.....
9. What is the floor type of the milking area? Soil Concrete

10. Hygienic status of the floor? Good ☐ Poor ☐

11. How is the cleaning quality of milking utensils? Good ☐ Poor ☐

12. Do you wash hands with a detergent/soap before milking? Yes ☐ No ☐

13. Do you wash cow's udder/teat before milking?

Yes ☐ No ☐

14. After milking each cow do you wash hands? Yes ☐ No ☐

15. How is the cleaning quality of milking utensils?

Good ☐ Poor ☐

D. Usage of Antimicrobials

16. Do you keep record of treatment for the cattle?

Yes ☐ No ☐

17. How do you know if the cow's udder is infected?.....……

18. What are the common diseases affecting your cattle?.……

19. How do you treat them?.....

20. Which antibiotics do use treat the cattle?.....

……

21. What do you do with the milk after administering the antibiotics?.……

22. Where do you get the antibiotics from?

Agrovet outlets ☐

Veterinarians ☐

Field officers ☐

Neighbor's ☐

23. Do you consult veterinarian or field officer or Doctor before administering antibiotics to the cattle/or yourself?

Yes ☐ No ☐

24. How do you dispose antibiotic containers?.....

……

25. Have you ever heard of antibiotic resistance?

Yes ☐ No ☐

THANK YOU

Appendix 2: Study permit