BOVINE MASTITIS: ESTABLISHING BACTERIAL DIVERSITY, ASSOCIATED RISK FACTORS, AND ANTIMICROBIAL RESISTANCE PROFILES OF THE ISOLATES IN EMBU AND KAJIADO COUNTIES, KENYA

CHRISTINE MINOO MBINDYO, BVM, MSc.

A THESIS SUBMITTED IN FULFILLMENT OF REQUIREMENTS FOR DOCTOR OF PHILOSOPHY DEGREE OF UNIVERSITY OF NAIROBI IN APPLIED MICROBIOLOGY (BACTERIOLOGY OPTION)

DEPARTMENT OF VETERINARY PATHOLOGY, MICROBIOLOGY AND PARASITOLOGY

FACULTY OF VETERINARY MEDICINE

UNIVERSITY OF NAIROBI

MARCH, 2022

DECLARATION

I declare that this thesis is my original work and it has not been presented for a degree in any other University

Christine Minoo Mbindyo

Signature  Date: 7th March, 2022

This thesis has been submitted for examination with our approval as University supervisors

Professor George Gitao (BVM, MSc, PhD)

Department of Veterinary Pathology, Microbiology & Parasitology, University of Nairobi

Signature:  Date: 10th March, 2022

Professor Paul Plummer (DVM, PhD, Diplomate ACVIM-LA)

College of Veterinary Medicine, Iowa State University

Signature:  Date: 11th March, 2022

Professor Charles Mulei (BVM, PhD)

Department of Clinical studies, University of Nairobi

Signature:  Date: 11th March, 2022

Dr. Rawlynce Bett (Bsc., MSc, PhD)

Department of Animal Production, University of Nairobi

Signature  Date: 11th March, 2022

DEDICATION

I dedicate this thesis to my lovely family:

My daughters Amara Atieno and Amy Adhiambo;

My Parents Philip Mbindyo and Rosina Nzembi and my sisters and brother.

This work would not have been possible without your prayers, love, encouragement, and support.

God bless you

ACKNOWLEDGEMENTS

Psalms 118: 23; This is the Lord's doing; it is marvelous in our eyes. First, I would like to thank the Almighty God for his favor, guidance, wisdom, and strength during my Ph.D. journey. Secondly, special thanks go to all my supervisors; Professor George C. Gitao, Professor Paul J. Plummer, Professor Charles M. Mulei, and Dr. Rawlynce Bett for their guidance, support, and encouragement through my Ph. D research. Your positive attitude and confidence in my research inspired me and gave me the strength to keep going even during the most trying times.

My special thank goes to the Department of Veterinary Pathology, Microbiology and Parasitology for offering me an opportunity to undertake my PhD. My deepest gratitude to the chairman of the department Professor Samuel Githigia, thank you for providing a comfortable working environment for me, and for your encouragement. You always told me to never give up no matter what. Special thanks to Dr. Felix Kibwegwa, Dr. Ovokeraye Oduaran and Helen W. Kariuki for their support during the molecular data analysis. My special gratitude goes to my colleagues Dr. Mahacla Odongo and Professor Lilly C. Bebora for their moral support, encouragement and reviewing my thesis.

My special thanks go to Consortium for Advanced Research Training in Africa (CARTA) for offering me a Ph. D fellowship without which this work would not have been possible. I am forever indebted.

To our laboratory technicians; Dimbu, Ann, Charity and Mainga thank you for supporting me during my laboratory work. A special thank you; to my daughters for their patience and understanding during the many days away working. To my parents; for supporting me and praying

for me during my Ph. D journey. To my sister, Mercy Mwongeli, for being a second mother to my daughters while I worked on my Ph.D. I am forever indebted and may God bless you all.

My friends; Dr. Getrude Shepelo, thank you for being my great friend, supporter, mentor and for your encouragement throughout my study, may God bless you richly. Stella Kichoi; thank you for your great friendship, prayers and words of wisdom during my Ph.D. journey. God bless you richly.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AMR	-	Antimicrobial resistance
BORSA	-	Borderline Oxacillin resistant *Staphylococcus aureus*
CLSI	-	Clinical and Laboratory Standards Institute
CM	-	Clinical mastitis
CMT	-	California mastitis test
CNS	-	Coagulase negative Mastitis
DNA	-	Deoxyribonucleic acid
EUCAST	-	European Committee on Antimicrobial Susceptibility Testing
FAO	-	Food and Agriculture Organization
GARP	-	Global Antibiotic Resistance Partnership
GDP	-	Gross Domestic Product
IEBC	-	Independent Electoral Boundaries Commission
IHBS	-	Internal household survey networks
KDB	-	Kenya Dairy Board
KNBS	-	Kenya National Bureau of Statistics
KIHBS	-	Kenya intergrated household and budget survey
MALFI	-	Ministry of agriculture, livestock, fisheries and irrigation
MDR	-	Multidrug-resistant
MRCNS	-	Methicillin-resistant CNS
MRS	-	Methicillin-resistant staphylococci
MRSA	-	Methicillin-resistant *Staphylococcus aureus*
MRSE	-	Methicillin-resistant *Staphylococcus Epidermidis*
NMC	-	National Mastitis Council
PCR	-	Polymerase Chain Reaction

rRNA	-	ribosomal Ribonucleic acid
SCC	-	Somatic cell count
SDGs	-	Sustainable development goals
SCM	-	Subclinical mastitis
UNFPA	-	United Nations Population Fund
USA	-	United States of America
WHO	-	World health Organization

ABSTRACT

Bovine mastitis is one of the most important global diseases of cattle in which it adversely affects animal and human health, quality and quantity of milk, and the economics of almost every country. Despite the global challenge of bovine mastitis, studies on prevalence, bacterial diversity of mastitis-causing pathogens, risk factors and antibiotic resistance profiles of the isolate in dairy cows in Kenya remain limited. This cross-sectional study was undertaken in Embu and Kajiado counties of Kenya with the following objectives; (1) To determine the prevalence of clinical and subclinical mastitis in dairy cows, (2) To isolate and characterize the bacterial communities from clinical and subclinical mastitic cow milk using culture and 16S rRNA metagenomics analysis, (3) To establish the phenotypic and genotypic antimicrobial susceptibility profiles of the isolates (4) To determine the risk factors associated with subclinical mastitis in dairy cows. The study was conducted among 395 randomly selected dairy cows from 154 smallholder farms. In each of the farms, a semi-structured questionnaire was used to collect data on mastitis management practices and cow level risk factors associated with mastitis. A total of 1574 milk samples were aseptically collected from each mammary quarter of the 395 cows. Six quarters were blocked and hence did not produce any milk. The milk was initially checked for clinical mastitis and screened for subclinical mastitis using the California Mastitis Test (CMT) before being analyzed for bacterial infection using standard bacterial culture methods. Sixty-six (66) mastitic milk samples based on their culture results were selected and further analyzed using 16S rRNA metagenomics analysis to further understand their bacterial diversity. Additionally, phenotypic antimicrobial susceptibility profiles for *Staphylococcus* species (n=183), *Streptococcus* species (n=22) *Escherichia coli* (n=12), and *Pseudomonas aeruginosa* (n=19) were determined against 10 antimicrobial drugs using the

disc diffusion method. Investigation of seven resistance genes to the various antimicrobial drugs was further done on the 183 *Staphylococcus* isolates using polymerase chain reaction (PCR) amplification and partial sequencing.

Overall, the farm-level, cow-level and quarter-level prevalence of mastitis were at 76.6% (118/154), 80.0% (316/395) and 67.8% (1068/1574) respectively. Of the mastitic cows, 8.5% (27/316) were clinical and 91.4% (289/316) had subclinical mastitis. On culture, eight genera of bacteria were identified where Coagulase-negative *Staphylococcus* (CNS) at 42.8% (435/1016) were the most prevalent bacteria. Twenty percent, (217/1068) of the mastitic milk samples yielded no bacterial growth on aerobic culture-based methods. Failure to milk mastitic cow last (p=0.04) and previous history of mastitis (p=0.03) were significantly associated with subclinical mastitis. Alpha and beta diversity comparison showed that there were no significant differences in bacterial number and diversities in mastitic milk from quarters based on the region, clinical/subclinical status and culture growth status. Genera level analysis using 16S rRNA metagenomics analysis revealed that 11 genera dominated by Pseudomonas (2.6%-83.8%) were shared among the three categories. An increased relative abundance of some phyla and genera which could not be identified using standard culture methods such as *Chlamydiae*, *Mycoplasma* and *Solibacillus* in culture-negative mastitic milk were also reported.

Overall, antimicrobial resistance (AMR) among the staphylococci ranged between 3.5% (8/183) for fluoroquinolones and 66.1% (121/183) for ampicillin. Strikingly, 25.0% (23/91) of *S. aureus* and 10.8% (10/92) of the Coagulase-negative *Staphylococcus* (CNS) isolates, were methicillin-resistant staphylococci (MRS) phenotypically. Among the *Streptococcus* species AMR ranged between 31.8% (7/22) for ampicillin and zero for fluoroquinolones, *E. coli* showed the highest

phenotypic resistance to ampicillin at 75.0% (9/12) while no resistance to fluoroquinolones. *Pseudomonas aeruginosa* showed the highest phenotypic resistance in cefaclor 89.5% (17/19) while lower resistance was reported in ciprofloxacin 5.3% (1/19). Unexpectedly 13.6% (3/22) vancomycin-resistant streptococci and 21.0% (4/19) carbapenem-resistant *P. aeruginosa* isolates were reported in this study. Higher multidrug resistance (MDR) was present in 66.0% (8/12), 78.9% (15/19) of the *E. coli* and *P. aeruginosa* isolates respectively. The most common antimicrobial resistant genes in *S. aureus* and CNS was *blaZ* at 44.3% (35/79) and 75.3% (55/73) respectively. This study shows a high prevalence of subclinical mastitis both at the farm and animal levels. Both the clinical and subclinical mastitis were predominantly associated with Coagulase-negative *Staphylococcus* and *Pseudomonas* species based on culture and 16S rRNA metagenomics analysis respectively. There was an increased relative abundance of some bacterial phyla and genera which could not be identified using standard culture-based methods in culture-negative mastitic milk implying the usefulness of using more sensitive techniques in the diagnosis of mastitis. There is a need to improve on management of the dairy farms through culling of cows with a previous history of mastitis, use of individual udder drying towels, and milking mastitic cows last as control measures for mastitis. The bacterial isolates revealed high resistance to beta-lactams with high *blaZ* genes being detected in staphylococci signifying a public health concern and a challenge to bovine mastitis therapy hence the need to prevent the emergence and control the spread of AMR in dairy farms. This is the first study to report on the presence, methicillin-resistant Coagulase-negative *Staphylococcus*, carbapenem-resistant *P. aeruginosa* and vancomycin-resistant *Streptococcus* in cow mastitic milk from Kenyan dairy farms and therefore further monitoring is recommended.

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Background information

The dairy industry is an important sub-sector and forms an integral part of Kenya's agricultural production system (Omore *et al.,* 1999). This fast-rising sub-sector contributes 44% of the livestock Gross Domestic Product (GDP), 14% agricultural GDP, and 4% of the country's GDP (FAO, 2011). Apart from producing 80% of the milk consumed in Kenya, this industry forms a pivotal livelihood for about 2.6 million small-scale farmers (FAO, 2011). Currently, the total dairy cattle population in Kenya is estimated at 4.5 million, which produces about 5.2 billion liters of milk annually (KNBS, 2019). Nevertheless, milk production from these animals is significantly below the national demand for milk and milk products (KIHBS, 2018). Moreover, the Kenyan human population, which presently stands at 47.5 million, is projected to double by 2050 (KNBS, 2019, UNFPA, 2020). As a consequence, the demand for milk and dairy products will exponentially increase (FAO, 2017). Further, this industry presents a huge opportunity for improved health, nutrition, and job creation, if it is fully optimized (FAO, 2011). However, the full potential of this industry is yet to be achieved; this is due to several factors which include: the high cost of production, poor access to quality inputs and services, environmental degradation, climate change impacts, and diseases such as mastitis, which is a significant contributor (Rademaker *et al.,* 2016, Maina *et al.,* 2019)

Bovine mastitis defined as inflammation of the mammary gland is arguably one of the most critical diseases in the dairy industry globally with high economic and public health impacts (Benić *et al.,* 2012, Abebe *et al.,* 2016). Mastitis has significant adverse effects on milk production, animal

health, welfare, and food security (Vakkamäki *et al.,* 2017). The economic losses due to mastitis are high worldwide (Nielsen, 2009). Although economic estimates in African countries, including Kenya are sparsely documented, studies in developed countries have estimated losses due to the disease at USD 1.7–2 billion annually (Nielsen, 2009, Liang *et al.,* 2017). The financial losses occur through reduced milk quality and quantity, increased treatment cost, culling, and even death of the animal (Jamali *et al.,* 2018, Romero *et al.,* 2018). In the African context, two studies one in South Africa and another one in Ethiopia estimate the cost of losses due to subclinical mastitis to be USD 78 and USD 38 respectively for each cow per lactation (Mungube *et al*., 2005, Man'ombe, 2014). However, Mungube *et al*. (2005) study in Ethiopia only quantified economic losses caused by reduced milk volumes during subclinical infection and therefore it underestimated the total cost of mastitis in the region. The economic impact of mastitis has been shown to vary between farms and herds and therefore it should be calculated at the farm or herd level especially in small and medium-scale farmers in developing countries (Romero *et al.,* 2018).

In addition, there is strong scientific evidence that mastitic milk acts as a reservoir of potentially zoonotic multidrug-resistant bacteria (Oliver & Murinda, 2012, Smith, 2015). The possibility of these strains of bacteria especially from subclinical mastitis entering the food chain and the resulting public health consequences are enormous and call for urgency in the intervention (Verraes *et al.,* 2014, Tilahun & Aylate, 2015). These consequences include severe zoonotic untreatable infections, high cost of treatment, increased deaths, reduced livelihoods, and food insecurity (WHO, 2015, FAO, 2016). In Kenya, however, extensive information on the prevalence and

distribution of these key mastitis causative bacteria is limited even though nearly 36% of milk produced is consumed raw (FAO, 2004, Njarui *et al.,* 2011).

Mastitis is the foremost reason for antimicrobial use in dairy cows (Saini *et al.,* 2012). It is estimated that treatment and prevention of mastitis make up to 86% of antibiotics used in dairy cows (Oliver & Murinda, 2012, Sharma *et al.,* 2018). Studies have consistently indicated that overuse and misuse of antibiotics in food animals has significantly contributed to the evolution and development of superbugs/multidrug-resistant strains transferable to humans (Schmidt *et al.,* 2015, Sharma *et al.,* 2018).

Indeed, a recent report projected that the use of antimicrobials in food animals has nearly tripled that of humans, with a higher increase of the use in developing countries including Kenya which was listed as a new hot spot for antibiotic resistance (AMR) (Sriram *et al.,* 2021). This increase in antibiotic use in food animals is driven by the fast-growing demand for animal proteins to feed the growing population in these regions (Sharma *et al.,* 2018). Global trends projects AMR as a global pandemic accounting for over 700,000 deaths worldwide per year (WHO, 2019). Therefore, a multidisciplinary One Health approach is necessary to prevent and control further emergence and spread of antibiotics resistance bacterial infections (WHO, 2019).

Further, evidence is accumulating that the routine culture method, which is the gold standard for the diagnosis of mastitis is inadequate (Oikonomou *et al.,* 2012, Kuehn *et al.,* 2013). About 10%-40% of mastitis cases remain culture-negative in routine culture assays (Kuehn *et al.,* 2013).

Several reasons for non-growths in mastitic milk have been highlighted which include, non-bacterial causative agents, low bacterial load in time of culture, or inability to culture the bacteria due to limitation of media or growth requirements (Kuehn *et al.*, 2013, Oikonomou *et al.*, 2014). These limitations have prompted the use of culture-independent techniques such as metagenomics analysis which has recently provided more insights into the bacterial diversity of mastitic milk, consequently improving intervention strategies (Kuehn *et al.*, 2013, Bhanderi *et al.*, 2014).

Metagenomics analysis which involves the analysis of genes contained in an uncultured sample has enabled researchers to genetically characterize and study the microbial population in culture-negative clinical mastitis cases in a culture-independent manner (Oikonomou *et al.*, 2020). This new and thriving field is likely to solve problems and create new knowledge in the world of diagnostics (Garrido-Cardenas & Manzano-Agugliaro, 2017). However, such studies are lacking in Africa, including Kenya (Motaung *et al.*, 2017). This study is therefore aimed at investigating the prevalence of clinical and subclinical mastitis and associated risk factors, bacterial diversity of mastitis-causing pathogens, and antimicrobial resistance (AMR) profiles of the isolates in dairy cows in Embu and Kajiado Counties in order to improve mastitis therapy and control emergence and the spread of AMR in Kenya.

1.2 Objectives

1.2.1 Overall objectives

The overall objective was to investigate the prevalence, bacterial diversity of mastitic milk, associated risk factors, and Antimicrobial resistance (AMR) of the isolates in dairy cows in Embu and Kajiado Counties, Kenya.

Specific objectives

1. To determine the prevalence of clinical and subclinical mastitis in dairy cows in Embu and Kajiado Counties, Kenya.
2. To isolate and characterize the bacterial communities from clinical and subclinical mastitic cow milk using culture and 16S rRNA metagenomics analysis in Embu and Kajiado Counties, Kenya.
3. To establish the phenotypic and genotypic antimicrobial susceptibility profiles of the isolates.
4. To determine the risk factors associated with subclinical mastitis in dairy cows in Embu and Kajiado Counties, Kenya.

1.3 Hypotheses

1. There is a high prevalence of clinical and subclinical mastitis in dairy cows in Embu and Kajiado Counties, Kenya.
2. The 16S rRNA based metagenomics analysis of culture-negative clinical and subclinical mastitic cow milk samples do not identify an increased relative abundance of one or more fastidious bacterial genera that are associated with mastitis.

3. Bacterial isolates from mastitic milk of dairy cows from Embu and Kajiado Counties, Kenya, have high resistance to antibiotics.
4. There are various risk factors associated with subclinical mastitis in Embu and Kajiado Counties, Kenya.

1.4 Research problem

The threat of zoonosis, the existence of antibiotic-resistant bacteria, and economic losses associated with the burden of bovine mastitis continue to be reported globally (FAO, 2016, Romero *et al.*, 2018, Gussmann *et al.*, 2019). Given the scales of mastitis and the associated burden of antibiotic resistance globally, new effective control and preventive strategies are urgently needed (Abdel-rady & Sayed, 2009, Gussmann *et al.*, 2019). Current literature shows that in Kenya and other resource-limited countries there is a paucity of studies on distribution, diversity of mastitis-causing pathogens, associated risk factors and antibiotic resistance profiles of the isolates in dairy cows (Gitau *et al.*, 2014, Motaung *et al.*, 2017). This limitation of studies is a serious problem because the inability to correctly identify the mastitis-causing pathogen leads to difficulty in selecting the appropriate pathogen-specific treatment or control measure (Koskinen *et al.*, 2009). Moreover, most of these organisms are zoonotic and have the potential to transfer their antimicrobial resistance genes to bacteria in humans, denoting a public health hazard (Oliver & Murinda, 2012, Kalińska *et al.*, 2017).

Bacteria are the primary cause of mastitis; more than 140 different pathogenic bacterial species have been implicated as causative agents of the disease worldwide (Motaung *et al.*, 2017).

Remarkable differences in the distribution of mastitis and mastitis-causing bacteria among countries, regions, and farms has been reported (Verbeke *et al.,* 2014, Taponen *et al.,* 2017). Yet previous studies in Kenya using the culture method managed to identify only a few of these mastitis-causing pathogens (Gitau *et al.,* 2014, Mureithi & Njuguna, 2016). These studies were of limited sensitivity because only culture methods were used to identify the pathogens. Significant inadequacy has been reported in the culture method including lack of growth in mastitic milk and failure to identify all the organisms involved in mixed infections (Oikonomou *et al.,* 2012, Kuehn *et al.,* 2013). The lack of growth from mastitic milk samples has remained a challenge in the diagnosis of mastitis worldwide including in Kenya (Richards *et al.,* 2019). As a consequence, effective treatment and prevention of the disease have been hindered (Kuehn *et al.,* 2013).

Several diversity studies using 16S rRNA metagenomics in the USA (Oikonomou *et al.,* 2012, Kuehn *et al.*, 2013), Finland (Taponen *et al.,* 2019), and India (Bhatt *et al.,* 2012), have shown evidence that there tend to be more bacteria associated with mastitis than are normally reported. The missed bacteria are normally not detected because they are mostly fastidious; being almost impossible to identify them using culture methods (Oikonomou *et al.,* 2014). This current study used culture and 16S rRNA metagenomics analysis to detect and characterize the bacterial species responsible for clinical and subclinical mastitis in dairy cows in Embu and Kajiado Counties, Kenya.

Further, multidrug-resistant (MDR) isolates associated with bovine mastitis, including methicillin-resistant staphylococci (MRS), are an emerging global public health problem (Oliver & Murinda,

2012, Anjum *et al.,* 2019). Mastitic milk has been documented as an important reservoir of these MDR strains (Lee, 2003, Boireau *et al.,* 2018). Increased risk of clonal transmission of antibiotic-resistant determinants between dairy cow herds and persons in constant contact with animals or raw milk products has also been described (Sharma *et al.,* 2018). The risk is even higher in low-resource countries including Kenya where 36% of milk produced is consumed raw, and there is a high burden of mastitis (Njarui *et al.,* 2011, Gitau *et al.,* 2014). In addition, in these countries, there is unregulated use of antimicrobial agents and intimate contact between humans and animals is common (Gitaka *et al.,* 2020). To address this challenge, therefore, more AMR studies in most low-resource countries including Kenya are needed (Van *et al.,* 2020). This information is key to improving antibiotic stewardship and mitigating the emergence and spread of AMR.

1.5 Justification of the study

This study was designed so that it contributes toward achieving sustainable development goals (SDGs); 1-poverty alleviation), 2-zero hunger, 3-healthy lives, and wellbeing and as well as Kenya's vision 2030 on poverty alleviation, zero hunger and healthy lives (FAO, 2018).

Dairy cows are a significant source of food, nutrition, and a mean of livelihood for many especially to 2.6 million disadvantaged small-scale farmers in Kenya (Rademaker *et al.,* 2016, FAO, 2017). In addition, this industry is a major contributor to the agricultural Gross Domestic Product (GDP) of Kenya, contributing about Ksh 100 billion (National Dairy Master Plan, 2015). For sustainable production and income to farmers, quality milk production is important (FAO, 2011, FAO, 2017, Maina *et al.,* 2019).

Mastitis is one of the most prevalent diseases of dairy cows affecting food safety, food security, animal welfare, public health, as well as heavy economic losses in many countries worldwide including Kenya (FAO, 2011). Despite the importance of mastitis, the microbial diversity of mastitic milk, antibiotic resistance, and associated risk factors in Kenya has not been fully elucidated (Gitau *et al.,* 2014). Previous studies have shown that the prevalence of cow level mastitis in Kenya remains high (50%-80%) and threatens both animal and human health (Gitau *et al.,* 2014, Mureithi & Njuguna, 2016, Ondiek & Kemboi, 2018). Therefore, determination of bacterial diversity of mastitic milk, risk factors, and antimicrobial resistance of the isolates would improve on mastitis treatment and control as well as support antibiotic stewardship and mitigate the emergence and spread of AMR in Kenya.

Controlling mastitis would significantly contribute to healthy animals and improved milk production. In addition, this would lead to increased support to the growing demand of milk, improve the farmers' income and nutrition. Further, controls of mastitis would reduce the prevalence of zoonotic multidrug-resistant bacteria, as well as reduce poverty in the country (Grace, 2015, FAO, 2017).

CHAPTER TWO: LITERATURE REVIEW

2.1 Overview of dairy industry in Kenya

Kenya has one of the rapidly growing dairy cow industries in Sub-saharan Africa, producing about 30% of milk of the 5% that Africa produces globally (Rademaker *et al*., 2016, FAO, 2017). The significant roles played by the Kenyan dairy cow sector include a source of food and nutrition, the contribution of about 4% of the country's Gross Domestic Product (GDP), and the creation of numerous job opportunities (FAO, 2011, Kiambi *et al.,* 2020). Currently, this industry which is growing at an average rate of 5%–7% per year employs about 1.2 million citizens (Rademaker *et al*., 2016). Moreover, it is a main form of livelihood to about 2.6 million small-scale farmers who own one to three cows in total, they produce about 90% of the milk consumed in the country (FAO, 2017, KDB, 2020). This industry presents a huge opportunity for improved health, food, and nutrition security especially for children and women, and poverty alleviation, if its full potential, is optimized (FAO, 2011, Rademaker *et al.,* 2016, Dominguez-salas *et al.*, 2016).

The present dairy cattle population in Kenya is estimated at 4.3 million cattle producing about 5.5 billion liters of milk annually (FAO, 2017, MALFI, 2019). About 80% of these animals are reared by small-scale farmers as their source of family income (FAO, 2011). The main dairy cow breeds kept in Kenya are Holstein-Friesian, Ayrshire, Guernsey, Jersey, several crossbreeds and several indigenous breeds (FAO, 2011, Odero-Waitituh, 2017). At the production level, the industry consists of large, medium, and small scale producers, with the latter dominating the industry in Kenya (Odero-Waitituh, 2017, Maina *et al.*, 2019).

Milk yields of small-scale producers are about 5–8 liters per cow per day, with medium and large-scale farm yields ranging between 17–19 liters per cow per day (FAO, 2011, Rademaker *et al.,* 2016). Nevertheless, the milk production from these animals is still way below the national demand for milk and milk products in Kenya (KIHBS, 2018). About 55% of the milk produced in these farms is marketed unchilled raw through informal channels while the rest is in part consumed in the family and the reminder given to calves (Bebe *et al.,* 2015, KDB, 2020).

In Kenya, like in most African countries, the production system is widely dominated by small-scale family-run farms (FAO, 2014, Ndahetuye *et al.*, 2019). Most of these farms are marked by minimal milk production due to diseases, poor management practices, inadequate quality and quantity of feed, poor genetics of cows, and seasonal fluctuations (Maina *et al.,* 2019). In addition, farmers lack the resources to attain modern production technologies, diagnostics, and practices to improve their enterprises (Rademaker *et al.,* 2016). Poor hygiene and poor production systems further compromise the industry from achieving its full potential (Mburu, 2016, Rademaker *et al.,* 2016).

By 2050 the demand for milk and milk products is expected to triple in sub-Saharan Africa, including in Kenya (FAO, 2017). This increase will be driven by the increasing human population growth, demand for animal proteins, expanding urbanization, and a growing middle class (Bebe *et al.*, 2015, FAO, 2017). However, despite the projected growth in milk production and the significant role played by the dairy industry in Kenya, the industry is still faced with major challenges that limit its full potential (FAO, 2004). Among the multiple challenges highlighted above, diseases such as mastitis are a significant contributor (FAO, 2011, Maina *et al.*, 2019). The

findings of this study will contribute to the improvement of mastitis therapy in dairy cows as well as control the spread and emergence of Antimicrobial resistance in Kenya.

2.2 Impact of bovine mastitis

Mastitis is a disease of major impact due to the associated economic losses at the different production levels (Motaung *et al.*, 2017, Ruegg, 2017). Adverse effects on animal health, welfare, and significant public health hazards due to mastitis have been described (Romero *et al.*, 2018). This highly prevalent disease is a global problem that has been documented in almost every country in the world (FAO, 2011) with incidences of clinical and subclinical mastitis ranging between 10%-40% and 19.2%- 83% respectively per cow (Jamali *et al.*, 2018, Kumari *et al.,* 2018). Estimation of economic losses due to mastitis has been carried out mainly in developed countries, with the value ranging between USD 1.7–2 billion annually (Nielsen, 2009, Liang *et al.,* 2017). However, variation of the economic impacts due to mastitis between regions has been described and therefore it should be calculated at the farm level and based on region and prevailing economic conditions (Romero *et al.,* 2018).

Reports on the economic impact of mastitis in most low-resource countries, including Kenya, are lacking (Motaung *et al.,* 2017). Lack of this information limits effective control of the disease, especially subclinical mastitis which is associated with about 80% of all the losses (Kumari *et al.,* 2018, Romero *et al.,* 2018). Respective financial losses occur mainly through a significant decrease in milk quality and quantity, which accounts for about 78% of all the losses (Jamali *et al.*, 2018). Other losses are incurred through high treatment costs and even death of the animal (Romero *et al.,*

2018, Jamali *et al.,* 2018). Further, clinical mastitis is a fundamental cause for the culling of dairy cows (Stevens *et al.,* 2016). Therefore, for sustainable milk production and improved income, it is critical to determine the financial impact due to mastitis in Kenya (FAO, 2011, Maina *et al.*, 2019).

Reports of antibiotic-resistant zoonotic bacteria isolated from cases of bovine mastitis are increasing globally and require urgent intervention (Sharma *et al.,* 2018, Adamu *et al.,* 2020). The main reason for this seems to be the rampant antibiotic usage towards treatment and prevention of mastitis in cows (Oliver *et al.,* 2011). The widespread usage of antibiotics in cows has contributed to in the emergence of multi-drug resistance strains globally, leading to non-treatable infections (Sharma *et al.,* 2018). Antibiotics resistance is projected to increase substantially, causing millions of deaths and costing trillions of dollars by the mid-21st century, if interventions are not taken (WHO, 2015, FAO, 2016). Being zoonotic, most of these organisms can easily be transferred to humans, denoting a public health hazard (Ayele *et al.,* 2017). Hence, the establishment of the microbial diversity of mastitic milk and respective antibiotic resistance profiles will help in combating the impact of mastitis in animals and potential zoonotic infection in humans (Sharma *et al.,* 2018).

2.3 Mastitis in dairy cows

Mastitis is defined as the inflammation of the mammary gland predominantly occurring due to invasion by pathogenic bacteria (Motaung *et al.,* 2017). In dairy cows, mastitis is primarily classified in two forms which are clinical and subclinical mastitis, based on the clinical signs (Ruegg, 2017).

Clinical mastitis (CM), which is less prevalent, is characterized by systemic signs in the cow such as fever and anorexia, in addition to visible abnormalities in the udder and milk (Bradley, 2002, Jamali *et al.*, 2018). In CM, the udder is usually characterized by swelling, tenderness, redness, pain and generally, the milk production from the affected quarter is reduced (Ruegg, 2017, Jamali *et al.*, 2018). The presence of clots, flakes, or watery milk is usually common in severe cases and can be observed, making diagnosis easy (Kibebew, 2017, Ndahetuye *et al.*, 2019). Clinical mastitis can be further categorized as peracute, acute, or subacute mastitis depending on the severity of symptoms (Motaung *et al.*, 2017). The severity of the disease is usually determined by factors such as the causative agent, age of the animal, immunological health, and lactation stage (Ashraf & Imran, 2018).

On the other hand, subclinical mastitis (SCM), commonly referred to as the 'hidden disease', is more common and causes more economic losses to farmers (Kumari *et al.*, 2018). Due to a lack of observable clinical signs on the cow or abnormalities in the udder or milk, subclinical mastitis is challenging to diagnose (Ruegg, 2017). Moreover, this type of mastitis persists longer in the herd and is associated with higher economic losses than clinical mastitis (Ruegg, 2017, Kumari *et al.*, 2018). Body of evidence shows that subclinical mastitis occurs 15 to 40 times more often than the clinical form and is characterized by increased somatic cell count (SCC) of more than (>) 200,000 cells/ml. The somatic cell constitutes, predominantly of leucocytes and some epithelial cells (Cobirka *et al.*, 2020). In addition, milk yields in affected quarters are greatly reduced (Suleiman *et al.*, 2018, Romero *et al.*, 2018). Cow infected with SCM serves as a reservoir of pathogens and

a source of udder infection among animals within the herd (Cobirka *et al.,* 2020). When unpasteurized infected milk is consumed, the pathogens are passed to humans (Ayele *et al.,* 2017).

2.4 Prevalence of dairy cow mastitis

Mastitis is one of the most critical diseases of the dairy industry, endemic to almost every country worldwide (FAO, 2014). Most of the well-documented mastitis incidence and prevalence reports come from developed countries, with limited ones from developing countries, including Africa (Motaung *et al.,* 2017, Adamu *et al.,* 2020). Studies have indicated great variability in the incidence of mastitis among countries and herds, influenced by management practices and environmental factors (Zadoks & Fitzpatrick, 2009, Taponen *et al.,* 2017).

Only 30% of the African countries have well-documented data on mastitis (Motaung *et al.,* 2017). The prevalence of clinical and subclinical mastitis in most countries including Kenya is estimated at 10% and more than 50% respectively (Gitau *et al.,* 2014, Motaung *et al.,* 2017). The low documentation on mastitis in most African countries, including Kenya, is of particular concern since it limits estimation of the economic impact of the disease and hinders effective control of mastitis (Motaung *et al*., 2017, Adamu *et al*., 2020). Apart from the limitation of studies, low reporting has also been associated with a lack of awareness and proper diagnosis of mastitis, especially subclinical mastitis cases where confirmatory laboratory testing is needed (Duarte *et al.,* 2015).

Prevalence of clinical and subclinical mastitis have been reported in a few different counties in Kenya (Gitau *et al*., 2014). Based on previous studies, clinical and subclinical mastitis is estimated

to range between 1-9% and 43%-83%, respectively (Gitau *et al*., 2014, Mureithi & Njuguna, 2016). Variation in the prevalence of mastitis reported in these studies could be attributed to differences in animal breeds, geographical location, the season of the study, management systems, and husbandry (Taponen *et al*., 2017). However, these studies were limited by the fact that they used standard routine culture methods, which has shown to be less sensitive in the diagnosis of mastitis (Oikonomou *et al*., 2012, Kuehn *et al.,* 2013). In addition, these studies focused on high potential areas, not on arid and semi-arid regions, despite some of these areas having a high population of dairy cows like in Kajiado County, Kenya (KNBS, 2019). Furthermore, these studies focused on the prevalence of mastitis and few risk factors; thus, extensive studies on mastitis and risk factors are lacking in Kenya (FAO, 2011, Mureithi & Njuguna, 2016).

