PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF ANTIMICROBIALRESISTANT NON-PATHOGENIC *STAPHYLOCOCCUS* SPECIES IN RAW CAMEL MILK FROM GARISSA COUNTY, KENYA

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DECLARATION

I declare that this thesis is my original work and has not been presented in this or any other University for the award of a degree. The various books, journals and other sources consulted for information have been duly acknowledged.

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DEDICATION

This thesis is dedicated to my parents Silvester and Alice Bittok, whom I consider a pillar in my education, my sister Hildah, my brothers Abel, Noah and Dominic and my friend Faith Summey.

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TABLE OF CONTENTS **Page**

LIST OF TABLES

Page

LIST OF FIGURES

Page

ABBREVIATIONS

AMR Antimicrobial Resistant

BPW Buffered Peptone Water

CoNS Coagulase Negative *Staphylococcus*

CoPS Coagulase Positive *Staphylococcus*

CLSI Clinical and Laboratory Standards Institute

DNA Deoxyribonucleic acid

MHA Mueller-Hinton Agar

MGE Mobile Genetic Elements

MDR Multidrug Resistant

PCR Polymerase Chain Reaction

RNA Ribonucleic Acid

SCC Staphylococcal Chromosomal Cassette

TSB Trypticase Soy Broth

XDR Extensively Drug Resistant

XDR-TB Extensively Drug Resistant Tuberculosis

ABSTRACT

Staphylococci bacteria are grouped into coagulase-positive *Staphylococcus* (CoPS) and coagulase-negative *Staphylococcus* (CoNS) by their ability to produce coagulase. CoNS are traditionally non-pathogenic but recent studies have reported CoNS to cause mastitis and septicaemia in livestock. In humans, CoNS play a role as opportunistic pathogens often involved in catheter-related infections, osteomyelitis, bacteraemia, endocarditis, boils, skin abscesses, cellulitis and urinary tract infections. Both *S. aureus* and CoNS species harbour resistant genes that confer antimicrobial resistant (AMR) phenotypes which are encoded by a range of genes. Some of these genes are found in mobile genetic elements which facilitate transfer of resistance genes from the non-pathogenic to pathogenic species. As a result of the genetic transfer, both groups can harbour multidrug-resistance (MDR) genes. This study investigated the phenotypicand genotypic AMR profiles of non-pathogenic *Staphylococcus* species contaminating raw camel milk from Garissa County in Kenya. A total of 231 raw camel milk samples were randomly collected from lactating camels. Smallholder camel farmers were randomly selected from the list of camel farmers provided by the clan heads in each of the sub-Counties. Bacteria were recovered in Buffered Peptone Water (BPW). *Staphylococcus* isolates were cultured on Mannitol Salt agar (MSA) and Blood Agar (BA). Coagulase and catalase tests were used to characterize the isolates. Thereafter the isolates were confirmed as *Staphylococcus* species by PCR and sequencing. Antimicrobial resistance profiles of the confirmed isolates were established by Kirby Bauer disk diffusion method. The antimicrobialsused included; 10ug ampicillin, 10ug streptomycin, 30ug cephalexin, 15ug erythromycin, 5ugciprofloxacin, 30ug cefoxitin, 30ug tetracycline and 30ug chloramphenicol. Mueller Hinton Agar (MHA) was streaked with a swab of isolates cultured on Trypticase Soy Agar. Antibioticdisks dispensed on the surface of the MHA using a disk dispenser in duplicates. The plates were incubated at 37°C for 24 hours after which diameters of zones inhibition (mm) were measured using a Vernier calibre and an average obtained. The readings were recorded as eithersusceptible, intermediate,

or resistant based on the interpretative breakpoints by the Clinical Laboratory Standards Institute (CLSI) guidelines. Genetic determinants responsible for the resistance phenotypes of *Staphylococcus* species were determined by Polymerase ChainReaction (PCR), sequencing, and Blast analysis. Genes encoding aminoglycoside (streptomycin) resistance (*aph(6)-Id* (*str*B), beta-lactams (*mec*A, *mec*C, *bla*Z and *bla*TEM) were analysed. Overall 122 (52.8%) isolates showed yellow mucoid (83.1%) while 83 (68%) were catalase positive. β-haemolysis with clear zones around the yellow colonies was observedin 122 (91.7%) of the isolates cultured in Blood agar (BA) indicating *Staphylococcus* species.On molecular analysis, 122 (91.7%) isolates were identified as *Staphylococcus* species at 900bp by amplification of 16S rRNA gene while 102 (83.6%) isolates were confirmed as *S. aureus* after PCR amplification of *nuc* gene at corresponding band of 323bp. Highest resistance among the isolates was observed against Cephalexin (81.9%) and Streptomycin (72.1%) while lowest resistance was against Chloramphenicol (1.6%) and Tetracycline (3.3%). High levels of resistance was seen in Methicillin- resistant Coagulase negative *Staphylococcus* (MRCoNS) (15%) than in Methicillin-resistant *S. aureus* (MRSA) (9.8%) isolates. MDR was relatively high in all the isolates (43.4%) comprising of 6/20 (30%) MDR-CoNS and 47/102 (46%) MDR

S. aureus. Overall, 68 (75.6%) *Staphylococci* isolates harboured at least one of the antimicrobial resistant genes namely *mec*A, *aph(6)-Id* (*str*B) and *blaTEM* genes. The *aph(6)-Id* (*str*B) gene was detected in 28.3% of the isolates while 2.3% of the isolates harboured *mec*A gene. Majority of the isolates carried *bla*Z gene (88.6%) and *blaTEM* gene (46.6%) while one isolate harboured *mec*A gene. Both CoPS (4) and CoNS(2) harboured *aph(6)-Id* (*str*B) and *bla*TEM genes. The *mec*A containing isolate also harboured *bla*TEM gene. The *mec*C gene was not detected in all the isolates. The findings showed that CoNS and *S. aureus* isolates coexist contaminating raw camel milk and carry similar resistance genes that horizontally transfer between the *Staphylococcus* species. Therefore, continuous monitoring is recommended in order to prevent the spread of AMR.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The Food and Agriculture Organization (FAO) reports indicate that life in the Arid and Semi-Arid Lands (ASALs) is supported by 27 million camels around the world (FAO, 2013). The highest numbers are in tropical and sub-tropical Countries which include some Asian Countries, Arabian Peninsula, Eritrea, Ethiopia, Kenya, Somalia and Djibouti (El-Naga and Barghash, 2016). Kenya has a population of about 3 million camels (AU/IBAR, 2017) contributing to about 6%of the total camel population in Africa. The number has gradually increased over the years with a significant rise of 2.2 million heads between 1999 and 2009 (Kangunyu and Wanjohi, 2014).

Arid and Semi-Arid Lands in Kenya cover 89% of landmass supporting about 36% of the Country's population (KNBS, 2019). Communities living in the ASALs depend on camels as one of their main source of livelihood (Isako and Kimindu, 2019). The camels are used for transport, sport and source of milk in the ASALs. Camel milk not only contains higher amount of nutrients compared to cow milk but it has also medicinal properties (EL-Fakharany et al., 2012; FAO, 2013). Therefore, camels contribute in raising the economy and food security for humans with the national value of camel milk estimated to be Ksh. 87.6 billion (FAO, 2019).

Camels have adaptive features to the harsh climatic conditions hence becoming most valued stable source of livelihood in the arid lands (Watson *et al.,* 2016). They are also known to be less susceptible to many diseases (Francisco *et al.,* 2021). In pastoral communities, camel milk is mainly consumed raw without any heat treatment or varying degrees of sournesswhich poses a health hazard to humans directly or indirectly (Gitao *et al.,* 2014). The milk canharbour a variety of microorganisms including *Staphylococcus* and can be important sources of infections.

Staphylococci cause a wide spectrum of diseases in both humans and animals. The group comprises 49 species and 26 sub species (Han *et al*., 2015) which are classified into pathogenic and non-pathogenic groups. The non-pathogenic *Staphylococcus* is commonly termed as coagulase negative *Staphylococcus* (CoNS) since they cannot produce coagulase enzyme or digest rabbit plasma (Becker *et al.*, 2014). Coagulase positive *Staphylococcus* (CoPS) are the pathogenic *Staphylococci* with *S. aureus* being the dominant organism. CoNS were traditionally perceived as skin protectants against major pathogens (Waller *et al.*, 2011). However, today their etiology in causing human and animal diseases is increasing (Pyoralla and Taponen, 2009). The ability to produce coagulase enzyme by *Staphylococcus* bacteria is associated with various virulence factors including production of enterotoxins, of which are occasionally produced by non-pathogenic *Staphylococcus* (Yu *et al*., 2017).

In animals, coagulase negative *Staphylococcus spp.* have been reported to cause mastitis, septicemia, and arthritis in poultry (Pyzik *et al*, 2019; Mbindyo *et al.*, 2020). The organism's role in causing mastitis has globally increased resulting in significant economic losses (Simojoki *et al.*, 2012, Sampimon *et al.*, 2011). Sakwinsak *et al.*, (2011) reported possible transmission of CoNS in milk. In man, CoNS such as *S. epidermidis* and *S. haemolyticus* are responsible for a wide range of infections including osteomyelitis, bacteremia, endocarditis, boils, skin abscesses, cellulitis and surgical site infections (Tong *et al.*, 2015).

CoNS have been reported to be significant reservoirs of antimicrobial resistance genes (Podkowik *et al.*, 2013) as well as multidrug resistance and resistance genes for other bacteria such as *S. aureus*, *Streptococcus* and *E. coli* (Otto, 2013). Besides transferring resistance determinants, the non-pathogenic *Staphylococcus spp.* can mobilize and recombine with non-coagulative plasmids to form new plasmids or acquire and transfer resistance transposons (Khan *et al*., 2011). Both pathogenic and non-pathogenic *Staphylococcus* species habour resistant genes which confer AMR phenotypes.

The AMR phenotypes are encoded by a range of genes such as *mec*A/C, *tet*-k/m among other genes (Ruaro *et al*, 2013). Some of these genes are found in mobile genetic elements (MGE). The MGE are known to facilitate transfer of resistance genes from the non-pathogenic to pathogenic species (Foster *et al*., 2014). As a result of the genetic transfer, both groups can harbour multidrug-resistance (MDR) genes (Frey *et al*., 2013; Otto, 2013). The resistant genes-transfer to antimicrobial susceptible pathogenic species can make the isolates resistant. There is risk of human infection by the resistant pathogenic isolates when contaminated milk is consumed. Among other CoNS, *S. epidermidis*, *S. hominis* and *S. haemolyticus* are often reported to be resistant to multiple antibiotics (Bouchami *et al*., 2011; Becker *et al*., 2014). Infections of humans with the resistant isolates cause treatment failure resulting in prolonged hospital admissions, increased virulence, high cost of treatment, reduced therapeutic efficacy of the antimicrobial agents, and even death (Moghadam *et al.,* 2020)

Recently, there has been increased global reported cases of CoNS contamination in food, more importantly the methicillin resistant CoNS (MR-CoNS) (Bhargava and Zhang, 2012; Osman *et al.*, 2016; Fowoyo & Ogunbanwo, 2017; Yang *et al.,* 2016). For instance, bacteria isolated from raw camel milk in India indicated both *S. aureus* and CoNS (Verma and Prakash,2016), cow milk in Finland and Brazil (Simojoki *et al*., 2012 ; Soares *et al.,* 2012; Silva *et al.*, 2014) including proteins of animal origin in Poland (Chajęcka-Wierzchowska *et al.*, 2015).

Coagulase-negative *Staphylococcus spp.* containing various antimicrobial resistance genes are frequently detected in milk (Verma and Prakash, 2016; Osman *et al.,* 2016 and Yang *et al.,* 2016). This fact emphasizes the importance of identifying CoNS in apparently healthy udders because of the potential risk of lateral transfer of resistant genes among *Staphylococcal* species and other pathogenic bacteria.

Therefore, there is a need to know the potential role of the non-pathogenic isolates in the transmission of AMR through camel milk. To address the gap, this study demonstrated the risk of acquiring non-pathogenic AMR *Staphylococcal* bacteria from raw camel milk in Garissa County, Kenya.

1.2 Problem Statement

The continuous emergence of various multidrug resistant bacteria with narrow spectrum effective antibiotics to clinically challenging situations has become a global concern including Kenya (Becker *et al.*, 2014). Little attention has been paid to the non-pathogenic *Staphylococcus spp.* yet they pose a feasible threat to available therapeutic agents as reservoirs of antimicrobial resistance genes and resistance- associated mobile genetic elements which can transfer between *Staphylococcal* species (Chajecka- Wierzchowska *et al.*, 2015; Foster *et al.*, 2014). In Africa, including Kenya, the role of non-pathogenic *Staphylococci* clones in camel milk is not well documented and therefore determining their antimicrobial resistance patterns and associated genes may address this challenge that poses great threat to Public Health.