Indeed, according to a recent review on the importance of mastitis in Africa by Motaung *et al.* (2017), in their results, it has been shown that subclinical mastitis is on the rise in most African countries, including Kenya. Therefore, given the huge impact of mastitis in the dairy cow industry, there is a need for extensive research using advanced technologies in order to determine the true prevalence of the disease in Kenya. Such information will be key in improving therapeutic and control strategies for the disease.

2.5 Etiology of mastitis

A wide range of microorganisms has been documented as causative agents of mastitis globally with more than 140 pathogenic bacteria having been described, in addition to fungi, algae, and viruses (Motaung *et al*., 2017, Jamali *et al*., 2018). Evidence-based studies have shown major diversity in

the distribution of mastitis and mastitis-causing bacteria among countries, regions, and farms (Verbeke *et al.*, 2014, Gao *et al.*, 2017). These variations are influenced by management practices and environmental factors in those particular regions and farms (Kumar *et al.*, 2016, Amer *et al.*, 2018).

Bacteria are the most common causes of cow mastitis (Bradley, 2002, Motaung *et al.*, 2017). Based on origin, mastitis-causing bacteria have been classified as either contagious or environmental (Bradley, 2002, Cobirka *et al*., 2020). Contagious bacteria are defined as pathogens adapted to live in the cow's skin or udder, transferrable from one infected quarter or animal to another uninfected animal or quarter during the milking process, either via milking machine, through milker's hands, or udder clothes (Zadok *et al.*, 2011, Motaung *et al*., 2017). According to Cobirka *et al*. (2020), the most common bacteria classified under this group include *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae*, *Streptococcus uberi*, *Mycoplasma* spp. *Corynebacterium* spp. *Arcanobacterium* spp. and T*rueperella* spp. These bacteria have very resilient adhesive properties that enhance their invasion into the inner lining of the mammary glands and therefore evading the immune response (Ashraf & Imran, 2018).

On the other hand, environmental pathogens are primarily found in the cows' habitat (Cobirka *et al*., 2020). These pathogens are transmitted from the environment primarily through beddings, soil, and water sources contaminated with feces and urine (Motaung *et al*., 2017). Frequently reported pathogens that exploit this form of transmission include coliforms such *Escherichia coli* and

Klebsiella species; *Streptococcus dysagalactiae*, Coagulase-negative *Staphylococcus* (CNS), Gram-positive bacilli, *Pseudomonas* species, yeasts, and algae (Ndahetuye *et al*., 2019). Over the last decade, mastitis cases caused by environmental pathogens have been on the increase whereas a significant decrease in cases caused by contagious pathogens continues to be reported (Nam *et al.,* 2009, Zadoks *et al.,* 2011, Gomes & Henriques, 2016). This shift has been majorly driven by the improved mastitis control measures towards contagious pathogens at the farm level in most developed countries (Zadoks &Fitzpatrick, 2009).

Nevertheless, in the current era of molecular epidemiology and the evolution of bovine mastitis pathogens, the classification of mastitis based on either contagious or environmental has been challenged (Cobirka *et al.,* 2020). Recent evidence shows that organisms such as *S. aureus*, *Streptococcus uberis,* and other streptococcal species, previously purely considered contagious, can be transmitted from the environment (Zadoks *et al.,* 2011, Gomes &Henriques, 2016). In addition, the evidence further suggests that environmental pathogens can adapt better in the udder than previously thought (Klaas & Zadoks, 2018). Thus indicating a lack of a clear distinction between contagious and environmental mastitis-causing bacteria (Klaas & Zadoks, 2018).

2.6 Evolution of mastitis pathogens

Over the last decade, mastitis pathogens have progressively evolved (Ruegg, 2017). This evolution has largely been attributed to management practices, new breeds, changes in mastitis causative agent's virulence mechanisms, host-adaptation, increasing societal and economic pressure (Zadoks *et al*., 2011, Ndahetuye *et al.,* 2019). For decades, studies had documented major pathogens of

mastitis as *Staphylococcus aureus* (*S. aureus*) *and Streptococcus agalactiae* (Zadoks & Fitzpatrick, 2009, Ruegg, 2017). The word "major" was used to reflect the economic, productivity, and animal health impact associated with those pathogens (Bradley *et al.,* 2007). However, studies continue to report a change in the trend of the causative agents of mastitis from the ones designated as major pathogens to those designated as minor pathogens (Zadok & Fitzpatrick, 2009, Taponen *et al*., 2017).

One of the key factors influencing this shift, in most countries, has been improved mastitis management and husbandry measures (Taponen *et al*., 2017). Although these measures have led to the reduction of the major pathogens of mastitis, they have resulted in an absolute increase in incidences of the minor pathogens (Ruegg, 2017). Indeed, current reports show that coagulase-negative staphylococci (CNS) and other bacilli such as *Corynebacterium bovis*, previously classified as minor pathogens, are emerging as important mastitis-causing pathogens in most countries (Piessens *et al.,* 2011, Wald *et al.,* 2019, Rahil, 2019). These studies have shown that these ''minor'' pathogens of mastitis play a significant role in the pathogenicity of mastitis (Thorberg *et al.,* 2009, El-jakee *et al.,* 2013). Apart from this, some of these organisms have a zoonotic potential and have been associated with the spread of antibiotic resistance determinants to other bacteria (Oliver *et al.,* 2011, Kalińska *et al.,* 2017).

The distribution and diversity of mastitis-causing pathogens have been shown to vary between countries and herds (Taponen *et al*., 2017). For instance, in most African countries, *S. aureus* has been reported as the predominant mastitis pathogen with an estimated prevalence of 70% (Motaung

et al., 2017). Similarly, previous studies in Kenya, examining the distribution of mastitis pathogens in different regions, reported *S. aureus* as the dominant mastitis-causing agent (Gitau *et al*., 2014, Muriethi & Njuguna, 2016, Ondiek & Kemboi, 2018).

However, in recent reports, minor pathogens such as CNS, *Bacillus* species, *Corynebacterium* species are increasingly being isolated in mastitis cases in the East African region (El-Jakee *et al*., 2013, Ndahetuye *et al*., 2019). For instance, several studies in Ethiopia (Tolosa *et al.,* 2015, Mekonnen *et al.,* 2017), Rwanda (Mpatswenumugabo *et al.,* 2017, Ndahetuye *et al.,* 2019), and Tanzania (Suleiman *et al.,* 2018) have reported the prevalence of CNS at 16%-40%. Despite the rising prevalence of CNS in the region, *S. aureus* still remains a challenge in cow mastitis in most African countries including Kenya, mostly due to inadequate effective control programs (Gitau *et al.,* 2014). Therefore, investigating the epidemiology and the specific factors contributing to the emerging/evolving mastitis pathogens are necessary (Gitau *et al.,* 2014, Motaung *et al*., 2017).

2.7 Important mastitis pathogens

2.7.1 *Staphylococcus aureus* (*S. aureus*)

Staphylococcus aureus, a Gram-positive bacterium, is one of the leading economically significant causative agents of dairy cow mastitis worldwide (Zadok *et al.,* 2011, Wang *et al.,* 2018). It is shown to be responsible for up to 70% of all clinical mastitis cases globally (Gomes & Henriques, 2016). This important pathogen has been isolated in a wide range of infections in humans including mastitis, soft tissue infections, food poisoning, endocarditis, septicemia (Holmes & Zadoks, 2011, Hennekinne *et al.,* 2012). Successful control of *S. aureus* mastitis in well-managed farms has been

described (Zadoks *et al.,* 2011). As a result of this, the prevalence of *S. aureus* mastitis has significantly reduced in developed countries which have adopted and complied to mastitis control programs (Barkema *et al.,* 2009). However, over the last decade, due to widespread use and misuse of antimicrobials in animals and humans (for therapeutic, prophylactic, and growth promotion reasons) worldwide, increased selection pressure has resulted in the development of multidrug-resistant (MDR) strains (Mišic *et al.,* 2017).

The increasing development of resistance to most of the available antibiotics by *S. aureus* is a key public health concern (Smith, 2015, Kalayu *et al.,* 2020). Following the introduction of penicillin, the first penicillin-resistant *S. aureus* was reported 2 years later (Hansen *et al.,* 2017). Since that time, penicillin-resistant *S. aureus* strains have increased and spread extensively worldwide (Sharma *et al.,* 2018, Kalayu *et al.,* 2020). Multi-drug-resistant *S. aureus* strains have been associated with reduced efficacy to most antibiotics, consequently challenging management of associated infections including bovine mastitis (Qu *et al.,* 2019, Kyany'a *et al.,* 2019). Alarming, however, is the increasing emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) strain which has become a major threat to public health and the dairy industry (Wang *et al.,* 2015, Asiimwe *et al.,* 2017, Hansen *et al.,* 2017). The number of publications reporting MRSA strains isolated from mastitic milk and dairy products with the potential zoonotic transmission has grown rapidly worldwide (Smith, 2015, Mistry *et al.,* 2020, Yang *et al.,* 2020). These organisms have shown resistance to major classes of antibiotics including all types of β-lactam antibiotics currently available for mastitis therapy (Vanderhaeghen *et al.,* 2010, Yang *et al.,* 2020).

In 1961 the first MRSA strain was reported in a hospital setting, two years after the introduction of methicillin (Hansen *et al.,* 2017). Methicillin resistance is conferred by the acquisition of the *mecA* or its homologue *mecC,* gene, carried by mobile genetic elements referred to as staphylococcal cassette chromosome *mec* (SCC *mec*). Further, the most recently described plasmid-borne encoded *mecB* and *mecD* genes of *Micrococccus* species origin have also been reported in *S. aureus* (Becker *et al.,* 2018, Becker, 2021). To date, about eleven staphylococcal cassette chromosome (SCC) elements grouped into types and subtypes, differing in structural organization and genetic content, have been described (Gagetti *et al.,* 2019). *Mec* genes code for alternative penicillin-binding proteins, PBP2a, that have reduced affinity to most β-lactam antibiotics (Kalayu *et al.,* 2020). Since the discovery of hospital-associated MRSA (HA-MRSA), various clones including community-associated MRSA (CA-MRSA) and livestock-associated MRSA (LA-MRSA) strains, with different genetic backgrounds and SCC *mec* types, have been discovered and disseminated across the globe (Smith, 2015). Several studies have shown that the prevalence and epidemiology of this organism differ within the geographical regions, with a higher prevalence of the diverse LA-MRSA strain frequently shared between livestock and humans being reported in Asia, Europe, and the USA (Smith, 2015, Anjum *et al.,* 2019).

Previously, MRSA was thought to have host-specific lineages; however, recent literature shows that these strains are transferable between humans and animals signifying a public health concern (Smith, 2015, Sharma *et al.,* 2018, Yang *et al.,* 2020). These pathogens have been shown to harbor resistant genes transferable to the human population in close interaction with animals through

consumption of contaminated milk, contact, or through the environment (Smith, 2015, Mcmillan *et al.*, 2016).

Mastitic milk has been well documented as an important reservoir of these multidrug-resistant strains (Lee, 2003, Verraes *et al.*, 2014). Indeed, evidence has been presented elsewhere linking the newly emerging livestock-associated methicillin-resistant *S. aureus* clonal complex 398 (LA-MRSA CC398) isolated from humans to cow milk origin (Smith, 2015, Wang *et al*., 2018). *S. aureus* showing low susceptibility to oxacillin/cefoxitin and lacking *Mec* genes or their allotypes commonly referred to as borderline oxacillin resistant *Staphylococcus aureus* (BORSA) have been reported in animals and humans (Hryniewicz & Garbacz, 2017, Becker, 2021). BORSA has remained a challenge in the treatment of staphylococcal infections (Hryniewicz & Garbacz, 2017). On average, the prevalence of BORSA in human clinical isolates has been estimated to be 5% (Krupa *et al.*, 2015, Hryniewicz & Garbacz, 2017).

However, higher prevalence rates have also been described (Hryniewicz & Garbacz, 2017). Khorvash *et al*. (2008), in Pakistan, reported that up to 25% of their MRSA strains, isolated from different surgical sites in humans, were without the *mec A* gene. In animals, BORSA has been documented in cattle, horses, and pigs (Hryniewicz & Garbacz, 2017, Scholtzek *et al.,* 2019). Krupa *et al*. (2008), in Poland, documented a prevalence of BORSA in 14% and 10 % of isolates from pig nasals and pork, respectively. Incidences of BORSA have also been reported in cow raw milk and porcine minced meat at a prevalence of 5.7% (Bystroń *et al*., 2010). Further, BORSA are

also increasingly being reported in horses (Scholtzek *et al*., 2019) and a prevalence of up to 25% have been reported in isolates from horses treated at a Swiss clinic (Sieber *et al.,* 2011).

Infrequent identification, misdiagnosis, and discrepancies of BORSA prevalence in different studies have largely been associated with diagnostic methods used, amongst other factors (Hryniewicz & Garbacz, 2017). Lack of a routine diagnostic method for the detection of BORSA remains a challenge in the identification of these strains (Hryniewicz & Garbacz, 2017). Intrinsic methods such as point mutation genes of the penicillin binding protein (PBP) genes and overproduction of β– lactamases have been described as the main mechanisms for BORSA phenotype determinant (Hryniewicz & Garbacz, 2017, Scholtzek *et al*., 2019).

In Africa little is known about MRSA carriage in milk and milk products (Asiimwe *et al*., 2017). This is despite previous studies in Africa, as well as in Kenya, reporting that *S. aureus* is the major udder pathogen causing intramammary infection in dairy cows (Gitau *et al*., 2014, Motaung *et al*., 2017, Ondiek & Kemboi, 2018). A few studies from African countries have demonstrated the existence of MRSA in cow milk and other dairy products (Elemo *et al.*, 2017, Asiimwe *et al*., 2017, Omwenga *et al.*, 2020). However, the presence of *mecA* or *mecC* in the *S. aureus* remains understudied. In a recent study in Kenya by Omwenga *et al*. (2020), they reported a prevalence of 41% *mecA* positive *S. aureus* in apparent healthy dairy animals which is very concerning. In addition, in their study the authors only screened for *mecA* genes, the isolates could have had other genes (Omwenga *et al*., 2020). Therefore, more studies are required to establish the prevalence of

MRSA and also characterize the strains in terms of subtype and genetic mechanism of resistance in Kenyan Dairy.

2.7.2 Coagulase Negative Staphylococci

Coagulase Negative Staphylococci (CNS) comprises a heterogeneous group of bacteria that consist of more than 50 species and subspecies previously considered to be opportunistic pathogens of animals and humans (Taponen *et al.,* 2017, Klibi *et al.,* 2019, Rahil, 2019). However, recent research indicates that CNS are potentially pathogenic and they are increasingly being identified in animal and human infections worldwide (Gomes & Henriques, 2016, Sender *et al.*, 2017). In humans, CNS has been linked to endocarditis, nosocomial infection, and septicemia (Becker *et al.,* 2014) while in cows these organisms are increasingly emerging as important pathogens of mastitis globally (Boireau *et al.,* 2018, Cheng e*t al.,* 2019).

Until recently, CNS were classified as minor pathogens, and their importance as independent causative agents of subclinical or clinical mastitis taken to be limited (Schukken *et al.,* 2009). However, these organisms have now been shown to cause intramammary infections in cows that increase somatic cell count (SCC), reduced milk quality and quantity (Schukken *et al.*, 2009, Frey *et al.,* 2013). In addition, CNS is considered as the main reservoir of resistance genes, with the potential to develop multidrug resistance and subsequent transfer of these traits (genes) to other bacteria including *S. aureus* (Klibi, *et al*., 2019, Santos *et al.,* 2020). Of great concern, however, is the emerging methicillin-resistant CNS (MRCNS) strain, which is increasingly being isolated from bovine mastitic milk (Becker, *et al.*, 2014). As mentioned earlier, methicillin resistance is usually

conferred by the presence of *mecA* gene, located on a mobile genetic element called Staphylococcal Cassette Chromosome *mec* (SCC*mec*), transferrable to *S. aureus* (Frey *et al.*, 2013, Kot *et al.*, 2020).

Similar to MRSA, *mecA* positive CNS have shown to have resistance to most available beta-lactam antibiotics including other classes of antibiotics such as aminoglycosides and macrolides (Gentilini *et al.*, 2002). This is further complicated by the ability of certain CNS to form biofilms within the mammary gland which impairs the local defense mechanism and drug activity (Gentilini *et al*., 2002, Mahato *et al.*, 2017).

Due to their zoonotic and multidrug resistance nature, CNS threatens the dairy industry and poses a great public health risk since they can easily be transferred between animals and humans (Becker *et al.*, 2014, Kim *et al.*, 2019). However, the global consequence and economic impact of CNS remain limited and under investigated in many countries (Frey *et al.*, 2013). Methicillin-resistant Coagulase negative Staphylococcus (MRCNS), specifically, methicillin-resistant *Staphylococcus epidermidis* (MRSE), have been isolated in mastitis in different countries worldwide (Klibi *et al*., 2019, Kim *et al.*, 2019). Nevertheless, only scanty information is available on the emergence of specific CNS species associated with bovine mastitis, their role in the pathogenesis of mastitis, their reservoirs, and mechanism of resistance in many countries including Kenya (Kim *et al*., 2019). Therefore, surveillance of CNS species and their molecular characterization need to be implemented to minimize mastitis and the risk for the development and spread of antibiotic resistance.

2.7.3 *Streptococcus* species

Members of the genus *Streptococcus,* classified as Gram-positive facultative anaerobic bacteria, are a significant etiology of clinical and subclinical bovine mastitis (Nam *et al*., 2009). Moreover, in humans, these organisms cause invasive diseases with *S. dysagalactiae* recently being reported as an emerging zoonotic pathogen (Gomes & Henriques, 2016). Over the last decades, *S. agalactiae* (*S. agalactiae*), an obligate udder bovine mastitis pathogen, was responsible for 90% of all bovine mastitis cases (Ruegg, 2017).

However, in the recent past, driven by improvements of mastitis control programs, particularly in developed countries, contagious pathogens of mastitis including *S. agalactiae* have significantly gone down (Saed & Ibrahim, 2020). These control programs however have shown to have minimal effect on environmental mastitis pathogens, including other streptococcus species (Nam *et al*., 2009). Recent reports indicate a low prevalence of *S. agalactiae* in many countries including North America (Oliveira *et al.,* 2013) Europe (Verbeke *et al.*, 2014), and Africa (Motaung *et al*., 2017). However, *S. agalactiae* is still a key etiology of bovine mastitis in countries like Brazil and Colombia (Tomazi *et al.*, 2019).

S. dysagalactiae and *S. uberis* are among the common causative agents of environmental bovine mastitis (Saed & Ibrahim, 2020). However, other *Streptococcus* species such as; *S. bovis, S. equinus, S. mitis, S. salivarius, S. equi subsp. zooepidemicus* have also, to a lesser extent, been linked to bovine mastitis (Nam *et al*., 2009, Zadoks *et al.,* 2011). Cows' habitat is well known as

the main reservoir of environmental streptococci, however, the presence of *S. uberis* in the cows' body such as the skin and around the muscles has also been described (Zadoks *et al*., 2011, Cobirka *et al.*, 2020).

Streptococci strains are mostly associated with subclinical mastitis, however, cases of acute clinical mastitis have also been reported, especially due to *S. dysagalactiae* (Ruegg, 2017). Incidents of environmental mastitis due to streptococci are consistently being reported worldwide (Zhang *et al.*, 2018). This increase has been in part associated with poor environmental hygiene in cows' surroundings, contaminated dirty bedding such as recycled manure/slurry solids which is increasingly being used as cow bedding (Klaas & Zadoks, 2017, Cobirka *et al*., 2020). Transmission of environmental streptococci in dairy cows occurs mostly between milking (Klaas & Zadoks, 2017). Therefore, providing cows with a clean environment through frequent removal of slurry and use of teat dips after milking have been shown to reduce streptococcal mastitis (Klaas & Zadoks, 2017).

Streptococcus species such *S. agalactiae* and other environmental *Streptococcus* species have been isolated in mastitis in dairy cows in Kenya (Gitau *et al.*, 2014, Ndirangu *et al.*, 2017). However, like in many countries worldwide, in Kenya, the prevalence of *S. agalactiae* mastitis remains low following improvements of standard mastitis control programs (Nam *et al.*, 2009). For instance, studies have shown that due to the use of specific control programs aimed towards the control of contagious mastitis, the prevalence of *S. agalactiae* in North America and Europe is extremely low

(Tomazi *et al.,* 2019). Similarly, in recent studies in Kenya by Gitau *et al.* (2014) and Mureithi & Njuguna, (2016) a low prevalence of *S. agalactiae* at (5.2%) and (8.4%) respectively were reported.

In contrast, the prevalence of environmental streptococcus mastitis has shown to be increasing as evidenced by recent studies in Kenya. For instance, Gitau *et al.* (2014), Mureithi & Njuguna (2016), and Ndirangu *et al.* (2017) in their studies reported prevalence of environmental *Streptococcus* species at (4.5%) (9.8%) and (38.9%) respectively. Environmental reservoirs such as dirty bedding, unhygienic water source, and dirty cows' surroundings significantly contribute to the spread of environmental streptococcus mastitis (Cobo-Angel *et al.,* 2018, Amer *et al.,* 2018). The discrepancies in the prevalence of streptococcal mastitis rates reported in these studies could be due to factors such as management practices, environmental, epidemiological, and cow breeds which may have been different in each region (Taponen *et al*., 2017). Further, molecular characterization of the environmental streptococci has indicated that dominant differences exist among streptococcus strains among countries and continents (Tomazi *et al.,* 2019).

2.7.4 *Trueperella pyogenes*

T. pyogenes formally known as *Arcanobacterium pyogenes* is a known pathogens of mastitis in domestic animals including in dairy cows (Nagib *et al.,* 2014). According to Jost & Billington, 2005, these grams positive, non-motile, non-spore forming short rods bacteria are known cause of other pyogenic infections in other species of animals. Although infections due to these opportunistic bacteria in humans have been reported they are quite rare (Kavitha *et al.*,2010).

T. pyogenes are mostly found in the animal body mostly on the skin, respiratory and gastrointestinal tract (Quinn *et al.,* 2011). In addition, in dairy cows in cases of mastitis, transmissions through contaminated milking utensils and biting flies have been described (Ribeiro *et al.,* 2015). In dairy cows these organisms are associated with suppurative clinical mastitis although cases of chronic mastitis have also been reported (Radostits *et al.,* 2007).

Identification of the *Trueperella* species remains to be one of the main limitation in the treatment and control of these bacteria in mastitis cases (Nagib *et al.,* 2014). The convectional identification of *T. pyogenes* rely on biochemical tests and sugar fermentation are time consuming and laborious (Nagib *et al.,* 2014) Other non-conventional methods such spectroscopy and molecular analysis are expensive and require highly skilled labour and further limiting identification and control of the disease (Wenning & Scherer, 2013).

2.7.5 Gram-negative bacteria

Gram-negative bacteria are key environmental mastitis-causing pathogens frequently associated with acute mastitis cases (Metzger & Hogan, 2013). Coliforms and non-coliform bacteria contribute to about 40% of all acute clinical mastitis cases in dairy cows worldwide (Schukken *et al.,* 2012). Most common coliform bacteria include members of the genus; *Escherichia*, *Klebsiella*, *Citrobacter*, *Enterobacter* while the non-coliform bacteria include; *Pseudomonas*, *Serratia* and *Proteus* (Nam *et al*., 2009, Schukken *et al.,* 2012, Cobirka *et al*., 2020). These bacteria are naturally

found in cow bedding materials such as soil, feces, and other organic matter in the environment of cows (Hogan & Smith, 2003).

Improvement of mastitis control practices has significantly led to the reduction of contagious mastitis in modern farms (Klaas & Zadoks, 2017). However, these practices have proven to be unsuccessful in controlling environmental pathogens (Ruegg, 2017). Indeed, studies have shown that clinical mastitis associated with Gram-negative bacteria contributes to the highest risk of culling in Holstein dairy cows (Hertl *et al*., 2011, Jamali *et al.*, 2018). Therefore, control measures need to target the relevant infection risks (Klaas & Zadoks, 2017).

In Africa, countries like Uganda and Egypt have reported a high level of mastitis caused by gram-negative bacteria compared to other countries (Katetee *et al.*, 2013, Ameen *et al.*, 2019). Studies by Katetee *et al.* (2013), in Uganda, and Ameen *et al.* (2019) in Egypt reported a prevalence of 29% and 33% of all the gram-negative mastitis respectively. In contrast, a lower prevalence level of coliform and non-coliform mastitis has previously been reported in Kenya. For instance, previous reports by Odongo *et al.* (2012) and Gitau *et al*. (2011) estimated the prevalence of coliforms mastitis at (17.2%-16.8%) and (9.7%-15.4%) for *Escherichia coli* (*E. coli*) and *Klebsiella* species respectively. Non-coliform mastitis (*Pseudomonas* species) has also been reported in Kenya at a prevalence of 8%-7% (Gitau *et al.*, 2011, Odongo *et al.*, 2012). However, in more recent studies in Kenya, a lower prevalence of coliform and non-coliform mastitis was reported indicating a decrease in the prevalence of these isolates as causative agents of mastitis. For instance, in Kenya, a prevalence of 0.7 % in *Klebsiella* species and zero in the *E. coli* and *Pseudomonas* species were

reported by Gitau *et al.,* (2014). Similarly, Mureithi & Njuguna, (2016) isolated *E. coli* and *Klebsiella* species at a prevalence of 2% and 1% respectively.

2.7.5.1 *Escherichia coli*

Escherichia coli (*E. coli*) classified as a Gram-negative environmental opportunistic bacterium, is frequently isolated in clinical and subclinical mastitis in dairy cows (Zadoks *et al.,* 2011, Cobirka *et al*., 2020). Studies have shown that 80% of coliform mastitis are due to *E. coli* (Liu *et al*., 2018). The organism commonly causes severe short-lived clinical mastitis and if untreated it can lead to lethal consequences (Bradley, 2002). Hyperacute mastitis cases due to *E. coli* have been described in cattle and are commonly associated with the death of the animal (Zadok *et al.,* 2011). Mild repeated subclinical and persistent forms of *E. coli* mastitis have also been described (Zadoks *et al*., 2011, Cobirka *et al*., 2020). These subclinical forms have shown to be more significant than the clinical form since they are difficult to diagnose (Katetee *et al.,* 2013). In addition, the infected cows act as reservoirs of the bacteria and hence a constant source of herd transmission (Marashifard *et al*., 2018).

Interaction between factors such as host immune system, stage of lactation, vaccination status, and pathogenicity of the specific *E. coli* pathogen has shown to influence the clinical outcome of the mastitis (Burvenich *et al*., 2003, Zadoks *et al.,* 2011)**.** The plasticity of *E. coli* genomes has been shown to allow the acquisition of diverse pathogenicity factors such as adhesins, invasins, toxins, capsule production, which influence the disease severity (Alawneh *et al*., 2020). Geographical variations among mastitis-causing *E. coli* have been described (Gomes & Henriques, 2016).

However, studies on genetic diversity and virulence factors in many countries, including Kenya, are scarce (Alawneh *et al.*, 2020).

High incidences of clinical mastitis due to *E. coli* are common during early lactation (Bradley & Green, 2001, Hertl *et al.*, 2011). New infections leading to these high incidences tend to be acquired during the drying-off periods rather than during the early lactation period (Hogan & Smith, 2003). Dry cow therapy and non-lactational therapy have been used effectively to reduce early incidences of clinical mastitis and new intramammary infection (Ashraf & Imran, 2018).

Antibiotics are an important part of the treatment and control of Gram-negative mastitis (Oliver *et al.*, 2011). Currently, the focus is on *E. coli* due to their widespread antibiotics resistance which is a major public health concern (Alawneh *et al*., 2020). For instance, *Escherichia coli* harboring extended-spectrum β-lactamase strains (ESBLs) gene are increasingly being reported in food animals including in cow milk (Ali *et al*., 2016, Castanheira, *et al.*, 2021). Horizontal gene transfer through plasmid and other mobile genetic elements between *E. coli* and other bacteria are well documented (Castanheira, *et al.*, 2021). Enzymes such as β-lactamases (ESBLs) and plasmids such as *Amblerclass C cephamycinases* and blaCMY-*2* been identified as the key mediators of resistance in ESBLs *E. coli* strains (Ali *et al*., 2016).

In Africa, including Kenya, studies on phylogenetic, phenotypic, and molecular antibiotic resistance genes in mastitis-causing *E. coli* in dairy animals are scarce (Founou *et al*., 2016). The few available studies have described ESBLs *E. coli* in feces of healthy animals, mostly from

chickens and pigs in the Northern African countries (Alonso *et al*., 2017). Similarly, in Kenya, ESBL-producing *E. coli* were isolated from feces of healthy poultry and camels (Langata *et al*., 2019, Nüesch-Inderbinen *et al*., 2020). In addition, ESBL-producing *E*. *coli* have also been isolated from raw and fermented camel and cow milk in Kenya (Njage *et al.*, 2012, Ngwaya *et al.*, 2019). However, molecular studies on *E coli* in Africa, including Kenya, are needed in order to improve knowledge on the genetic mechanism of *E*. *coli,* their phenotypic and genotypic AMR profiles (Alonso *et al*., 2017).

2.7.5.2 *Klebsiella* species

Klebsiella species including *K. pneumoniae* (*K. pneumoniae*) and *K. oxytoca* are associated with bovine mastitis (Zadoks *et al.*, 2011). In addition, *K. pneumoniae* is a key human pathogen that causes fatal pneumonia and septicemia (Podder *et al*., 2014). These Gram-negative organisms are mostly found in the environment like water or soil (Tzouvelekis *et al*., 2014). Drug-resistant *K. pneumoniae,* especially ESBL producing strain, is a major public health concern (Sriram *et al*., 2021). Indeed, the organism is among the WHO-listed priority pathogens with a high antibiotics resistance rate (WHO, 2019, Sriram *et al*., 2021). Extended-spectrum beta-lactamase (ESBL)-producing *K. pneumoniae* has shown to have limited therapeutic options and can easily collect and spread multidrug resistance plasmid(s) among other bacteria (Tzouvelekis *et al.,* 2014).

In dairy cows, *K. pneumoniae* is the leading cause of environmental clinical mastitis associated with high economic losses (Podder *et al*., 2014). However, mild subclinical mastitis due to *Klebsiella* species has been described (Cobirka *et al.,* 2020). Cow bedding, soils, and water are the

primary reservoirs of these important mastitis pathogens (Zadoks *et al*., 2011). Although *K. pneumoniae* is primarily transmitted from the environment, transfer from one infected cow to a healthy cow has been described (Schukken *et al.*, 2012). Similar to the other coliform mastitis, *Klebsiella* mastitis starts early during the dry period and progresses to clinical mastitis during the early lactation period (Cobirka *et al.*, 2020).

2.7.5.3 *Pseudomonas aeruginosa*

P. aeruginosa is an important psychotropic opportunistic Gram-negative bacterium associated with acute and chronic infection in humans and animals (Park *et al.*, 2014, Cobirka *et al.*, 2020). These organisms are predominantly found in the environment (Meng *et al*., 2020). In humans, *P. aeruginosa* is associated with septicemia, pneumonia, and necrosis in immune-compromised persons (Schauer *et al.*, 2021). In cows and other ruminants, the organism is occasionally isolated from mastitic milk (Park *et al*., 2014).

At dairy farms, contaminated water, soiled beddings, udder wipes, teat dips, and infusion equipment are the commonly reported sources of udder infection (Aguayo *et al.*, 2020, Schauer *et al*., 2021). These environmental pathogens enter the cow through the teat canal and produce a wide range of virulence factors such as exotoxin A, exoenzyme, and protease, which induce an inflammatory response and damage mammary gland tissues (Park *et al*., 2014).

Clinical, subclinical, and chronic mastitic cases in dairy cows due to *P. aeruginosa* have been described (Cobirka *et al.*, 2020). In other ruminants like sheep and goats, gangrenous mastitis with

high cases of fatality has been reported (Scaccabarozzi *et al.*, 2015). Mastitis cases by *P. aeruginosa* are commonly progressive and associated with high somatic cell counts (Aguayo *et al.*, 2020). In addition, decreased milk quality, and poor response to antibiotics are very common in pseudomonas mastitis (Park *et al.*, 2014). Non-clinical *P. aeruginosa* mastitis outbreaks in dairy herds have also been described. Such cases were shown to be associated with repeated exposure of teats to low *P. aeruginosa* loads (Kirk & Bartlett, 1984). Apart from intermittent intramammary infection, herd outbreaks of pseudomonas mastitis associated with heavy economic losses have been described (Schauer *et al*., 2021).

Pseudomonal organisms, including *P. aeruginosa*, have been categorized as high risk for antibiotics resistance (Devarajan *et al*., 2017). They are among the WHO-listed priority pathogens with a high antimicrobial resistance rate (WHO, 2019, Sriram *et al*., 2021). The high antibiotics resistance rate in *P. aeruginosa* organisms is partly due to their innate intrinsic resistance mechanism (Devarajan *et al*., 2017). Mechanisms such as reduction in outer-membrane permeability, efflux systems (mexABoprM), and chromosomal (*AmpC* cephalosporinase) have shown to be the main drivers of innate resistance (Ohnishi *et al.*, 2011). Moreover, *P. aeruginosa* organisms can produce biofilm, hindering antibiotic efficacy (Park *et al*., 2014). Further, some strains can survive in harsh environments for a long period thereby increasing their ability to spread resistance genetic elements between pathogenic and non-pathogenic bacteria (Meng *et al*., 2020).

Treatment of pseudomonas bovine mastitic cases using antibiotics remains a challenge (Schauer *et al*., 2021). Improved farm practices remain largely ineffective in controlling incidences of

pseudomonas mastitis (Aguayo *et al*., 2020). Therefore, culling sick animals and the use of other alternative treatment options such as chitosan nanoparticles should be considered (Aguayo *et al.,* 2020, Schauer *et al*., 2021).

2.8 Transmission of mastitis pathogens

Two modes of transmission of cow mastitis have been described and include; contagious transmission (cow to cow transmission), and environmental transmission (Zigo *et al*., 2021, Cobirka *et al.,* 2020). In contagious transmission, the bacteria are usually found on the cow's skin, udder, or teats (Cobirka *et al.*, 2020). Therefore, the mastitic cows/quarters are the main sources of the infection for uninfected cows/quarters (Kibebew, 2017). Pathogens that are mostly transmitted through this way include *Staphylococcus aureus* and *Streptococcus agalactiae* (Idriss *et al.,* 2013, Bakhat *et al.,* 2020). Transmission of these pathogens occurs primarily during milking, where they are spread via the milking machine, udder, cloths, or milker's hands (Zadoks *et al*., 2011). Transmission can also spread during milking when infected milk contacts an uninfected mammary gland and bacteria then penetrate the teat canal (Cobirka *et al*., 2020).

Another version of contagious transmission is via vectors, especially flies and wasps (Klaas & Zadok, 2017). This type of transmission can occur in *S. aureus, S. agalactiae* and *Trueperella pyogenes* during the early dry period in heifers (Kibebew, 2017). Scholars have classified this form of transmission as contagious since the vector transfers the pathogens from host to host (Klaas & Zadoks, 2017).

On the other hand, environmental transmission occurs from the cows' environment (Motaung *et al*., 2017). This type of transmission involves both Gram-positive and Gram-negative bacteria (Cobirka *et al*., 2020). Soil, bedding materials, manure, and other organic matter remain the main sources of environmental pathogens of mastitis (Ruegg, 2017, Kibebew, 2017). These organisms are established in the teat during milking, between milking, or after milking, when the teat canal remains open for one-two hours (Cobirka *et al.,* 2020). However, there can also be further, microbial contamination of the udder before and after udder preparation for milking (Tancin *et al.*, 2007, Cobirka *et al.,* 2020).

The human-to-animal form of transmission of mastitis also referred to as reverse zoonosis has been described (Munoz *et al*., 2007, Zadok *et al.,* 2011). People are the natural hosts for two important staphylococcal species: *S. aureus* and *S. epidermidis* (Zadok *et al*., 2011). Studies have shown that these bacteria in humans can directly contaminate the udder during milking and cause mastitis (Munoz *et al*., 2007).

Overall, intramammary infection predominantly occurs through the teat canal (Kibebew, 2017). However, hematogenous spread through systemic diseases such as tuberculosis, leptospirosis, and brucellosis have been described (Radostitis *et al.,* 2007). Therefore, a deeper understanding of the pathogenicity of mastitis is key for the successful improvement and development of suitable detection techniques and control programs (Kibebew, 2017).

2.9 Pathogenesis of mastitis

Generally, three stages are involved in the development of bovine mastitis; invasion, infection, and inflammation (Radostits *et al*., 2007). The invasive stage refers to the time in which pathogens enter the teat canal and lactiferous sinus through the teat opening (Radostits *et al*., 2007, Paduch & Kromer, 2011). The invasion stage may lead to infection or lack of it (Gomes & Henriques, 2016). The infection stage is the stage in which the pathogens multiply rapidly and invade the alveoli and mammary tissue (Alnakip *et al.*, 2014). Lastly, inflammation stage where varying degrees of clinical and subclinical abnormalities of the udder and milk, as well as systemic effects in clinical mastitis, become visible (Radostits *et al*., 2007, Kibebew, 2017). The severity of mammary gland inflammation is influenced by the host immune system reaction, pathogen virulence factors, and strain-specific characteristics such as some particular strains are more infectious than others (Zadoks *et al.*, 2011).

2.10 Insight into microbial diversity of bovine mastitis

Despite the diverse range of microorganisms documented as causative agents of bovine mastitis globally, reports of mastitis-causing pathogens in Africa, remain scarce (FAO, 2011, Motaung *et al*., 2017). Evidence-based studies have shown significant variability in the type and number of pathogens causing mastitis in different countries (Zadoks & Fitzpatrick, 2009, Motaung *et al*., 2017). In Africa, for instance, studies on the etiology of mastitis using the classical culture methods in Kenya and Niger reported an average of six genera of mastitis (Gitau *et al.*, 2014, Motaung *et al.*, 2017). However, reports from South Africa, Tanzania, and Uganda reported fewer than six different genera (Motaung *et al.*, 2017).

On the other hand, countries in the developed world have reported high numbers of genera and species of mastitis-causing organisms (Oikonomou *et al.*, 2020). Studies in China, and the USA using metagenomics analysis reported between 50 and 106 different mastitis-causing organisms with varying abundance (Oikonomou *et al.*, 2012, Kuehn *et al*., 2013, Pang *et al.*, 2018). The inconsistencies in reporting of bacteria from these studies have been linked to use of an effective reporting system as well as the use of advanced techniques to diagnose mastitis, which is lacking in Africa, including Kenya (Motaung *et al.*, 2017).

Building on microbial diversity, in the last decade, the development of culture-independent techniques has led to the characterization of mastitis-causing pathogens at great depths (Oikonomou *et al.*, 2012, Taponen *et al.*, 2019). Metagenomics profiling has not only allowed the discovery of more genera of mastitis (Kuehn *et al.*, 2013, Patel *et al.*, 2019), but also it has enhanced the exploration of functional traits of these microorganisms (Escobar-zepeda *et al.*, 2015). Further, this method has given new insights, especially on the bacterial composition of culture-negative clinical mastitis cases which has remained a challenge in mastitis (Oikonomou *et al.*, 2012, Taponen *et al.*, 2019). These cases have been shown to inhabit a highly diverse and rich bacterial community (Falentin *et al.*, 2016). Apart from reporting high numbers of non-cultivable bacterial species, other microorganisms reported by metagenomics were found to be completely new in the phylogeny of mastitis-causing microbial agents (Falentin *et al.*, 2016, Taponen *et al.*, 2019). While most of these studies are from developed countries, the question remains as to whether the microbial diversity of mastitic milk in developing countries is similar to those in developed

countries? Answering this question is critical in informing the treatment and prevention of the disease.

Further, considerable variability has been reported on the distribution and abundance of bacteria, associated with mastitis (Oikonomou *et al*., 2020). These differences are influenced by host-microbial adaptation, environmental factors, and herd management practices (Taponen *et al.,* 2019, Oikonomou *et al*., 2020). These factors have been shown to contribute significantly in shaping the bovine milk microbial diversity (Derakhshani *et al.,* 2018, Oikonomou *et al*., 2020). Therefore, great effort is needed to understand risk factors that influence mastitic milk microbial diversity and their interactions (Derakhshani *et al*., 2018).

2.11 Risk factors associated with mastitis

Over the years, risk factors associated with both clinical and subclinical mastitis have been described (Piepers *et al*., 2011, Gordon *et al*., 2013, Jamali *et al*., 2018). Clinical and subclinical mastitis, are multifactorial key diseases of dairy cows with incidence rates of (10%-40%) and (19%-83%) in cows per year respectively worldwide (Jamali *et al*., 2018, Bhakat *et al*., 2020). Due to the multifactorial nature of the disease, several factors such as host, pathogen, and management have been shown to directly influence the occurrence and recurrence of mastitis in dairy cows (Ramírez *et al.*, 2014).

Host factors such as parity (older cows) and early lactation periods have been described as key risk factors influencing the development and recurrence of clinical mastitis in dairy cows (Jamali *et al*.,

2018). Indeed, studies have shown that multiparous cows are 2.6 times likely to get clinical mastitis during their first 30 days of lactation compared to the rest of the lactation period (Hammer *et al*., 2012, Elghafghuf *et al*., 2014). On the other hand, however, cow breed, higher parity, and late stage of lactation are significant factors associated with subclinical mastitis (Ramírez *et al*., 2014).

Other factors such as high milk production, teat callosity, host immune system, cleanliness of the teat, and genetic resistance have also shown to significantly contribute to the occurrence and recurrence of clinical and subclinical intramammary infections in dairy cows (Elghafghuf *et al*., 2014, Jamali *et al*., 2018, Bhakat *et al*., 2020). High recurrence of mastitis, especially clinical mastitis, has also been described (Zadoks *et al.,* 2001). Cows with a previous history of clinical mastitis were found to be more likely to come down with the disease again (Jamali *et al*., 2018).

Virulence factors, number of organisms, type of pathogen, and bacteriological cure rates are major pathogen factors associated with the occurrence and recurrence of clinical mastitis in dairy cows (Elghafghuf *et al.,* 2014, Jamali *et al*., 2018). Bacteria such as *Staphylococcus aureus, Escherichia coli, Klebsiella* species, have been shown to cause the highest milk losses in primipara cows (Jamali *et al*., 2018). Moreover, high mastitis losses due to standard culture-negative mastitis cases in primipara animals have also been described (Grohn *et al.,* 2004). In older cow's however, bacteria such as *Streptococcus* species, in addition to *S. aureus* and *Klebsiella* species are mostly associated with high milk losses (Grohn *et al.,* 2004). Further, a higher risk of recurrence has been observed in intramammary infections with specific pathogens such as *S. aureus*, *E. coli* and *Streptococcus dysagalactieae* (Jamali *et al*., 2018). Higher death rates and culling rates in multiparous cows due

to clinical mastitis caused by Gram-negative bacteria have also been described (Schukken *et al*., 2009, Hertl *et al*., 2011).

Several management risk factors associated with bovine mastitis have been described (Neave *et al.*, 1969). Factors such as poor udder hygiene, poor teat condition, poor environmental hygiene, and improper teat dipping are among the major mastitis risk factors (Gordon *et al*., 2013, Jamali *et al*., 2018). Other factors such as large herd size, improper milking technique, and nutrition (vitamin E and selenium deficiency) have also been described as mastitis risk factors (Piepers *et al*., 2011, Gordon *et al*., 2013, Jamali *et al*., 2018). Due to the high number of management risk factors, control programs should be based on local specific risk factors within an industry or set up (Ramírez *et al*., 2014, Jamali *et al*., 2018).

In Kenya where the cow dairy industry is dominated by smallholder farmers, only a few studies have described risk factors associated with cow mastitis. Mureithi & Njuguna, (2016), in Thika, found that multiparous cows, breed, mid-lactation, dirty udders and muddy/soil floor were significantly associated with subclinical mastitis. Mahlangu *et al*. (2018) in their study in dairy goats in Thika also reported that frequent cleaning of the stalls and parity were significantly associated with subclinical mastitis. Due to the evolution of mastitis-causing pathogens, continuous reviewing of the responsible risk factors is highly recommended (Zadoks & Fitzpatrick 2009, Ruegg, 2017).

2.12 Metagenomics sequencing technique

Metagenomics, a high-throughput sequencing technique, has enhanced the investigation of microbial diversity, including in the bovine mastitis microbiome (Oikonomou *et al*., 2020). The advent of this new technique in the last decade has remarkably revealed a new perspective of the bovine microbiome not previously regarded (Taponen *et al*., 2019). Metagenomics has enabled the characterization of microorganisms in a culture-independent way, providing a powerful tool to study uncultivable milk microbes and their dynamics (Addis *et al*., 2016).

There are currently two main types of metagenomics approaches; Shotgun and 16S rRNA metagenomics (Alves *et al*., 2018). Shotgun involves sequencing of random fragments of the genome, while 16S rRNA targets a particular single gene used as a taxonomic marker (Fouhy *et al*., 2016). The 16S rRNA partial gene sequencing approach remains the most commonly used phylogenomic survey tool for studying the mastitic milk microbiome (Addis *et al*., 2016). This approach has shown to be exceptionally valuable, as even poor quality or low concentrations of DNA and uncultivable bacteria can successfully be sequenced (Fouhy *et al*., 2016). In addition, the use of metagenomics approaches has overcome the disadvantages of the culturing method (Oikonomou *et al.*,2012, Kuhn *et al*., 2013). This technique has enabled researchers to genetically characterize and study the microbial population in culture-negative clinical mastitis cases in a culture-independent manner (Addis *et al*., 2016).

Moreover, metagenomics methods have shown superiority in unraveling novel antimicrobial genes, and taxonomic groups (Alves *et al*., 2018). This new and thriving field is likely to solve

problems and create new knowledge in the world of diagnostics (Fouhy *et al*., 2016). With the relative availability and the sequencing cost remarkably affordable, this is an opportunity for developing countries, including Kenya, to study unknown etiological agents in cow mammary gland infections (Oikonomou *et al*., 2020). However, it remains uncertain as to the feasibility of this approach for routine diagnosis of bovine mastitis in limited-resource countries (Oikonomou *et al*., 2020). Taponen *et al*. (2019) cautioned that, although this method remains critical in the provision of new information, critical assessment is required. Due to the high sensitivities of the technique, high prone to contamination, and other drawbacks during sample processing, interpretation of results need to be done cautiously (Taponen *et al*., 2019).

2.13 Diagnosis of the bovine mastitis

Accurate diagnosis of mastitis is fundamental for successful treatment and control (Sharun *et al*., 2021). Diagnosis of clinical mastitis is less complicated because of observable clinical signs such as swollen udder and clotted milk from the udder (Jamali *et al*., 2018). However, subclinical mastitis cannot be visually diagnosed and therefore requires laboratory diagnostic support (Gomes & Henriques, 2016, Kumari *et al.,* 2018). The wide range of causative agents of mastitis further complicates the diagnosis process, and hence specific diagnostic techniques targeting specific pathogens are needed (Kalińska *et al*., 2017). The use of conventional methods as well as advanced diagnostic techniques have been described (Sharun *et al*., 2021). Methods routinely used for diagnosis of subclinical mastitis include; Increased Milk SCC such as California Mastitis Test (CMT), electron somatic cell counter and culture methods (Duarte *et al.,* 2015). Most recently, polymerase chain reaction (PCR) and metagenomics analysis have opened a new field of

investigation that has made it possible to diagnose uncultivated bacteria associated with mastitis (Oikonomou *et al*., 2012, Oikonomou *et al.,* 2020).

2.13.1 Somatic Cell Counts (SCC)

Somatic cell count (SCC) is well established indicator of cow udder health (Halasa & Kirkeby, 2020). SCC is a measure of total number of immune cells and studies have shown that increase in these cells is indicative of inflammation in the udder mostly due to subclinical mastitis (Damm *et al.*,2017). These immune cell are mainly lymphocytes, polymorphonuclear neutrophils (PMN) and macrophages (Halasa & Kirkeby, 2020). According to Persson & Olofsson, 2011, the distribution of these immune cells have shown to differ in normal and mastitic milk. In milk obtained from healthy udders, higher number of lymphocytes and macrophages have been reported while mastitic milk is usually dominated by higher numbers of PMN (Damm *et al.,* 2017). In early cases of subclinical mastitis, the elevation of PMN into the milk is triggered by the resident immune cell (lymphocytes, macrophages, and epithelial cells). These immune cell have shown to be involved in attacking the pathogens, defending the udder and early udder inflammation (Rivas *et al.,* 2001, Hand *et al.,* 2012).

The measure of SCC is routinely used to identify subclinical mastitis in dairy cows (Damm *et al.,* 2017). The two techniques commonly used in measuring the SCC are classified as either direct or indirect (Rivas *et al.,* 2001) These techniques include direct such automatic cell counter and indirect methods such as CMT have been documented as effective ways of diagnosis subclinical mastitis at the farm level and or at the laboratory level (Hand *et al.,* 2012).

2.13.2 California Mastitis Test (CMT)

This method is used to detect subclinical mastitis early, especially at the farm level (Duarte *et al*., 2015). It is a simple cow-side indicator test commonly used to indirectly measure the number of somatic cells in milk (NMC, 2017). Somatic cells mainly comprise macrophages, lymphocytes, erythrocytes and epithelial cells. Somatic cells are used as an indicator of the health status of the udder (Lam *et al.,* 2009). Therefore, Somatic Cell Count (SCC) indicates the presence and extent of inflammation of the udder tissue. The test is very simple, rapid, and economical (NMC, 2017). However, despite this method being recommended for routine use, it has several limitations (Sharun *et al*., 2021). CMT lacks universal standards and it is usually subjective when it comes to interpreting the results (Duarte *et al.,* 2015). Secondly, it does not identify the type of bacteria that are causing mastitis (Lam *et al.,* 2009). Therefore, this method makes it harder to initiate treatment requiring more sensitive methods such as the culture method to be used before the start of treatment (Lam *et al.,* 2009, Sharun *et al*., 2021).

2.13.3 Automatic somatic cell count

Many studies have indicated that early direct measuring of SCC in subclinically mastitic milk using an automatic counter is more accurate, sensitive and reliable compared to use indirect methods such as California Mastitis Test technique (CMT) (Persson & Olofsson, 2011). This technique requires use of an automatic cell counter machine at the farm or at the laboratory (Hand *et al.,*

2012). However, beside the its benefits over CMT, these machine are lacking in most laboratory and farms in developing countries including Kenya and therefore limits there use (Persson & Olofsson, 2011). The other disadvantages of this technique is that it is time consuming and the machines are expensive (Persson & Olofsson, 2011). Therefore, CMT remains the most commonly used alternative methods for screening subclinical mastitis in many dairy cows' herds (Middleton *et al.*, 2004)

2.13.4 Culture method and cultivation of bacteria

Bacterial culture is still considered a gold standard of diagnosing bovine mastitis despite many shortcomings associated with the technique (Ashraf & Imran, 2018). Culturing of bacteria mainly involve growing them in a solid nutrient media in plates and there after incubating them in room temperature or in an incubator at 37^0C (Markey *et al.*, 2013). General media such nutrient agar, tryptic soy agar, and brain heart infusion agar are routinely used in primary isolation of bacteria in bacteriology laboratories (Markey *et al.*, 2013). These media consist of a wide variety of nutrients that supports the growth of diverse bacteria (Markey *et al.*, 2013).

However, when it comes to fastidious bacteria, such as *Mycoplasma* spps, *Chlamydia* spps among others, specialized media and enriched media are required (Erkmen, 2021). Enriched media have more added nutrients compared to general media and have shown to support the growth requirement of many bacteria including the fastidious which require complex nutrients (Markey *et al.*, 2013, Erkmen, 2021). However, despite the benefits of using specialized/ enriched media to

grow bacteria, many clinical samples including mastitic milk remain culture negative and hence the rise in use of culture independent techniques (Kuehn *et al.*, 2013, Bhanderi *et al*., 2014).

One of the very positive aspects of the culture method is that it enables the isolation of bacteria that can be used for phenotypic antimicrobial resistance (AMR) testing (CLSI, 2016). However, there are significant inadequacies of the culture method which include lack of bacterial growth in mastitic milk and failure to identify all the organisms involved in mixed infections (Oikonomou *et al*., 2012, Bhanderi *et al*., 2014). Indeed, studies have reported that approximately 25% of clinical and 30% of sub-clinical mastitis cases lack any bacterial growth using standard culture methods (Kuehn *et al*., 2013, Bhanderi *et al*., 2014). Besides, the procedure is usually time-consuming and labor-intensive (Bhatt *et al*., 2012). These limitations of the culture method make it unreliable for the diagnosis of mastitis and have spurred the use of culture-independent metagenomics analysis for mastitis diagnosis (Oikonomou *et al*., 2012).

2.13.5 16S rRNA Metagenomics analysis

The dawn of high throughput sequencing techniques such as 16S rRNA metagenomics over the last decade has enhanced the accuracy and efficiency of mastitis diagnosis (Oikonomou *et al*., 2012, Falentin *et al*., 2016). These techniques have made it possible to genetically analyze microorganisms at greater depth in uncultured environmental samples overcoming the limitation of the culture method (Alves *et al*., 2018). In addition, the use of other techniques such as shotgun metagenomics has shown superiority in studying the genetic variations responsible for antibiotic resistance determinants (Taponen *et al*., 2019).

Currently, pyrosequencing of the 16S rRNA genes is the metagenomic approach commonly used to describe the microbial diversity including in bovine milk samples from health and mastitic quarters (Oikonomou *et al.,* 2012, Oikonomou *et al.,* 2020). Pyrosequencing is a relatively new molecular technique with an incredible potential for metagenomics analysis (Alves *et al*., 2018). It is based on what is known as a "sequencing-by-synthesis" method, utilizing specific enzymes to record each nucleotide inserted into a complementary DNA strand (Ahmadian *et al.,* 2006).

16S rRNA metagenomics approach involves sequencing and analysis of hypervariable regions within the 16S rRNA gene in the 30S subunit present in the bacteria (Taponen *et al*., 2019). The 30S subunit region has shown to accurately differentiate between bacterial genera and has been used in 16S rRNA gene studies of samples from mammalian hosts (Alves *et al.,* 2018). This technique successfully provides a relatively rapid and cost-effective way of assessing bacterial diversity, abundance, inventory and pathogen identification (Sundquist *et al.,* 2007). Studies on bovine milk using 16S rRNA metagenomics analysis have demonstrated its usefulness in understanding the microbial diversity of healthy and mastitic milk (Oikonomou *et al.,* 2020).

Nevertheless, concerns are rising about the usefulness of 16S rRNA metagenomics in routine diagnosis (Addis *et al*., 2016, Hoque *et al*., 2019). First, the technique is relatively expensive and remains unavailable in most developing countries (Addis *et al*., 2016). Secondly, metagenomics detects the presence of DNA from bacteria in milk, whether dead or alive, and does not confirm its

pathogenicity (Oikonomou *et al*., 2020). Hence, as per the current situation, sequencing will serve more as a surveillance tool (guiding empirical treatment) rather than as a routine diagnostic tool.

2.14 Control and prevention of mastitis

The overall goal of any mastitis control program is to improve the quality of milk (Gussmann *et al*., 2018). For a successful mastitis control program, three factors need to be considered: elimination of existing infections, prevention of new infections, and monitoring of udder health status (Radostits *et al.*, 2007). This involves considering the cow, the surrounding environment, and the microorganism for an effective control program (Derakhshani *et al.*, 2018). Incidences of mastitis in some of the developed countries have drastically gone down (Ruegg, 2017). Control measures such as improved hygiene measures, blanket dry cow therapy, strategic culling, post-milking teat disinfection, therapeutic and prophylactic antibiotics have been shown to effectively control mastitis (Jamali *et al.*, 2018, Cobirka *et al.*, 2020). Specifically, these control programs have been shown to significantly reduce contagious mastitis-causing pathogens namely: *Streptococcus agalactiae, Staphylocccus aureus,* and *Mycoplasma* spp. (Ruegg, 2017). However, there are cases where control of environmental pathogens of mastitis using the same strategies has been less successful (Saed & Ibrahim, 2020).

Vaccination is one of the promising strategies for controlling mastitis (Bradley, 2002). However, upto date, despite decades of research, no truly effective commercial vaccine for mastitis is available worldwide (Bradley, 2002, Sharun *et al.*, 2021). Vaccines targeting organisms such as *E. coli, S. aureus. Streptococcus agalactiae* are available in developed countries such as the USA and

Europe (Ismail, 2017). Progressive reduction of new infections of mastitis in cows vaccinated with *E. coli* containing vaccines has been reported (Kawai *et al*., 2021). However, the efficacy of most available vaccines in protection against future infection remains a challenge (Ismail, 2017, Kawai *et al*., 2021).

2.15 Antibiotic use and antibiotic resistance in dairy cows

2.15.1 Antibiotic use in dairy cows

Antibiotic agents play a major part in the treatment, control of diseases and as growth promoters in dairy cows worldwide (Grace, 2015, Van *et al*., 2020). It is undeniable that the rational use of antibiotics plays a critical role in dairy cows' welfare, health and production (Oliver *et al*., 2011, Sharma *et al*., 2018). However, evidence supports that the irrational and irresponsible use of antibiotics in food animals has significantly contributed to the development of antibiotic-resistant strains (Ruegg, 2017). Of particular concern is the expected future increase in the usage of antibiotics within the animal production sector in developing countries, driven by urbanization and population growth (Oliver & Murinda, 2012, Sharma *et al*., 2018).

Indeed, the use of antibiotics in food animals is expected to rise by over 67% by 2030 worldwide, driven by global demand for animal protein (WHO, 2019). According to Sriram *et al*. (2021), China and India represent the highest consumers of veterinary antibiotics and the largest hotspots for AMR. In addition, Kenya and Brazil were listed as emerging new AMR hot spots due to the increased antimicrobial use in animals reported in these countries (Sriram *et al*., 2021). In the last five years, efforts in understanding and use of One Health approaches to control AMR worldwide

have been evident. However, the burden and impact of AMR from food animals remain less emphasized (Cheng *et al*., 2019, Sriram *et al.*, 2021).

A few studies on the use of antibiotics in cows have been done globally (Hoelzer *et al*., 2017). Most of the available literature on antibiotic use in cows is mainly from developed countries, with minimal information from Africa, including Kenya (Grace, 2015, Van *et al*., 2020). This is despite the wide distribution of dairy cattle in Africa with the largest numbers of dairy farms reported in South Africa, Nigeria, and Kenya (Motaung *et al*., 2017). Furthermore, in many low and middle-income countries including Kenya, the use of antibiotics is highly unregulated and there is easy access to antibiotics over-the-counter even without prescription (GARP, 2011, Van, *et al*., 2020). In these countries, there is generally a lack of stringent measures on drug withdrawal periods (Mutua *et al*., 2020, Van *et al.*, 2020).

In addition, the majority of animal health providers and farmers fail to adhere to the recommended mastitis diagnosis and treatment guidelines (Shitandi, & Sternesjö, 2004, Mutua *et al*., 2020). The reasons for this have been attributed to limited veterinary professional access, poverty, and inadequate knowledge on antimicrobial agent use and AMR among these groups (Grace, 2015). Also, animal health providers do not routinely perform simple diagnostic tests before the administration of antimicrobial agents due to the unavailability of laboratories and their lack of capacity (Mutua *et al.*, 2020). Farmers tend to carry out self-diagnosis and treatment of cows for mastitis; many of them only seeking professional treatment when the mastitis cases fail to respond (GARP, 2011, Van *et al*., 2020). As a consequence, these practices have significantly contributed

to the growing emergence and spread of antibiotic-resistant bacterial strains and antibiotic residues in foods of animal origin (Grace, 2015, Van *et al*., 2020). Given this, there is an urgent need for research and policy on antibiotic use in order to regulate antibiotic abuse in these countries (FAO, 2016, Sharma *et al.*, 2018).

Globally it is estimated that about 86% of antibiotics used in dairy cows are for treatment and prevention of mastitis (Oliver & Murinda, 2012). In Kenya, like in many other developing countries, 90% of antibiotics used in dairy cows are mainly used for the treatment of intramammary infections (Shitandi & Sternesjö, 2004, Pol & Ruegg, 2017). According to Omwenga *et al.* (2020) in Kenya, commonly used antimicrobials in dairy cows include tetracyclines, beta-lactams, sulphonamides, and aminoglycosides. Studies have elsewhere reported a rise in the use of third and fourth-generation cephalosporins in cows (Du *et al.*, 2019, Cheng *et al.*, 2019). The rise in resistance rate to clinically important antibiotics, such as quinolones and third/fourth-generation cephalosporins in commensal bacteria in healthy animals has been reported in Africa (Van *et al*., 2020, Omwenga *et al*., 2020). This is of great concern because such drugs are usually reserved for use in the treatment of chronic bacterial infections in humans (Becker, 2021).

2.15.2 Antimicrobial use in dairy cows and its contribution to Antimicrobial resistance

Antimicrobial resistance (AMR) is a global health threat and could change the gains long achieved by antibiotics in public health care (Sharma *et al.*, 2018, WHO, 2019, FAO, 2021). AMR is defined as the ability of a microorganism to resist the effects of an antibiotic that once could successfully kill it (WHO, 2019). Currently, AMR causes 700, 000 deaths annually in humans and this is

estimated to rise to 10M and 100 Trillion USD in economic losses by 2050 if no urgent interventions are taken (WHO, 2016).

Antimicrobial use in animals and other farm settings contributes to the burden of AMR, more so, to increased AMR levels in humans (FAO, 2016, Sharma *et al.*, 2018). However, the quantification of the impact of antimicrobial use in food animals and the development of AMR in humans is yet to be established (Hoelzer *et al*., 2017, Van *et al.*, 2020). This lack of consensus has subsequently delayed the uptake of the judicious use of antibiotics in animals, including in dairy cows (Hoelzer *et al.*, 2017). Therefore, immediate action must be taken to limit further emergence and spread of animal-associated- antibiotic-resistant bacteria (FAO, 2016).

2.15.3 Development of antibiotic resistance

Naturally, antibiotic-resistant strains develop through selection pressure and adaptation to the environment (Sharma *et al.*, 2018, Yang *et al.*, 2020). However, this process has been accelerated in part by the inappropriate and excessive usage of antibiotics in food animals including in dairy cows (Qu *et al.*, 2019, Cheng *et al.*, 2019). The selective pressure among bacteria within the animals has led to the mutation of the existing genes (vertical transfer) or acquiring resistance genes (horizontal transfer) from related or unrelated bacterial strains (Sharma, *et al.*, 2018, Anjum *et al.*, 2019).

Resistance transfers between bacteria occur through mobile genetic elements mainly phages, plasmids, and transposons (Van *et al*., 2020, Becker, 2021). When this happens, the consequences are treatment failure of infections caused by the resulting resistant strains increased cost of treatment, and increased mortalities (WHO, 2015, FAO, 2016). Several mechanisms are involved in the development of AMR in bacteria (Meng *et al.*, 2020). These include; antibiotic inactivation, alterations to modification enzymes, modification in the metabolic pathways to overcome antibiotic effect; and minimizing entry and/ or promoting active efflux of the antibiotics (Sharma *et al*., 2018). Horizontal and vertical transfer of antibiotic resistance genes among bacterial species being identified as one of the major mechanisms leading to resistance (Smith, 2015, Sharma *et al.*, 2018, Becker, 2021).

CHAPTER THREE: PREVALENCE, ETIOLOGY AND RISK FACTORS OF MASTITIS IN DAIRY CATTLE IN EMBU AND KAJIADO COUNTIES, KENYA

3.1 INTRODUCTION

Bovine mastitis remains one of the most prevalent and economically challenging diseases in the dairy industry globally (FAO, 2014, Abebe *et al*., 2016). While only 30% of the African countries have well-documented data on mastitis, the prevalence of mastitis in most countries including Kenya is estimated at 10% and more than 50% for clinical and subclinical mastitis respectively (Gitau *et al*., 2014, Motaung *et al*., 2017). The limitation of research data on mastitis from these countries is concerning because accurate estimation of economic impacts and effective management of the disease is hindered (Adamu *et al*., 2020). Apart from the substantial economic losses, mastitis is of zoonotic importance and is association with increasing antimicrobial resistance bacteria (Nielsen, 2009, Ayele *et al.,* 2017, Sharma *et al.,* 2018, FAO, 2021).

The complexity of mastitis is magnified by the high number of causative agents of the disease (Motaung *et al.,* 2017). Currently, a bacterial culture is the gold standard method for the identification of mastitis-causing microorganisms (Ashraf & Imran, 2018). However, this method has been faced with several limitations (Kuehn *et al.,* 2013). For instance, long turnaround time (24–48 hours) to obtain results (Catozzi *et al.,* 2017). Furthermore, about 25%-30% of mastitis remains culture-negative using the convectional culture method (Kuehn *et al.,* 2013, Oikonomou *et al.,* 2014).

The absence of growths in clinically mastitic milk samples may have been described in several explanations (Pang *et al.,* 2018). These include low concentration of bacteria in the milk, the microorganism may require special media and growth requirement and presence of antibiotics in milk hence inhibiting microbial growth and causative agent may not be a bacterium (Kuehn *et al.,* 2013, Taponen *et al.,* 2019). Therefore, identifying the correct causative agents in culture-negative mastitis cases is crucial for effective treatment and control of mastitis as well as mitigation of antimicrobial resistance associated with bovine mastitis (Ruegg, 2017, Cattozi *et al.,* 2017).

To overcome the limitations associated with culture methods in the diagnosis of bovine mastitis, culture-independent techniques such as the 16S rRNA metagenomics analysis have been used (Kuehn *et al.,* 2013). These technique has provided great insight into the bacterial composition and diversity of bovine mastitis milk where a wide range of microbial agents have been described (Falentin *et al.,* 2016, Taponen *et al.,* 2019). However, In the African set up including Kenya, such studies are lacking even though 20%-37% of mastitic cases remain culture-negative cases (Motaung *et al.,* 2017, Richards *et al.,* 2019). Hence, understanding the bacterial composition and diversity in bacterial communities in clinical and subclinical mastitis culture-negative cases will provide insights on strategic ways of improving mastitis treatment and control in dairy cows in Kenya (Kuehn *et al.,* 2013, Taponen *et al.,* 2019).

In this context, the objectives of the current study were: (1) to establish the prevalence of clinical and subclinical mastitis in dairy cows in Embu and Kajiado Counties in Kenya, (2) To isolate and identify bacteria from the milk of the dairy cows studied in (1), using conventional culture method,

and investigate the bacterial diversity of the milk of the dairy cows studied in (1) using 16S rRNA metagenomics sequencing.

3.2 MATERIALS AND METHODS

3.2.1 Study area

This study was conducted in two counties in Kenya, namely Embu and Kajiado. These two counties were purposefully selected based on the high populations of dairy cows in the regions, and the increasing demand for cow milk due to the rapidly growing human population in the regions and country as a whole. In addition, the counties had different production systems however they were predominantly small holder farms. In Embu the study was conducted in Runyenjes and Kyeni North region while in Kajiado the study was conducted in Rongai, Ngong and Kiserian regions as shown in Figure 3.1 &3.2.