1.3 Objectives of the study

1.3.1 General objective

To determine the phenotypic and genotypic AMR profiles of non-pathogenic *Staphylococcus spp.* contaminating raw camel milk from Garissa County, Kenya.

1.3.2 Specific objectives of the study

The following were the specific objectives of the study;

i. To identify non-pathogenic *Staphylococcus* species in raw camel milk intended for human consumption in Garissa County, Kenya
ii. To characterize antimicrobial resistance profiles of the non-pathogenic S*taphylococcus* isolates in camel milk.

iii. To determine the genetic determinants of the antimicrobial resistant phenotypes characterized from milk collected from Garissa County.

1.4 Hypothesis

Non-pathogenic *Staphylococcal* isolates contaminating camel milk do not habour AMR genes associated with resistant phenotypes.

1.5 Justification

Coagulase negative Staphylococcus (CoNS) cause of mastitis in animals and nosocomial as well as surgical site infections in humans (Tong et al., 2015; Pyzik et al, 2019). These organisms were traditionally perceived as non-pathogenic commensals, however, today their etiology in causing human and animal diseases is increasing. CoNS species harbour resistant genes that confer antimicrobial resistant (AMR) phenotypes which are encoded by a range of genes which transfer resistance from the non-pathogenic to pathogenic *Staphylococcus* species. *Staphylococcus* species isolates with AMR phenotypes can contaminate milk meant for human consumption. There is risk of human infection by the resistant pathogenic isolates when contaminated milk is consumed and resultant treatment failure, economic burden due to prolonged hospitalization and even death. This study can form a good basis for further studies on molecular epidemiology of CoNS organisms' potential guide for food safety experts, Veterinarians, Health care professionals and policy makers in designing biosecurity measures for managing transmission of AMR CoNS through milk consumed in pastoral areas. Ultimately, this may aid in controlling AMR acquired through food chain and its spread in communities.

Data obtained from the study will guide in establishing antimicrobial resistance control measures and policy framework for antimicrobial use. This will be disseminated through clan heads who helped in identification of the small holder camel farmers as well as the local County government through veterinarians and other food experts or interested parties in the community

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Management of various infectious diseases with the discovery of antimicrobials has greatly boosted health and life expectancy of humans and welfare of production animals (GARP, 2011). The prolonged use of the antimicrobial drugs has however resulted in a global problem of antimicrobial resistance hence a threat to Human and Animal Health. This can be through intrinsic resistance by microorganisms or acquired resistance through transfer of resistance plasmids (Azounwu *et al.,* 2019). Various inanimate objects and surfaces including food may habour antimicrobial resistance genes encoding resistant phenotypes that may be transferred to humans (Depoorter *et al.,* 2012). This establishes the link between the increasing use of antimicrobial agents in food and animal production to boost yield and the current rising trend of resistance to most therapeutic agents (Van *et al*., 2020).

Resistance to antimicrobials can be achieved by bacteria through various mechanisms including enzymatic degradation of antibiotics, antibiotic target modification, changing the bacterial cell wall permeability and alternative pathways to escape the activity (Verraes *et al.,* 2013). Van Boxstael *et al.,* (2012) established possible transfer of antimicrobial resistant *Salmonella enterica* strains through consumption of pork and poultry meat products. Recent studies have also demonstrated increasing concern in antimicrobial use in aquaculture and their resistance (Zou *et al*., 2012). Therefore, ingestion of antimicrobial resistant bacteria by humans may result in short and long term effects hence a Public Health risk. Commonly studied organisms with their ability to transfer AMR phenotypes between animals and humans through food include *Salmonella*, *Staphylococcus* and *Campylobacter* species (Varma *et al.,* 2005).

The mobile genetic elements (MGE) present increase the gene pool from which pathogenic microorganisms may transfer the resistance genes to other non-pathogenic bacteria including commensals such as coagulase negative *Staphylococcus* (CoNS). This results in increased virulence risk due to co-selection of resistance and integration of virulence and resistance plasmids (Guerra *et al.,* 2014) coupled with limited antimicrobials available hence impeding treatment.

2.2 Global Camel distribution

The Food and Agricultural Organization (FAO) estimates the global camel population to be 27 million (FAO, 2013) with some regions in Asia and in the Arabian Peninsula, Ethiopia, Kenya, Djibouti, Eritrea, Ethiopia and Somalia having the highest numbers (Tarek *et al*., 2012). However, it is difficult to estimate the exact camel global population because the animal is kept mainly by nomadic pastoralists who keep moving from one place to another in search of pasture and water (Faye, 2015).

Africa hosts about 80% of this camel population and about 60% are in the Horn of Africa. The global camel numbers have since been on a 3.4% annual growth since 1963, the first statistics, hence are animals of great role in pastoral communities (FAO,2013).

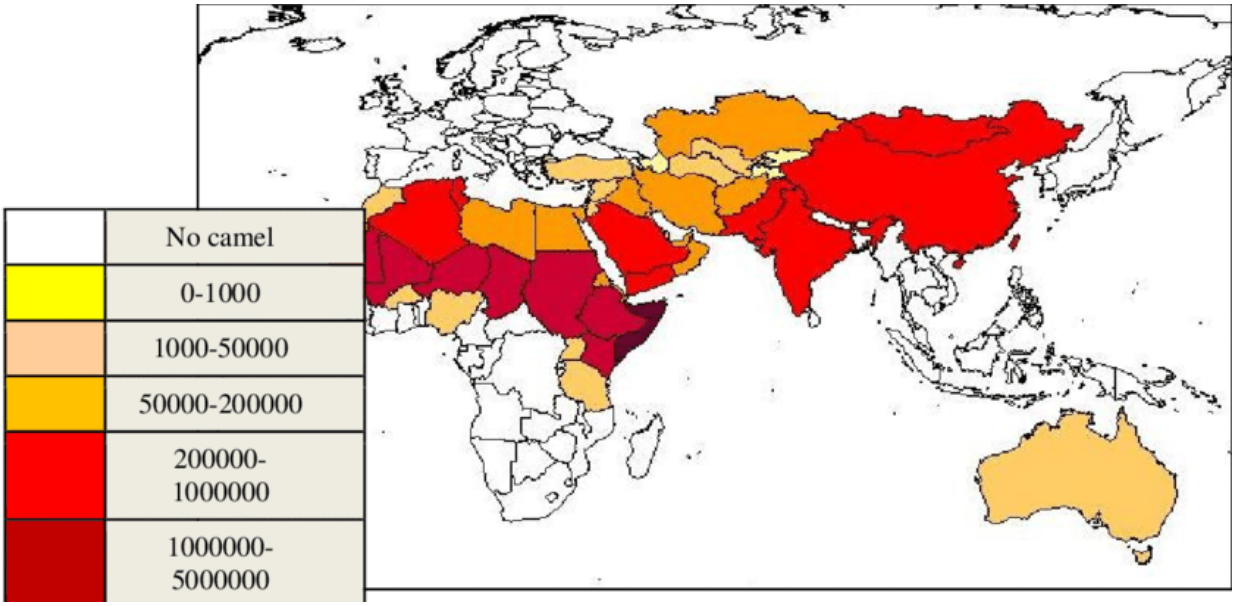

Figure 2.1Camel distribution of the world (Faye and Bonnet, 2012).

2.3 Camel production in Kenya

Kenya has an estimated 2.97 million dromedary camel herd (*Camelus dromedaries*) traditionally kept by the Somali, Rendille, Gabbra, and Turkana communities living in the country's harsh arid and semi-arid areas of North and North-Eastern Kenya which are also ranked as the poorest parts of the country (KNBS, 2015).

Camels support about 30% of Kenyan pastoral communities living in Arid and Semi-Arid lands (ASALs) (Noor *et al.,* 2013) that cover over 83% of the Kenyan land mass. The physiological adaptations of camels to the harsh environmental conditions in the ASALs help them to thrive and cope with the harsh conditions. Their ability to drink less water and stay longer without water compared to other livestock is due to high tolerance of dehydration while keeping normal blood volume (Guliye, 2010). The communities in Northern Kenya mainly keep camel herds in line with their pastoral way of life which is characterized by constant movement in search of water and pasture (Noor *et al*., 2013). Clan feuds and terrorism is also a contributing factor to their constant movement from place to another. This ensures efficient utilization of the vast rangelands in the ASALs (Guliye *et al*., 2007).

Kenyan camel milk production has significantly increased with the emergence of new camel keepers in the ASALs. Most of the milk produced does not reach the vast markets in the cities and towns (88%) but rather for subsistence use by the pastoral communities. However, 38% of this milk is consumed while 50% gets spoilt due to absence of appropriate storage facilities (Akweya *et al*., 2012). Camel milk is mainly consumed raw without any heat treatment or as fermented milk called *Suusac* which is stored in smoked guards.

2.4 Significance of Camels

The camel is a multipurpose animal which is kept for milk, hides, skin, meat, transport, barter trade and sociocultural events such as racing, tourism and beauty contests (Noor *et al.,* 2013). Camel milk is the white gold of the desert with its market expanding especially among the middle class population in the urban areas. This has been attributed to various qualities of camel milk such as low cholesterol and sugar levels, high minerals and vitamin C as well as protective proteins such as lactoferrins, lactoperroxidases, immunoglobulins and lysozymes (EL-Fakharany *et al.,* 2012). Kenyan camel milk production in 2017 was estimated to be over 876,224 tonnes valued at over Ksh. 87.6 billion at the farm level (FAO, 2019).

Camels are important animals in the many pastoral communities with their sedentary life ensuring food security through provision of milk and meat. These well adaptable beasts of burden have been found to produce more milk compared to goats, sheep and cattle in thesame environmental conditions (Seifu *et al.,* 2019). Camels also serve as source of cash, transport and for cultural purposes and an indicator of status in the society, especially the Somali community (Mahmoud, 2010). The therapeutic value of camel milk has increasingly raised its global valueand resultantly expanded its market (Abbas and Mahasneh, 2014). Its therapeutic properties include antioxidant, antihypertensive, antithrombotic and antimicrobial properties (Agrawel *etal.,* 2011).

2.5 Bacterial diseases of Camels in Kenya

Camels are considered resistant to many diseases due to their adaptations to the harsh weather conditions, however, current research have scoredvarious infections in camels (Hughes and Anderson, 2020). The most important limiting factor to livestock production is diseases and this also applies to camel rearing in the ASALs in Kenya (Dabelo, 2012). Camels are more resistant to infectionssuch as respiratory diseases and Foot and Mouth disease compared to other livestock (Hughes and Anderson, 2020). The disease-causing microorganisms in camels in Kenya include bacteria such as *Brucella spp* (Wanjohi *et al.,* 2013), *Coxiella brunetti*, the causative agent of Q-fever and *Dermatophilus congolensis* causing skin conditions reported in five studies in Laikipia, Samburu and Turkana (DePuy *et al.,* 2014; Browne *et al.,* 2017). Mutual *et al., (*2017) reported *Brucella spp*., CoNS, *S. aureus* and *Streptococcus agalatiae* from nasalswabs of camels in Samburu, Nakuru and Isiolo Counties. Recent studies have shown *S. aureus*as the most isolated mastitis causing organism from camel milk in the Northern pastoral region of Kenya (Akweya *et al.,* 2012; Njage *et al.,* 2013; Omwenga *et al.,* 2019; Omwenga *et al*., 2021).

2.5.1 Camel Mastitis in Kenya

Mastitis is a significant constraint to camel production in various parts of the world, including Kenya, Tunisia, Ethiopia, Pakistan and Middle East (Wanjohi *et al.,* 2013; Abdelgadir, 2014; Toroitich *et al*., 2017; Klibi *et al.,* 2018). Camel mastitis has botheconomical and medical importance affecting human and animal health (Akweya *et al*., 2012;Njage *et al.,* 2013). Mastitis occurs in clinical and subclinical forms mainly through colonization of the teat canal or infection of the udder (Younan, 2013). Generally, the disease occurs as a contagious or environmental form. Contagious mastitis is caused by organisms in the udder or on the skin. These include *S. aureus*, coagulase negative *Staphylococcus*, *Streptococcus agalactiae* and *Streptococcus uberis* (Iyer *et al.,* 2014; Khan *et al.,* 2011). The organisms contaminate milk during milking through splashes or through milker's hands. Environmental mastitis is caused

by microorganisms such as *Streptococcus dysgalactiae, E. coli* and *Klebsiella* spp. in the animal's surrounding (Khan and Khan, 2006)

The dominant mastitis causing organisms in camels reared in ASALs of Kenya are *Streptococcus agalactiae* and *Staphylococcus aureus* (Wanjohi *et al., 2*013). However, other microorganisms have also been reported to cause mastitis. These include *Streptococcus uberis, Streptococcus dysgalactiae, Streptococcus pyogenes, Escherichia coli, Bacillus cereus, Pasteurella spp., Mycobacterium tuberculosis, Klebsiella spp., Corynebacterium pseudotuberculosis* and *Candida albicans (*Lamuka *et al.,* 2017).

Communities living in the ASALs of Kenya use a variety of traditional herbs in the management of camel infections including mastitis (Bornstein and Younan, 2013). However, the efficacy and clinical cure of these herbs has not been established. Both systemic and intramammary antibiotic infusions are used in treatment of camel mastitis, although intramammary infusions are seldom used due to the camel teat anatomy that complicates administration of the drugs (Saleh and Faye, 2011). Systemic antimicrobials are used in treating acute mastitis include penicillin, aminoglycosides, trimethoprim –sulfamethoxazole, sulphonamides and anti-inflammatories such as flunixin meglumine (Jilo *et al.*, 2017). Regular milking of camels as well as hydrotherapy helps to reduce udder edema. Chronic mastitis is difficult to treat and may lead to the loss of affected quarter (Khan and Khan, 2006).

2.6 The Genus Staphylococcus

Staphylococcus bacteria belongs to Micrococcacae family of gram positive, non-motile, encapsulated cocci occurring in pairs, short chains or singly. They are catalase positive, facultatively anaerobic and are differentiated by their ability to produce coagulase (Bennet *et al.,* 2013). The organisms occur singly, in tetrads or in bunches and produce opaque white to cream coloured colonies that are 2–3 mm in diameter blood agar (Kloos *et al.,* 1998).

The genus *Staphylococcus* are found on varied surfaces in the environment including the skin

and mucous membranes of warm-blooded mammals as commensals and in soil, sand, air and water. They are also isolated from various sources of animal protein (Even *et al.*, 2010). There are 49 known species and 26 subspecies within the genus *Staphylococcus* (Han *et al.*, 2015). Classification of the *Staphylococcus* species is often based on various genotypic and phenotypic characteristics, including nuclease production, antibiotic resistance, and DNA similarity (Becker *et al.*, 2014). However, production of the enzyme, coagulase is the basis of classification of the 49 known *Staphylococcus* species. This groups them into: coagulase-positive *Staphylococcus* (CoPS) and coagulase-negative *Staphylococcus* (CoNS). Therefore, the different *Staphylococcus* species can be differentiated based on their haemolytic activity, patterns of carbohydrate utilization, components of cell wall and patterns on anaerobic growth in thioglycolate medium (Götz, 2006)

Staphylococcus species are known to have a variety of virulence factors, including lipases, deoxyribonuclease (DNase), leukocidin, exfoliative toxins A and B, toxic shock syndrome toxin 1 (TSST-1), emetic pyrogenic superantigens (SAgs) proteases, coagulase and haemolysins (α, β, γ and δ) (Bartolomeoli *et al*. 2009). The ability to produce coagulase is typically associated with the production of enterotoxins. However, CoNS isolates, on occasion, have also been shown to produce enterotoxins (Zell *et al.* 2008).

Staphylococcus pathogens account for 30% of nosocomial infections with *S. aureus* and *S. epidermidis* being the mainly isolated CoPS and CoNS respectively (Otto, 2013). *S. aureus* is the most studied of the *Staphylococcus* group due to its high virulence (Foster *et al.*, 2014). However, it is believed that CoNS are significant reservoirs of resistance associated MGE which can transfer between the staphylococcal species. Multi-drug resistance has been reported in CoNS such as *S. epidermidis, S. hominis* and *S. haemolyticus* (Bouchami *et al*., 2011; Becker *et al*., 2014)

2.6.1 Coagulase-negative *Staphylococcus* (CoNS)

Coagulase negative *Staphylococci* (CoNS) are considered commensals on skin and mucosal

membranes of the humans and animals (Becker *et al.,* 2014). These organisms are thought to be opportunistic pathogens that only cause disease when there is damage of skin or mucus membranes (Schoenfelder *et al,* 2010). Recent studies have however indicated that CoNS can directly or indirectly be pathogenic (Bhargava and Zhang, 2012).

There are about 41 recognized CoNS species with 34% colonizing humans for example *S. saprophyticus, S. hominis, S. warneri, S. epidermidis* and *S. hominis* (Grace *et al.,* 2019). *S. epidermidis* is the most isolated organism due to its pathogenicity and virulence (Qin *et al.,* 2017).

Most nosocomial infections have been identified to be caused by *S. epidermidis, S. saprophyticus* and *S. haemolyticus* (Mazzariol *et al.,* 2012). In animals, the commonly isolated disease-causing CoNS include *S. chromogenes, S. simulans* and *S. xylosus* (Unal and Cinar, 2012). CoNS isolates are frequently encountered in human clinical samples (Natsis & Cohen, 2018) as well as milk and other animal products (Simojoki *et al.,* 2012; Mbindyo *et al.,* 2020). In humans, the population at risk of CoNS infections include, premature neonates, cancer patients, post-transplant patients as well as intravenous drug abusers (Winderstrom *et al.,* 2012). However, these coagulase-negative species have since been perceived to be a part of the human and animal microbiota and not the primary disease causing pathogens (Waller *et al.,* 2011).

Some CoNS are used in various industries such as food processing, where it adds to flavour, aroma formation and colour functionalities, particularly in fermented foods. These strains include the non-enterotoxin-producing *Staphylococcus carnosus* and *Staphylococcus xylosus* (Even *et al.,* 2010; Podkowik *et al.,* 2013). The true extent of the potential and actual virulence of these CoNS species is limited. Coagulase negative *Staphylococcus* are increasingly being recognized as important cause of mastitis in cattle in Kenya, Tanzania, Egypt, Tunisia, Czech Republic and other parts of the world including Europe, South America and the Middle East (Mbindyo *et al.,* 2020; Soares *et al.,* 2012; Abd El-Razik *et al.,* 2017; Srednik *et al.,* 2017;

Pyatov *et al.,* 2017; Mohammed *et al.,* 2018 and Melo *et al.,* 2018). The organisms cause subclinical intra-mammary infections and resultant increase in somatic cell count, reducing the quality of milk (Pyorala and Taponen, 2009; Malinowski *et al.,* 2011).

CoNS can habour drug resistance genes of other bacteria such as S. aureus, Streptococcus and *E. coli* (Frey et al., 2013; Otto, 2013) with their main adhesive matrix molecules coded by a group of *ses* and by the *aae*, *atlE*, *sdrG* and *Embp* genes (Christiner *et al.,* 2010; Sender *et al.,* 2017). Bhargava and Zhang, (2012) demonstrated conjugative transfer of tet (M) from 10 Tetracycline resistant CoNS to Enterococci strains, therefore affirming pathogenic potential of CoNS. The organisms are often multi-drug resistant than S. aureus and generally respond weakly to antimicrobial therapy hence persistence of CoNS infections in both humans and animals (Frey *et al*., 2013). CoNS have largely been overlooked as a potential animal and human pathogen which can transfer resistant genes and mobile genetic elements to S. aureus (Osman *et al.,* 2015).

2.7 Antimicrobial Resistance

The availability of antibiotics for treating infectious diseases significantly improves health and life expectancy of humans as well as the health and welfare of animals. However, the use of antibiotics has resulted in a selection for antimicrobial resistance by bacteria (GARP, 2011). The World Economic Forum Global Risks has identified antimicrobial resistance as a major threat to human and animal health (Walker and Fowler, 2011). Resistance genes have been maintained in various environmental bacteria that have designed means to retain, accumulate and cross transfer resistant genes among various bacteria including the commensals (Manyi-Loh *et al.,* 2018).

Resistance by microorganisms is a result of selective pressure established in all organisms however, bacteria in particular, pose great threat to Public Health due various reasons. These include increased abuse of antibacterial drugs as compared to antifungals or antiviral agents. Bacterial genetics and rapid evaluation also contribute immensely coupled with abundant

nature of bacteria in the environment compared to other organisms (WHO, 2014). Antimicrobial resistance has both economic and health implications in the economies of the world yet no new drugs are being developed. For instance, about 23,000 annual deaths due to antimicrobial resistant bacteria are observed in the United States with a population of over 2 million citizens infected with methicillin resistant bacteria (WHO, 2014).

In Kenya, antimicrobial resistance is mainly reported in hospital based infections, enteric and respiratory infections with most focus being on antimicrobial resistance in humans (GARP, 2011). The resistance has been attributed to inappropriate dosing, ease of access to antimicrobials, self-prescription and poor quality antimicrobials (Mukokinya *et al.*, 2018). In the last two decades, research has demonstrated the various mechanisms with which bacteria have developed to counter antimicrobials. These include global response to environmental stresses such as the oxidative stress response system, established *mar*-regulon among fluoroquinolone resistant isolates and the s*ox*RS regulon of *E. coli* and *Salmonella eterica* (Demple, 2005). Biofilm formation acts as playgrounds for concentrating and conferring resistance traits. This is also advanced by plasmid mediated resistance interdependency and exchange of mobile genetic elements such as integrons, gene cassettes and transposons (Fey and Oslon, 2010).

The commonly isolated antimicrobial resistant bacteria include penicillin-resistant *Streptococcus pneumoniae*, vancomycin resistant Enterococcus, methicillin-resistant *Staphylococcus aureus*, resistant *E. coli* and non-Typhi *Salmonella* (GARP, 2011). Tetracyclines and Sulphonamides are the most commonly used antimicrobials in the ASALs (Omwenga *et al.,* 2021). These are followed by Aminoglycosides, Beta-lactams, Macrolides and Trimethoprim. In Kenya, antimicrobials are mostly used in poultry especially chicken consisting 20% of the total antimicrobials used. This is greatly associated to the growing middle class with preference of white meat, hence providing avenue for antimicrobial misuse leading to antimicrobial resistance and resultant transfer of AMR genes from animals to humans

(GARP, 2011).

Coagulase negative *Staphylococci* (CoNS) are more often multi-drug resistant than the common *Staphylococcus aureus* and generally respond weakly to most therapeutic agents (Frey *et al.,* 2013). Strommenger *et al*., 2003, demonstrated erythromycin resistance in CoNS genomes cultured from bovine milk. The main adhesive matrix in the CoNS species are coded by a group of *ses* genes and the *aae*, *atl*E, *sdr*G and *Embp* genes, which have shown transmission of the same genes to pathogenic *Staphylococcus* (Azara *et al.,* 2021).

CoNS increasing antibiotic resistance has been attributed to various factors including injudicious antibiotic use (Srednik *et al.,* 2017) as well as *mecA* mediated oxacillin resistance (Mahato *et al.,* 2017) and biofilm forming genes (Trembley *et al.,* 2014; Srednik *et al.,* 2017). Feßler *et al.,* (2010) linked the non-pathogenic *Staphylococcus* isolated from cow's udder to be reservoirs on of antimicrobial resistant genes which could be transferred as zoonotic pathogens hence a potential Public Health threat with regard to antimicrobial resistance and development of multidrug resistance.

2.7.1 Mechanism of Resistance by Coagulase Negative Staphylococcus (CoNS)

2.7.1.1 Resistance against ß-lactams

Beta-lactams are a group of antibiotics which include penicillins, cephalosporins, monobactams and carbapenems. They are the most prescribed class of antimicrobials with several clinical indications with annual expenditure of about $15 billion which is 65% of the antibiotic market (ThaKurla and Lahon, 2013).

Bacteria act by various mechanisms including; inactivation of beta-lactams by the production of beta-lactamases, decreased penetration to the beta-lactams to the target site, alteration of target site penicillin binding proteins and efflux from the periplasmic space through specific pumping mechanisms (Ibrahim *et al.,* 2019). CoNS resistance to ß-lactams occurs by two mechanisms namely; production of penicillinases that degrade ß-lactams and production of

penicillin binding protein 2α (PBP2α). Penicillinase enzymes are encoded by *bla*Z gene while PBP2α is coded by *mec*A gene. Methicillin resistance is mainly due to the presence of the *mec*A gene, which encodes a PBP2α that has low affinity for β-lactams. The *mec*A gene is carried within a mobile genetic element called the *Staphylococcal* cassette chromosome *mec* (SCC*mec*) (Duran *et al.,* 2012). . SCCmec elements are highly diverse in their structural organization and genetic content, and have been classified into types and subtypes from type I to type XI with the *mec*A gene variant/ homologue , mecALGA251, which the *mec*C and which shares only 70% similarity to *mec*A. The proteins encoded by *mec*C and *mec*A may differ from each other in terms of their structure and function. For instance, *mec*C encoded by PBP2c which has a four-fold higher binding affinity for oxacillin than that of PBP2α (Aslantaş, 2020).