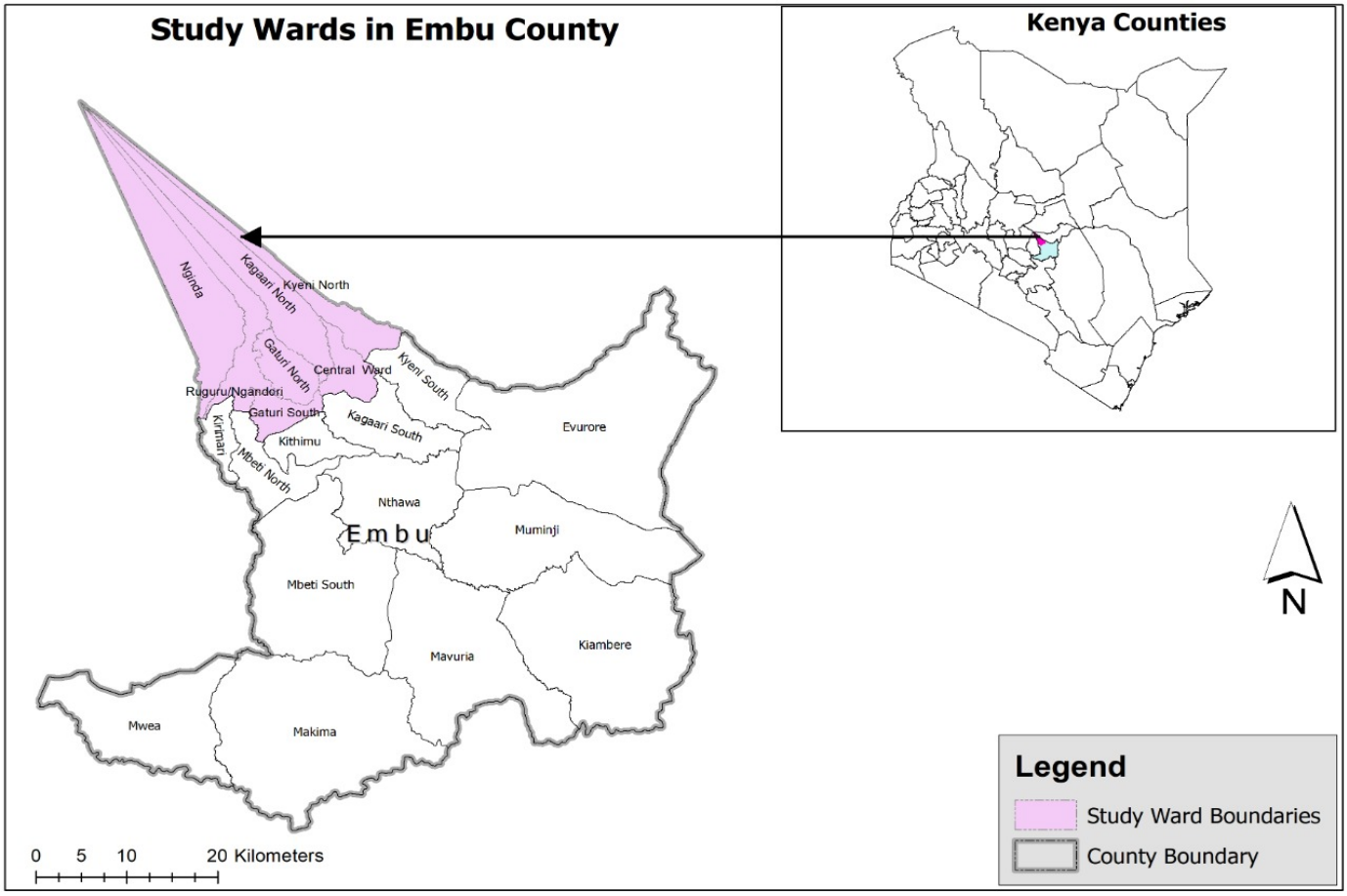


Figure 3.1: Map of the Embu showing the study sites (source: Kenya IEBC 2012 ICPAC geoportal).

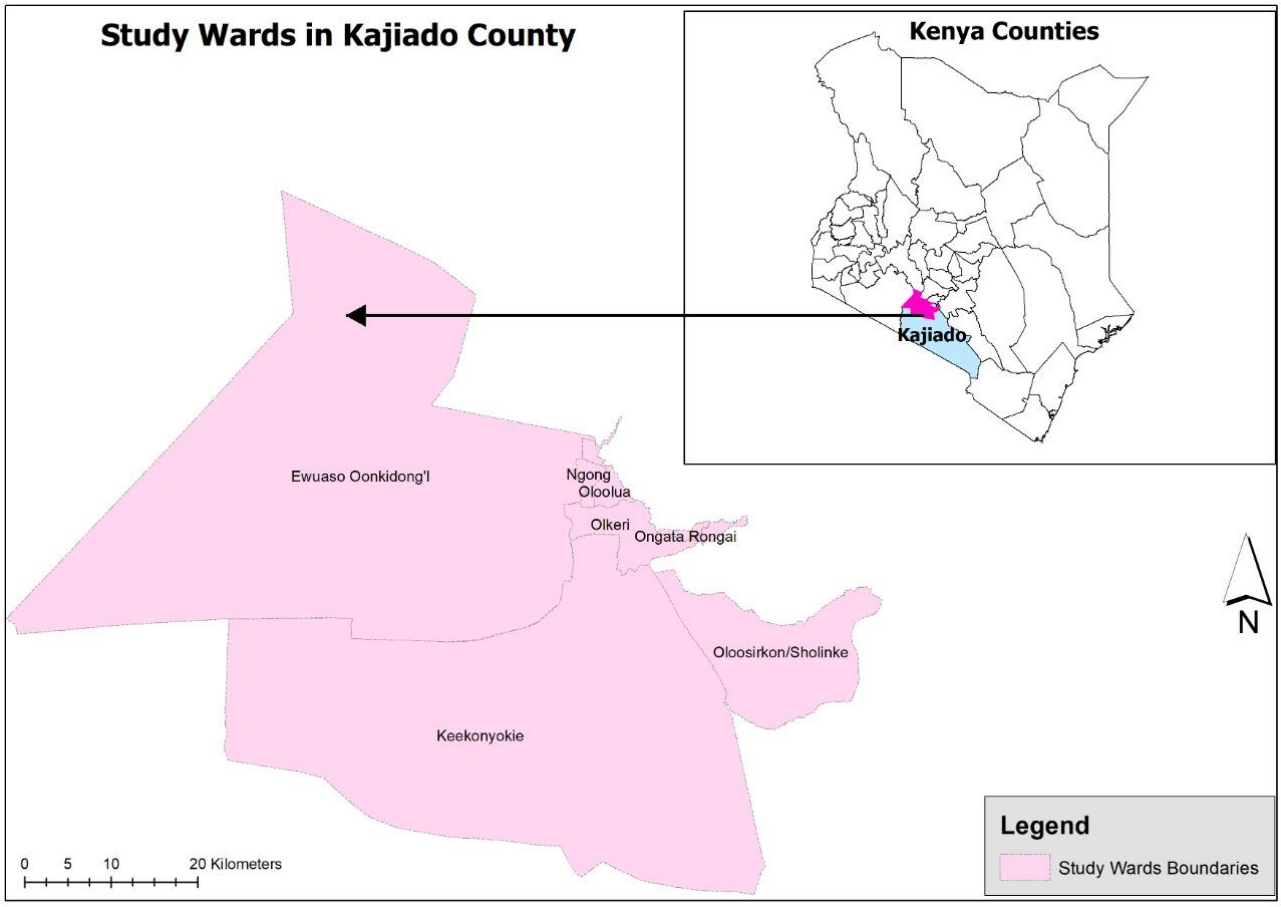


Figure 3.2: Map of the Kajiado showing the study sites (source: Kenya IEBC 2012 ICPAC geoportal).

The study areas broadly fall into two agro-climatic zones. Kajiado County zone has both hot and humid tropical climates, while Embu County is under the trimodal rainy and humid tropical climate. Smallholders' farms in this study were defined as farms with 1-10 lactating dairy cows.

Embu County is a high potential area that consists of both highlands and lowlands. This county lies between, 37.7238° E and 0.6560° S. It rises from about 515m above sea level at the Tana river

basin in the East to over 4,570m above the sea in the North West which is part of Mt. Kenya. It covers an area of a population of 608,599 persons, most of whom are small-scale farmers.

Kajiado County lies between 2.0981° S, 36.7820° E. It covers a 21,292.7 km area, and in 2019, the human population was at 1,117,840, most of whom are traditional pastoralists. The county borders the capital city Nairobi to the West and Tanzania to the South (Independent Electoral Boundaries Commission, 2012).

3.2.2 Study animals

The study animals were lactating dairy cows of exotic types (Fresian, Jersey, Guernsey) and crosses (mainly crossbreed of exotic and the zebus, a local breed). The cows were in different parities, and stages of lactation from smallholder farmers randomly selected from the two counties.

All cows from Embu were intensively kept, whereas, in Kajiado, cows were semi intensively and intensively reared. Intensively reared cows in Embu and Kajiado counties were predominantly fed on kikuyu grass, nappier grass, and hay. However, the semi-intensively reared cows were fed on both nappier grass and pasture mainly maasai love grass (*Eragrostis Superba*). All dairy cows were supplemented with mineral salt licks and dairy meal during the milking period. In this study, all cows were milked twice daily in the morning and evening.

3.3 Study design

A cross-sectional design was used in this study. A total, 395 dairy cows from 154 smallholder farms were randomly selected and sampled in both Embu and Kajiado Counties. The sampling was

done between November 2018 and June 2019. Of the 395 cattle, 206 cows in from 74 farms from Kajiado County while 189 cows in 80 farms were from Embu. A total of 1574 milk samples were collected from each cow's quarter and analyzed in the laboratory using the standard culture method and 16S rRNA metagenomics analysis.

3.3.1 Sample size determination

The sample size was calculated using the formula as given by Thrushfield (2005).

$$n = \frac{(1.96^2)(\mathrm{P_{exp}})(1 - \mathrm{P_{exp}})}{\mathrm{d}^2}$$

where n is the sample size, 1.96 is the Z statistic for a level of 95% confidence, P_{exp} is expected prevalence, and d is the desired absolute precision, which is equal to 5% (0.05). With an expected prevalence of 54.2% as average from previous studies in Kenya (Gitau *et al.*, 2014, Muriethi & Njuguna, 2016) a sample size of 381 was obtained. Therefore, in this study 395 animals recruited to cater for study withdraws. These animals were distributed in 154 smallholder dairy farms (<10 cows).

3.3.2 Sampling procedure

Study farms were randomly selected from lists of farmers provided by the veterinary county officers from each of the counties. Using the list given a random number generator was used to select the farms to be included in the study. For a farm to be included in the study, it had to have at least two lactating dairy cows and the farmer had to be willing to take part in the study and allow sample collection and provide access to the animals' data through milk production records. In each farm, a minimum of two and a maximum of 6 lactating cows were randomly selected and sampled.

To facilitate the random sampling of the cows in the farms with more than two cows, each cow was given a number and the numbers were randomly chosen from the list until the required number of cows was sampled (Dohoo *et al.,* 2003).

3.3.3 Case definitions

In this study, mastitis was characterized initially as clinical and subclinical. Clinical mastitis was defined as an animal that showed either systemic or localized signs of mastitis. CMT was done on all milk samples not showing clinical signs and when CMT was positive the cow was considered to have subclinical mastitis.

Clinical signs observed for clinical mastitis were inflammation on the udder, teat, fever, and the presence of clots, blood or and flakes in the milk. A cow was considered positive for clinical mastitis if it had clinical signs of mastitis in one or more of the quarters. A quarter was considered positive for subclinical mastitis if it had a CMT score reading of (+1, +2, +3). A cow was considered positive for subclinical mastitis if one or more of the quarters had a CMT positive.
A farm was considered positive for mastitis if it had one or more cows with clinical or subclinical mastitis.

At culture, a quarter was considered positive if it was clinically sick or had a CMT score positive of +1 or higher and subsequently confirmed by isolating one or more bacteria from the milk sample. A cow was considered positive for mastitis if one or more quarters was culture positive. A quarter was defined as negative of mastitis if it had no signs of clinical mastitis and had a CMT score of 0

or trace and no bacteria were isolated from the milk samples following routine aerobic culturing. A cow was defined as negative if all the quarters were negative for CMT and bacterial isolates.

3.3.4 Detection of clinical and subclinical mastitis

A total of 395 cows were screened, and milk samples were collected from each quarter. At the farm level, all udders and teats for each cow were physically examined. Detection of clinical mastitis was done by examining the udder and the teats for any inflammation, fever in the animals, and checking the milk's consistency for the presence of clots, blood, and flakes (Radostits *et al*., 2007). Following physical examination, screening for subclinical mastitis (SCM) using the California Mastitis Test (CMT) was done as described below.

3.3.5 California mastitis test (CMT)

This was done based on the guidelines described by National Mastitis Council's Laboratory Handbook on Bovine Mastitis (NMC) (NMC, 2017). Briefly, the milk sample from each quarter was milked into a clean separate cup in the four CMT paddle. The paddle was tilted nearly to vertical to equalize the milk in each cup to about 5ml. Equal amounts of CMT solution were added to each cup of the paddle. The paddle was then swirled to mix the milk samples with the reagent and the result read within 10 seconds. The CMT results were interpreted subjectively as either negative (0), trace, 1+, 2+, or 3+, as described by NMC handbook (2017). Based on the CMT, cows were considered positive for SCM if they had readings of (1+, 2+, 3+) whereas negative and trace reactions were taken as negative. The cows were then grouped and results were recorded as mastitic or non-mastitic. A cow was regarded as mastitis positive if at least one of the quarters was CMT

positive. Subclinical mastitis positive milk samples selected for 16S rRNA metagenomics analysis had to have a score of 3+ in this study.

3.3.6 Sample collection, handling, and transportation

Milk samples were collected from 395 dairy cows. Before sampling the udder was thoroughly washed with water and dried. After disinfecting the teats with 70% ethyl alcohol swabs, followed by discarding 4-5 streams of milk, 5mls of milk was collected from each quarter aseptically and put in separate universal bottles while held at a slightly horizontal position to avoid contamination from the udder (Radostits *et al*., 2007, NMC 2017). Concurrently, approximately 2ml of milk samples from each quarter were collected in sterile vials for 16S rRNA metagenomics analysis. The sample universal bottles and the vials were then appropriately sealed and labeled. The milk samples in vials were immediately frozen in liquid nitrogen (-196^0C) while the samples in the universal bottles were refrigerated in ice-boxes with cold packs. All samples were transported with their accompanying history to the University of Nairobi, Department of Veterinary Pathology, Microbiology and Parasitology (VPMP), Bacteriology Laboratory, for further processing. The samples were cultured immediately or stored in the refrigerator at 4°C for a maximum of a day, awaiting culture. The milk in the vials were then transferred to -20°C and stored there until they were further processed.

3.3.7 Bacterial isolation and identification using conventional culture method

All bacteriological examinations were done at the bacteriology laboratory, department of VPMP, according to standard methods described by the National Mastitis Council Laboratory Handbook

on Bovine Mastitis, USA (NMC, 2017). The samples were first brought out of the fridge, left outside to warm up to room temperature (24ºC-26ºC).

Briefly, a 0.01 ml aliquot of each milk sample was aseptically streaked onto the surface of 5% sheep Blood agar and Mac Conkey agar plates (Oxoid, England). The plates were incubated aerobically at 37°C for 24 hours after which the colony morphology was observed and recorded. The plates with no colony growth after initial incubation were further incubated for up to 72 hours, after which they were recorded as 'No growth'. Samples yielding more than one colony were referred to as mixed cultures; distinct colonies were identified and sub-cultured separately to obtain respective pure colonies. Sub-culturing was repeated where need be. Identification of resultant, respective single colonies (pure cultures) was done macro-morphologically, then microscopically and later through biochemical testing in order to determine the respective genus and species of the organism. Samples of the pure cultures were also sub-cultured onto Tryptose Soy Broth (TSB) with 20% glycerol and stored at -20 º C for further use and referrals.

Staphylococcus species were further identified using Gram stain, growth characteristics on Mannitol Salt Agar (Oxoid, England), catalase test and tube coagulase testing and polymerase chain reaction (PCR) in the case of *S. aureus*. *Streptococcus* species were further identified using catalase test and growth characteristics on Edward's medium (Oxoid, England) and, within the group, differentiation was done using the CAMP (Christie–Atkins–Munch-Peterson) test. CAMP test positive isolates were classified as *Streptococcus agalactiae* in this study. Gram-negative bacteria were identified using colony morphology and lactose fermentation on Mac Conkey agar,

oxidase test, sugars fermentation, and Indole, Methyl red, Voges-Proskauer and Citrate-utilization (IMViC) tests (Oxoid England).

3.3.8 16S rRNA metagenomics analysis of clinical and subclinical mastitic milk samples with growth and no growth from Kajiado and Embu Counties

For purposes of 16S rRNA metagenomics analysis, milk samples were grouped into three categories namely disease status of the milk, region and culture growth status. Disease status was defined as milk samples from quarters exhibiting clinical and subclinical mastitis. For the 16S rRNA metagenomics analysis, 12 milk samples which had clinical mastitis and 54 milk samples with subclinical mastitis with a CMT score of 3+ were included. The region referred to samples from either Embu or Kajiado Counties. Growth status was defined as milk samples from quarters with clinical or subclinical mastitis with the presence or absence of growth on classical culture (indicated as yes) or absence of growth on culture (indicated as no).

To establish the bacterial diversity and assess the comparison in the three categories, 66 milk samples from 53 cows were purposefully selected from the available 1574 quarter milk samples (six quarters were blind so did not produce any milk) collected in this study and analyzed using 16S rRNA metagenomics sequencing. The distribution of the 66 milk samples in each category were as follows; region; Kajiado (n=35) and Embu (n= 31); diseases status; clinical mastitis (n=12) and subclinical mastitis (n=54) and growth status; presence of bacterial growth on culture (culture positive) (n=34) and absence of bacterial growth on culture (culture-negative) (n=32).

3.3.8.1 DNA extraction, library preparation and sequencing

One ml of each milk sample was centrifuged for 10 minutes at room temperature using Eppendorf 5415R Centrifuge at 16000 RCF (relative centrifuging force) (Oikonomou *et al.*, 2014). The supernatant was discarded and the remaining pellet was re-suspended in 750µl of ZymoBiomics bead tube lysis solution of ZymoBiomics^{TM} DNA extraction mini kit and used for DNA extraction as described by the manufacturers (Zymoresearch, USA).

After elution, the DNA quality and quantity were assessed by visualization with UV light on 1% agarose gel electrophoresis, Pico green fluorescence-based quantifications Picogreen (Invitrogen) and Qubit dsDNA HS Assay (Life Technologies Corporation, Grand Island, NY, USA). The sequencing libraries were prepared according to the Illumina MiSeq system protocol with a few modifications (Amplicon, 2013). Briefly, the V3 and V4 regions of the 16S rRNA gene were amplified using a two-step polymerase chain reaction (PCR) protocol. In the first step, the V3 and V4 region was amplified in a PCR reaction mixture and the amplification program as described by the manufacturer except that 35 cycles of amplification were used. Additionally, 63° C was used as the annealing temperature. The primers used were 341F 5'-CCTACGGGNGGCWGCAG-3′ and 805R 5′-GACTACHVGGGTATCTAATCC-3′. For the second PCR, dual-index barcodes and Illumina sequencing adapters were added to each amplicon. Nextera XT index primers were used and the amplification cycles were increased to 9 from the manufacturer's recommendations of 8 cycles. After each of the PCR steps, Amplicons were cleaned using AMPure XP beads (A63881, Beckam Coulter, Brea, CA, USA). Libraries were assessed with the Qubit dsDNA HS assay kit (Life Technologies Corporation, Grand Island,

NY, USA), and Agilent 2100 Bioanalyzer high sensitivity kit (Agilent Technologies Inc., Santa Clara, CA) before being pooled in and sequenced (200 cycles) using the Illumina MiSeq v3 (Illumina) System at the Macrogen South Korea.

3.3.8.2 Bioinformatics data analysis

To facilitate comparison of milk samples sequence results were classified based on disease status, region and culture growth status. Quantitative Insights Into Microbial Ecology (QIIME version 1.91) was used to analyze the raw sequenced fastaQ reads as described by (Caporaso *et al.*, 2010). The forward and reverse sequences were merged after which Quality filtering was done at a pred quality score of above Q20. The vsearch method (Rognes *et al.*, 2016) was used to detecting and removing of chimera. Next, using the Greengenes database 13_8 as reference (De Santis *et al.*, 2006), Ribosomal Database Project (RDP) classifier (Wang *et al.*, 2007) was used to assign bacterial taxonomy for each sequence. The generated Operational Taxonomic Units (OTUs) table from the above-described process with counts per sample was imported into the Phyloseq R package (1.30.0) (Navas-Molina *et al.*, 2013). Reads with low abundance <0.01% of total OTUs were also filtered.

To analyze the bacterial diversities of the mastitic milk samples, Chao 1 and ACE were used to detect species richness in the samples while Fisher's alpha, Shannon, and Simpson diversity indices were used to detect the diversity of the species within the samples in the three groups (Navas-Molina *et al.*, 2013).

Statistical analyses were performed on alpha-diversity indices with regard to the different groups and sequences using one-way ANOVA where a p-value of less than 0.05 was considered significant. Non-parametric Monte Carlo test (999 permutations) was used to assess the statistical significance of alpha diversity (Oksanen *et al.,* 2007). The microbial composition in relation to different categories was assessed using ANOVA with a p-value of 0.05 using the Adonis function of the Vegan R package (Mcmurdie &Holmes 2013).

On the other hand, beta diversity (a measure of differences between taxa among samples) was done was calculated using unweighted UniFrac distance matrices, presented using Principal Coordinates Analysis (PCoA) (Catozzi *et al.,* 2017).

3.3.9 Data entry and statistical analysis

Data entry and management were done using Microsoft excel 2016, while data analysis was done using the STATA version15. Descriptive statistics were used to calculate the prevalence of clinical and subclinical mastitis. Prevalence of mastitis was calculated as the proportion of clinically sick animals or subclinical mastitis over the total population analyzed. The chi-square test or Fisher's exact test were used where applicable, to compare the prevalence and the statistical significance level set at 0.05 ($p < 0.05$).

3.4 RESULTS

3.4.1 Prevalence of clinical and subclinical mastitis in dairy cows

Overall, 1574 quarter milk of 395 cows from 154 farms in Kajiado and Embu counties were analyzed for mastitis in this study. The overall prevalence of mastitis based on California Mastitis Test (CMT) and clinical examination at farm, cow and quarter levels were at 76.6% (118/154) (95% confidence interval [CI 74.6%-78.5%]), 80% (316/395) (95% [CI 79%-80.9%]) and 67.8% (1068/1574) (95% [CI 66.7%-69.8%]) respectively. Of the mastitic cows, 8.5% (27/316) (95% [CI, 7.5%-9.4%]) had clinical signs of mastitis while 91.4% (289/316) (95% [CI 90.4%-94.3%]) were CMT positive as an indication of sub-clinical mastitis (Figure 3.3).

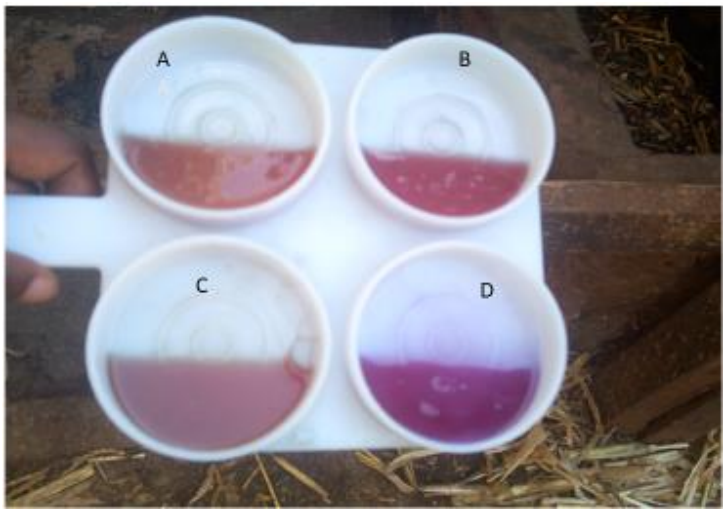


Figure 3.3: California mastitis test (CMT) showing a positive reaction in the four quarters of a cow as indicated in A, B, C, D.

Embu County reported a higher farm-level prevalence of both clinical and subclinical mastitis at 78.7 % (63/80) and cow-level at 82% (155/189) as compared to farms in Kajiado which had 72.9 % (54/74) and 78.1% (161/206) respectively (p=0.3, p=0.8). Moreover, Embu reported a significantly higher quarter-level prevalence of mastitis at 73.6% (552/750) as compared to Kajiado which had 62.6% (516/824) (p=0.04).

Of the mastitic cows in Embu, 10.3% (16/155) and 89.6% (139/155) were clinical and subclinical mastitis respectively. In Kajiado County, 6.8% (11/161) and 93.1% (150/161) of cows had clinical and subclinical mastitis respectively. However, there were no statistically significant differences in the distribution of clinical and subclinical mastitis in the two counties at the cow level (p=0.34).

Based on bacterial culture results, the overall prevalence of mastitis in this study, at farm, cow, and quarter levels were at 76.6% (118/154) (95% [CI 75.6%-77.5%]), 74.4% (294/395) (95% [CI 73.4%-75.3%]), 53.2% (841/1574) (95% [CI 52.2%-54.1%]) respectively. Embu County reported a higher prevalence at the farm level 78.7% (63/80) compared to Kajiado County which had 74.3 % (55/74). Embu County reported a significantly higher prevalence of cow level mastitis at 79.3% (150/189) as compared to cows in Kajiado County which had 69.9% (144/206) (p=0.03). Similarly, Embu County reported a significantly higher prevalence of quarter-level mastitis at 60.1% (455/756) as compared to Kajiado at 43.6% (360/824) (p=0.01).

3.4.2 Prevalence of bacteria isolated from mastitic cow milk

Out of the 1580 quarters examined, 0.3% (6/1580) of them were blocked and did not produce any milk. The remaining 1574 quarters were then cultured for bacterial isolation and of these, 67.8% (1068/1574) were mastitic while 32.2% (506/1574) were negative. Out of the mastitic samples, 78.7% (841/1068) yielded bacteria while 20.3% (217/1068) had no bacterial growth, as per the growth media and conditions used in this study. All CMT negative samples did not yield any bacterial growth on standard culture methods.

A total of 1016 bacterial colonies some of which are shown in Figure 3.4 were yielded from the 53.4% (841/1574) milk samples. Among the culture-positive samples, 62% (522/841) had a single colony while the rest 37.9% (319/841) had mixed cultures.

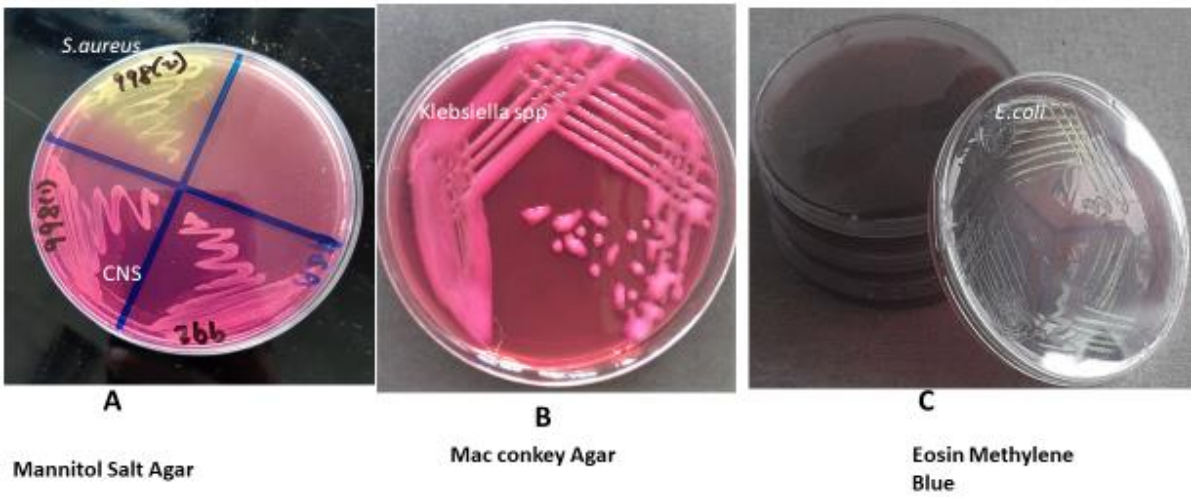


Figure 3.4: Showing bacterial growth characteristics on various media; (A) Mannitol Salt agar (MSA) representing mannitol fermenter (yellow- no. 998 (2) for *S. aureus*) and non-mannitol fermenter (pink–998(1) and 992 for CNS), (B) Mac Conkey agar, showing lactose fermenter for *Klebsiella* species, (C) Eosin Methylene blue (EMB) showing greenish metallic sheen for *Escherichia coli*.

Overall, Coagulase-negative *Staphylococcus* (CNS) isolated at 42.8% (n=435) was the most prevalent bacteria in this study. This was followed by other *Streptococcus* species at 22.2% (n=226) and *Staphylococcus aureus* isolated at 15.7% (n=160). *Micrococcus* species at 0.9% (n=9) and *Enterobacter* species at 0.7% (n=7) were the least recovered bacterial isolates from the mastitic samples (Table 3.1). Notably, from this study, *Micrococcus* species and *Streptococcus agalactiae* were not recovered from any of the clinically sick quarters. However, all other mentioned bacteria were isolated in subclinical mastitis (Table 3.1).

Table 3.1: Distribution of the 1016 bacteria isolated from mastitic milk from 294 dairy cows in Embu and Kajiado Counties

	Clinical mastitis		Subclinical mastitis	
	Embu County (n^1=44)	Kajiado County(n^1=18)	Embu County (n^1=547)	Kajiado County (n^1=407)
Bacteria	n (%)	n (%)	n (%)	n (%)
CNS[2]	20 (45.4)	4 (22.2)	227 (41.4)	184 (45.2)
other *Strep* spp[2]	10 (22.7)	7 (38.8)	110 (20.1)	99 (24.3)
S. agalactiae	0 (0)	0 (0)	1 (0.1)	3 (0.7)
S. aureus	6 (13.6)	4 (22.2)	76 (13.8)	74 (18.1)
Bacillus spp	4 (9)	0 (0)	58 (10.6)	15 (3.6)
P. aeruginosa	1(2.2)	0 (0)	29 (5.3)	22 (5.4)
E. coli	3(6.8)	1 (15.5)	23 (4.2)	5 (1.2)
Klebsiella spp	0 (0)	1 (5.5)	11(2)	2 (0.4)
Micrococcus spp	0 (0)	0 (0)	6 (1)	3 (0.7)
Enterobacter spp	0 (0)	1(5.5)	6 (1)	0 (0)

, [1]n refers to the number of bacteria isolated in each county [2]Coagulase negative *Staphylococcus*, [2]other *Streptococcus* are isolates not identified as *Streptococcus agalactiae*

Unexpectedly, this study did not find any significant differences in the distribution of mastitis causing bacterial species isolated in the two counties. However, frequencies of isolation for some bacteria differed between the counties. For instance, In Embu County, the frequently isolated bacteria in both clinical and subclinical mastitis was CNS at 45.4% and 41.4% respectively. This was followed by other *Streptococcus* species isolated at 22.7% for clinical mastitis and 20.1% for subclinical mastitis. However, interestingly, in Kajiado County, the most prevalent bacteria in clinical mastitis was other *Streptococcus* species isolated at 38.8% while CNS at 45.2% was frequently isolated in the subclinical mastitis (Table 3.1).

Significantly higher prevalence of *Bacillus* species and *Escherichia coli* were recovered in cows from Embu County compared to those from Kajiado (p=0.03). However, there were no statistically significant differences in the distribution of the other bacterial species isolated from the two counties (p > 0.05).

3.4.3 Bacterial diversity of mastitic milk of dairy cows in Embu and Kajiado Counties using 16S rRNA metagenomic analysis.

3.4.3.1 Summary of culture growth results of 66 quarter milk samples

The bacterial culture results of all 1574 mastitic milk samples have been presented in detail in section 3.3.2. In summary, the bacterial culture growth results of the 66 representative quarter milk samples used for 16S rRNA metagenomics analysis were as follows; Coagulase-negative *Staphylococcus,* 28% (n=19); other *Streptococcus* species 10.6% (n=7); *Staphylococcus aureus,* 9% (n=6); *Bacillus* species, 9% (n=6) and *Pseudomonas aeruginosa,* 6% (n=4) and 48.4% (n=32) of the samples were described as culture negative following aerobic standard culture.

3.4.3.2 Sequencing results

Analysis of the same 66 milk samples using 16S rRNA metagenomics sequencing yielded a total of 18, 464,046 million reads with an average of 279,183 reads per sample and an average of 301bp in read length. Of these sequences, about 40% (7,435,372) sequences were eventually analyzed using QIIME following quality control and yielded a total of 3047 operational taxonomic units (OTUS). For comparison, the sequence results were grouped based on study county (Kajiado or Embu), disease status (clinical /subclinical mastitis) and culture growth results (culture-positive or

culture-negative). As shown in Table 3.2 there were no statistically significant differences in terms of the number of and percentages of reads that were removed after quality control across the three groups.

Table 3.2: Distribution of the number of original sequences and percentages of reads that were removed after quality control in each group

			Original sequences (n±SD/SE)	**% reduction Pairing and filtering**	**% reduction after chimera removal**
Embu	clinical	no	258986±0	55.359±0	1.103±0
		yes	283200.67±14831.46	57.312±3.28	6.779±5.753
	Subclinical	no	294667.78±15798.11	61.071±3.008	1.801±0.727
		yes	282186.11±8400.17	60.164±1.476	1.324±0.309
Kajiado	clinical	no	255653±5766.76	55.04±0.701	5.094±2.73
		yes	276567.5±16357.16	62.335±4.713	2.239±1.098
	Subclinical	no	270744.67±5810.59	56.457±0.724	1.667±0.519
		yes	287090.22±10689.77	58.533±1.158	1.873±0.48
p-value	Regions (R)		0.553	0.856	0.975
	Status (S)		0.219	0.468	0.056
	Culture Growth (CG)		0.317	0.223	0.544
	R*S		0.853	0.2	0.818
	R*CG		0.601	0.329	0.066
	S*CG		0.399	0.343	0.463
	R*S*CG		0.511	0.781	0.132

3.4.3.2.1 Alpha and Beta diversity indices

Alpha diversity of clinical mastitic milk was significantly higher than subclinical mastitic milk based on Shannon (p=0.025) and Simpson indices (p=0.013). However, there were no other significant differences in richness and diversity in bacteria in the other groups (Table 3.3 & Figure 3.5).