The expression of PBP2α in *Staphylococcus* including CoNS, results in a total ß-lactam ring resistance due to the reduced binding affinity of PBP2α to ß-lactams as opposed to the intrinsic set of *Staphylococcal* PBPs (PBP 1 to 4) (Becker *et al.*, 2014). *Staphylococcus spp.* resistance to ß- lactams is however much more complex and is marked by the diverse polymorphisms at the gene level and the SCC*mec* element. Widespread Methicillin Resistant CoNS (MR-CoNS) hasbeen reported in nosocomial infections with the exception of *S. lugdunensis* with which its firstresistance was reported in 2003 (Starlander *et al.*, 2014). Kotsakis *et al.*, 2011, described methicillin resistance by *S. lugdunensis* by mutational alteration of PBP1A/1B. The selection pressure within CoNS organisms has resulted to increased resistance to multiple antibiotics and biocidic compounds especially in hospitalized and recovered patients (Cherifi *et al.*, 2013).

Point mutations in the *mec*A gene affect the function of the mecA-encoded PBP2α, leading to a change in the methicillin resistance activity and resultant reduced affinity to the binding sites. For instance, mutations in the allosteric site disrupts the allosteric opening and may play a vital role in creating resistance against ceftaroline (Ali *et al.*, 2021).

2.7.1.2 Resistance against glycopeptides

The commonly used glycopeptides in management of CoNS infections include vancomycin and teicoplanin (Périchon and Courvalin, 2012). These antimicrobials are used as the last resort in CoNS and nosocomial infections due to the increased resistance to methicillin, hence it's resistance among CoNS in a significant risk to Public Health.

Glycopeptides act by inhibiting peptidoglycan synthesis through binding to precursor molecules which are substrates for enzymes that cross-link the peptidoglycan (Périchon and Courvalin, 2012). The mechanisms of resistance of CoNS to gylcopeptides is not clear but teicoplanin resistance has been attributed to cell wall thickening and cellular aggregation (Cremniter *et al.,* 2010). Resistance among CoNS has been observed in *S. hameolyticus, S. epidermidis, S. warnei* and *S. capitis* (Sujatha and Praharaj, 2012).

2.7.1.3 Resistance against aminoglycosides

Aminoglycosides are mainly used alone or in combination with glycopeptides in management of nosocomial infections caused by CoNS. Aminoglycoside resistance to CoNS is mainly through the aminoglyocide modifying enzymes (AME) which interrupts the inhibitory protein synthesis (Sabzehali,*et al.,* 2017). The AMEs are encoded by *aac*-(6')-*le-aph*(2''), *aph*(3')-*IIIa* and ant*(4')-la* haboured in transposons and plasmids which facilitate rapid horizontal transfer hence conferring resistance to closely related species (Duran *et al.,* 2012).

Fey and Olson, (2010), observed that the organisms produce antimicrobial peptide sensors coded by *aps* and *gra* genes in order to avoid the influence of neutrophil antimicrobial proteins and capsule production coded by *cap* genes (Otto, 2013). The genes coding for biofilm production in most *Staphylococcal* organisms are located in the same region as the production of heat stable enterotoxins which contribute to *Staphylococcal* food poisoning, hence a threat to human health (Fijalkowski *et al.,* 2014).

Trembley *et al.,* (2014) established that CoNS growing within biofilms demonstrate a lesser

susceptibility to antimicrobials including Penicillins, Gentamycin –Novobiocin combinations and Cetiofur, therefore indicating that biofilm formation by CONS could impede antimicrobial therapy. Biofilm formation in *S. epidermidis* is encoded by *ica* ADBC operon with which the biofilm protects the bacteria and allowing horizontal gene transfer and increased bacterial virulence (Madsen *et al.,* 2012). Other mechanisms of antimicrobial resistance by non-pathogenic *Staphylococcus* include acquisition of plasmids /transposons (Lozanoa *et al.*, 2012), mobilization of non-conjugate plasmids as well as formation of new plasmids by recombinant non-conjugate plasmids to acquire and transfer resistance transposons (Khan *et al.*, 2011).

2.7.2 Methicillin Resistant Coagulase Negative Staphylococci (MRCoNS)

The *mec*A and *mec*C genes isolated from various *Staphylococcus* organisms have been shown to mediate resistance to first choice of drugs which are used in veterinary and human medicine, the beta lactams (WHO, 2014).

2.8 Multi drug resistance

Multi-drug resistance (MDR) is a growing global challenge in the management of infectious diseases with the reduced spectrum or absence of antibiotics that target these microorganisms. MDR is the insensitivity or resistance of microorganism(s) to antimicrobial agents which are structurally unrelated and of varied molecular targets despite earlier insensitivity to the antimicrobial under test (Tanwar *et al.*, 2014).

The continuous emergence of various multidrug resistant bacteria with narrow spectrum effective antibiotics to clinically challenging situations has become a global concern including Kenya (Medina and Pieper, 2016). This has led to ineffective treatment and prolonged hospitalization as well as spread of infections. Further spread of MDR has been exacerbated by the extensive emergence of immunocompromised conditions and nosocomial infections caused by the coagulase negative *Staphylococcus* (Becker *et al.*, 2020).

Surveillance of antimicrobial resistance in different regions of the world including Africa,

America, Europe and the West Pacific have indicated significant evolution of microorganisms and the looming MDR and resultant Public health threat (Opintan *et al.,* 2015; WHO, 2014; Van Kinh *et al.,* 2017). Antimicrobial resistance has both social and economic impacts including high mortalities and medical costs due to the ineffectiveness of antimicrobial agents (WHO, 2014). The high costs of medical treatment has been attributed to MDR as most microbial agents have become resistant to commercially available drugs hence the option of more expensive therapeutic forms (Fishbain and Peleg, 2010). The ineffectiveness of most therapeutic agents has also been attributed to quality of public hygiene as well as difference in resistance profiles of various pathogens (Tanwar *et al.,* 2014).

Multi-drug resistance is broadly classified into three broad categories; primary, secondary and clinical resistance. Primary resistance results when a new organism encountered a particular drug of interest for the first time while secondary resistance also known as acquired resistance results in an organism after exposure to a drug (Tanwar *et al.,* 2014). Secondary resistance is further classified into intrinsic and extrinsic resistance. Intrinsic resistance is insensitivity of common first line drugs used for a particular disease condition to a single species of microorganisms. For instance, multi-drug resistance of fluconazone in management of *Candida* spp. (Loeffler and Stevens, 2003). Extrinsic resistance is as a result of ability of organisms to withstand the inhibitory effects of one or two most effective antimicrobial agents (Lee *et al.,* 2013). This is also referred to as Extensively drug resistance (XDR) and commonly occurs in patients after therapeutic failure of first line drugs used in management of a particularcondition, for example, fluoroquinolone resistance in XDR-TB. Additionally, clinical resistance results from ineffectiveness of an antimicrobial agent to a certain organism at a concentration which is higher than could be safely achieved with normal dosing (Loeffler andStevens, 2003).

2.8.1 Mechanisms of Multidrug resistance

The development of resistance to commercially available therapeutics is increasingly gaining

global concern while microorganisms constantly develop mechanisms to avoid drug inhibition. These mechanisms include chromosomal mutations or conjugative exchange of extrachromosomal DNA elements (WHO, 2014) which alter bacterialcell wall composition leading to lack of active target sites for microbial agents.

Microorganisms have also established MDR by overexpression of drug target enzymes hence modification of metabolic pathways and resultant target bypass (He *at al.,* 2014). Enzymatic degradation of antimicrobial agents and chemical transformation of amide and ester bonds have also greatly contributed to emergence of MDR (Méndez-Vilas, 2013). Oxidation and reduction processes by resistant strains to antimicrobial agents also contribute to the observed MDR. Resistance to the inhibitory effects of microbial agents can occur by conformational changes or altered binding of substrate as well as mutations in the reverse transcriptase domain of polymerase gene (Tanwar *et al.,* 2014). MDR mediated by drug efflux pumps is still the main mechanism of MDR in most microorganisms through overexpression of genes encoding for ATP-binding cassette transporter membrane proteins (Nikaido, 2009). MDR can result from accumulation of genes coding for an organism or by expression of genes encoding formultidrug efflux pumps (Nikaido, 2009). MDR mainly occurs through horizontal gene transferin a mass from one bacteria to another. This is facilitated by intergrons which attract severalresistant genes located in a range of plasmids or in the chromosomal DNA (Emamalipour *etal*., 2020).

The MDR phenotype is a characteristic of most *Staphylococcus spp.* including the MRSA strains (Egyir *et al.*, 2014). The *mec*A gene encoding resistance in β–lactamases phenotypic resistanceis in the *Staphylococcal* cassette chromosome *mec* (SCC*mec*) (Siiriken *et al.*, 2016). The SCC*mec* subtypes I, II, III, IV, and V are known to harbour other AMR genetic determinants,such as *tet*K, *tet*M, *bla*Z, *aac* (6¢)/*aph* (2¢¢), *aph* (3¢)-IIIa, *msr*A, and *erm*A. These genetic determinants are believed to be responsible for the emergence of MDR MRSA.

2.9 Antimicrobial susceptibility testing

Antimicrobial susceptibility tests (ASTs) are methods used to establish the resistance of an organism to a particular antimicrobial agent/antibiotic or resistance to individual isolates (Walker and Fowler, 2011). ASTs are performed to direct clinicians on selection of appropriate targeted antibiotic to fulfil therapeutic cure in both animals and humans. This reduces mortalities and infections related to the specific organisms when the particular antibiotic established to be sensitive is administered.

The increasing interest in research and development of new antimicrobials to curb antimicrobial resistance, has resulted in many researchers focusing on the various antimicrobial screening methods. The discovery of most antimicrobials in the early 1960s solved main problems of therapeutics but the current threat is in the increased microbial resistance of the same antimicrobials. This has led to treatment failures and mortalities hence a global Public health concern (Guschin *et al.,* 2015). The diffusion methods are qualitative tests of AST and may be prone to degree of error depending on the organism tested and the drug (Kuper *et al*., 2009). Quantitative methods include the broth dilution and agar dilution method because they can measure the Minimum Inhibitory Concentration (MIC) of a drug. MIC is the lowest concentration of an antibiotic that inhibits visible growth of a microorganism. Both dilution and diffusion methods are the reference susceptibility methods due to their highreproducibility.

2.9.1 Phenotypic antimicrobial susceptibility testing.

2.9.1.1 Diffusion methods

Diffusion methods work with the principle that antibiotic molecules of a known concentration diffuse out from a disk into the agar, creating a dynamically changing gradient of antibiotic concentrations while the tested organism divides and progressively grows towards the critical mass (Kuper *et al.,* 2009). The concentration of the antibiotic starts to inhibit the organism's growth reaching an overwhelming cell mass at the zone edge. At this stage, antibiotic density

is high to absorb the test antibiotic in the vicinity hence creating a concentration equilibrium at sub inhibitory levels and enable the organism to grow. The diameter of the zone of inhibition is directly proportional to susceptibility of the organism to the tested antimicrobial (CLSI, 2008).

i) Agar disk-diffusion method.

This is one of the oldest method developed in 1940 although still recognized globally because of the reproducibility of its results. In the method, agar plates are inoculated with a standard inoculum test organism. The inoculum is prepared by suspension of isolated bacterial colonies in in broth to a turbidity of McFarland standard (1×10^8 colony forming units/ml). Standardization of the solution concentration is crucial because an inoculum with too high colony forming units result in false resistance registered by the smaller zone sizes while low inoculum gives falsely larger zones indicating some false susceptibility (Balouiri *et al.*, 2015). Antibiotic disks are then manually or with dispensing apparatus, placed on the dried agar plate surface. The number of disks placed depends on the agar plate size, for instance, most organisms require 12 disks on a 150mm agar plate or 5 disks on a 90mm agar plate. CLSI, (2017) recommends that in order to avoid overlapping, disks with predictably small zones should be placed next to disks with predictably larger zones of inhibition. Inhibition overlap can also be avoided by optimally placing the disks at a distance of 30mm apart and not closer than 24mm apart (Kuper *et al.*, 2009).

The agar plates are incubated in an inverted position under suitable conditions appropriate to a particular organism. The period of incubation is varied ranging between 16-18hours or longer depending on the bacteria (CLSI, 2017). After incubation, the agar plates are examined for growth by establishing semi confluent or even ‘lawn’ growth. The zone diameter is read to the nearest millimetre once the lawn growth is satisfactory. This can be done by using a ruler or sliding callipers with the inhibition zone identified as the area with no bacterial growth as seen by the naked eye. The antibiogram illustrates qualitative results identified as either susceptible,

intermediate or resistant as interpreted using the CLSI guidelines (Jorgensen and Turnidge, 2015; Bauer *et al*., 1966).

Disk diffusion is commonly used as a typing tool for empirical therapy but since bacterial inhibition does not mean bacterial death, the method cannot be used to establish the bacteriostatic or bactericidal nature of an antibiotic (Balouiri *et al.,* 2015). Additionally, the method cannot establish the Minimum Inhibitory Concentrations (MICs) since one cannot quantify the amount of antibiotic which diffuses into the agar (Nijs *et al,* 2003). The major advantages of disk diffusion are its simplicity, cost effectiveness, inexpensive equipment and can test a variety of antibiotics (Jorgensen and Turnidge, 2015).

ii) Antimicrobial gradient method (E-test)

This method works on a combined principle of both dilution and diffusion methods in the determination of the MIC in order to create a concentration gradient of the tested antimicrobial in the selected agar. Strips impregnated with a higher concentration gradient of the test antimicrobial is placed on the agar surface with pre-inoculated test organism creating a steep gradient (Balouiri *et al.,* 2015). MIC value is established at the strip intersection with the growth inhibition ellipse.