Table 3.3: Alpha diversities showing changes in microbial communities by clinical status, region and culture growth status

			Chao1	**ACE**	**Shannon**	**Simpson**	**Fisher**
			Mean±SD/SE	**Mean±SD/SE**	**Mean±SD/SE**	**Mean±SD/SE**	**Mean±SD/SE**
Embu	clinical	no	332.5±0	293.8±0	3.4±0	0.9±0	49.1±0
		yes	315.8±182.9	344.2±193.92	1.6±1.38	0.4±0.29	59.3±46.72
	Subclinical	no	186.4±35.65	197.6±39.16	0.8±0.31	0.2±0.09	22.1±6.8
		yes	254.1±30.37	266.9±32.54	1.1±0.3	0.3±0.08	28.1±3.67
Kajiado	clinical	no	132.6±19.66	147.8±17.77	1.0±0.56	0.4±0.19	14.9±1.54
		yes	233.8±65.29	238.3±64.14	2.2±0.66	0.7±0.13	34.8±12.01
	Subclinical	no	266.4±32.78	280.6±36.7	1.3±0.38	0.3±0.09	32.6±5.33
		yes	342.6±48.25	351.5±50.44	0.5±0.11	0.2±0.03	32.0±4.78
p -value[1]	Region (R)		0.583	0.704	0.344	0.515	0.21
	Status (S)		0.866	0.744	**0.025**	**0.013**	0.222
	Culture Growth (CG)		0.27	0.209	0.581	0.436	0.314
	S * CG		0.773	0.997	0.933	0.769	0.482
	S * R		**0.032**	0.063	0.354	0.722	**0.041**
	CG * G		0.54	0.851	0.35	0.22	0.929
	S * CG * R		0.596	0.862	**0.04**	**0.029**	0.643

[1]indicate statistical differences compared within each two or three groups

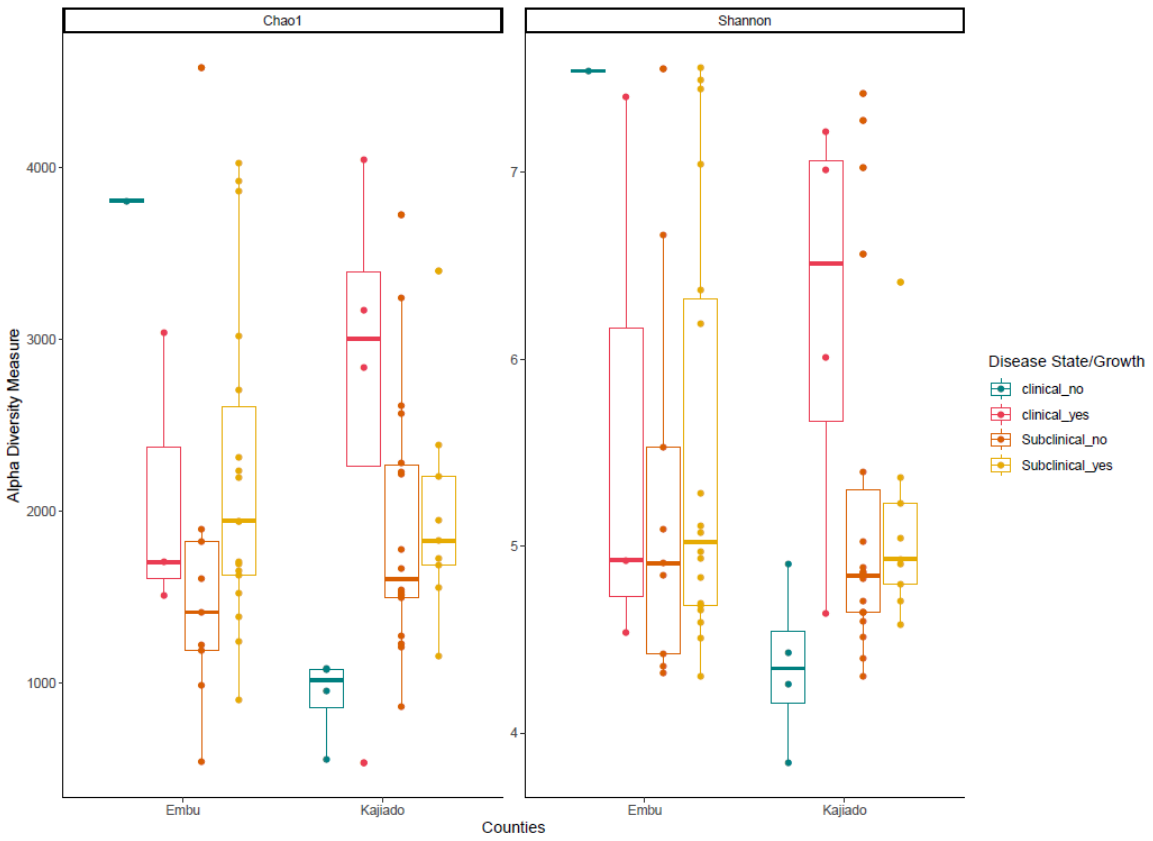


Figure 3.5: Plot boxes showing Alpha diversities measures (Chao1 and Shannon) showing bacterial richness and diversity in the three categories; clinical status (clinical or subclinical), region (Embu or Kajiado), and culture growth status (Yes/No).

As shown in (Figure 3.6) the Bray-Curtis Principal Coordinate Analysis (PCoA) plots revealed that bacterial communities from Kajiado and Embu Counties clustered closely together along the Y-axis. Further, there was no clear separation between bacteria taxonomies from mastitic milk samples with culture-negative and those with culture-positive in the (PCoA) plots.

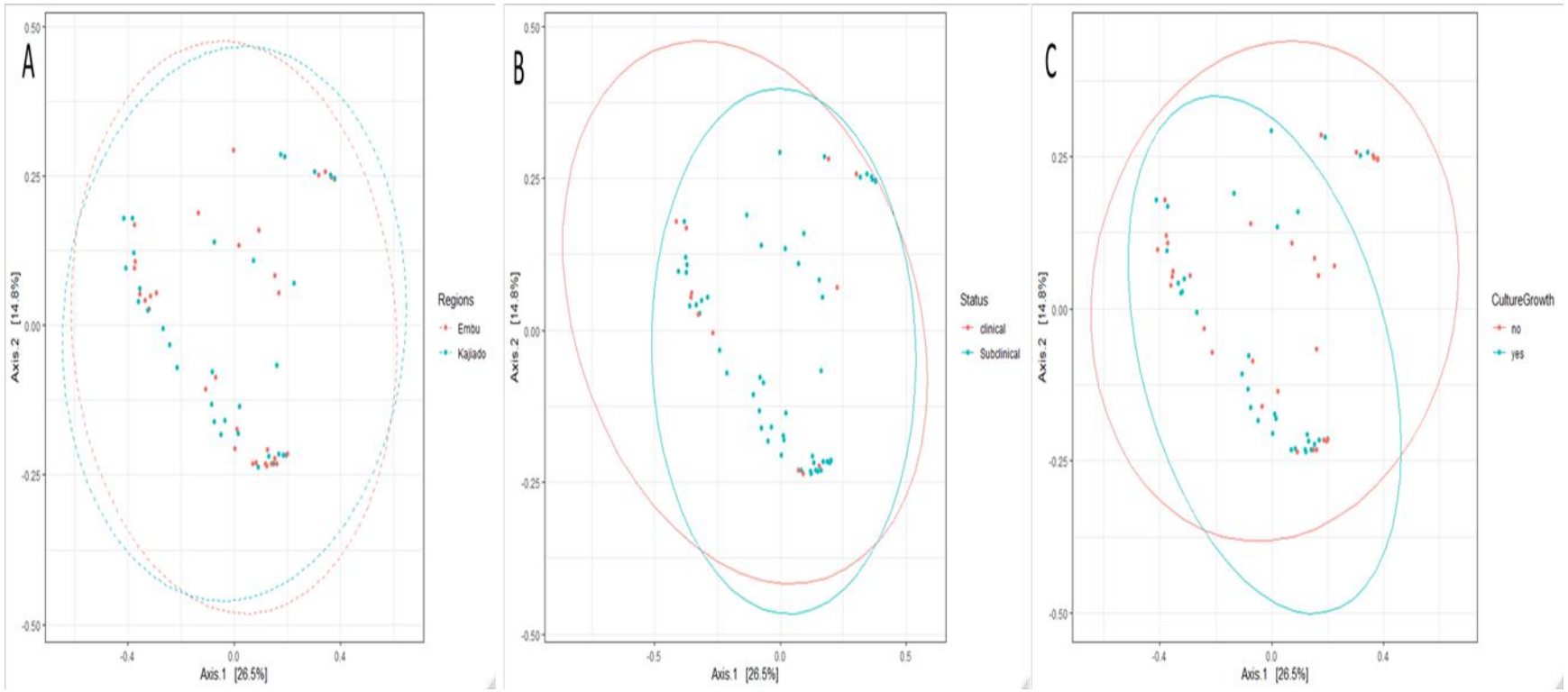


Figure 3.6: Principal Coordinate Analysis (PCoA) (Bray Curtis distance) plots A, B, C using unweighted UniFrac distances clustered samples comparing changes in bacterial communities by region, clinical status, and growth status on culture. The different colors in the PCoA analysis represent different groups, and the closer the sample distance is, the more similar the microbial composition between the samples is, and the smaller the difference is.

3.4.3.2.2 Bacterial communities identified using 16S rRNA metagenomics analysis

On taxonomic analysis of 3047 operational taxonomic units (OTUs) with an average relative abundance of more than 0.01% count, 16 different bacterial phyla were identified from 77% (2359) of the OTUs while 22.5% (688) of them were unassigned. The three most abundant bacterial phyla identified across all the groups were *Proteobacteria* (49%-88%), *Firmicutes* (4%-54%), and *Spirochaetes* (0.004%-0.9%). Other phyla with relatively lower abundance reported in mastitic samples in this study were *Chlamydiae* (0-9.2%), *Acidobacteria* (0-0.8%), *Actinobacteria* (0-0.3%), *Bacteroidetes* (0-0.004%), *Gemmatimonadetes* (0-0.003%), *TM6* (0-0.03%), *Tenericute* (0-0.7%), *Verrucomicrobia* (0-0.2%) and *WWE1* (0-0.1%) (Table 3.4 and Figure 3.7 A&B).

Table 3.4: ANOVA Table of 12 Most Abundant Phyla and Genera recovered from dairy cows with mastitic milk using 16S rRNA metagenomics analysis from Kajiado and Embu counties

Phyla/Genera	Regions								P-Value						
	Embu County				Kajiado County										
	Clinical		Subclinical		clinical		Subclinical								
	No Mean±SD/SE	Yes Mean±SD/SE	No Mean±SD/SE	Yes Mean±SD/SE	No Mean±SD/SE	Yes Mean±SD/SE	No Mean±SD/SE	Yes Mean±SD/SE	S	CG	R	S * CG	S * R	CG * R	S * CG * R
Proteobacteria	49.8±0	88.3±10.976	86.8±6.811	74.3±7.764	85.819±13.755	45.323±12.79	86.802±4.486	86.246±9.832	0.10	0.70	0.90	0.78	0.63	0.09	**0.02**
Pseudomonas	2.7±0	66.9±30.307	81.0±10.395	63.3±10.283	66.908±21.834	14.702±11.102	71.145±9.579	83.822±10.421	**0.01**	0.90	0.70	0.77	0.98	0.14	**0.01**
Acinetobacter	30.6±0	11.9±11.73	2.97±2.765	4.6±2.174	2.057±1.792	10.291±4.668	6.771±3.619	0.472±0.252	**0.02**	0.36	0.07	0.73	0.07	0.25	**0.04**
Rickettsiella	0±0	0±0	0.002±0.002	0.009±0.005	0±0	0.011±0.007	0.028±0.028	0.004±0.002	0.74	0.95	0.73	0.78	0.91	0.84	0.67
Ralstonia	0.4±0	0.005±0.005	0.1±0.057	0.3±0.147	0.876±0.863	0.616±0.432	0.529±0.287	0.029±0.021	0.50	0.48	0.33	0.78	0.44	0.62	0.52
Enhydrobacter	0.4±0	0.02±0.006	0.04±0.023	1.0±0.717	0.136±0.12	0.479±0.316	0.24±0.127	0.032±0.008	0.91	0.77	0.83	0.76	0.69	0.85	0.46
Rheinheimera	0.4±0	0.7±0.717	0.03±0.023	0.02±0.011	0.002±0.001	0.159±0.107	0.216±0.191	0.004±0.003	0.19	0.69	0.31	0.33	0.14	0.59	0.98
Firmicutes	48.5±0	9.5±9.026	12.4±6.4	24.9±7.607	4.44±4.131	53.635±12.741	11.053±3.627	13.018±9.508	0.13	0.49	0.71	0.91	0.71	0.03	0.01
Staphylococcus	22.8±0	0.4±0.19	8.7±4.572	16.9±7.06	2.568±2.444	21.745±20.141	2.144±0.852	1.126±0.724	0.52	0.89	0.46	0.72	0.42	0.26	0.08
Solibacillus	18.5±0	3.0±3.01	2.5±2.013	0.893±0.412	0.002±0.001	0.591±0.453	2.353±1.253	0.305±0.103	**0.01**	**0.00**	**0.00**	**0.05**	**0.00**	**0.01**	**0.01**
Facklamia	1.4±0	1.9±1.903	0.16±0.103	0.396±0.162	0.133±0.121	1.062±0.881	0.999±0.408	0.317±0.09	0.16	0.61	0.46	0.32	0.12	0.80	0.46
Streptococcus	0.2±0	0.2±0.231	0.2±0.117	2.013±1.033	0.381±0.368	23.918±13.149	1.086±0.647	9.383±9.339	0.51	0.07	0.08	0.46	0.39	0.10	0.35
Turicibacter	0.8±0	0.3±0.309	0.2±0.121	0.6±0.295	0.127±0.123	0.569±0.198	0.366±0.21	0.17±0.08	0.68	0.91	0.64	0.87	0.87	0.78	0.25
Tenericutes	0.7±0	0.6±0.647	0.06±0.039	0.03±0.022	0±0	0.184±0.152	0.241±0.121	0.067±0.04	**0.04**	0.92	0.09	0.51	**0.01**	0.89	0.50
Mycoplasma	0.03±0	0.005±0.005	0±0	0.002±0.002	0.012±0.03	0±0	0±0	0.001±0.001	0.13	0.60	0.12	051	0.23	0.71	0.25
Acholeplasma	0±0	0±0	0.002±0.002	0.009±0.005	0±0	0.011±0.007	0.028±0.028	0.004±0.002	0.95	0.91	0.8	077	0.12	0.36	0.73
Other Unassigned	0.4±0	0.8±0.551	0.4±0.305	0.683±0.181	0.448±0.346	0.279±0.113	0.637±0.222	0.435±0.093	0.83	0.78	0.70	0.89	0.71	0.36	0.93
Acidobacteria	0.1±0	0±0	0.001±0.001	0.018±0.013	0±0	0.039±0.036	0.012±0.01	0.003±0.002	0.17	0.58	0.44	0.41	0.53	0.14	**0.02**
Chlamydiae	0.04±0	0.005±0.005	0±0	0.002±0.002	9.282±9.282	0±0	0±0	0.001±0.001	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Actinobacteria	0±0	0.027±0.027	0.02±0.015	0.026±0.011	0.003±0.002	0±0	0.013±0.006	0.009±0.004	0.47	0.62	0.38	0.68	0.99	0.45	0.70
Bacteroidetes	0±0	0.004±0.002	0.004±0.003	0.004±0.001	0.004±0.002	0±0	0.007±0.006	0.003±0.001	0.59	0.83	**0.88**	0.87	0.93	0.54	0.87
Gemmatimonadetes	0.03±0	0±0	0.002±0.002	0.001±0.001	0±0	0.009±0.009	0.001±0.001	0±0	**0.00**	**0.01**	**0.01**	0.02	0.03	0.00	0.00
Spirochaetes	0.3±0	0.5±0.465	0.26±0.163	0.05±0.029	0.004±0.002	0.496±0.381	0.986±0.963	0.207±0.189	0.95	0.91	0.86	0.63	0.72	0.95	0.78
TM6	0±0	0±0	0±0	0.026±0.026	0±0	0±0	0±0	0±0	0.77	0.77	0.77	0.77	0.77	0.77	0.77
Verrucomicrobia	0.2±0	0.2±0.241	0.052±0.039	0.017±0.013	0±0	0.034±0.025	0.151±0.135	0.01±0.005	0.64	0.86	0.54	0.58	0.32	0.79	0.86
WWE1	0±0	0.016±0.016	0.002±0.002	0±0	0±0	0±0	0.096±0.09	0±0	0.79	0.79	0.80	0.71	0.72	0.72	0.80

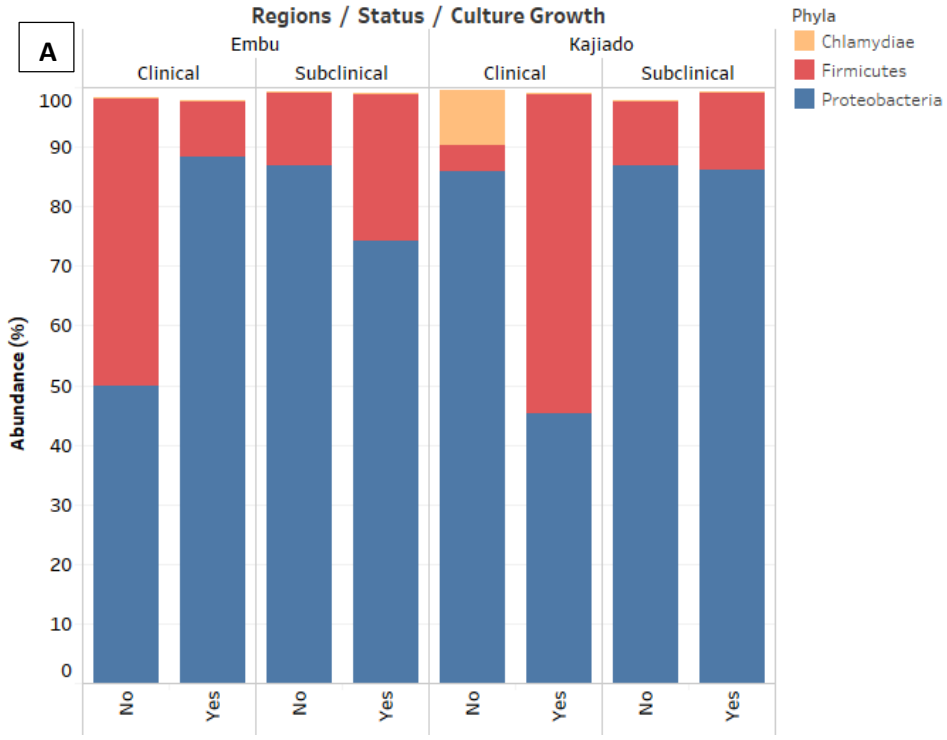


Figure 3.7 A: Taxonomic profile at the phyla level showing the three of the sixteen most abundant bacterial phyla identified in mastitic cow milk using 16S rRNA metagenomics analysis, classified based on region (Embu and Kajiado), disease status (clinical and subclinical) and culture growth (Yes -presence of growth on culture) (No-absence of growth on culture).

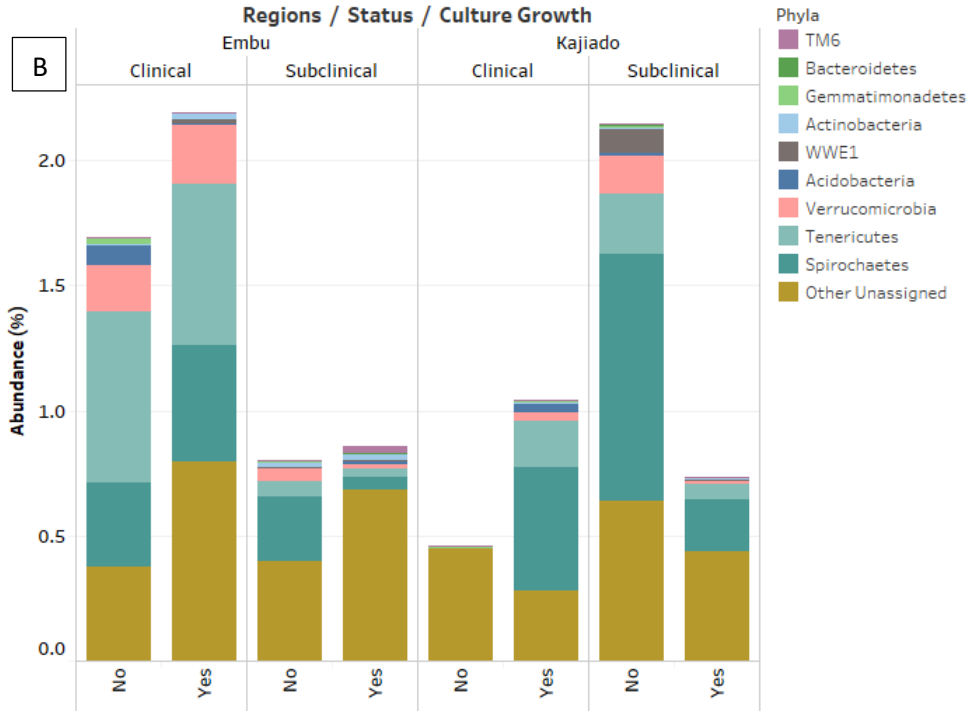


Figure 3.7 B: Taxonomic profile at the phylum level showing ten of the thirteen abundant bacterial phyla identified in mastitic cow milk using 16S rRNA metagenomics analysis, classified based on region (Embu & Kajiado), status (clinical & subclinical) and culture growth (Yes -presence of growth on culture) (No-absence of growth on culture).

Of the identified bacterial phyla, *Proteobacteria* was the most dominant across all categories with the highest relative abundance of 86% observed in culture-positive clinical mastitis samples from Embu. *Firmicutes* were the second abundant phylum with the highest abundance observed in culture-positive clinical mastitic samples from Kajiado at 53%. Further, although no statically significant differences were observed, the phylum *Chlamydiae* had a relatively higher abundance in clinical mastitis cases compared to subclinical mastitis. More specifically, the highest relative abundance of 9.2% was observed in clinical mastitic culture-negative samples from Kajiado.

The phylum *Tenericutes* were also significantly higher in abundance in clinical mastitic samples compared to those from subclinical mastitis ($p=0.04$). More specifically higher relative abundance of 1.3% was reported in clinical samples from Embu County. Further analysis of this phylum revealed only two genera namely; *Mycoplasma* at (0-0.03%) and *Acholeplasma* at (0-0.02%) were associated mastitis in this study. The relative abundance of *Gemmatimonadetes* significantly differed across the three categories ($p<0.05$) while the phylum *TM6* was only observed in culture-positive subclinical samples from Embu in this study (Table 3.4 and Figure 3.7 A&B).

The mastitis cow milk microbiome was dominated by 11 the bacterial genera that had a relative abundance greater than 0.01% in at least one sample in this study. Of the bacterial genera reported, the three most abundant were *Pseudomonas* at (2.6%-83.8%), *Acinetobacter* (0.4%-30.5%) and *Staphylococcus* (0.4%-22.8%). Other genera with lower abundance reported in this study included; *Streptococcus* (0.2%-23.9%), *Rickettsiella* (0-0.3%), *Ralstonia* (0.005%-0.2%), *Enhydrobacter* (0.2%-0.98%), *Rheinheimera* (0.002%-0.8%), *Facklamia* (0.1%-2.0%) ad *Turicibacter* (0.2%-0.8%) (Table 3.4 and Figure 3.8).

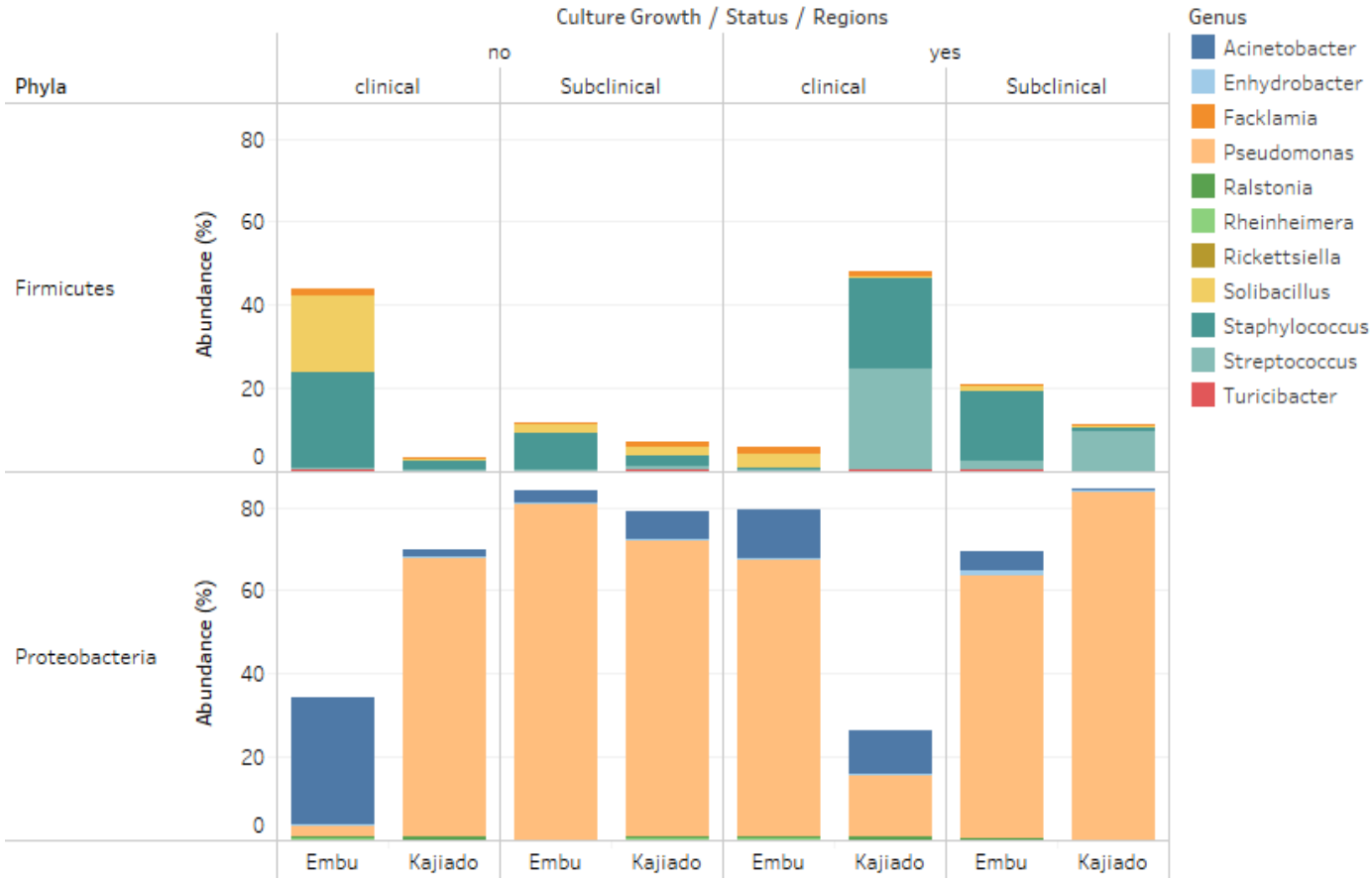


Figure 3.8: Taxonomic profile at genus level showing all the 11 bacterial genera identified in mastitic cow milk using 16S rRNA metagenomics analysis, classified based on region (Embu or Kajiado), status (clinical or subclinical) and culture growth (Yes -presence of growth on culture) or (No-absence of growth on culture).

Based on the disease status, three bacterial genera were found to be significantly different between clinical and subclinical mastitis samples in this study which included *Pseudomonas* (p=0.01), *Acinetobacter* (p=0.02), and *Solibacillus* (p=0.01). The genus *Pseudomonas* was significantly higher in the subclinical mastitis samples compared to the clinical mastitis ones (p=0.04). More specifically, the highest relative abundance of *Pseudomonas* was reported in culture-positive subclinical mastitis samples from Kajiado at (83%). Further, significantly higher relative abundance in *Acinetobacter* and *Solibacillus* were observed in samples with clinical mastitis compared to subclinical mastitic ones (p=0.02; p=0.01). The relative abundance of both *Acinetobacter* (30%) and *Solibacillus* (18%) was significantly increased in culture-negative clinical mastitic samples from Embu County (Table 3.4 & Figure 3.8).

Notably, from the 32 milk samples defined as culture-negative using the aerobic culture method in this study, 16S rRNA analysis identified phyla and genera of bacteria that are known to cause mastitis in dairy cows. These genera included; *Pseudomonas* (2.6%-81%), *Staphylococcus* (2.5%-22.8%), *Streptococcus* (0.2%-0.3%), *Mycoplasma* (0%-0.03%) and phylum *Chlamydiae* (0.004%-9.2%). As shown in Table 3.4 the genera, *Pseudomonas*, *Staphylococcus*, and *Streptococcus* were prevalent in almost all the culture-negative samples. However, for the phylum *Chlamydiae* increased relative abundance was observed in clinical mastitic milk samples from Kajiado County at 9.2%. Similarly, the genera *Mycoplasma* was reported higher in clinical samples from Embu at 0.03%. Unexpectedly, a high relative abundance of the genus *Staphylococcus* at 22% was observed

in culture-negative clinical mastitis samples from Embu compared to the other categories in this study (Table 3.4).

Further, based on the study counties, the 32 culture-negative clinical mastitic milk samples from Embu and Kajiado were similarly dominated by four main bacterial genera. These genera were *Pseudomonas* (2.6%-66.9%), *Acinetobacter* (2.6%-30.5%), *Staphylococcus* (2.5%-22.8%) and *Solibacillus* (0.002%-18.5%). However, their relative abundance between the two counties differed. Higher abundance in *Acinetobacter* (30.5%), *Staphylococcus* (22.8%) and *Solibacillus* (18.5%) being observed in milk samples from Embu as compared to those from Kajiado County. On the other hand, milk samples from Kajiado County showed a higher relative abundance of *Pseudomonas* (66.9%) compared to those from Embu which had (2.6%) (Table 3.4).

In this study, 34/66 samples were culture positive. Of these, CNS was reported in (28%, n=19) of the samples followed by *Streptococcus* species at (10.6%, n=7), *Staphylococcus aureus* at (9%, n=6), *Bacillus* species (9%, (n=6) and *Pseudomonas aeruginosa* (6%, n=4). However, 16S rRNA metagenomics analysis of the same samples revealed a higher percentage of some of those taxa which included *Pseudomonas* (14%-83.8%), followed by *Streptococcus* (0.3%-23%) and *Staphylococcus* (0.4%-21%). In addition, two other genera, *Acinetobacter* (0.4%-14%) and *Solibacillus* (0.4%-3%) not identified using culture method were reported in increased abundance in the 16S rRNA metagenomics analysis. Moreover, other genera with lower abundances reported in metagenomics analysis of culture-positive samples included *Ralstonia, Rheinheimera,*

Enhydrobacter, Facklamia, Turicibacter, Mycoplasma, Rickettsiella and Acheloplasma (Table 3.4).

3.5 DISCUSSION

This study investigated the prevalence, bacterial diversity of mastitic milk from dairy cows in Embu and Kajiado Counties. The knowledge on the prevalence of mastitis and the bacterial diversity associated with the disease development would greatly improve control, prevention and guide on respective treatment.

3.5.1 Prevalence of clinical and subclinical mastitis based on California Mastitis Test (CMT) in Embu and Kajiado Counties

Overall, a relatively high cow level prevalence of mastitis (80%) was reported in Embu and Kajiado counties in this study. Ondiek & Kemboi (2018), reported a slightly higher prevalence of mastitis at (82.9%) in their study in Njoro, Kenya. High prevalence of cow mastitis in other African countries such as Uganda (86.5%), Ethiopia (76%) and Tanzania (70.9%) and Rwanda (76.2%) have been reported (Abrahmsén *et al.*, 2014, Abebe *et al*., 2016, Suleiman *et al*., 2018, Ndahetuye *et al.*, 2019). On the contrary, a slightly lower prevalence of mastitis was reported by Mureithi & Njuguna, (2016) (64%) and Gitau *et al*. (2014) (54.2%) in studies carried out in different parts of Kenya. The discrepancies in the prevalence of mastitis in these studies could be linked to differences in management practices, geographical and breed factors which have been shown to directly influence the occurrence and recurrence of mastitis (Ramírez *et al*., 2014, Taponen *et al*., 2017). The high prevalence of mastitis reported in this study could be indicative of inadequate

monitoring and lack of specific mastitis control programs in the study areas as evidenced during field sampling and has been reported in other studies in Kenya and Africa (Mureithi & Njuguna, 2016, Adamu *et al*., 2020).

Clinical mastitis was reported at 6.8% in this study. These findings were closely related to what was reported by Sarba & Tola, (2017) and Zeryehun & Abera (2017) both in Ethiopia who reported a prevalence of (9.9%) and (10%) respectively. However, a slightly higher prevalence of clinical mastitis has been reported in other countries such as Japan at (12%) (Amer *et al*., 2018) and Canada at (23%) (Levison *et al*., 2016). A lower prevalence of clinical mastitis ranging between 0.5% to 0.9 % was reported in different parts of Kenya (Gitau *et al*., 2014). Accordingly, Gao *et al*. (2017), also reported a prevalence of 3.3% in China.

The prevalence of subclinical mastitis in this study was about 74%. These findings were in agreement with studies in Ethiopia and Rwanda which reported prevalence of subclinical mastitis at (76%) and (76.2%) respectively (Abebe *et al.,* 2016, Ndahetuye *et al*., 2019). A higher prevalence of subclinical mastitis has also been reported in Kenya at (82.9%) (Ondiek & Kemboi, 2018) and Ethiopia (85%) (Tolosa *et al.,* 2015). However, slightly lower results have been reported previously in different parts of Kenya at (49.6%-64%) (Mureithi & Njuguna 2016, Gitau *et al.,* 2014), Ethiopia (34.1%-40.1%) (Bihon *et al.,* 2018), and Rwanda (50.5%) (Mpatswenumugabo *et al.,* 2017). The prevalence of mastitis has been shown to vary from country to country and among farms and cows (Motaung *et al*., 2017). The high prevalence of subclinical mastitis observed in this study may be due to the poor milking and environmental hygiene standards observed in the study areas (Ramírez *et al*., 2014, Bihon *et al*., 2018, Ismael, 2018). Moreover, the high number of

older cows with a previous history of mastitis, reported in this study, could be a contributing factor (Jamali *et al*., 2018).