Antimicrobial gradient method is relatively simple to implement but the strips are costly, hence expensive if many antimicrobials are tested (Reller *et al.,* 2009). The method is reproducible with standard results observed when compared with broth dilution and disk diffusion method (Gupta *et al.,* 2015).E-test can be used to determine the combined effect of two antibiotics where one strip pre-impregnated with one antibiotic is placed on the agar for a while before the second pre-impregnated strip is placed on the same agar. The synergy is established by a decrease of combined MIC by at least two dilutions compared to the most effective antibiotic tested alone (Balouiri *et al*., 2015).

iii) Agar well diffusion method

Agar well diffusion is mainly used in order to establish antimicrobial activity of a plant or microbial extracts (Valgas *et al.,* 2007). The method uses diffusion principle where the bacterial inoculum is spread on agar plate with which a 6-8mm hole is punched at the centre and a volume of the desired antimicrobial agent is introduced into the hole. This is incubated under suitable conditions to allow the agent to diffuse into the agar and inhibit growth of the antimicrobial tested.

iv) Agar plug diffusion method

This method is mainly used to establish antagonism between microorganisms (Balouiri *et al.,* 2015). An agar culture of the organism on its specific medium is made by streaking on its surface and incubated. An agar plot is aseptically cut and placed on agar plate surface of another plate pre-impregnated with the test organism. This allows substances to diffuse from plug to agar medium and the antimicrobial activity determined by inhibition zone around the agar plug.

2.9.1.1 Dilution methods

Dilution methods are the mostly recommended tests in establishing MIC values because of estimation of the concentration of the tested antimicrobial agent in the agar thus considered qualitative methods of antimicrobial sensitivity testing. They include; broth and agar dilution methods.

i) Broth dilution method

The broth dilution technique works on the principle of establishing bacterial sensitivity against varied concentrations of the microorganism, usually a serial two-fold dilution of the antimicrobial (Reller *et al.,* 2009).

Broth micro – or micro – dilution method involves preparation of the dilutions of an antimicrobial agent in tubes containing about 2ml of the liquid growth medium or smaller volumes when using 96 well micro-titration plate. Each tube is inoculated with bacterial

inoculum and is adjusted to 0.5 McFarland scale of turbidity and mixed then incubated under suitable conditions depending on the test organism.

Results are interpreted as minimum inhibitory concentration (MIC) in mg/mL and they are determined by the antibiotic that inhibits or kills the organism as marked by turbidity. The advantages of micro-dilution include its reproducibility, space due to miniaturization of test and reduced costs as compared to macro-dilution. However, the final MIC values include inoculation size, type of growth medium, incubation time and methods used in preparation of the inoculum (CLSI, 2017)

ii) Agar dilution method

Agar dilution involves testing susceptibility of a bacteria against varying desired concentrations of an antimicrobial agent in an agar. The method is used for both antifungal and antibacterial susceptibility and is the method of choice in testing susceptibility of fastidious organisms (CLSI, 2017) and anaerobes as well as antifungal agent –drug combinations against Candida species, Aspergillus, Fusarium and dermatophytes (Menon *et al.*, 2001).

2.9.2 Genotypic antimicrobial susceptibility testing

Genotypic susceptibility testing is mainly encouraged due to its accuracy and rapidity of susceptibility testing (Datar *et al.*, 2022). These methods include; nucleic acid amplification techniques such as PCR provides high sensitivity, specificity and speed in detection of known resistance genes (Trinh *et al.*, 2021). PCR is used in detection of various resistant genes including *mec*A gene for the detection of methicillin/oxacillin resistance in *Staphylococcus aureus* and CoNS (Khan *et al.*, 2019). Additionally, Matrix-assisted Laser Desorption Time-of-Flight mass spectrometry (MALDI-TOF MS) is also used in antimicrobial susceptibility (Angeletti, 2017) The method is not however not commonly used as a diagnostic tool due to its affordability.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was conducted in Garissa County, in the five Sub-Counties of Fafi, Garissa, Ijara, Lagdera and Balambala. Garissa County lies in the North Eastern part of Kenya, between latitude 1^0N and 2^0S and longitude 39^0E and 41^0E, with an estimated area of 44,175km^2. The County has a population of 623,060 people who are mainly pastoralists and agro-pastoralists along river Tana as shown in figure 3. The County is one of the ASAL lands of Kenya and is classified between IV-VI Agro ecological zones. The vast land experiences frequent droughts which are marked by high terrestrial temperature of 40^0C and erratic rainfall patterns (KNBS, 2015). Garissa County has a population of 234,683 camels (Toroitich. 2012). The common breed of camel is the dromedary camel (*Camelus dromedaries*).

Figure 2: A map of Kenya highlighting the study area - Garissa County: ArcGIS version 10.1

The targeted sub-Counties selected based on their camel populations included; Fafi, Garissa,Ijara Lagdera and Balambala .

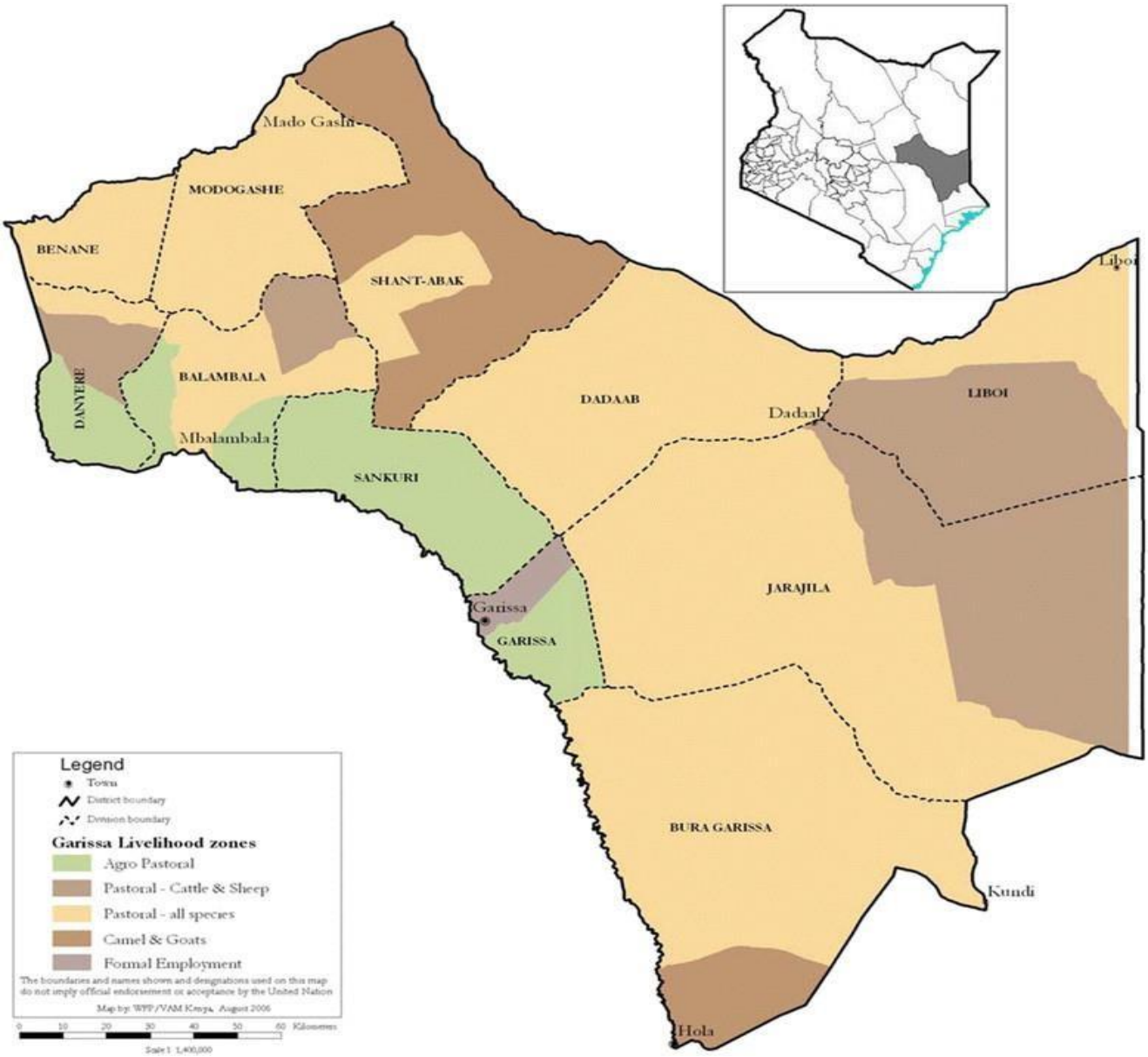

Figure 3: A map of Sub counties in Garissa County: ArcGIS version 10.1

3.2 Study Design

This study was part of an on-going Feed the Future project implemented by the International Livestock Research Institute (ILRI) in collaboration with Department of Public Health

Pharmacology and Toxicology (PHPT) of the University of Nairobi. A cross-sectional study with convenient sampling was done in the selected Sub-Counties in Garissa County. This involved exclusion criterion which included avoiding all non-lactating camels and clinically sick camels during milk sampling. Smallholder camel farmers were randomly selected from the list of camel farmers provided by the clan heads in each of the sub-Counties. Each farmer had to have at least two lactating camels and willing to participate in the study.

3.3 Sample size determination

The sample size for the study was determined by using a formula described by Pfeiffer, (2013). n=Z2 P (1-P)/L2]; Where, n=sample size, Z= the value of z that gives 95% confidence, with 25% expected prevalence (Younan et al., 2013), and 5% desired precision. n= (1.962 x 0.25 x 0.75)/ (0.05)2. The number of samples per Sub County was done proportionally to the population of camels in that Sub County.

3.4 Sample collection and recovery of bacterial cells

Two hundred and thirty-one raw camel milk samples were aseptically collected from apparently normal udders into sterile screw falcon tubes after discarding the first three streams of milk. This was done after washing the udders with water and then cleaning the teats using cotton wool soaked in 70% ethanol. Geo-referencing of the camel households was done using Garmin ETrex GPS units. The sample tubes were labelled using reference numbers and taken to the laboratory for bacteriological analysis.

The milk samples were transported to the department of Public Health, Pharmacology and Toxicology (PHPT), Faculty of Veterinary Medicine, University of Nairobi, in cool boxes and kept in a deep freezer maintained at -20^{0}C until time for processing. On due time for laboratory work, the samples were kept at room temperature (25^{0}C) overnight in order to thaw. The samples were then enriched by inoculation in buffered peptone water (BPW) which was prepared by dissolving 10g of powdered BPW in 500mls of distilled water. The prepared

solution was divided into aliquoted 4mls test tubes and sterilized by autoclaving at 121^0C for 15 minutes. Thereafter, 1ml of each sample was added onto the 4ml aliquots of BPW and incubated at 37^0C overnight. *Staphylococcus* bacteria were then phenotypically confirmed by culture on Mannitol Salt Agar (MSA) and Blood Agar (BA) as well as coagulase and catalase tests.

The main challenge during sampling was the distance covered in the vast pastoralist lands of Garissa County. Camel keeping househods in the various sub Counties were distant from each other in accordance with their pastoral way of life. Other challenges included funding as well as language barrier but this was helped with the clan heads in each sub County

3.5 Culture on Mannitol Salt Agar

A weighing balance was used to measure 111g of powdered Mannitol Salt Agar (Oxoid) which was dissolved in 1000ml of pure distilled water. The solution was then sterilized by autoclaving at 121^0C for 15 minutes and further aseptically (20mls) dispensed onto each petri dishes after cooling. This was left to solidify before streaking a loop-full of the inoculum from the samples incubated in the buffered peptone water. The plates were incubated at 37^0C for 18hours after which yellow grape-like shape colonies of bacteria were thought to be *Staphylococcus* species. These colonies were further cultured on Blood Agar to test for beta-haemolysis, a typical attribute of *S. aureus*.

3.6 Culture on Blood Agar

Twenty (20) grams of powdered Blood Agar (Oxoid) was dissolved into 500mls of pure distilled water. The solution was then sterilized by autoclaving at 121^0C for 15 minutes. Thereafter, the media was cooled in a water bath at 55^0C then 7% sterile sheep blood was added and mixed thoroughly. About 20mls of the solution was aseptically dispensed onto sterile petri dishes and left to cool at room temperature (25^0C). A loop-full of the cultured yellow bacterial colonies was streaked on each petri dish under a bunsen flame and incubated overnight at 37^0C. Haemolysis was observed by clearing around the colonies and identified as *S. aureus*. These

colonies were further enriched on Trypticase Soy Agar before subsequent coagulase and catalase biochemical tests.