A significantly higher prevalence of sub-clinical mastitis (74%) was reported in this study compared to clinical (6.8%) mastitis. Other studies in Tanzania (Zeryehun & Abera, 2017) and Kenya (Gitau *et al*., 2014) also reported a higher prevalence of subclinical mastitis compared to clinical mastitis. Indeed, incidences of subclinical mastitis have been reported to be 40 times more prevalent compared to clinical mastitis (Ruegg, 2017). The lower prevalence of clinical mastitis has been linked to the fact that clinical mastitis can easily be diagnosed and treated (Motaung *et al.*, 2017). On contrary, the sub-clinical form lacks physical abnormalities; hence it is hardly diagnosed by the farmers and continues to be a source of infection on the farm (Abebe *et al*., 2016, Ismael, 2018). In addition, ineffective mastitis control programs and poor hygiene standards in the study areas could also have been key contributors (Abrahmsén *et al.,* 2014). Mastitis control programs tailed based on local specific risk factors within the study areas should be implemented.

In the present study, Embu County had a significantly higher cow and quarter-level prevalence of mastitis compared to Kajiado County. The higher prevalence of mastitis reported in Embu County could be associated with poor hygiene and the wet weather experienced between March and June 2019 during the sampling period. Increased prevalence of mastitis has been reported in wet seasons compared to dry seasons (Moosavi *et al.,* 2014, Kurjogi & Kaliwal, 2014). Moreover, in Embu County, it was observed that cows stayed in dirty beddings and poorly drained houses. Poor drainage, dirty beddings and muddy floor have been linked to high bacterial contamination of the udder with environmental mastitis pathogens (FAO, 2014, Mureithi & Njuguna, 2016).

Improvement of hygiene measures for successful control of mastitis in the region is therefore, highly recommended.

3.5.2 Causative bacteria of mastitis in Embu and Kajiado Counties

The predominance of Coagulase Negative mastitis (CNS) in clinical and subclinical mastitis have been reported in several studies (Mekonnen *et al*., 2017, Amer *et al*., 2018, Ndahetuye *et al*., 2019). Epidemiological studies have shown that CNS are emerging bacteria, increasingly being isolated from cases of cow mastitis worldwide (Piessens *et al*., 2011, Sender *et al*., 2017, Vakkamäki *et al.*, 2017). However, this study's findings, contrasted with those of Gitau *et al*. (2014), Mureithi & Njuguna, (2016) and Ondiek & Kemboi, (2018) all in Kenya, who reported that *Staphylococcus aureus* was the dominant mastitic causative bacteria.

The present results demonstrate the emergence of CNS as the main causative bacteria of mastitis in dairy cows in Kajiado and Embu counties. CNS were initially classified as minor pathogens of mastitis (Piessens *et al.,* 2011). This transition from *Staphylococcus aureus* to CNS may imply the need to revise control procedures. The high prevalence of CNS in this study might be explained by the fact that the bacteria which are normal flora of the skin, could be originating from milker's hands or cow skin during milking (Piessens *et al.,* 2011, Frey *et al.,* 2013). The environmental origin of CNS has also been described (El-jakee *et al*., 2013, Kim *et al.*, 2019). Therefore, epidemiological characterization of CNS isolates will be necessary to confirm circulating species and the specific factors contributing to the increase in CNS mastitis in Kenya.

Streptococcus agalactiae, a key contagious mastitis-causing pathogen, was reported at a low

prevalence of 0.4% in this study. Similarly, previous studies in Kenya, investigating the prevalence of mastitis-causing pathogens by Gitau *et al*. (2014) and Mureithi *et al*. (2017), reported a prevalence of 5.2% and 8.4% respectively. This low prevalence indicates that *S. agalactiae* is no longer a major problem in bovine mastitis in the study areas. Improvement of specific control programs targeting contagious pathogens such as milking and udder hygiene has significantly led to the reduction of *S. agalactiae* and *S. aureus* in most countries (Zadoks & Fitzpatrick 2009, Tomazi *et al*., 2018).

Mastitis due to environmental *Streptococcus* species (other than *S. agalactiae*) was reported in this study at 22.2%. These findings were comparable to findings by Birhanu *et al*. (2017) at (22%) in Ethiopia and those by Gitau *et al*. (2014) at 20.6% in Kenya. A higher prevalence of environmental streptococcal mastitis at (36%) has been reported previously in Kenya (Ndirangu *et al*., 2017). In contrast, a lower prevalence of environmental streptococcal mastitis has also been reported in various countries including Kenya (10.8%) (Odongo *et al*., 2012) Finland (14%) (Vakkamäki *et al.*, 2017), and Japan (9%) (Amer *et al*., 2018). The inconsistencies in prevalence in these studies could be due to managemental, environmental, epidemiological and breed factors (Taponen *et al*., 2017).

A rise in the prevalence of environmental mastitis due to *Streptococcus* species are consistently being reported worldwide (Zhang *et al*., 2018). This high prevalence of environmental streptococcal mastitis in this study could be due to udder contamination from the cow's environment or the water source (Zadoks *et al*., 2011, Amer *et al.*, 2018). Providing a clean environment, frequent removal of slurry and use of teat dips after milking have shown to

significantly reduce the rate of streptococcal mastitis (Klaas & Zadoks, 2017). Therefore, such practices should be adopted in the study areas.

Further, *Bacillus* species were reported in this study at a prevalence of 7.6%. This was slightly higher compared to a previous report by Odongo *et al.* (2012) in Kenya who reported a prevalence of 4.6%. Other studies have reported *Bacillus* species as important pathogens of mastitis (Sadashiv *et al*., 2014). A higher prevalence of bacillus mastitis has been reported in Japan (22%) (Amer *et al*., 2018). However, a lower prevalence of bacillus mastitis has also been reported in Ethiopia (1.3%-4.2%) (Zeryehun & Abera, 2017, Fisseha *et al*., 2020). *Bacillus* organisms have been reported as important emerging mastitis-causing pathogens (Sadashiv *et al.,* 2014). In this study, the presence of *Bacillus* organisms could be due to environmental contamination of the udder by mud or manure (Sadashiv *et al*., 2014). Therefore, improving environmental hygiene, milking hygiene and the use of teat sealants could reduce such infections (Kromker *et al*., 2014).

In this study, the prevalence of *Bacillus* spp. and *Escherichia coli* were significantly higher in Embu County compared to Kajiado County. Studies elsewhere have shown that such differences are commonly linked to geographic variations (Taponen *et al.*, 2017). Since these organisms are environmental mastitis-causing pathogens, soiled bedding, manure and dirty cow environment, observed in Embu County, are important sources of infections (Schauer *et al*., 2021).

Similar to a finding in Canada (Levison *et al.*, 2016), lack of growth was reported in 20% of the samples. These findings were lower than those reported by Richards *et al*. (2019) in Kenya who reported lack of growth in 37.5% of the mastitis cases, but higher than those reported by Gitau *et*

al. (2014) at (10%), also in Kenya. Globally, about 10-40% of mastitis cases remain culture-negative in aerobic classical routine culture method (Kuehn *et al*., 2013, Bhanderi *et al*., 2014). The lack of growth in samples has remained a challenge in the diagnosis of mastitis worldwide (Oikonomou *et al.,* 2012). Limitations of the culture method, low level of bacteria in milk, cow pretreated with antibiotics, and non-bacterial causative agents of mastitis have been described as key reasons for lack of growth in mastitic milk (Oikonomou *et al*., 2012, Kuehn *et al*., 2013).

3.5.3 16S rRNA metagenomics analysis

Unlike most cow milk studies using 16S rRNA metagenomics analysis that have focused on microbial diversity between healthy and mastitic milk, this study being the first one in Kenya explored the bacterial diversity of clinical and subclinical mastitic cow milk from two different counties based on culture growth diagnosis (Oikonomou *et al.,* 2012; 2014, Kuehn *et al.,* 2013).

Three bacterial genera namely; *Staphylococcus*, *Streptococcus* and *Pseudomonas* diagnosed by culture were also reported in higher percentages in 16S rRNA metagenomics. However, the results of these three genera did not compare well between the two methods. This is because the percentages of those taxa were reported higher in the 16S rRNA metagenomics analysis compared to culture. Furthermore, eight other abundant genera were recovered using metagenomics analysis and not in culture. This lack of direct comparison between culture and 16S rRNA metagenomics analysis has been described (Oikonomou *et al.,* 2012; 2014). These results demonstrate that classical culture methods fail to describe the true bacterial complexity of mastitic cow milk (Oikonomou *et al.,* 2012; 2014; Kuehn *et al.,* 2013).

3.5.3.1 Alpha and Beta diversity indices

Most of the alpha and beta diversity comparisons showed that there were no significant differences in bacterial number and diversities in milk from quarters among the three groups except for Shannon and Simpson diversity indices. These results indicate that most of the bacteria were shared among the different three groups in this study. Consistent with this study's findings, Catozzi *et al.* (2017), found no cluster discrimination between samples from clinical and subclinical mastitis in their study in water buffaloes in France. Similarly, Oikonomou *et al.*, 2012, in their study in the USA found no significant differences between bacterial diversity in clinical and subclinical mastitis in bovine milk. However, in the same study, they reported discriminate clustering between samples from the healthy quarters from those with clinical mastitis but culture-negative (Oikonomou *et al.*, 2012).

Further, the variation observed in Shannon and Simpson diversity indices in this study could be explained by the significantly increased relative abundance of the genera *Acinetobacter* and *Solibacillus* in clinical mastitis samples in comparison to those from the other groups. These results indicate disease status of the milk could have had more impact on bacterial diversity of mastitic milk than region and culture growth status. However, the lack of clear separations of the samples in the beta analysis using the Principal coordinate analysis (PCoA) plots across the three categories indicate that there were no significant differences in the number of different bacterial communities in mastitic milk in this study.

3.3.3.2 Bacterial communities identified using 16S rRNA metagenomics analysis

Similar to other studies, phyla *Proteobacteria* and *Firmicutes* were the most abundant across the groups. Pang *et al.* (2018) in China found that *Proteobacteria* and *Firmicutes* were the most abundant phyla in mastitic and healthy milk. Zhong *et al.* (2018), China also reported that *Proteobacteria* (33.6%), followed by *Firmicutes* (28.8%) were the dominating phyla in manure from dairy cows. Another study in the USA reported a higher abundance of *Proteobacteria* in mastitic milk driven by *Enterobacteriaceae* and *Pseudomonas* when compared to milk from healthy quarters in cows (Ganda *et al.*, 2016).

In contrast, Hoque *et al.* (2019) reported a higher relative abundance in *Proteobacteria* followed by *Bacteroides* in clinical mastitis milk samples as compared to those from healthy quarters in cows in the USA. Falentin *et al.* (2016), found that *Firmicutes, Bacteroides*, and A*ctinobacteria* were the most abundant bacterial phyla in mammary gland microbiota in the bovine quarters with a history of mastitis in France. The dominance of *Proteobacteria* and *Firmicutes* in mammary glands has been linked to the existence of a potential endogenous entero-mammary pathway facilitating the migration of gut bacteria into the mammary gland (Hoque *et al.*, 2019). These results indicate that similar to other studies globally, *Proteobacteria* and *Firmicutes* were the main bacteria in mastitic milk from Kajiado and Embu, Kenya across all groups and provide confidence in this approach.

In this study, increased relative abundance of the phylum *Chlamydia*e was observed in culture-negative clinical mastitis samples from Kajiado County compared to those in the other categories. These findings were consistent with those by Falentin *et al.* (2016), who reported an increased

relative abundance of phylum *Chlamydia*e in quarters with a previous history of clinical mastitis in France. *Chlamydia* species such as *C. abortus* and *C. pecorum* are well-known pathogens of chronic and subclinical mastitis in cows and can be identified using specific culturing method or by the use of polymerase chain reaction (PCR) (Biesenkamp-Uhe *et al.,* 2007, Reinhold *et al.,* 2011). Although the dawn of molecular techniques has led to the improvement in the diagnosis of most of the *Chlamydia* species, difficulties in identifying some of the species have been reported (Reinhold *et al.,* 2011). Further, the zoonotic potential of some *Chlamydia* spp. including *C. abortus* has been described (Harkinezhad *et al.,* 2009). Therefore, the increased relative abundance of *Chlamydiae* in culture-negative clinical mastitic samples in this study is a public health concern and warrants further investigation and surveillance (Reinhold *et al.,* 2011).

In this study, the phylum *Tenericutes* were also significantly higher in relative abundance in clinical mastitis compared to samples from subclinical mastitis (p=0.04). The presence of *Tenericutes* in mastitic milk has been described in several studies (Hoque *et al.,* 2019, Sokolov *et al.,* 2021). Among the *Tenericutes,* an important genus *Mycoplasma* spp. a known causative agent of mastitis was reported in increased abundance in the culture negative mastitis samples from Embu County. Although, the prevalence of *Mycoplasma* spp. was low (0.03%) in this study, it is the first report from the Kenyan dairy. *Mycoplasma* species are important pathogen of mastitis that are difficult to identify using culture methods and therefore their prevalence remains unknown in most countries (Dudek *et al.,* 2020, Sokolov *et al.,* 2021). Furthermore, mycoplasma mastitis is a contagious organism which is hard to treat, manage (Dudek *et al.,* 2020). Future studies should consider the

use of more sensitive molecular techniques to further characterize *Mycoplasma* species in culture-negative mastitic samples (Sokolov *et al.*, 2021).

Three genera namely; *Pseudomonas, Acinetobacter* and *Solibacillus* were found to be significantly different between clinical and subclinical mastitic samples in the current study. A significantly higher relative abundance of *Pseudomonas* was reported in subclinical mastitis samples compared to clinical samples (p=0.01). *Pseudomonas* spp are well-known causative agents of mastitis in ruminants including in dairy cows (Zadoks, 2017). Kuehn *et al.* (2013), however, found a higher abundance of *Pseudomonas* in healthy cow milk samples compared to clinical mastitic ones in their study in the USA. Similarly, Liu *et al.* (2020) reported a higher relative abundance of *Pseudomonas* spp. in dairy raw milk in the USA. The high level of *Pseudomonas* in culture-negative mastitis samples in this study is surprising since *Pseudomonas aeruginosa* can easily be cultured in the laboratory. These findings suggest that other *Pseudomonas* spp. not easily cultured could be associated with mastitis (Kuehn *et al.,* 2013). In the USA, *Pseudomonas* has been isolated in water sources and studies have reported infection of the udder or contamination of samples due to water positive for *Pseudomonas* (Kuehn *et al.,* 2013, Liu *et al.,* 2020). However, characterization of *Pseudomonas* to species level will beneficial and their role in milk warrants further investigation.

In this study, significantly higher *Acinetobacter* was reported in clinical mastitis with no culture growth in Embu compared to the other groups. Catozzi *et al.* (2017) found a higher abundance of *Acinetobacter* genera in subclinical mastitis in water buffalos compared to the healthy group in Italy. Studies in China by Pang *et al.* (2017) and India by Patel *et al.* (2017) found that

Acinetobacter was more prevalent in healthy milk compared to mastitic milk in cows and humans respectively. *Acinetobacter* is frequently identified on the skin of the teats (Derakhshani *et al.,* 2018). However, *Acinetobacter* has also been described as the core microbiota of milk with its pathogenesis of mastitis been described as minimal (Catozzi *et al.,* 2017, Derakhshani *et al.,* 2018). Although the role of *Acinetobacter* in milk is still unclear, these organisms are well known for multidrug resistance and a clinically relevant human pathogen (Hoque *et al.,* 2019, Chapartegui-González *et al.,* 2021). Therefore, the presence of a high relative abundance of *Acinetobacter* in clinical culture-negative mastitic samples in this study is a public health concern and warrants further investigation and surveillance (Pang *et al.,* 2017).

Solibacillus (30%) was significantly higher in culture negative clinical mastitic samples from Embu (p=0.05). Kusumawati *et al.* (2021) in their study in Indonesia, reported the presence of *Solibacillus* in subclinical milk at 8%. However, unlike in our study, Catozzi *et al.* (2017), Italy, reported an increased abundance of *Solibacillus* in healthy milk and decreased abundance in clinical mastitis in water buffaloes. *Solibacillus* has been regarded as a fecal bacterium in dairy cows (Catozzi *et al.,* 2017, Kusumawati *et al.,* 2021). Although fecal contamination of milk during sample collection, and possibly interfering with microbial diversity of milk have been described, it is unlikely that it occurred in these samples since *Solibacillus* seem to decrease significantly in the other categories. Therefore, its role in milk should be further investigated. Further, our findings suggest that geographical location had an impact on the relative abundance of *Solibacillus* since the increased abundance was only reported in Embu County (Liu *et al.*, 2020).

A high number of unassigned bacteria (22.5%) were reported in this study. High numbers of unassigned bacteria have been reported previously (Ganda *et al.,* 2016, Falentin *et al.,* 2016, Hoque *et al.,* 2019). The presence of a higher number of bacteria not assigned to any pathogenic group could be indicative of dysbiosis (Hoque *et al.,* 2019). Dysbiosis in mammary glands has shown to not be caused by a change in microbial composition but also by an increase in nonspecific intramammary infectious bacteria (Falentin *et al*., 2016, Ganda *et al.,* 2016,).

Interestingly in this study, genera *Staphylococcus* which are aerobic bacteria and easily cultivable were identified in high abundance in culture-negative clinical mastitis. The genus staphylococci are well described as a dominant bovine and human mastitis pathogens in most microbial diversity studies (Falentin *et al.,* 2016, Oikonomou *et al.,* 2020). The presence of this bacteria in culture-negative samples could indicate that the quarters could have been pretreated with antibiotics before culture (Oikonomou *et al.,* 2012). In addition, since the volume of milk samples used for metagenomics analysis is 100 times more as compared to that used in the culture method, a higher likelihood of finding pathogenic bacteria in metagenomics studies compared to culture has been described (Oikonomou *et al.,* 2012). Further, although the culture method is only able to identify bacteria that are alive and reproducing at the time of analysis, metagenomics analysis can detect bacteria that are in low numbers including DNA from dead bacteria (Oikonomou *et al.,* 2012).

In this study, a relative increase in abundance, of *Acinetobacter, Staphylococcus*, and *Solibacillus* in culture-negative clinical samples from Embu compared to Kajiado was observed. On the other hand, samples from Kajiado County had a higher abundance of *Pseudomonas* compared to those

from Embu. Variation of microbiota based on geographical areas and herds has been described (Hoque *et al.,* 2019). Further, although the relative abundance between culture-negative differed between the two counties, similarities were observed in the number of abundant genera common to both counties suggesting a great degree of similarities within the two geographical regions.

3.6 CONCLUSION AND RECOMMENDATION

The current study reported a high prevalence of subclinical mastitis dominated by Coagulase-negative *Staphylococcus* (CNS) using culture and *Pseudomonas,* by 16S rRNA metagenomics. Therefore, action need to be taken prevent bovine mastitis in the two counties in Kenya.

Alpha and beta diversity indices analysis showed that there were no significant differences in number and diversities of bacterial communities in milk from mastitic quarters based on the study counties, culture growth and disease status. These lack of differences may indicate the similarities of management practices within the two counties.

The 16S rRNA metagenomics analysis identified increased relative abundance of some phyla and genera which are important mastitis pathogens that are hard to culture such as *Chlamydiae, Mycoplasma, Solibacillus* in culture-negative mastitic milk. These findings highlight the usefulness of using more sensitive technique such 16S rRNA metagenomics in the diagnosis of mastitis.

16S rRNA metagenomics analysis revealed more phyla and genera than conventional culture methods showing that culture methods alone do not adequately identify mastitis-causing bacteria since the majority appear to be unculturable.

Based on the high prevalence of subclinical mastitis dominated by CNS and *Pseudomonas* in the two counties there is a need to create awareness to the farmers regarding subclinical mastitis and the respective control measures such as milking mastitic cow last, using udder cloth for each cow, and culling mastitic cows. Additionally, CNS and *Pseudomonas* be monitored routinely and further characterized to species level in order to improve mastitis management in the counties.

CHAPTER FOUR: PHENOTYPIC AND GENOTYPIC ANTIBIOTIC RESISTANCE PROFILES OF BACTERIA ISOLATED FROM MASTITIC MILK IN THIS STUDY

4.1 INTRODUCTION

Numerous pathogens have been documented as causative agents of clinical and subclinical mastitis in dairy cows globally (Motaung *et al.*, 2017). Bacteria are the most common cause of mastitis, and more than 140 different pathogenic species have been reported (Ruegg, 2017). Several reports globally including Kenya have reported that the main frequently isolated organisms include; CNS, *S. aureus Streptococcus* spp, *E. coli*, and *P. aeruginosa* (Gao *et al.*, 2017, Cheng *et al.*, 2019).

Antimicrobial therapy is the primary tool for controlling mastitis in dairy cows in most countries globally (Oliver *et al.*, 2011, Nobrega *et al.*, 2019). However, the efficacy of antimicrobial therapy against most mastitis-causing pathogens including *Staphylococcus* spp., *Streptococci* spp., *E. coli,* and *Pseudomonas* spp. is on the decline (Qu *et al.*, 2019). This low cure rate of mastitis-causing pathogens is, in part, associated with an increase in multi-drug resistance accelerated by overuse and misuse of antimicrobials in veterinary practice (Wang *et al.*, 2018, Cheng *et al.*, 2019). Of great concern, is the high level of resistance to β-lactam antibiotics and other antibiotics including antimicrobial of last resort being reported among mastitis-causing bacteria from bovine milk (Vanderhaeghen *et al.*, 2010, Yang *et al.*, 2020).

Multidrug-resistant (MDR) bacteria, including methicillin-resistant staphylococci (MRS), and carbapenem-resistant *P. aeruginosa* are emerging global public health problems (Smith, 2015, Anjum *et al.*, 2019). Mastitic milk has been documented as an important reservoir of these

multidrug-resistant strains (Verraes *et al*., 2014, Liu *et al.,* 2018, Schauer *et al.,* 2021). Increased risk of clonal transmission of multidrug bacteria between dairy cows and persons in contact with the animals has also been described (Sharma *et al*., 2018, Schauer *et al.,* 2021). This growing trend presents a serious threat to mastitis management and poses a potentially significant public health risk to humans consuming or handling raw milk (Sharma *et al.,* 2018).

While poorly quantified, the burden of antimicrobial resistance (AMR) attributable to livestock is comparatively higher in low-income countries, including Kenya and wider sub-Saharan Africa (Grace, 2015, Van *et al*., 2020). One of the key drivers of this is the largely unregulated use of veterinary antimicrobials in Kenya (Gitau *et al*., 2014, Van *et al.,* 2020). In addition, Kenyan farmers often self-diagnose and treat cows suffering from mastitis without laboratory confirmation to guide therapy, and there is a general lack of stringent measures on drug withdrawal periods (GARP, 2011, Gitau *et al*., 2014). As a consequence, these practices have significantly contributed to the emergence and spread of antibiotic-resistant bacterial strains resulting in treatment failures (Van *et al*., 2020). Given this scenario, there is an urgent need for evidence-informed policy on antibiotic use and the reduction of antimicrobial resistance in dairy farm bacteria in Kenya (FAO, 2016).

Although, a few studies in Kenya have investigated the prevalence of phenotypic antimicrobial resistance in *Staphylococcus aureus* and *Streptococcus agalactiae* (Shitandi & Sternesjö, 2004, Odongo *et al.,* 2012, Gitau *et al.,* 2014, Mureithi *et al.,* 2017, Ndirangu *et al.,* 2017); no studies have comprehensively investigated the phenotypic and genotypic antimicrobial resistance profiles

of CNS, *E. coli* and *P. aeruginosa* the key mastitis pathogens prevalent in Kenya. This is a problem that requires serious attention because increasing antimicrobial resistance continues to cause heavy economic losses and many deaths in animals and humans (Sriram *et al.,* 2021, FAO, 2021).

This study aimed to establish phenotypic antimicrobial susceptibility profiles of *Staphylococcus* species, other *Streptococcus* species, *E. coli*, *P. aeruginosa* and also to determine the presence of selected antimicrobial resistance genes in staphylococci isolates from mastitis milk from dairy cattle in Embu and Kajiado, Kenya. This information is key to improve mastitis therapy and control the emergence and spread of antimicrobial resistant bacteria in Kenya.

4.2 MATERIALS AND METHODS

4.2.1 Study area

As described in Chapter 3

4.2.2 Selection of the isolates for antibiotic susceptibility testing

For detection of phenotypic antibiotic susceptibility testing (AST), only five types of bacteria were analyzed in detail out of the 10 different bacteria obtained in this study. These five included; *Staphylococcus aureus*, Coagulase-negative staphylococci, other *Streptococcus* species (defined as non agalactiae streptococcus species in this study), *Pseudomonas aeruginosa* and *Escherichia coli.* The other five types of bacteria isolated from mastitic quarters in this study were not analyzed for AST due to their low prevalence rate in mastitic samples and limitation of finances (section 3.2.3). Further, molecular characterization of the bacteria isolate was only done on *S. aureus* and detection of antibiotic resistance genes was carried out in *Staphylococcus* isolates alone in this study.

4.2.2.1 *Staphylococcus aureus* and Coagulase-negative Staphylococci (CNS)

Overall a total of 183 *Staphylococcus* organisms (91 *S. aureus* and 92 CNS) isolated from milk samples as described in Chapter 3 above was used. The isolates were randomly selected from the available 595 staphylococcal isolates recovered from 395 dairy cows in 154 smallholder farms. The distribution of the 183 staphylococci isolates among the counties was as follows: for *S. aureus*: 58 isolates from 31 cows in Embu County; 43 isolates from 29 cows in Kajiado County. For CNS; 53 isolates from 48 cows in Embu County; 29 isolates from 34 cows in Kajiado County.

4.2.2.2 Other Streptococcus species

A total of 22 isolates classified as other *Streptococcus* species in this study, were randomly selected from the available other streptococcal isolates (n=226) previously isolated in the current study as described in Chapter 3. These 22 streptococcal isolates were recovered from 22 dairy cows in 22 smallholder farms in both counties. Eleven of the isolates came from 11 cows from Embu and other 11 isolates from 11 cows from Kajiado County.

4.2.2.3 *Escherichia coli* and *Pseudomonas aeruginosa*

A total of 31 Gram-negative isolates, comprising of *E. coli* (n=12) and *P. aeruginosa* (n=19) were randomly selected from the available *E. coli* (n=32) and *P. aeruginosa* (n=52) isolates recovered in this study as described in Chapter 3. These isolates used for the determination of the antibiotic susceptibility profile were isolated from 31 dairy cows in 29 smallholder farms. The distribution of the isolates among the counties was as follows; Kajiado; *P. aeruginosa* (n=11 isolates were from 11 cows in 9 farms. Among the *P. aeruginosa* four isolates came from four different cows in two farms). In Embu, P. *aeruginosa* (n=8) isolates were from 8 cows from 8 different farms. For *E. coli* the isolates (n=10), were from 10 cows from different 10 farms while in Kajiado the isolates (n=2) were from 2 cows from 2 farms.

4.2.3 Genotypic confirmation of staphylococci isolates

4.2.3.1 Extraction of Bacteria genomic DNA

Extraction of genomic DNA was done using the boiling method as described by Monday *et al.* (2006). Briefly, a loopful from bacterial colonies grown overnight on Tryptone Soy Agar (TSA) was added to 1.5ml Eppendorf tubes containing 100 μl of nuclease-free water. The tubes were boiled in the water bath at 100^0C for 25minutes. After centrifugation at 30,000 ×g for 5minutes, in a microcentrifuge (Thermo Scientific™) the supernatant was transferred to a new 1.5 microcentrifuge tube. The extracted DNA was stored in a freezer at -20^0 C until used for PCR analysis.

4.2.3.2 *Staphylococcus aureus nuc* gene amplification

A total of 91 biochemically identified *Staphylococcus aureus* were confirmed using Polymerase chain reaction (PCR) through the detection of the staphylococcal thermonuclease (*nuc*) gene. The oligonucleotide primers and protocol used in this study as shown in Table 4.1. The PCR was performed using Taq polymerase (Qiagen, German) following manufacturers' instruction. Briefly, A PCR reaction mixture volume of 25 µL containing 12.5 µL of master mix (DNA Taq polymerase, dNTPs, MgCl2 and PCR buffer, (Qiagen, German), 5 µL DNA template, 0.625µL of the forward and reverse primers each, and 6.25 µL of nuclease-free water was used. PCR was performed using Thermo Cycler (T100™ Bio-Rad) and the amplification conditions were as follows; initial one cycle of 94 °C for 15 sec followed by a total of 35 PCR cycles run under the following conditions: DNA denaturation at 94°C for 3 secs, primer annealing at 50 °C for 10 sec and DNA extension at 74 °C for 2 min. The final cycle was at 45 °C for 2 sec. *Staphylococcus aureus* ATCC 29213 (*nuc*

positive strains) (CLSI, 2016) and DNase deionized water was used as the positive and negative control respectively. Electrophoresis for each PCR amplicon was performed using Ethidium Bromide stained 1.5% agarose gel in Tris-Borate-EDTA (TBE) buffer and visualized using UV-illuminator (GelMax® Imager, UK). A molecular ladder was used to determine the sizes of the amplicon (Gelpilot 1 kb plus ladder (100), Qiagen, Germany). Positive cases were determined by an amplicon size of 276 bp.

Table 4.1: Details of primers and annealing temperatures used to detect Antibiotic Resistance Genes in the study

Target Gene	Primer sequence (5′- 3′)	Annealing temperature (°C)	Amplicon size (bp)	Reference
nuc	[1]F-GCGATTGATGGTGATACGGTT	50	276	Wang *et al*.,1997
	[2]R-CAAGCCTTGACGAACTAAAGC			
blaZ	F- ACT TCA ACA CCT GCT GCT TTC	54	173	Martineau *et al*., 2000
	R- TGA CCA CTT TTA TCA GCA ACC			
strB	F-CGGTCGTGAGAACAATCTGA	60	313	Pyatov *et al.*, 2017
	R-ATGATGCAGGATCGCCATGTA			
ermB	F- ACGACGAAACTGGCTAA	55	409	Gao *et al.*, 2011
	R-TGGTATGGCGGGTAA			
msrA	F- AAGGCTTGTCCGCAATACAC	60	320	Pyatov et al.,2017
	R- CCATTACCCCCAATAAGTGC			
tetM	F-GTCCGTCTGAACTTTGCGGA	59	662	Gunga *et al.*, 2018
	R- GCGGCACTTCGATGTGAATG			
tetK	F- TTAGGTGAAGGGTTAGGTCC	59	718	Gunga *et al.*, 2018
	R-GCAAACTCATTCCAGAAGCA			
ermC	F- AATCGGCTCAGGAAAAGG	55	562	Pérez-Serrano, 2020
	R- ATCGTCAATTCCTGCATG			

[1]F- forward [2] R-reverse

4.2.4 Phenotypic antibiotic susceptibility profiling

4.2.4.1 Antibiotics and medium used in this study

For *Staphylococcus* isolates, 10 antibiotic discs belonging to seven classes of antibiotics (respective concentrations given in parenthesis) were studied; aminoglycosides (gentamicin 10 µg, streptomycin 10 µg), fluoroquinolone (ciprofloxacin 5 µg and norfloxacin 10 µg), tetracycline (tetracycline 30 µg), Folate pathway inhibitors (sulphonamide + trimethoprim 25 µg), macrolides (erythromycin 15µg), beta-lactams (ampicillin 25 µg and cefoxitin 30 µg) and phenicols (chloramphenicol 10µg). The choice of antibiotics was guided by drugs that are commonly used in dairy veterinary practice in Kenya and some of which are important to human medicine (Omwenga *et al.*, 2020).

For other streptococcal isolates, 10 antibiotic discs belonging to eight classes of antibiotics at the following concentration (respective concentrations given in parenthesis) were analyzed; aminoglycosides (gentamicin 10 µg, streptomycin 10 µg), fluoroquinolone (ciprofloxacin 5 µg and norfloxacin 10 µg), tetracycline (tetracycline 30µg), folate pathway inhibitors (sulphonamide + trimethoprim 25µg), macrolides (erythromycin 15µg), penicillin (ampicillin 25µg), and glycopeptide (vancomycin 30 µg) and phenicols (chloramphenicol 10 µg).

For *P. aeruginosa* and *E. coli*; nine antibiotic discs belonging to seven classes of antibiotics (respective concentrations given in parenthesis) were studied; Aminoglycosides (gentamicin 10 µg, streptomycin 10µg), fluoroquinolone (ciprofloxacin 5µg and norfloxacin 10µg), tetracycline

(tetracycline 30µg), folate pathway inhibitors (sulphonamide + trimethoprim 25µg), macrolides (erythromycin 15µg), beta-lactams (ampicillin 25µg), phenol (chloramphenicol 10µg). Further, for *P. aeruginosa* four extra antibiotics were tested which included; beta-lactams (cefepime 30µg), carbapenems (imipenem (10µg), penicillin (piperacillin, 100 µg), and lipopeptide (colistin 10µg). The medium used to perform all the antibiotic susceptibility testing for all isolates in this study was Mueller Hinton agar (Oxoid, Basingstoke, England, UK).

4.2.4.2 Antibiotic susceptibility testing procedure

Phenotypic antibiotic susceptibility testing was carried out using the Kirby-Bauer disc diffusion method following the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines where applicable (CLSI, 2016, EUCAST, 2021). Briefly, fresh pure cultures of the bacterial isolates were separately suspended in sterile physiological saline and set at turbidity equivalent to 0.5 McFarland turbidity tube. Sterile cotton wool swabs were then used to inoculate the standardized bacterial suspension onto Mueller-Hinton (Oxoid) agar plates. In order to get a confluent growth, the cotton swabs containing the culture were spread evenly on the Mueller Hinton agar and allowed to dry. Respective antibiotic discs were then placed on the inoculated agar and the plates were incubated at 35°C - 37°C for 17 hours. Susceptibility was demonstrated by the presence of an inhibition zone around the respective disc; interpretation of which was guided by CLSI (2016) and EUCAST (2021).

Since most of the antibiotics used have no approved bovine-defined breakpoints for staphylococci, streptococci, *E. coli*, and *P. aeruginosa*, human-derived interpretive criteria described by CLSI,

(2016) and EUCAST (2021) were used. For antibiotics that had appropriate breakpoints given, the isolates were interpreted, based on the inhibition-zone size, as either susceptible, intermediate, or resistant to the tested antibiotic agent. However, in this study, all strains that fitted in the "intermediate" category were considered as resistant. *S. aureus* ATCC 25923, *S. pneumoniae* ATCC 49619, *E. coli* ATCC 49619 and *P. aeruginosa* ATCC 27853 were used as the quality control strains. Isolates were classified as being multidrug-resistant (MDR) if they were found to be resistant to at least one antibiotic in three or more different antimicrobial classes (Magiorakos *et al*., 2012).

Staphylococcal isolates (*S. aureus* and CNS) resistant to cefoxitin were presumptively identified as methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant Coagulase negative staphylococcus (MRCNS) respectively (CLSI, 2016, EUCAST, 2021). Due to the lack of approved CLSI clinical breakpoints for streptomycin for the staphylococcal isolates, likely sensitivity was interpreted using the interpretive criteria of class representative antibiotics (gentamicin 10µg) in this study (CLSI, 2016). Similarly, there are no clinical breakpoints for aminoglycoside in streptococci, the likely sensitivity of the isolates was interpreted using the interpretive criteria of aminoglycoside of other gram positives (staphylococci) in this study as suggested by EUCAST for isolates with no interpretive criteria (EUCAST, 2021).

Due to the lack of approved CLSI clinical breakpoints for *P. aeruginosa* for trimethoprim-sulphamethoxazole and tetracycline antibiotics, likely sensitivity was interpreted using the

interpretive criteria of *Acinetobacter* species. Further, for *P. aeruginosa* for phenicols and macrolides, isolates were interpreted using other gram negatives bacteria as suggested by EUCAST for isolates with no defined clinical breakpoints (EUCAST, 2021).

4.2.5 Detection of antibiotic resistant genes

All staphylococcal isolates (*S. aureus* & CNS) showing phenotypic resistance to beta-lactams (ampicillin, cefoxitin), erythromycin, tetracyclines, and streptomycin were analyzed by Polymerase Chain Reaction (PCR) for genes that confer resistance to penicillin (*blaZ*), erythromycin (*ermB*, *ermC, msrA*), streptomycin (*strB*) and tetracycline (*tetK*, *tetM*)). Details on primers and conditions used in the study to detect the seven antimicrobial-resistant genes are provided in Table 4.1. The PCR mixtures (25 μL) used to detect the antimicrobial resistance genes in all strains contained 12 μL reaction mixture (DNA Taq polymerase, dNTPs, MgCl2 and PCR buffer, Qiagen, German), 1 μL of the forward and reverse primer of each, 5 μL genomic DNA and 6 μL ddH2O.

PCR products (10 μL) were electrophoresed using Ethidium Bromide stained 1.5% agarose gel in Tris-Borate-EDTA (TBE) buffer and visualized using UV-illuminator (GelMax® Imager, UK). A molecular ladder was used to determine the sizes of the amplicon (Gelpilot 1 kb plus ladder (100), (Qiagen, Germany, GmbH, Hilden Germany). In all PCR reactions, positive and negative controls were included.

4.2.6. *nuc* gene and antibiotic resistance gene sequencing and analysis

A subset of the PCR amplicon of the *nuc* and the antibiotic resistant genes were sequenced to confirm identities of the detected organisms and genes using Sanger DNA sequencing approaches (Schaumburg *et al.*, 2014). Quality control, assembly, and editing of nucleic sequence trace files were performed using SnapGene version 5.2.4 (snapgene.com) and customized UNIX shell scripts. Sequences identities were confirmed using the Basic Local Alignment Search Tool (BLASTn) (NCBI).

Sequencing and BLASTn analysis targeting the specific *S. aureus nuc* gene was done as previously described by Schaumburg *et al*. (2014). Purified DNA samples were sequenced with an Automated DNA sequencing Analyzer at Macrogen Europe Laboratories (Amsterdam, Netherlands) where forward and reverse primers similar to the ones used for the PCR reaction were used.

4.2.7 Statistical Data analysis

Data entry and management were done using Microsoft excel 2016 while antibiotic susceptibility testing data were analyzed using STATA version 15. Descriptive statistics were used to calculate the proportion and frequencies of all variables. The chi-square test (χ^2 test) or Fisher's exact test were used when applicable to compare categorical variables. Statistical significance level was set at 0.05 ($p < 0.05$).

4.3 RESULTS

4.3.1 Confirmation of *Staphylococcus aureus* with PCR

All 91 (100%) *Staphylococcus aureus* isolates yielded an amplicon for the *nuc* gene as shown in Figure 4.1. A subset (8) of the *nuc* gene was sequenced and revealed 97%-100% homogeneity to *Staphylococcus aureus*.

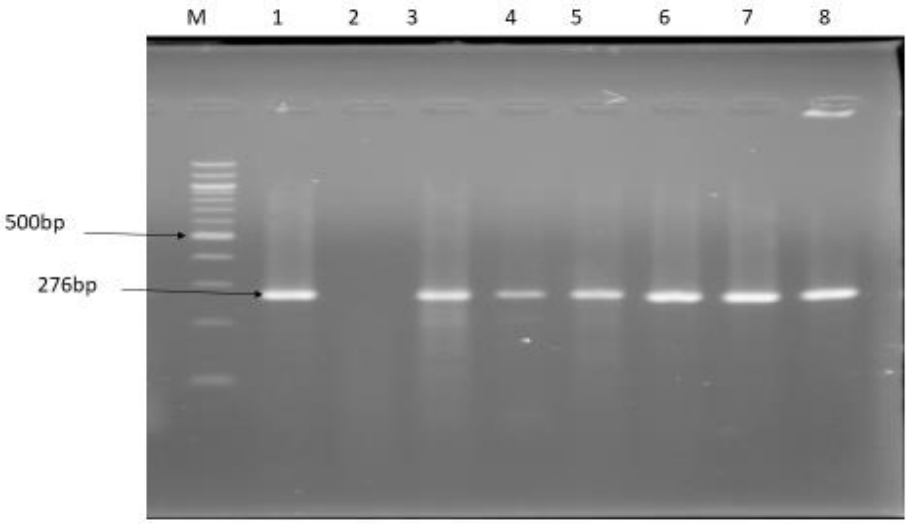


Figure 4.1: PCR amplicon showing *nuc* gene. M represents DNA ladder, lanes (1) -shows positive control, lanes 3,4,5, 6, 7,8 showing positive amplicon for *nuc* gene at approximately at 276bp, lane 2 negative control.

4.3.2 Phenotypic antimicrobial resistance patterns of the isolated *S. aureus* and CNS

Overall, the highest phenotypic resistance was reported to ampicillin 66.1% (n=121) followed by tetracycline 23% (n=42). However, lower resistance rates to fluoroquinolones 4% (n=8) and gentamicin 5.4% (n=10) were noted among the isolates. *S. aureus* showed significantly higher resistance to cefoxitin and ampicillin compared to CNS (p=0.009, p=0.014) respectively. However, there were no statistically significant differences in the resistance frequencies for the other antibiotics between *S. aureus* and CNS.

Among the *S. aureus*, 75.8% (n=69) of the isolates were resistant to at least one of the antibiotics agents tested. As shown in Table 4.2, ampicillin was the one which had most of the isolates resistant to at (74.7%), followed by erythromycin at (25.2%) while a lower resistance rate was reported to fluoroquinolones (3-4%). Further, 25% of the isolates were phenotypically methicillin-resistant (MRSA) based on the cefoxitin disc diffusion test. In addition, all MRSA isolates (100%) showed phenotypic resistance to ampicillin; 52% were resistant to erythromycin, 48% to tetracycline, and 30% to trimethoprim-sulfamethoxazole.

Table 4.2: Antibiotic susceptibility patterns of 183 staphylococci isolated from mastitic cow milk in the two study counties

Antibiotic class	Disk concentration (µg)	Disc diffusion interpretive criteria (mm)		*S. aureus*	CNS
		S[1]	R[2]	R n(%) (n=91)	R n(%) (n=92)
β – lactams					
Cefoxitin	30	≥22	≤21	23 (25.3)	10 (10.9)
Ampicillin	25	≥29	≤28	65 (71.4)	53 (57.6)
Aminoglycosides					
Gentamicin	10	≥15	≤12	6 (6.0)	4 (4.3)
Streptomycin	10	≥15	≤12	23 (25.3)	18 (19.6)
Fluoroquinolones					
Ciprofloxacin	5	≥21	≤15	3 (3.2)	3 (3.0)
Norfloxacin	10	≥17	≤12	4 (4.3)	3 (3.0)
Tetracycline					
Tetracycline	30	≥19	≤14	23 (25.3)	21(22.8)
Folate pathway inhibitors					
Trimethoprim-sulfamethoxazole	23.75/1.25	≥1	≥10	17 (18.7)	16 (17.4)
Macrolides					
Erythromycin	15	≥18	≤13	23 (25.3)	14 (15.2)
Phenicols					
Chloramphenicol	10	≥18	≤12	8 (8.8)	7 (7.6)

[1]sensitive, [2]resistant; Disc diffusion interpretive criteria for CNS for cefotixin was done based on EUCAST 2021 (S ≥25, R<25) and the rest according to CLSI M100 2016.

In this study, significantly higher resistance to various antimicrobials was observed in methicillin-resistance *Staphylococcus aureus* (MRSA) compared to methicillin-sensitive *Staphylococcus*

aureus (MSSA) ($p<0.05$) except for ciprofloxacin and trimethoprim-sulfamethoxazole (Figure 4.2).

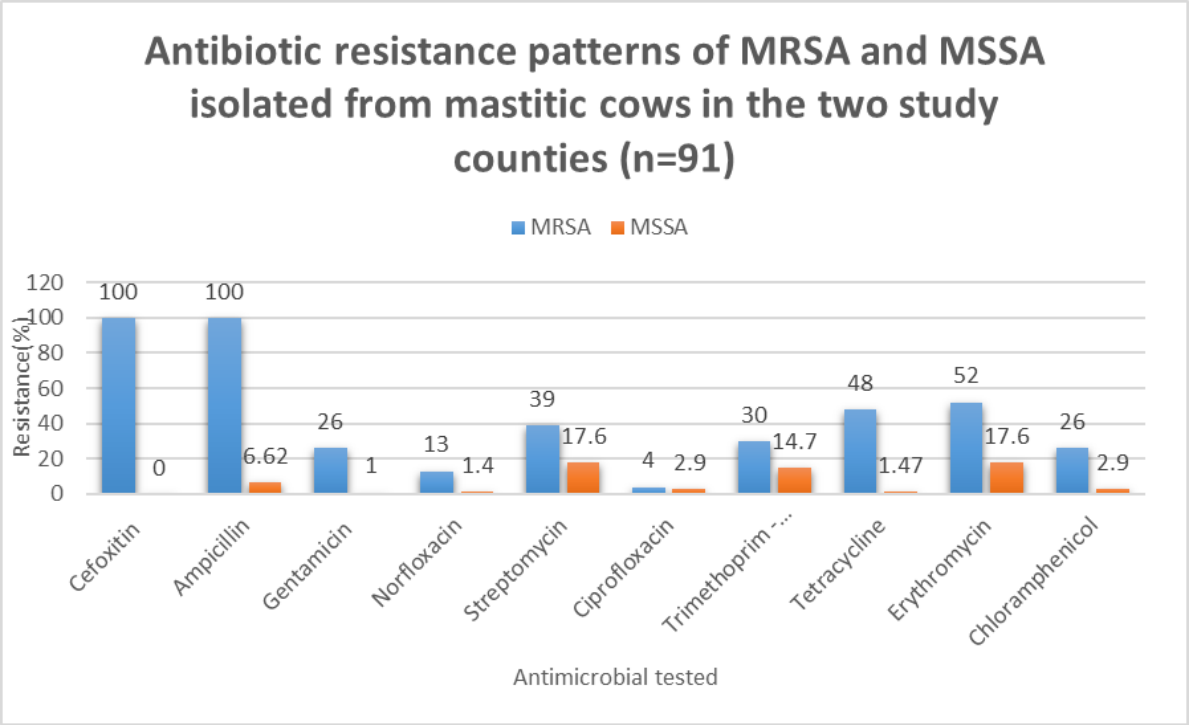


Figure 4.2: Phenotypic Antibiotic resistance profiles of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *Staphylococcus aureus* (MSSA) isolated from cow mastitic milk from Kajiado and Embu Counties (n=91).

Among the CNS, 68.5% (n=63) of the isolates were resistant to at least one antimicrobial agent tested. Ampicillin was the one that had most of the isolates resistant-to (57.6%), followed by tetracycline (22.8%), while resistance to fluoroquinolones was at 3.0%. In this study, (10.8%) of

CNS were resistant to methicillin and hence classified as phenotypic methicillin-resistant Coagulase-negative *Staphylococcus* (MRCNS) (Table 4.2).

4.3.2.1 Prevalence of multidrug resistance in the staphylococcal isolates

Multidrug-resistance (MDR) defined as isolates showing resistance to three or more classes of antibiotics was detected in 22.9% (n=42) of the staphylococcal isolates. Significantly, higher proportion of MDR isolates were reported among *S. aureus* at 29.6% (n=27) compared to CNS 16.3% (n=15) (p=0.032). Among the *S. aureus*, MRSA and MSSA showed MDR at 56.5% (n=13) and 20.5% (n=14) respectively. Among the MRCNS, 70% (n=7) were MDR. Further, 3.2% (n=6) of staphylococcal isolates showed resistance to more than seven antibiotics tested out of which 83.3% (n=5) of them were among the MRSA while 2 were MRCNS.

4.3.3 Detection of resistant genes from the staphylococcal isolates

Overall, 57.2% (95/166) isolates harbored at least one resistance gene of the 7 different genes detected in different combinations. As shown in Table 4.3, 1 isolate carried 4 genes, 8 isolates carried 3 genes and 20 isolates carried 2 genes. In addition, 66 isolates carried 1 gene of which 98% (63/64) of these isolates carried *blaZ* genes (Figure 4.3). Notably, 22 of the isolates carrying more than ≥2 genes were multidrug resistant (MDR). All the isolates carrying more than (≥) 2 antimicrobial resistance genes showed high resistance to ampicillin (96%) and tetracycline (75%) (Table 4.3).

Table 4.3: Antimicrobial resistance profiles, with respect to genes present, of the Staphylococci recovered from dairy cows' mastitis cases from the two study counties

Isolate ID	Antibiotic Resistance gene expressed	Phenotypic resistance profile
457(1)[1]	*blaZ, tetM, ermB, msrA*	AMP, FOX, ERY, TET
803(1)[2]	*blaZ, strB,msrA*	AMP, FOX, STR, ERY, NX
657[1]	*blaZ, tetM, ermB*	AMP, FOX, TET
379[1]	*blaZ,strB,msrA*	AMP, SXT, STR, ERY, TET
525[1]	*blaZ,strB,msrA*	AMP, STR, ERY, TET
1510(1)[2]	*blaZ,tetM,msrA*	AMP, FOX, C, ERY, TET, CIP
245[2]	*tetM,strB,ermB*	AMP, FOX, SXT, STR, GEN, TET, C
1530[2]	*blaZ, strB, ermB*	AMP, FOX, SXT, STR, GEN, ERY, TET, CIP, C
683[2]	*blaZ,tetM,strB*	AMP, FOX, STR, TET
37[1]	*blaZ,strB*	AMP, FOX, SXT, STR, ERY, TET
65(4)[1]	*blaZ, msrA*	AMP, FOX, SXT, STR, ERY, GEN, TET, C
242(2)[1]	*blaZ, strB*	AMP, FOX, STR, ERY, GEN, TET, C
247(2)[1]	*blaZ, ermB*	AMP, FOX, STR, GEN, ERY, TET, NX, C,
5(KRF)[1]	*blaZ, msrA*	AMP, FOX, SXT, ERY
482(2)[1]	*blaZ, tetM*	AMP, FOX, TET,
1338[1]	*blaZ,msrA*	AMP, SXT, ERY, CIP
908(1)[1]	*blaZ,tetM*	AMP, STR, SXT, TET
253(1)[1]	*blaZ,tetM*	AMP, TET
187[1]	*blaZ, strB*	AMP, STR, TET
474[1]	*blaZ,strB*	AMP, SXT, STR, ERY, TET
806[2]	*blaZ, strB*	AMP, TET
1512[2]	*blaZ, tetM*	AMP, TET

1604[2]	*blaZ, ermB*	AMP, FOX, SXT, ERY, TET
1579[2]	*blaZ, msrA*	AMP, SXT, STR, GEN, ERY, C
1247[2]	*blaZ, tetM*	AMP, TET, C
1189[2]	*blaZ, strB*	AMP, STR, NX
1609[2]	*blaZ, ermB*	AMP, ERY
472(2)[2]	*blaZ, strB*	AMP, FOX, STR
1178[2]	*blaZ, tetM*	AMP, ERY, CIP, TET

Only staphylococci isolates carrying ≥ 2 antibiotic resistant genes are shown in Table 4.4; [1]*Staphylococcus aureus,* [2]Coagulase-negative staphylococci, FOX cefoxitin, AMP ampicillin, ERY erythromycin, TET tetracycline, C chloramphenicol, GEN gentamicin, STR streptomycin, SXT trimethoprim-sulfamethoxazole, CIP ciprofloxacin and NX, norfloxacin.

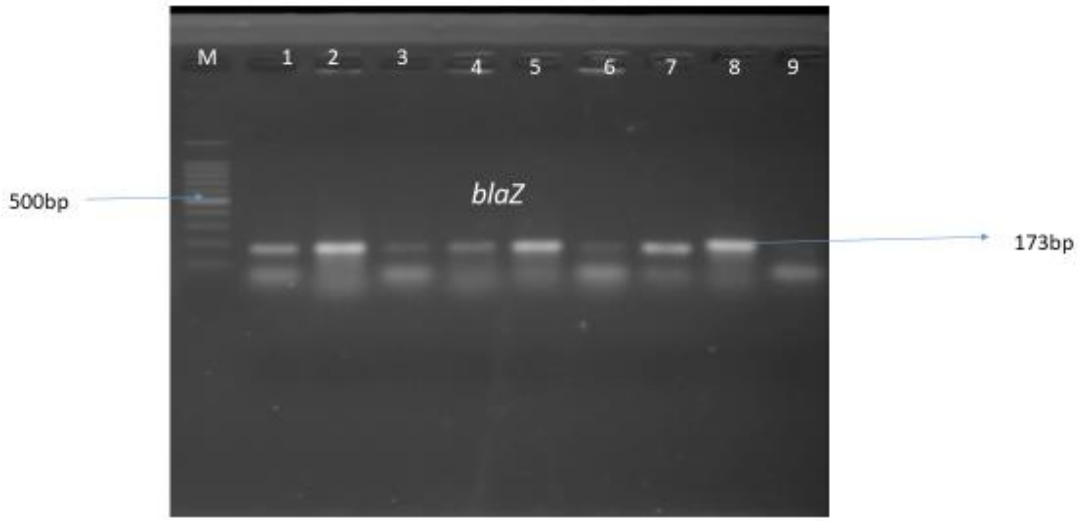


Figure 4.3: PCR amplicon showing *blaZ* gene, M-DNA ladder (Qiagen, German), Lanes; 1, 2, 3, 4, 5, 6,7 showing positive amplicon for *blaZ* at approximately at 173bp. Lanes; 8 positive control, lane 9 was negative for *blaZ*

Overall, more resistance genes were reported in CNS at a prevalence of 70.2% (n=59) compared to *S. aureus* at 42.3% (n=36) (p=0.001). The most prevalent resistance gene was the β-lactamase gene *blaZ* at 59.2% (n=90) (Table 4.4). The prevalence of *blaZ* gene was higher CNS at 65.4% (n=55) compared to *S. aureus* at 41.1% (n=35) (p=0.002). Strikingly, 20.5% (n=15) of methicillin sensitive *Staphylococcus aureus* (MSSA) strain had a *blaZ* variant. Further, one of the methicillin-resistant Coagulase-negative *Staphylococcus* (MRCNS) isolates carried three other resistance genes; namely *blaZ*, *msrA*, *strB* conferring resistance β-lactams, aminoglycosides and macrolides.

Table 4.4: Antibiotic resistance genes to various antibiotics detected in the staphylococci isolated from bovine mastitis in the two study counties

Species	β – lactams		Tetracycline			Streptomycin		Erythromycin			
	[1]No. R	*blaZ* R n(%)	No.R	*tetM* R n(%)	*tetK* R n(%)	No.R	*strB* R n(%)	No.R	*msrA* Rn(%)	*ermB* Rn (%)	*ermC* Rn(%)
S. aureus	79	35(41.1)	23	4(17.3)	-	23	6(26)	23	5(21.7)	4(17.3)	-
CNS	73	55(65.4)	21	3(14.2)	3(14.2)	18	9(60)	14	4(28.5)	4(28.5)	1(4.3)
Total	152	90(59.2)	43	7(16.2)	3(6.9)	42	15(35.7)	37	9(24.3)	8(21.6)	1(2.7)

[1] Number of phenotypic resistant isolates in each category.

Tetracycline resistance *tetM* (Figure 4.4) and *tetK* genes (Figure 4.5) were detected in *Staphylococcus* species at 16.2% and 6.9% respectively. Notably, all *tet K* detected in this study were from the CNS with none from *S. aureus*. Streptomycin resistance *strB* gene (Figure 4.6) was present in 35.7% of the staphylococcal isolates. Higher occurrence of *strB* genes was reported in CNS at 50.0% compared to *S. aureus* 13.0%. Among the erythromycin-resistant Staphylococcus isolates *msrA* (24.3%) and *ermB* (21.6%) (Figure 4.7 & 4.8) were the most prevalent. The frequencies of *msrA* and *ermB* in *S. aureus* and CNS were almost similar in this study (Table 4.4).

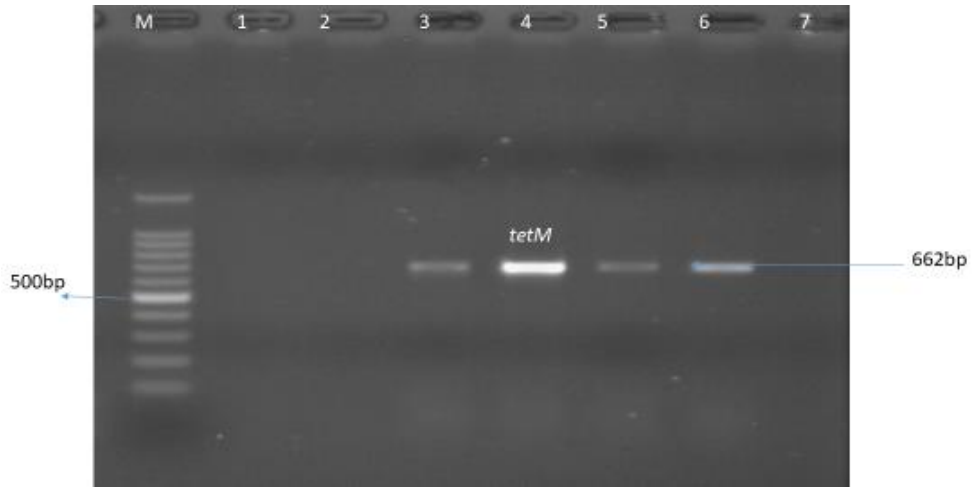


Figure 4.4: PCR amplicon showing *tetM* gene, M-DNA ladder (Qiagen, German), Lanes;3,4,5, showing positive amplicon for *tetM* at approximately at 662 bp, Lanes;6 positive and 7 negative control, lane 1 and 2 negative samples

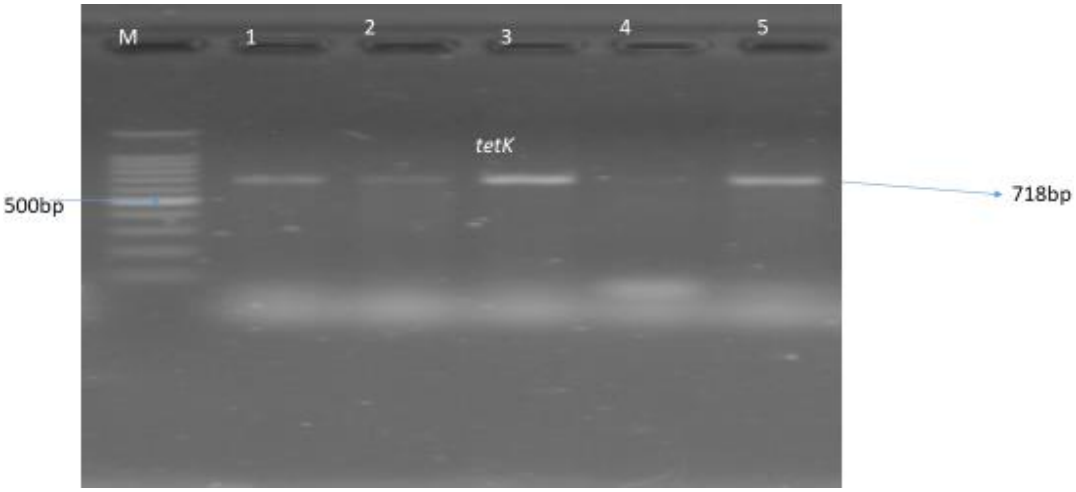


Figure 4.5: PCR amplicon showing *tetK* gene, M-DNA ladder (Qiagen, German), Lanes; 1,2,3 showing positive amplicon for *tetK* at approximately at 718bp, Lanes;5, positive control and 4 negative control.

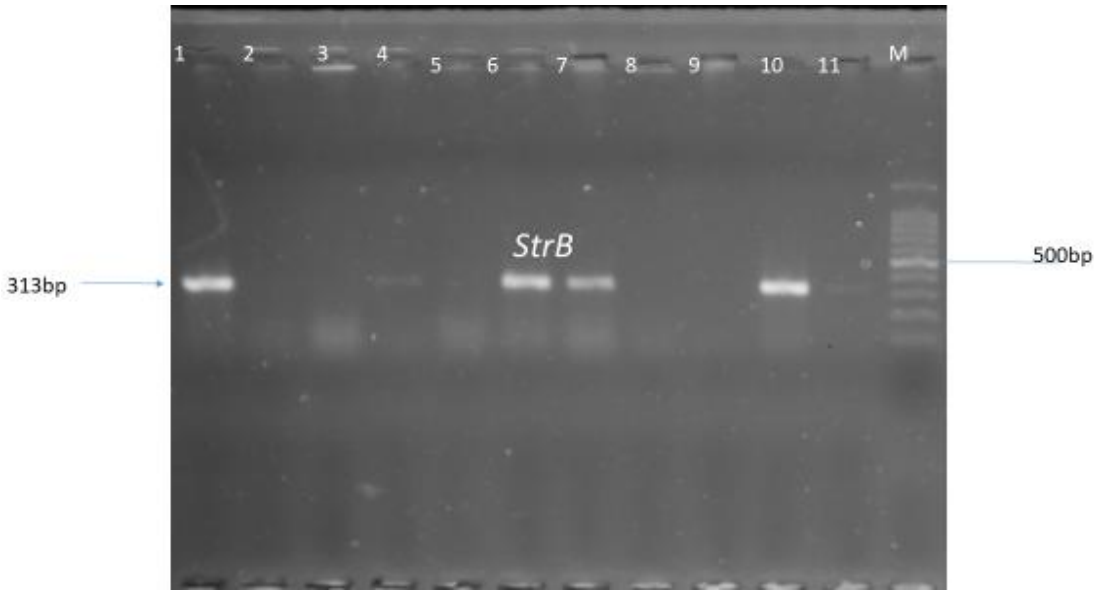


Figure 4.6: PCR amplicon showing *strB* gene, M-DNA ladder (Qiagen, German), Lanes; 1,6,7 showing positive amplicon for *strB* at approximately at 313 bp, Lanes;10 positive and 11 negative control.

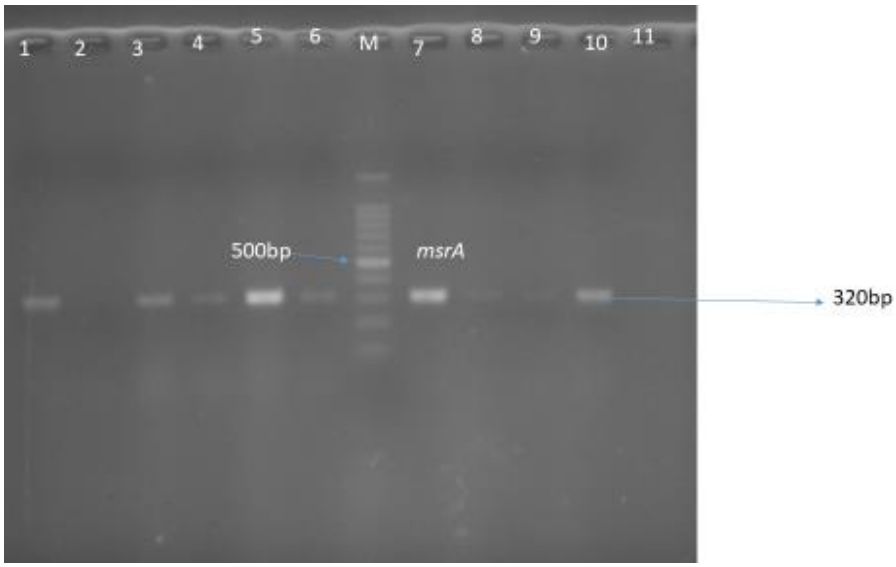


Figure 4.7: PCR amplicon showing *msrA* gene, M-DNA ladder (Qiagen, German), Lanes; 1,3,4,5, 6,7 showing positive amplicon for *msrA* at approximately at 320bp, lane 2 showing negative for *msrA* Lanes;10 positive and 11 negative controls

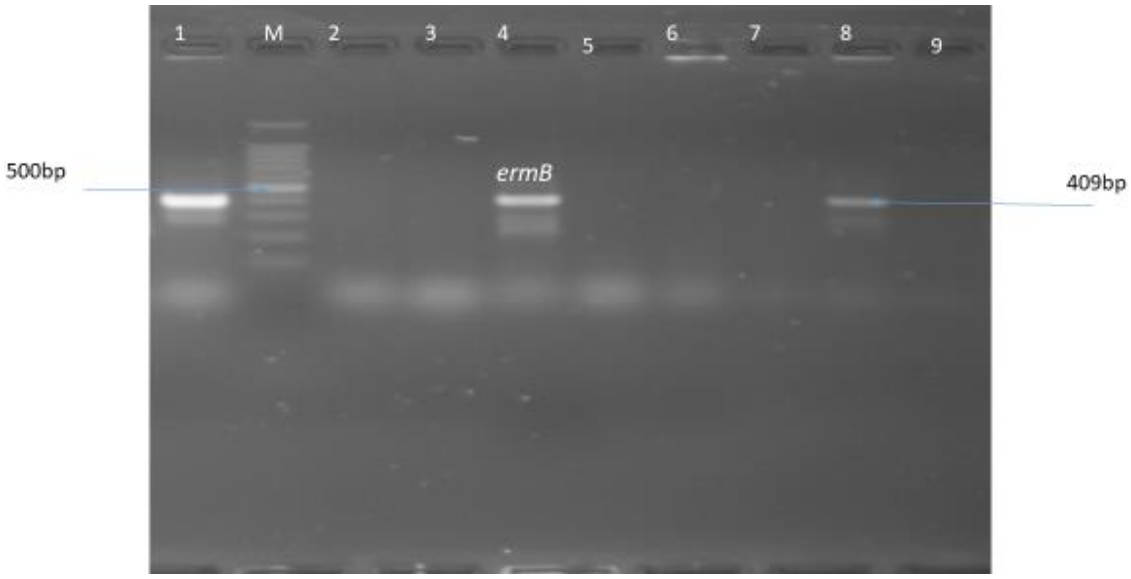


Figure 4.8: PCR amplicon showing *ermB* gene, M-DNA ladder (Qiagen, German), Lanes; 1,4,5, 8 showing positive amplicon for *ermB* at approximately at 409 bp, Lanes; 2,3,5,6,7, negative. Lane 9 negative control.

4.3.4 Phenotypic antibiotic resistance patterns of *Streptococcus* species

A total of 22 non *Streptococcus agalactiae* species were tested in this study, out of which 50% (n=11) showed phenotypic resistance to at least one of the 10 antimicrobial agents tested (Figure 4.9), while 13.6 % (n=3) were multidrug-resistant (MDR).

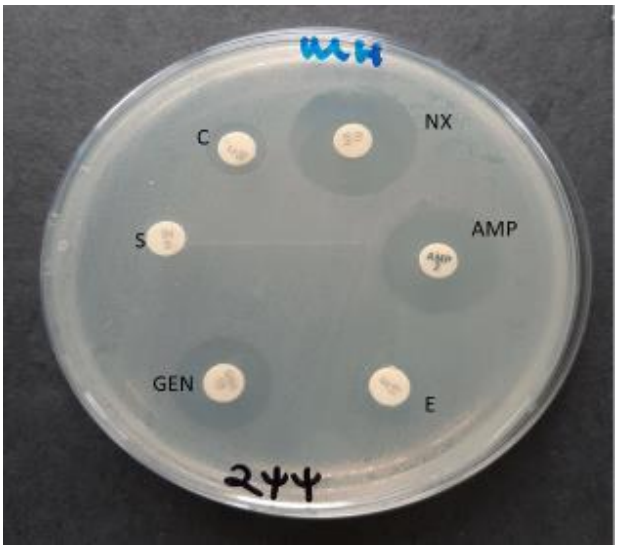


Figure 4.9: Disc diffusion method showing antibiotic susceptibility of *Streptococcus spp* (244) against C (Chloramphenicol) NX (Norfloxacin), AMP (Ampicillin), E (Erythromycin) GEN (Gentamicin), S (Streptomycin).

The highest phenotypic resistance was reported for ampicillin and tetracycline both at 31.5% (n=7), followed by moderate resistance levels to streptomycin at 22.7% (n=5) and sulfamethoxazole-trimethoprim at 18.2% (n=4). Lower phenotypic resistance to vancomycin at 13.6% (n=3) and erythromycin at 9% (n=2) were reported in this study. However, no isolates were resistant to fluoroquinolones and gentamycin (Figure 4.10).

Remarkably, all the three MDR streptococcus isolates showed a 100% resistance level to ampicillin and vancomycin. Notably, one of the streptococcus isolates showed resistance to six antibiotics tested.