3.7 Culture on Trypticase Soy Agar

An amount of 40g of powdered TSA (Himedia) was dissolved in 1000mls of pure distilled water and sterilized by autoclaving at 121^0C for 15 minutes. The media was then cooled and 20mls dispensed in each petri dishes and left to solidify. A loop-full of distinct colonies picked from Blood agar plates was streaked onto each TSA plate and incubated overnight at 37^0C.

3.8 Catalase and Tube coagulase tests

Catalase test was done by adding Trypticase soy agar (TSA) cultured bacterial colonies to a labelled microscope slides containing a drop of 3% hydrogen peroxide (H_2O_2). The microscope slides were placed against a dark background for observation of effervescence thatindicates catalase positive test (Reiner, 2010). Tube coagulase test was performed by adding two colonies of TSA cultured bacteria to labelled-test tubes containing 0.5ml of plasma and incubated at 37^0C. Coagulase positive test were observed by clot formation in each test tube at one hour intervals for the first 4 hours. Negative samples after the fourth hour were further incubated at room temperature (25^0C) for 24 hours.

3.9 Antimicrobial Susceptibility Testing

Antibiotic sensitivity of the isolated *Staphylococcus* species was determined by using the disk diffusion method described by Kirby Bauer (Bauer *et al.*, 1966) and results interpreted as per the Clinical Laboratory Standards Institute (CLSI) 2017 guidelines. Isolated bacteria were tested for susceptibility to selected antimicrobial drugs that included; 10ug Ampicillin, 10ug streptomycin, 30ug cephalexin, 15ug erythromycin, 5ug ciprofloxacin, 30ug cefoxitin,30ug tetracycline and 30ug chloramphenicol. The *Staphylococcal* isolates were cultured on Trypticase Soy Agar overnight after which five colonies of the organisms were picked using a sterile wire-loop from each plate and suspended in 5 ml of sterile normal saline which was then adjusted to a density approximately equal to McFarland Opacity Standard No. 0.5. A swab of

the suspension was used to streak the Mueller Hinton Agar (MHA) followed by dispensing antibiotic disks (OXOID®) on the surface of the MHA using a disk dispenser. Each sample was incubated in MHA in duplicates. The plates were incubated at 37°C for 24 hours after which diameters of zones inhibition (mm) were measured using a Vernier calibre and an average obtained. The readings were recorded as either susceptible, intermediate, or resistant based on the interpretative breakpoints by the Clinical Laboratory Standards Institute (CLSI) guidelines.

3.10 Detection of AMR genes

3.10.1 DNA Extraction

Staphylococcus colonies cultivated on MSA were randomly selected for determination of antibiotic resistance genes and DNA extracted as described by Diaz (2012). Two colonies of the presumptive isolates were obtained from 24 hours cultures inoculated on Tryptic Soy Agar (TSA) (4.1%) and transferred to 1.5 ml of Appendorf tubes containing 400µl of distilled water. This was followed by boiling at 95^0C for 7 minutes and centrifugation at 15000 rpm for 10 minutes. Conventional PCR technique was used to investigate antibiotic resistant genes of staphylococcus DNA supernatant extracts (Gao *et al.,* 2011).

3.10.2 Molecular Identification of *Staphylococcus* species by PCR

Primers for identification of resistant *Staphylococcus* isolates was determined by amplification of a portion of 16S rRNA gene as described by Woo *et al,* (2001). A total volume of 10µl containing 4.9µl of DNA as a template was used. The amplification cycles were done in a thermocycler and the reaction conditions optimized to 94^0C for 2 minutes as initial denaturation then followed by 33 cycles of 94^0C for 30 seconds, 58^0C for 30 seconds, 72^0C for 30 seconds and final extension step at 72^0C for 5 minutes.

3.10.3 Identification of *S. aureus* by PCR assay

The *nuc* gene of all isolates was amplified according to the protocol described by Javid *et al.,* (2018). A total volume of 20µl containing 5µl of DNA as a template was used with initial

denaturation done at 95^0C for 3 minutes, followed by 35 cycles at 95^0C for 30 seconds, primer annealing at 52^0C for 15 seconds, 720C for 1 minutes and final elongation at 72^0C for 10 minutes. Amplification of the 416 bp PCR products indicated the strain belonging to the genus *Staphylococcus.*

3.10.4 Identification of resistant phenotypes by PCR

Amplification of *mecA* gene was done as described by Pournajaf *et al.,* (2014) with slight modifications. A total volume of 10µl containing 4.6µl of DNA as a template was used. This was by initial denaturation at 94^0C for 3 minutes, followed by 33 cyles at 94^0C for 30 seconds, annealing at 50^0C for 30 seconds, 72^0C for 60 seconds and final extension at 720C for 5 minutes.

For *strB* gene PCR, primer pair in Table 1 used for typing were derived from Pyatov et al., (2017). The PCR mixture contained 20µl containing 5µl of DNA and concentration of 0.2µl each of the primers. PCR conditions included initial denaturation at 95 °C for 1 min, 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 60 °C for 30 seconds and elongation at 72 °C for 30 seconds and final elongation at 72 °C for 7 minutes.

A final volume of 10µl containing 4.8 µl of DNA as a template was used in amplification of bla*TEM* gene. The thermocycler conditions included initial denaturation at 95^0C for 5 minutes followed by 40 cycles of 95^0C for 1 minute, annealing at 64^0C for 30 seconds, 72^0C for 30 seconds and final extension at 72^0C for 2 minutes.

3.10.5 Gel Electrophoresis

DNA amplification products were confirmed by agarose gel electrophoresis. Agarose powder (0.6g) was dissolved in 40mls of 1 x Tris Acetate EDTA (TAE) to make 1.5% agarose gel. This was then allowed to cool and Ethidium Bromide added. Thereafter, 4µl of the PCR product mixed with the loading dye was loaded into the gels including the 100bp DNA ladder. Electrophoresis was done at 70volts for 45 minutes and resultant visualization was done under ultravioletillumination machine. UV trans-illuminator (BIORAD) was used to anaylze the

document photos of each gel.

Table 1: Nucleotide sequences and amplicon sizes for *Staphylococcus* genes specific primers used in the study

Target gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
16S rRNA	*16S rRNA(F) AGTTTGATCCTGGCTCAG* *16S rRNA(R) AGGCCCGGGAACGTATTCAC*	1284bp	Woo *et al,* (2001)
Nuclease gene	SA-(F) GCGATTGATGGTGATACGGTT SA-(R) CAAGCCTTGACGAACTAAAGC	276bp	Wang *et al., (*1997)
***mec*A gene**	*mec*A(F) AAAATCGATGGTAAAGGTTGGC *mec*A(R) AGTTCTGGAGTACCGGATTTGC	533bp	Pournajaf *et l.*, (2014)
***blaZ* gene**	*bla*Z(F) ACTTCAACACCTGCTGCTTTC *bla*Z (R) TGACCACTTTTATCAGCAACC	173bp	Martineau *et al.,* (2000)
***Str* Gene**	strB-(F) CGGTCG TGAGAACAATCTGA strB-(R) ATGATGCAGATCGCCATGTA	313bp	Pyatov *et al.,* (2017)
***bla*TEM**	*bla*TEM (F) GCTCACCCAGAAACGCTGGT *bla*TEM (R) CCATCTGGCCCCAGTGCTGC	686bp	Ojdana *et al.,* (2014)

3.8 Sequencing of PCR products

PCR products were purified with Qiaquick PCR purification kit (Qiagen, Germantown, USA) in order to remove excess primers, salt and Taq polymerase, which interferes with the sequencing reaction. The purified products and the primers previously used for the PCR were then submitted to Humanizing Genomics Macrogen Europe Laboratory- Netherlands for Oligonucleotide sequencing. The BLASTn tool of the NCBI Gene bank database (http:/blast.ncbi.nim.nih.gov/blast.cg) was used to analyze the sequenced DNAs.

3.10 Ethical Approval

Ethical approval of this study was sought from the University of Nairobi, Faculty of Veterinary Medicine Biosafety, Animal Use and Ethics Committee before the start of the study (FVM/BAUEC/2018/157).

3.11 Data analysis

Data was entered into Microsoft Excel sheet 2016, coded and outliers removed before exporting to STATA version 13 software. Comparisons of antimicrobial resistance profiles between CoPS and CoNS were conducted by using the χ^2 test; p values <0.05 was considered significant. Percentages of antibiotic resistant CoNS and CoPS including the multidrug resistant *Staphylococc*i were determined. Data was presented by using tables and graphs accordingly.

CHAPTER FOUR

RESULTS

4.1 Culture and Biochemical test results

A total of 231 raw camel milk samples were collected from camel farmers in five sub Counties of Garissa County; Fafi, Garissa, Ijara, Lagdera and Balambala. Out of the 231 samples cultured overnight on Buffered Peptone Water (BPW) 133 (57.6%) showed turbidity as a marker of successful recovery. A total of 122 (91.7%) samples had growth of round yellow colonies on MSA changing the colour of the media from pink to yellow as shown in Figure 4. These colonies were therefore, presumed to be *Staphylococcus* species. Out of all isolates further grown on Blood Agar, 102 (83.6%) samples showed clear zones around the colonies indicating β-haemolysis. All the 122 samples sub cultured on Trypticase Soy Agar (TSA) exhibited coccoid cream colonies while 102(83.6%) *Staphylococcus* isolates were coagulase positive as indicated by plasma clotting that remained intact when the test tubes were inverted and 20 (17.7%) were coagulase negative in tube coagulase test. From the catalase test, 83 (68.0%) of the isolates were catalase positive as shown by production of effervescence after reaction with hydrogen peroxide on the microscope slide. Table 2 summarizes the culture and biochemical test results.

Table 2: Recovery and biochemical tests for *Staphylococcus* species from raw camel milk

Culture and Biochemical Characterization tests	**Number of positive samples (%) (N=122)**
Growth on BPW*	133 (57.6%)
Growth on MSA*	122 (91.7%)
β-Haemolysis	102 (83.6%)
Growth on TSA*	122 (91.7%)
Coagulase	102 (83.6%)
Catalase	83 (68.0%)

*__BPW__- Buffered Peptone Water, **MSA-** Mannitol Salt Agar, **TSA-** Trypticase Soy Agar

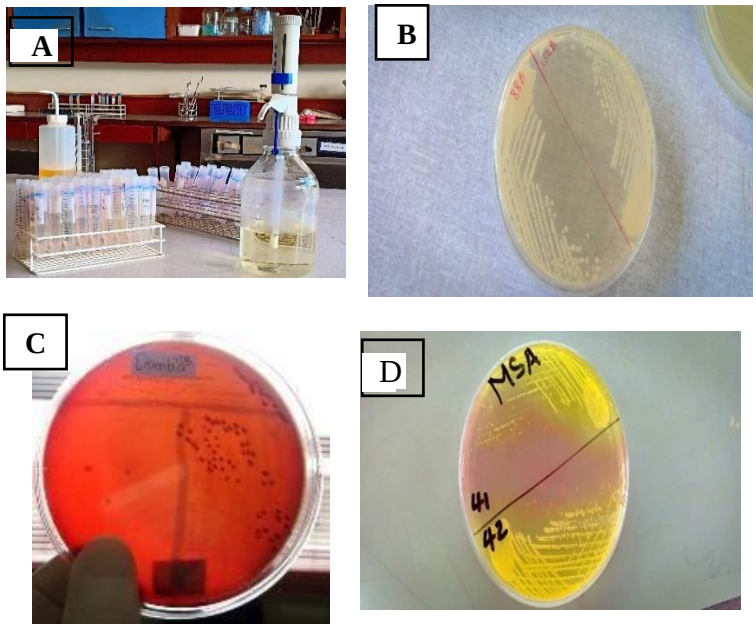

Figure 4: Recovery and Isolation of Staphylococcus species in BPW, TSA, BA and MSA; A shows prepared Buffered Peptone Water (BPW) for revival of bacterial cells in raw camel milk, **B:** shows enrichment of suspected *Staphylococcus* colonies in Trypticase Soy Agar (TSA) for further biochemical tests. **C**: shows clear zones around *Staphylococcus* colonies cultured on Blood Agar, suggestive to bet haemolysis, **D:** shows yellow coccoid colonies of suspected *Staphylococcus* isolates on MSA culture.

Table 3: Distribution of camels sampled from the sub counties in Garissa County n=231

Sub County	Camels samples
Fafi	58
Garissa (Township)	25
Ijara	23
Lagdera	83
Balambala	42
Total	**231**

4.2 Identification of *Staphylococcus* species by PCR assay

Staphylococcus isolates were determined by amplification of a portion of *16S rRNA* gene by conventional PCR. The *nuc* gene of *Staphylococci* isolates were amplified to establishthe *S. aureus* isolates. The primer sequences for the *16S rRNA* and *nuc* gene as shown in Table1 were used. All the isolates screened for presence of *16S rRNA* gene yielded strong bands at 323bp while 102 (83.6%) isolates were positive for *S. aureus* indicated by bands at 900bp on gel electrophoresis as shown in Figure 6.0 below.

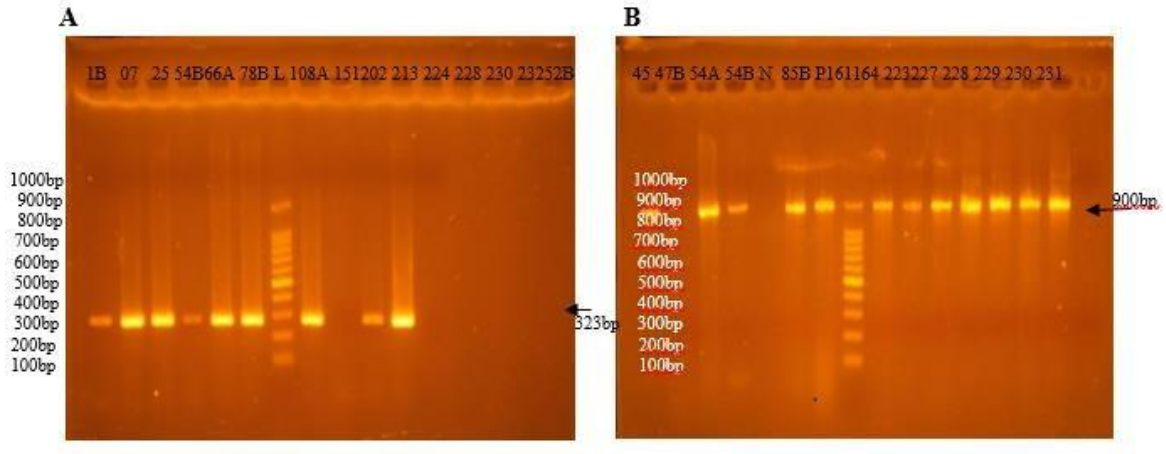

Figure 5: Conventional PCR amplification of *Staphylococcus-16S rRNA* gene and *nuc* gene fragment for identification of *Staphylococcus* species and particularly *S. aureus* in the isolates respectively. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel, stained with ethidium bromide. Gel image A shows *nuc* gene detection of *Staphylococcus sp.* at 323bp while gel image B shows isolate bands yielded by *16S rRNA* gene of 900bp. Lane L is DNA ladder while Lane P is the positive standard of *S. aureus* ATCC®25923™, Lane N is negative control and the numbered lanes are the test samples. The lanes with no bands are samples negative for *nuc* gene while the lanes with bands show the presence of the *nuc* gene indicating the presence of *S. aureus*. The arrows show the positions of the amplified genes in gel A and B at 323bp and 900bp respectively.

All the 122 isolates were identified by blast analysis of 16S rRNA using the National Centre of Biotechnology Information (NCBI) database: https://blast.ncbi.nlm.nih.gov/Blast.cgi and confirmed various *Staphylococcus* species as shown in Table 3.

Table 4: Results of *Staphylococcus* species identified by BLASTn analysis using *16S rRNA* sequences of the isolates from raw camel milk in Garissa County.

Samples	Target gene	Homologous Sequences	E.values	ID (%)
1B	16S Rrna	*S. agnetis*	0.0	98
7	16S Rrna	*S. aureus*	0.0	98
25	16S Rrna	*S. aureus*	0.0	99
54B	16S rRNA	*S. aureus*	0.0	98
78B	16S rRNA	*S. agenteus*	0.0	98
93	16S rRNA	*S. aureus*	0.0	98
108A	16S rRNA	*S. aureus*	0.0	100
151	16S rRNA	*S. aureus*	0.0	99
252B	16S rRNA	*S. agnetis*	0.0	98

4.3 Antimicrobial susceptibility Testing.

Table 5: Antimicrobial susceptibility of 122 *Staphylococcus* isolates in the 8 antibiotics.

Antibiotic	Resistant n (%)	Intermediate n (%)	Sensitive n (%)
Cephalexin	81.90	-	18.10
Streptomycin	72.10	-	27.90
Ampicillin	33.60	-	66.40
Cefoxitin	10.70	-	89.30
Erythromycin	5.70	10.70	83.60
Ciprofloxacin	4.10	8.20	87.70
Tetracycline	3.30	0.80	95.90
Chloramphenicol	1.60	2.50	95.90

Most of the isolates were highly sensitive to Chloramphenicol (95.9%) and Tetracycline (95.9%) but highly resistant to Cephalexin (81.9%). The order of decreasing resistance in the 8 antibiotics tested was; cephalexin (81.9%), streptomycin (72.1%), ampicillin (33.6%), cefoxitin (10.7%), erythromycin (5.7%), ciprofloxacin (4.1%), tetracycline (3.3%) and chloramphenicol (1.6%) as shown in Table 4. High levels of resistance was seen in Methicillin-resistant Coagulase negative *Staphylococcus* (MRCoNS) (15%) than in Methicillin-resistant *S. aureus* (MRSA) (9.8%) isolates. MDR was relatively high in all the isolates (43.4%) comprising of 6/20 (30%) MDR-CoNS and 47/102 (46%) MDR *S. aureus*.

4.3.1 Comparison of antibiotic susceptibility of CoPS and CoNS

Both coagulase positive and coagulase-negative *Staphylococci* isolates were highly sensitive to tetracycline and chloramphenicol. As shown in Table 8 below, CoPS were more resistant to most drugs when compared with coagulase-negative *Staphylococcus spp.*

A total of 72.5% (74/102) of the coagulase positive *Staphylococcal* isolates showed resistance to cephalexin and 70% (14/20) coagulase negative isolates phenotypically showed cephalexin

resistance. CoPS showed highest resistance to streptomycin (86.3%) while highest resistance among the CoNS was in cephalexin (70%).

The *Staphylococcal* isolates showed moderate resistance to Amoxicillin showing 34.3% and 30% resistance in CoPS and CoNS respectively.

Table 6: Comparison of overall resistance of (CoPS) *S. aureus* and CoNS (p>.05)

Antibiotic	% CoPS resistant	% CoNS resistant	χ2 test results	P value
Cephalexin	72.5	70	3.8979	<0.05
Streptomycin	86.3	60	6.0381	< 0.001
Erythromycin	10.8	20	4.012	<0.05
Ciprofloxacin	11.8	15	3.982	<0.05
Ampicillin	34.3	30	0.1607	<0.5
Cefoxitin	9.8	15	4.325	<0.05
Tetracycline	3.9	5	0.8411	<0.5
Chloramphenicol	20	15	40.0367	<0.05

The comparison of antimicrobial resistance in the isolated CoNS and CoPS showed significantly lower antimicrobial resistance in CoNS (mean = 18%) than in CoPS (mean = 32%). Antimicrobial drugs that showed statistical correlation as regards to percentage of resistant isolates and coagulase production were cephalexin, erythromycin, ciprofloxacin, cefoxitin and chloramphenicol (all with $p<0.05$) and streptomycin ($p<0.001$).The remaining tested antimicrobial drugs showed no significant association (Table 6)

4.4 Detection of antibiotic resistant genes by PCR

The isolates;1B, 47B, 54A, 60A, 66A, 103A, 109A, 114A, 122A, 140B, 152B and 164 were positive for *strB* (Figure 7). Only one isolate, 93 was positive for *mecA* (Figure 8) while 70.8% of the isolates (34/48) were positive for *bla*TEM gene. 87.1% of the isolates were positive for *bla*Z gene. All the isolates were negative for *mec*C, *bla*-CTX and *bla*-SHV gene even though phenotypically resistant. Isolate numbers 1B, 47B, 54A, 114A, 66A and 109A harboured both *strB* and *bla*-TEM genes (Figure 6).

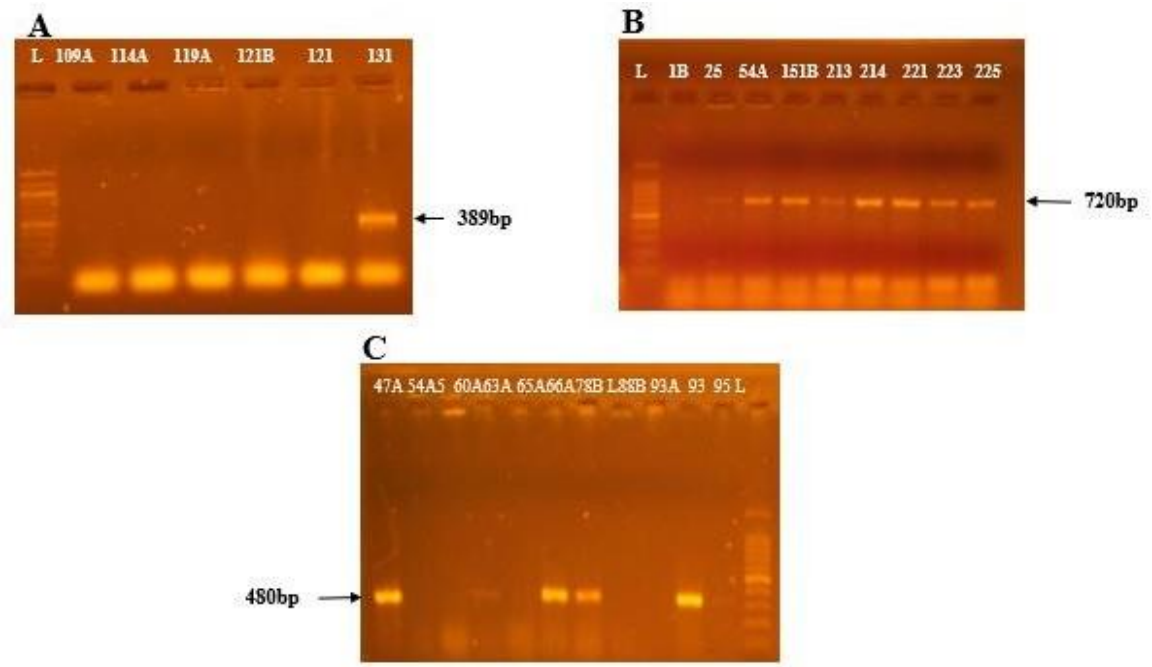

Figure 6: Polymerase chain reaction detection of resistant genes (A) *mec*A (389 bp), (B) *bla*TEM (720 bp and (C) *aph(6)-Id* (*str*B) (480bp). Lane L is the DNA Ladder, numbered lanes are the test sample and Lanes with no bands are samples negative for *mec*A, *bla*TEM and *str*B genes. The arrows shows the positions of amplified genes.

S. aureus isolates were confirmed by blast analysis of *nuc* gene using the NCBI as shown in Table 6.

Table 7: Results of *S. aureus* identified by BLASTn analysis using *nuc* gene

Samples	Target gene (nuc)	Homologous sequences	E.values	ID (%)
47B	nuc	*S. aureus*	2e-117	99
93	nuc	*S. aureus*	0.0	98
54A	nuc	*S. aureus*	3e-114	99
85B	nuc	*S. aureus*	4e-116	98
161	nuc	*S. aureus*	3e- 117	98
227	nuc	*S. aureus*	1e- 116	100
229	nuc	*S. aureus*	9e- 119	100
231	nuc	*S. aureus*	4e- 117	98
252	nuc	*S. aureus*	1e-117	99

CHAPTER FIVE

DISCUSSION

5.1 Discussion

The results of this study demonstrates the presence of multidrug resistant CoNS and *S. aureus* isolated from raw camel milk in the less studied ASALs of Kenya. The pastoral community in the area consume raw camel milk and this can pose a health hazard to the community. Management of infectious diseases with antimicrobial agents has significantly improved animals' health. However, the use of antibiotics has resulted in a selection for antimicrobial resistance by bacteria (GARP 2011). This can be through intrinsic resistance by microorganisms or acquired resistance through transfer of resistance plasmids (Azounwu *et al*., 2019). The increased resistance to antibiotics in the ASALs can be attributed to misuse, overdosing/under-dosing by pastoralists self –medicating their camels as well as easy access to antibiotics over the counter (Lamuka *et al.,* 2017).

In the current study, it was established that 52.6% (122/231) of the bacteria isolated from analysed camel milk samples were *Staphylococcus* species. This constituted 83.6% CoPS and 16.4% CoNS with CoPS being the most prevalent *Staphylococcus spp*. These findings were similarto those obtained by Amjad *et al.,* (2017) who found 74.5% *Staphylococcus spp.* from camel milk in Pakistan with 87.2% CoPS and 12.8% CoNS. The findings of the study were higher than those obtained by El-hag *et al.,* (2013) who isolated 28.69% of *Staphylococcus spp*. in camel milk from Bar- Khartoum, Sudan and lower than the findings of Abera *et al.,* (2016) who isolated 89.8% *Staphylococcus spp.* from raw camel milk in Ethiopian Somali region state.

Remaz and Nagwa (2015) in their study on *Staphylococcus* species in camel milk from Khartoum North, Sudan isolated 46.7% *Staphylococcus spp.* with 32.1% CoNS and 67.9% CoPS which agrees with the findings of this study with CoPS being the most common isolate. This was also similar to the findings of Varma and Prakesh (2016) who reported 62.5% CoPS

and (37.5%) CoNS from raw camel milk from different regions of India. Ehosseny *et al.,* (2018) in their study on evaluation of physiochemical properties and microbial quality of camel milk in Egypt isolated 42.8% *Staphylococcus spp.* with 38.5% being CoPS which is significantly lower than the findings in this study. Jaradat *et al.,* (2013) reported 48 isolates with coagulase gene and 16 CoNS in their study on camel meat and nasal swabs in order to establish difference in coagulase production, genotype and methicillin resistance.

Egyir *et al.,* (2014) in their study of *Staphylococcal* isolates from six healthcare facilities in Ghana established 65.9% CoNS in bacteremia, skin and soft tissue infections while 12.4% CoPS were isolated from bloodstream infections. The prevalence of *Staphylococcus* in the study was also lower than the findings of Njage *et al.,* (2013) who reported 62% *Staphylococcus* prevalence on raw and fermented camel milk from Kenya and Somalia. The high prevalence of CoPS isolated was significantly higher than those of Hany *et al.,* (2020) in their study in Saudi Arabia, where CoPS prevalence was 5% in pasteurized camel milk. This may be attributed to pasteurization process which eliminates most of the organisms in camel milk. However, this study is comparable to that of Al-Dughaym and Fadlelmul (2015) and Wanjohi *et al*. (2013). In contrast, Remaz and Nagwa (2015) in their study in Khartoum North, Sudan isolated 67.9% CoPS and 32.1% CoNS, mainly *S. aureus*. Mutua *et al.,* (2017) reported higher CoNS isolates from nasal cavity of camels from Nakuru (36.84%), Samburu (29.27%) and Isiolo (22.43%) Counties.

Comparing with isolation from other samples, Boamah *et al.,* (2017) isolated significantly higher CoNS at 63.8% from poultry farms in three different regions of Ghana while Sangeda *et al.,* (2017) isolated 17.5% of CoNS and 82.7% CoPS in their compilation of studies on wounds and blood borne infections from hospitalized patients in Nairobi, Kenya. Youssif *et al.,* (2021)in their study of determining genes conferring antimicrobial resistance in cattle with subclinical

mastitis in Cairo, Egypt, isolated *S. aureus* (66.6%) as the major pathogen. Multiple antibiotic testing of the isolated *Staphylococcus* showed highest resistance to cephalexin (81.9%) and streptomycin (72.1%) followed by ampicillin (33.6%), cefoxitin (10.7%), erythromycin (5.7%), ciprofloxacin (4.1%), tetracycline (3.3%) and chloramphenicol (1.6%). The high resistance in beta-lactamases and aminoglycosides is attributed to wide use and the sub therapeutic doses used by the pastoralist self-medicating their camels (Omwenga *et al*., 2021). These findings were to some extent agreeable to those of Mutua *et al.,* (2017) who found highest susceptibility of *Staphylococcus* to chloramphenicol, kanamycin and gentamycin (all at 100%), followed by co-trimoxazole and streptomycin (34%), ampicillin (23%) and tetracycline and sulphamethoxazole (12%) respectively.

Gitao *et al,* (2014) in their study of prevalence of common camel milk borne pathogens causing mastitis and their antibiotic resistance in North Eastern Province in Kenya, identified *S. aureus* to be resistant to Ampicillin (0.30), Co-Trimoxazole (0.25), and Sulphamethoxazole (0.13) but sensitive to Gentamicin (1.89) and Tetracycline (1.08). Multidrug resistance in the study (43.4%) was higher than that of Mutua *et al.,* (2017) who reported 30.5% MDR in *Staphylococus* isolates from nasal cavity of camels in Samburu, Nakuru and Isiolo Counties. Aqib *et al.,* (2017) reported overall resistance of 54.7% from camel milk in Pakistan with significant resistance to Penicillins (90%), Cephalosporins (77.5%), Quinolones (77.5%) and 92.7% to Sulphonamides. Al-Thani and Al-Ali, (2014) also reported that *Staphylococcus* from different farms in Qatar farms to be resistant to tetracycline, Penicillin and Ampicillin which did not concur with study findings since highest resistance recorded in Cephalexin (81.9%) and Streptomycin (72.1%). The difference is attributed to accessibility and wide use of beta-lactams in Garissa County, Kenya as compared to farms in Qatar which have restricted access to most antimicrobial drugs. Njage *et al.,* (2013) in their study on resistance patterns of *S. aureus* from raw and fermented camel milk from Kenya and Somali, established *Staphylococcus* resistance

to Ampicillin (11 isolates) followed by Streptomycin (5 isolates) and Tetracycline (5 isolates). Varma *et al*., (2005) reported high resistance by *Staphylococcus* to Ampicillin from camel milk from Bikaru District, India as compared to this study. Sangeda *et al.,* (2017) reported high resistance of both CoPS and CoNS to Penicillin (91.7%), Tetracycline (46.4%), Erythromycin (43.6%), Ciprofloxacin (30.8%) and Chloramphenicol (30.1%) from human wounds in Nairobi Kenya. This was significantly higher levels of CoNS than this study.

Most of the isolates in the study were generally sensitive to the antimicrobials tested except Cephalexin (18.1%) and Streptomycin (27.9%). Tetracycline (95.9%) and Chloramphenicol (95.9) had the highest susceptibility followed by Cefoxitin (89.3%), Ciprofloxacin (87.7%), Erythromycin (83.6%) and Ampicillin (66.4%). Aqib *et al.*, (2017) reported 100% *S. aureus* sensitivity to Ciprofloxacin in their study on prevalence and antibiogram of *Staphylococcus aureus* from camel milk in Pakistan. This was significantly higher than the findings in this study with which Ciprofloxacin had 87.7% sensitivity. Gitao *et al,* (2014) reported high sensitivity of isolates from raw camel milk to Tetracycline and Ampicillin.

Various studies have reported resistance in commonly used antimicrobial drugs by livestock keeping pastoral communities in Kenya (Omwenga *et al.*, 2021; Mutual *et al.*, 2017). These antimicrobials include maclorides, sulphonamides, beta-lactams and fluoroquinolones. The increasing resistance in these antimicrobials is mainly attributed to their first line use on empirical therapy with no consideration of appropriate dosages or withdrawal duration (Omwenga *et al.*, 2021). In the study, both CoPS and CoNS were highly sensitive to Chloramphenicol and Tetracyline but on the other hand, there was comparable high resistance to Ciprofloxacin (15%), Cefoxitin (15%) and Erythromycin (20%) among CoNS isolates when compared to CoPS isolates. This was in agreement with findings of Sangeda *et al.,* (2017) who reported high resistance to Erythromycin (69.6%) and Ciprofloxacin (52.2%) among CoNS as compared to CoPS from human wounds in Nairobi, Kenya.

Methicillin resistant *Staphylococci* (MRSA) have been reported in various foods of animal origin including camel and cattle milk (Soares *et al.,* 2012; Njage *et al.,* 2013; Silva *et al.,* 2014; Mbindyo *et al.,* 2020). However, the surveillance of this resistance trait is imperative toPublic Health and Veterinary medicine. In this study, one isolate carried the *mec*A gene, a putative genetic carrier in zoonotic pathogens. This finding was in tandem with that of Klibi *etal.,* (2018) and Silva *et al*., (2014) with relatively smaller cassette size as compared to other SCCmec types which may be essential for horizontal transfer among *Staphylococcus* species. Methicillin sensitive CoPS also isolate in the study samples, may act as reservoir of mobile genetic elements (MGE) carrying the antimicrobial resistance. The migrant SCCmec elements are transferable to other species including the pathogenic *S. aureus* (Dos Santos *et al.,* 2016)

The low levels of *mec*A detected in the study could be attributed to methicillin resistance by other mechanisms including increased production of beta-lactamases, coded by *bla*Z gene or the change in penicillin binding proteins or production of new methicillinases (Dos Santos *et al.*, 2016; Soares *et al.*, 2012). In the study, 87.1% of the isolates harboured *bla*Z gene and 46.6% of the isolates carried *bla*TEM gene. The significantly high presence of *bla*Z gene may be due to the frequent use of beta-lactams by the pastoral community in the management of various camel diseases. The *bla*Z gene in CoNS isolates from milk have also been reported in Brazil (16%), Tunisia (58.33%), Switzerland (90%) and China (30.3%) (Soares *et al.,* 2012; Klibi et al., 2018; Frey *et al.,* 2013 and Xu *et al.,* 2018).

Most of the isolates showed resistance to Cephalexin, Streptomycin and Ampicillin but only 6 isolates harboured both aph(6)-Id (*str*B) and *bla*TEM genes. This comprised 2 CoNS and 4 CoPS isolates. The *bla*TEM gene was the most prevalent resistant gene among both CoNS and CoPS isolates. The recorded high antibiotic resistance in camel milk can be avoided through restricting antibiotic sale and use to Veterinarians as well as civic education to the community on the role of antimicrobial resistance in their health and well-being.

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

5.2 Conclusion

The following conclusions were made from the study;

i. Raw camel milk in Garrisa County is contaminated with both *S. aureus* and CoNS.

ii. The high resistance of *Staphylococci* to beta-lactam drugs is observed in the studydemonstrates the extent AMR spread in camel milk.

iii. In this study, *mec*A, *bla*Z and *bla*TEM genes were established to be causing observed resistance in beta-lactam drugs.

5.3 Recommendations

The following recommendations were made from the study;

i. Continuous surveillance and monitoring of AMR *Staphylococcus* species, including the MDR-CoNS, in order to curb the emergence and spread of antimicrobial resistance in the ASALs.

ii. All animal health professionals and camel owners need to be made aware on appropriate antibiotic use and biosecurity strategies in order to curb the spread of antimicrobial resistance in camels.

iii. Large scale study is recommended to determine the extent of contamination and antimicrobial resistance of CoNS in camel milk from the entire ASALs of Kenya.

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APPENDIX I: PUBLISHED PAPER

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Antimicrobial resistance phenotypes of Staphylococcus aureus and Coagulase negative Staphylococci species isolated from raw camel milk from Garissa County, Kenya

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Abstract

The emergence of multidrug resistant bacteria in clinically challenging situations is a global concern. *Staphylococcus* resistance poses a threat to available therapeutic agents in management of camel diseases. S. *aureus* is often isolated from mastitic camel milk. Coagulase negative *Staphylococcus* (CoNS) can be pathogenic in humans and animals. This study investigated the antimicrobial resistance phenotypes of *Staphylococci* species in raw camel milk from Garissa County, Kenya. A total of 231 raw camel milk samples from healthy camels were collected. Disk diffusion was used to determine antimicrobial susceptibility of the isolates. Bacteria were revived in Buffered Peptone Water (BPW). *Staphylococcus* isolates were cultured on Mannitol Salt agar (MSA) and Blood Agar (BA). Coagulase and catalase tests were used to biochemically characterize the isolates. Antibiotic disks were placed on Mueller Hinton Agar and incubated at 37°C for 24 hours and diameters of zones inhibition measured. The readings were recorded as either susceptible, intermediate, or resistant based on the interpretative breakpoints by the veterinary Clinical Laboratory Standards Institute (CLSI) guidelines. Antimicrobial agents tested included; Ampicillin, Streptomycin, Cephalexin, Erythromycin, Ciprofloxacin, Cefoxitin, Tetracycline and Chloramphenicol. Out of the 231 raw camel milk samples cultured, 52.8% (122/231) *Staphylococci* isolates were recovered. Among the *Staphylococci* isolates 83.6% (102) were *S. aureus* and 16.4% (20) were CoNS. Overall, 83 (68%) isolates were catalase positive and 122 (91.7%) showed β-haemolysis on BA culture. Highest resistance was observed against Cephalexin (81.9%) and Streptomycin (72.1%) while the lowest resistance was seen against Chloramphenicol (1.6%) and Tetracycline (3.3%). MRSA and MRCoNS were reported at 9.8% and 15% of the isolates respectively. MDR was recorded in 43.4% of the isolates resistant to at least 3 or more antimicrobial groups while 39.3% isolates were resistant to 1 or 2 antimicrobial tested. In conclusion, the study showed that CoNS and *S. aureus* isolates coexist contaminating raw camel milk and are highly resistant to Cephalexin and Streptomycin. Continuous monitoring of resistance is recommended in order to prevent the spread of AMR.

Keywords: *Coagulase negative Staphylococcus; Staphylococcus aureus; Multidrug resistance; camel*

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