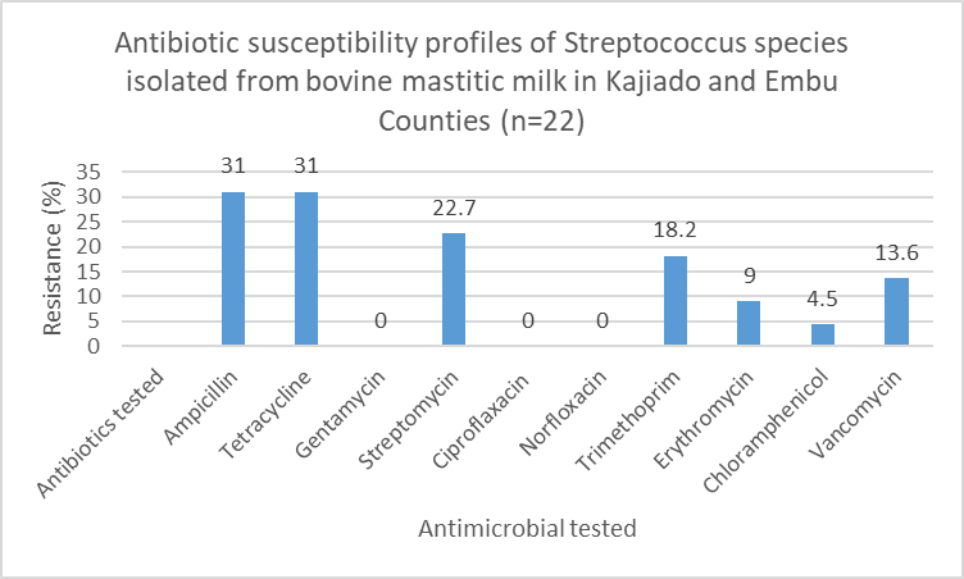


Figure 4.10: Phenotypic Antibiotic susceptibility profiles of *Streptococcus* species isolated from bovine mastitic milk in Kajiado and Embu Counties (n=22).

4.3.5 Phenotypic Antibiotic susceptibility patterns of the isolated *Pseudomonas aeruginosa* and *Escherichia coli*

All 31 isolates, showed resistance to at least one of the antibiotics tested (Figure 4.11A &B). Of the resistant isolates, 23% (n=7) were resistant to one class of antibiotics, 6% (n=3) were resistant to two classes of antibiotics while 70.9% (n=22) were MDR. Overall, the highest phenotypic resistance was reported to ampicillin at 83.9% (n=26), followed by cefaclor at 80.7% (n=25). In contrast, significantly low resistance rates to ciprofloxacin at 3.2% (1) were observed among the isolates.

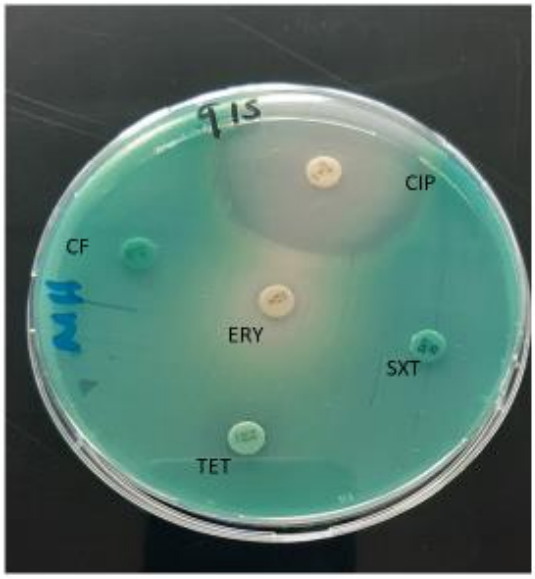


Figure 4.11A

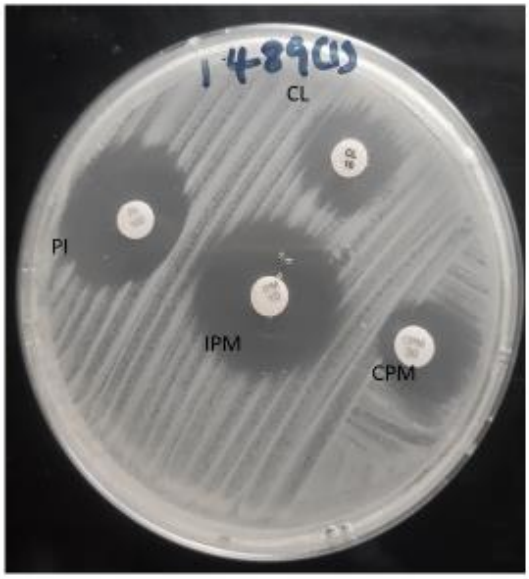


Figure 4.11B

Figure 4.11: (A) -Disc diffusion method showing antibiotic susceptibility of *P. aeruginosa* against trimethoprim sulphamethoxazole (SXT), erythromycin (E), tetracycline (TET), cefaclor (CF), ciprofloxacin (CIP). **(B)** Disc diffusion method showing antibiotic susceptibility of *P. aeruginosa* against imipenem (IPM), cefepime (CPM), colistin (CL), piperacillin (PI)

P. aeruginosa showed significantly higher resistance to chloramphenicol compared to *E. coli* (p =0.05). However, there were no statistically significant differences in the resistance frequencies to the other antibiotics between *P. aeruginosa* and *E. coli* (Table 4.5). Further as shown in Figure 4.12, 50% (n=11) of the MDR isolates were resistant to 5 classes of antibiotic tested while 4.5% of isolates showed resistance to 7 classes of antibiotics agents tested. The most commonly reported resistance pattern being trimethoprim-sulphamethoxazole, erythromycin, tetracycline, ampicillin, cefaclor, streptomycin.

Table 4.5: Antimicrobial susceptibility profiles of *Escherichia coli* (n=12) and *Pseudomonas aeruginosa* isolated (n=19) from mastitic cow milk in the two counties

Antibiotics tested	*E.coli* (n=12)	*P. aeruginosa* (n=19)	[3]p-value
	[1]R n (%)	R n (%)	
Cefaclour	8 (66.7)	17 (89.5)	0.14
Ampicillin	9 (75.0)	17(89.5)	0.28
Piperacillin	[2]NT	0 (0)	NT
Cefepime	NT	13 (68.4)	NT
Imipenem	NT	4 (21.1)	NT
Colistin	NT	14 (73.6)	NT
Streptomycin	8 (66.7)	13 (68.4)	0.61
Gentamicin	1(8.3)	5 (26.3)	0.22
Norfloxacin	0 (0)	2 (10.5)	0.36
Ciprofloxacin	0 (0)	1(5.3)	0.61
Sulphamethoxazole	7 (58.3)	13 (68.4)	0.40
Tetracycline	5 (41.7)	10 (52.6)	0.41
Erythromycin	7(58.3)	15 (78.9)	0.20
Chloramphenicol	1(8.3)	8 (42.1)	**0.05**

[1]Resistance, [2]NT not tested, [3]p-value refers to differences between *E. coli* and *P. aeruginosa* isolates resistant to the respective antibiotics

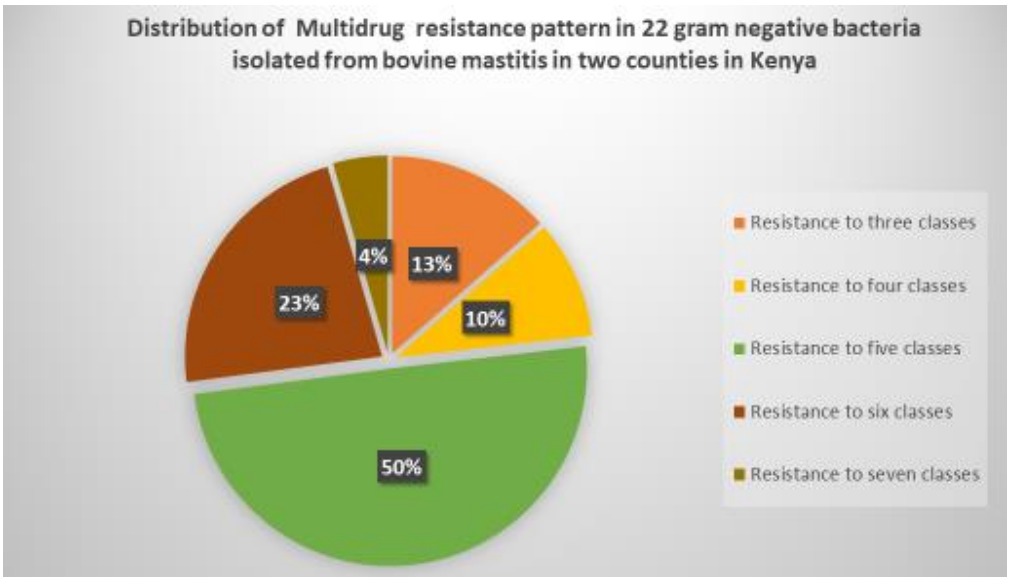


Figure 4.12: Pie chart showing the distribution of Multidrug resistance pattern in 22 Gram-negative bacteria isolated from bovine mastitis in the two counties

Among the *E. coli* isolates, the highest phenotypic resistance was to ampicillin (75%), followed by cefaclor and streptomycin each at (67%). These isolates demonstrated 100 % susceptibility to fluoroquinolones (Table 4.5) while MDR was observed in 66% (8) of the isolates. All *E. coli* isolates (8) showed resistance to ampicillin.

Among the *P. aeruginosa* isolates, the highest resistance was observed on both cefaclor and ampicillin each at 89.5%, followed by erythromycin (78.9%) and colistin 73.6%. However, the lowest resistance was to ciprofloxacin with 5.3%. Unexpectedly 21% (4) of the *P. aeruginosa* isolates were imipenem-resistant in this study.

MDR was present in 78.9% (15) of the *P. aeruginosa* isolates with all of them showing 100% resistant to cefaclor and ampicillin. Notably, 68% (13) of the *P. aeruginosa* isolates showed resistance to at least 5 of all the antibiotics tested. In this study, 4 isolates of *P. aeruginosa* came from 4 cows in two farms. The resistance profiles of those from the same farm were similar.

4.4 DISCUSSION

This study investigated the antibiotic susceptibility profiles of *Staphylococcus*, other *Streptococcus, Escherichia coli,* and *Pseudomonas aeruginosa* isolates recovered from mastitic cow milk in Kenya.

4.4.1 Phenotypic and genotypic antibiotic resistance profiles of staphylococci

4.4.1.1 Phenotypic antimicrobial resistance patterns of the isolated *Staphylococcus aureus* and Coagulase Negative *Staphylococcus* (CNS)

Overall, this study showed a high proportion of resistance (71.5%) to at least one antibiotic tested, with no differences between the *S. aureus* (75%) and CNS (68%). This was slightly lower than what was reported in other studies in Malaysia (96.2%) and South Africa (90%) in *S. aureus* and CNS respectively (Phopi *et al.*, 2019, Aklilu *et al*., 2020). In contrast, slightly lower resistance proportions in *S. aureus* (50%) and CNS (50%) were reported in Uganda (Majalija *et al*., 2020). High resistance levels observed in this study could be linked to the indiscriminate use of antibiotics for the treatment of mastitis in dairy cows by farmers and veterinarians (Omwenga *et al.*, 2020). Like in many developing countries, in Kenya, most of these antibiotics are cheap and readily available as over-the-counter (OTC) drugs and can be bought without any veterinary prescription (Majalija *et al*., 2020). However, further studies investigating antibiotic use and practices in dairy farms in Kenya are necessary to provide conclusive evidence on the extent to which such practices contribute the spread of antimicrobial resistance.

This study reported a much higher resistance rate in penicillins (ampicillin) than those reported previously in dairy cows in Kenya by Gitau *et al.* (2014) and Shitandi & Sternesjö, (2004) of about 30%. However, these findings were comparable to a recent report by Mureithi *et al*. (2017) who found a prevalence rate of 64% in ampicillin. These results indicate an increase in the antibiotic resistance in staphylococci over the years. High resistance levels to penicillin and other β- lactams among mastitis-causing staphylococci have been described (Frey *et al*., 2013, Liu *et al*., 2017, Mekonnen *et al*., 2018). In contrast, lower resistance to penicillin in staphylococci (0-20%) has also been reported in some European countries (Käppeli *et al*., 2019) and Canada (Nobrega *et al.,* 2018). Higher resistance rates are likely due to widespread use of penicillin in the treatment of mastitis in dairy cows as observed in the study farms and as evidenced by other previous studies in Kenya (Mitema *et al.,* 2001, Shitandi & Sternesjö, 2004). Further, changes over time, spatial sampling, differences in antibiotic use and practices might explain discrepancies in resistance levels between regions (Boireau, *et al*., 2018, Majalija *et al*., 2020).

S. aureus had a significantly higher proportion of ampicillin resistance when compared to CNS in this study. *S. aureus* has been described as a common cause of bovine mastitis in the study region (Gitau *et al*., 2014). This higher resistance could be because penicillin is still the first-line drug of choice for the treatment of mastitis in Kenya (Shitandi & Sternesjö, 2004, Omwenga *et al.*, 2020). Routine culture and identification coupled with antibiotic susceptibility testing should be adopted before treatment with antibiotics to avoid the selection pressure of antibiotics resistant *S. aureus* (Liu *et al*., 2017).

S. aureus had a significantly higher proportion of methicillin resistance compared to CNS in this study. A study in Korea found a slightly higher prevalence of MRSA compared to MRCNS (Moon *et al*., 2007). However, in contrast, Schnitt & Tenhagen, (2019) in their review highlighted several studies that have reported higher MRCNS compared to MRSA in mastitic milk samples. Noteworthy, in all these studies methicillin resistant staphylococci (MRS) was defined based on the presence of *mecA* gene unlike in this study, and could explain the discrepancy. However, although the reason for the higher prevalence of MRSA in the current study findings is unclear, lower virulence observed in the MRCNS might be a contributing factor (Schnitt & Tenhagen, 2019).

Low resistance levels to quinolones and chloramphenicol were reported in this study among the staphylococci. Studies in Ethiopia (Kalayu *et al*., 2020), South Africa (Phopi *et al*., 2019) and Canada (Nobrega *et al*., 2018), reported similar findings. These critically important human medicine antibiotics are restricted for use in the treatment of animal diseases in many countries including Kenya (Phobi *et al*., 2019). However, even the low resistance rates reported are of public health significance and control measures should be implemented to curb further spread (Nobrega *et al*., 2018).

In the present study, 25% *S. aureus* and 10.8% CNS isolates were phenotypically resistant to cefoxitin and were consequently classified as MRSA and MRCNS respectively. Cefoxitin disk test has shown in several studies to be a reliable marker for methicillin resistant *S. aureus* and CNS not identified to species levels (Boireau, *et al*., 2018, EUCAST, 2021). It is worth noting that all the

MRSA and MRCNS showed resistance to ampicillin. These findings on MRSA were in close agreement with reports by Liu *et al*. (2017) in China. A relatively higher prevalence of cefoxitin-resistant-MRSA has been reported in Malaysia (38.6%) and Ethiopia (58.1%) (Aklilu *et al.,* 2020, Elemo *et al*., 2017). Similarly, a higher prevalence of phenotypic MRCNS has been reported in South Korea (21.2%) (Kim *et al*., 2019). Tunisia (29.4%) (Klibu *et al.,* 2019), and Switzerland (47%) (Frey *et al*., 2013). Detection of methicillin-resistant staphylococci (MRS) in mastitic milk is a public health concern and should be further investigated as most of these organisms have shown to be potentially zoonotic in addition to multidrug-resistant, reducing the role of therapy in control of staphylococcal mastitis (Wang *et al*., 2015). Culling of infected cows to avoid further transmission would be of the highest importance (Liu *et al.,* 2017).

The presence of the *mecA* gene is considered the gold standard of defining MRSA, in addition to the new resistance genes *mecC* and *mecB* which are homologue to *mecA* (CLSI, 2016, Becker *et al.,* 2018, Scholtzek *et al.,* 2019). However, in this study, screening for *mec* genes in the phenotypically methicillin-resistant strains were not carried out which is a limitation in this study. A further investigation targeting *mec* genes and other mechanisms should be carried out in future studies in order to broaden the understanding on the genetic basis of antimicrobial resistance of these isolates in the current study (Panchal *et al.,* 2020). Molecular typing to assess the clonality of the isolates is also recommended.

Strikingly, the trimethoprim-sulfamethoxazole resistance rate in MRSA reported in this study was quite high (30%). However, higher resistance rates in *E. coli* to trimethoprim-sulfamethoxazole

have been reported in livestock especially in chickens in Kenya (Muloi *et al*., 2019). Although data supporting the use of trimethoprim-sulfamethoxazole directly in cows is scanty in Kenya, sulfonamides have been reported as the second commonly, used antibiotic in food animals after tetracycline (Mitema *et al*., 2001, Omwenga *et al*., 2020). Moreover, Muloi *et al*. (2019) in a different study on antibiotic practices and knowledge among antibiotics retailers in Nairobi, sulfonamides were reported to be amongst the most purchased class of drugs (at 63%) by the dairy farmers, from Agrovet shops in Nairobi county which neighbors the study counties indicating high usage of sulfonamides in dairy animals.

Furthermore, Mitema *et al.* (2001), reported sulfonamides to be the most extensively used drug in the poultry industry and the treatment of calf scours and pneumonia in Kenya. The role of horizontal transfer of AMR genetic determinants among different bacterial species between humans and animals has been described (Van *et al*., 2020). The high resistance rate reported in this study is very concerning since trimethoprim-sulfamethoxazole is heavily used for prophylaxis in HIV-infected patients, especially in the highly infectious disease setting including Kenya (Hamel *et al*., 2008).

4.4.1.2 Prevalence of Multidrug Resistance in staphylococci species

Similar to other studies, a significantly higher resistance rate to the various antibiotics tested was observed in MRSA strains than in MSSA strains (Wang *et al*., 2015, Liu *et al.*, 2017). MRSA has shown to have the potential to develop resistance to nearly all the antimicrobial agents (Wang *et*

al., 2018). This evolving trend and the rapid emergence of antibiotic resistance in *S. aureus* threatens disease management in both animal and human health (Sharma *et al*., 2018).

A higher proportion of multi-drug-resistant isolates were reported among *S. aureus* compared to CNS. In contrast, Dorneles *et al.* (2019) and Cheng *et al*. (2019) reported higher MDR rates in CNS compared to *S. aureus* in a similar study in Brazil and China respectively. Significantly higher MDR rates in *S. aureus* have been described (Wang *et al*., 2015, Liu *et al*., 2017). The significant MDR resistance of cattle-derived *S. aureus* in this study presents a serious challenge to bovine mastitis therapy and potential public health risk to humans in Kenya.

4.4.1.3 Detection of Resistant Genes from the Staphylococci species

Knowledge of the distribution of antibiotics resistance genes among pathogenic udder microbes is key to understanding the evolution of multi-drug resistant bacteria in dairy cattle. Higher levels of resistance genes were reported in CNS (70.2%) compared to *S. aureus* (42.3%) in this study. These findings support the hypothesis that CNS are the main reservoir of genetic elements transferrable to other species of bacteria including *S. aureus* (Frey *et al.,* 2013, Wang *et al.,* 2015).

In agreement with other studies, genes encoding for the beta-lactamases *blaZ* gene was the most common among staphylococcal isolates (95%) (Qu *et al*., 2019, Pekana & Green, 2018). It is likely that this has contributed to the high resistance level to some penicillin derivatives (ampicillin 64%) recorded in this study. High *blaZ* genes might indicate an increased use and possible misuse of β lactams in the study farms (Qu *et al*., 2019).

This study reported that 20.5% (n=14) of the MSSA isolates had an effective *blaZ* variant. A significantly higher prevalence of *blaZ* (91%) in MSSA has been reported in a hospital setting in Kuwait (Vali *et al*., 2017). Qu *et al*. (2019) also found that 83% of *S. aureus* isolates carried *blaZ* gene but lacked *mecA* gene. The presence of *blaZ* genes in *S. aureus* has been shown to play a significant role in promoting the acquisition and stabilization of *MecA* gene (Milheiriço *et al*., 2011). However, according to Vali *et al*. (2017) and Milheiriço *et al.* (2011) presence of *blaZ* gene in MRSA and MSSA may be responsible for encoding for resistance to only penicillin. Penicillin is still the first-line drug of choice for the treatment of mastitis in Kenya (Shitandi & Sternesjö 2004). Further, MSSA isolated from a hospital setting in urban areas in Africa have shown to have significantly higher resistance to penicillin compared to other MSSA isolates from elsewhere as described in a review by Schaumburg *et al*. (2014). Diversities between *blaZ* allotypes due to non-clonal evolutions in MRSA and MSSA isolates have also been observed in different geographical regions (Milheiriço *et al*., 2011). Therefore, evaluation of *blaZ* allele between MRSA and MSSA in the study isolates should be investigated in the future.

A low to moderate prevalence of antimicrobial resistance genes (ARGs) to tetracycline (*tetM& tet K*), macrolides (*msrA, emrB, ermC*), streptomycins (*strB*) compared to the phenotypic resistance was observed in this study. Low prevalence of ARGs has been reported by Pekana & Green, 2018 who found low expression of genotypic resistance in *S. aureus* in South Africa. Other studies by Gao *et al*. (2011) and Feng *et al*. (2016) both in China also reported low genotypic resistance compared to phenotypic resistance in staphylococcal isolates. Resistance mediated by other

independent mechanisms such as point mutations, biofilm formation, or antibiotic tolerance could explain these findings (Frey *et al*., 2013, Panchal *et al.,* 2020). Moreover, resistance genes not included in this study may account for phenotypic resistance observed (Pekana & Green, 2018). Further, the use of human disc diffusion interpretative criteria may have contributed to the misalignment between phenotypic and genotypic resistance observed in the isolates. Whole-genome sequencing is needed to expand our knowledge on staphylococci and their genetic basis of antibiotic resistance (Panchal *et al.,* 2020).

4.4.2 Phenotypic Antibiotic resistance patterns of the isolated *Streptococcus* species

In this study the most frequently reported antibiotics resistance was to ampicillin (31.8%). This agrees with a previous report in Kenya by Gitau *et al*. (2014), who reported a prevalence of 30% resistance in *S. agalactiae*. Higher resistance rates to ampicillin were previously reported in Kenya (42.9%), and Egypt (52%) (Muriethi *et al*., 2017, Saed & Ibrahim, 2020). In contrast, high susceptibility to penicillin has been reported in Germany, Poland, and Korea (Nam *et al*., 2009, Minst *et al*., 2012, Kaczorek *et al.,* 2017). The higher resistance level of streptococci to ampicillin in this study could be because β lactams are still considered the first line of mastitis treatment in Kenya leading to selection pressure to resistant isolates (Shitandi & Sternesjö, 2014). Differences in antibiotic use and practices might explain discrepancies in resistance levels between regions (Boireau *et al*., 2018).

Resistance rate to tetracycline at 31.5%, observed in this study, was comparable to previous findings in Kenya (42.9%) by Muriethi *et al*. (2017), Egypt (36%) (Saed & Ibrahim, 2020), and

China (33%) (Zhang *et al*., 2018). However, this study's findings were lower than reported by Cheng *et al*., (2019) in China (59%), Kaczorek *et al*. (2017) in Poland (63%), and Nam *et al*. (2009) in Korea (61.2%). Lower resistance rates tetracycline (12%) in *Streptococcus dysagalactie* have been reported in countries like Sweden (Bengtsson *et al.,* 2009). An extensive variation in tetracycline resistance to bovine streptococci in different countries has been described (McDougall *et al.,* 2021). The high resistance observed for tetracycline in this study might be linked to the extensive use of tetracycline for treatment and prophylaxis in bacterial infectious diseases in dairy cows in Kenya (Omwenga *et al*., 2020). The collection of more isolates and genomic analysis would provide more understanding of the mechanism of resistance between herds, regions, and countries (McDougall *et al.,* 2021).

This study reported no resistance of the *Streptococcus* species to gentamycin and moderate resistance to streptomycin (22.7%). Similar findings were reported by Ndirangu *et al*. (2017) in Kenya and Saed & Ibrahim, (2020) in Egypt in their different studies. However, these findings contrasted reports by Kaczorek *et al*. (2017) who reported a higher resistance level of streptococci isolated from clinical mastitic milk to gentamycin (50%-100%) in Polland. Naturally low susceptibility of *Streptococcus* spp. to aminoglycoside has been described (Zhang *et al.,* 2018). This low resistance level could be explained hypothetically by the lack of or low usage of aminoglycoside in livestock in some areas in Kenya hence little selective pressure (Omwenga *et al*., 2020). However, the use of aminoglycoside for therapy in streptococcal mastitis should be done with caution since there are no approved interpretive criteria for *Streptococcus* species (EUCAST, 2021).

Streptococcus species isolates resistant to erythromycin were reported at 10.5% in this study. Higher resistance levels to erythromycin have been reported in bovine mastitis studies by Tomazi *et al*. (2018) in Brazil (29.1%) and Zhang *et al*. (2018) in China (47.7%). Jisuve *et al*. (2020) found a higher resistance in clindamycin of 30% in group B Streptococcus isolates from pregnant women, Kenyatta national hospital, Kenya. Nonetheless, Orucho *et al*. (2020) reported a high susceptibility level to erythromycin in *S. pneumoniae* isolated from humans with pneumonia in Kisii hospital, Kenya. Increased resistance to macrolides and lincosamide in *Streptococcus* species continues to be reported globally (Cheng *et al.*, 2019). Horizontal transfer of resistance genes among *Streptococcus* species has also been described (Minst *et al*., 2012). Methylation of *erm* genes and efflux pumps mediated by *mefA*, ermTR and *linB* genes have been described as the key resistance mechanism used by the bacteria to confer resistance to macrolides and lincosamides (Minst *et al*., 2012, Jisuve *et al*., 2020).

Strikingly, 13.6% resistance to vancomycin was reported in this study. This was relatively higher than findings by Cheng *et al*. (2019) in China who reported a prevalence of 9%. Vancomycin is usually used as a last resort antibiotic in the treatment of multidrug Gram-positive infections in humans (Du *et al*., 2019, Cheng *et al.*, 2019). These critically important human medicine antibiotics are restricted for use in the treatment of animal diseases in many countries including Kenya (Phophi *et al*., 2019). Low resistance to vancomycin in streptococcus species associated with *VanG* gene has been described (Du *et al*., 2019). The presence of vancomycin-resistant isolates in streptococcal isolates in this study poses a severe public health concern and hence routine monitoring should be enhanced (Cheng *et al*., 2019).

This study found high susceptibility to fluoroquinolones (100%) among the *Streptococcus* isolates. This was in agreement with other similar studies by (Kaczorek *et al*., 2017) in Poland (100%) susceptibility in both *S. agalactiae* and *uberis*. Further, Ruegg *et al*. (2015) in Wisconsin reported (97%) susceptibility to fluoroquinolones in streptococci. Fluoroquinolones are highly restricted for use in animal diseases in most countries including Kenya (Kaczorek *et al.*, 2017). This may explain the high susceptibility reported in this study.

Multidrug resistance (MDR) among the *Streptococcus* species was reported at 13.6%. This was comparable to what was reported among *Streptococcus* spp in Germany (13%) (Minst *et al*., 2012.). However, Minst *et al*., (2012) reported a higher MDR rate in *Streptococcus uberis.* A relatively higher prevalence of MDR was reported in china (21%) (Cheng *et al*., 2019). Continuous monitoring of AMR-resistant *Streptococcus* isolates and antibiotic susceptibility testing before treatment should be implemented to prevent further spread and development of AMR in the dairy farms (Minst *et al*., 2012).

4.4.3 Phenotypic Antibiotic susceptibility patterns of the isolated *Pseudomonas aeruginosa* and *Escherichia coli*

In this study, all isolates exhibited phenotypic resistance to at least one of the antibiotics tested. This observation was consistent with that of Nam *et al*. (2009), who reported low susceptibilities among Gram-negative bacteria to almost all antibiotics tested. Srinivasan *et al*. (2007) also reported similar findings in their study in the USA where they reported that all 129 *E. coli* isolates showed resistance to one or two antibiotics drugs tested. Ismail & Abutarbush, (2020) reported

similar findings in Jordan where they found that all 14 *E. coli* isolates studied were resistant to six different classes of antibiotics. The high resistance profiles reported in this study are indicative of indiscriminate use of antibiotics in the study areas as reported in many countries in different studies (Nam *et al*., 2009). High usage of antibiotics leads to selective pressure for antimicrobial-resistant Gram-negative isolates and the progressive spread of antibiotic resistance genes through mobile genetic elements as described in other bovine mastitis studies (Liu *et al*., 2018, Zhang *et al*., 2018).

In the present study, high resistance to β lactams (ampicillin 83.9% and cefaclor 80.7%) was reported among the Gram-negative bacteria. High β lactam resistance level in Gram-negative has been reported in many countries such as Japan, Korea, Egypt, China and the USA (Ohnishi *et al.,* 2011, Nam *et al*., 2009, Ameen *et al.,* 2019, Srinivasan *et al*., 2007). Overexpression of the β -lactamase genes in Gram-negative bacteria are responsible for high phenotypic resistance reported in the beta-lactams antibiotics (Srinivasan *et al*., 2007, Nam *et al.,* 2009). However, detailed genotypic characterization is recommended to confirm this in this study.

Notably, a significantly higher resistance rate to chloramphenicol was reported in this study in *P. aeruginosa* (42%) compared to *E. coli* (8%). These findings closely agreed with those of Meng *et al*. (2020) who found that 45.3% of *P. aeruginosa* isolates were resistant to chloramphenicol, in China. Higher resistance rates in *P. aeruginosa* to chloramphenicol have been reported in Korea (99%) and Egypt (95%) (Nam *et al.,* 2009, Ameen *et al.,* 2019). However, unlike in Korea and Egypt where the drug is used in the treatment of mastitis, in Kenya and many other countries, chloramphenicol is restricted for use in animals (GARP, 2011). This finding could be indicative of

a horizontal transfer of AMR genes from human pathogens (Srinivasan *et al*., 2007, Alonso *et al.*, 2017). Therefore, this high resistance raises concern and should be further investigated.

Similarly, as observed in Egypt in camels by Elhariri *et al.* (2017), in this study, 21% of the *P. aeruginosa* isolates were carbapenem (imipenem) resistant. However, this contrasted with findings by Ameen *et al.* (2019) and Schauer *et al.* (2021) in Egypt and Austria respectively who reported that all their *P. aeruginosa* isolates were susceptible to imipenem. Lower resistance rate to imipenem has also been reported in Egypt (2.9%) by Ibrahim *et al.* (2017) in mastitic cow's milk and Falodun & Musa (2019) in cow dung in Nigeria (6.7%). However, a higher level of carbapenem resistance *P. aeruginosa* (100%) was recently reported in Kenya in a study by Musila *et al.* (2021) from human clinical samples. The high prevalence reported in this study is a serious clinical and public health threat and warrants close monitoring since the use of carbapenems such as imipenem in animals is highly restricted in many countries including Kenya (Falodun & Musa, 2019). These findings could be indicative of a possible horizontal gene transfer among bacteria in humans and animals.

A significantly low resistance level in *P. aeruginosa* and *E. coli* was observed against the fluoroquinolones and gentamycin in the present study. A similar finding was reported in *P. aeruginosa* in Japan, Korea, and Italy (Ohnishi *et al.*, 2011, Park *et al.*, 2014, Decimo *et al.*, 2016). This also agreed with the finding of Srinivasan *et al.* (2007) who found that only one *E. coli* isolate was resistant to fluoroquinolones in their study in the USA. In contrast, however, a study in Egypt

reported a higher resistance rate in *P. aeruginosa* (70%-95%) to quinolones and aminoglycosides (Ameen *et al.*, 2016).

In this study, multidrug resistance (MDR) in *E. coli* was reported at 66.7%. Higher MDR in *E. coli* has been reported in the USA (90%), China (98%) and Jordan (100%) (Srinivasan *et al*., 2007, Feng *et al.*, 2016, Ismail & Abutarbush, 2020). On the other hand, a considerably lower MDR in *E. coli* at 5.5% has been reported in Egypt (Ameen *et al.*, 2016). Overuse and misuse of antibiotics in the dairy industry are key contributors to the development of MDR *E. coli* (Alonso *et al.*, 2017). The increasing emergence of mastitis-associated MDR *E. coli* isolates globally is alarming and a public health concern that requires urgent intervention (Ismail & Abutarbush, 2020).

High MDR in *P. aeruginosa* was reported at 78.9% in this study. A higher prevalence of MDR in *P. aeruginosa* at (90%-96.7%) has been reported in Korea (Nam *et al*., 2009, Park *et al*., 2014). *P. aeruginosa's* high resistance to multiple drugs of clinical use is a serious problem to mastitis therapy and of public health concern (Nam *et al*., 2009, Meng *et al.*, 2020). The innate resistance mechanisms of *P. aeruginosa* such as impermeable cell walls, biofilm formation and enzymes further limit therapeutic options (Meng *et al*., 2020, Aguayo *et al.*, 2020). Hyper-mutation and horizontal gene transfer of the antibiotic resistance genes in *P. aeruginosa* have been described (Meng *et al.*, 2020). Genetic transfers of an antibiotic resistance gene from non-pathogenic *Pseudomonas* spp. to *P. aeruginosa* have been described which is a serious concern (Aguayo *et al.*, 2020, Schauer *et al*., 2021).

4.5 CONCLUSION

Staphylococci: This study revealed a high ampicillin resistance rate and low resistance rate to fluoroquinolones among the bovine mastitis-causing staphylococci. In addition, *blaZ* and s*trB* were the most prevalent genes among the isolates. Detection of various antibiotic resistance genes in these strains signifies a public health concern and a serious challenge to bovine mastitis therapy. Therefore, there is need to control the emergence and spread of AMR in dairy farms.

The presence of phenotypic methicillin-resistant staphylococci (MRS) in this study provides a baseline data for their further monitoring in Kenyan dairy farms. Further screening of the *mec* genes (*A, B, C*) and other intrinsic mechanisms encoding for resistance to MRS should be considered in future studies.

PCR was used to determine the genotypic resistance in this study, this technique targets fewer AMR genes, restricting the results to screened elements. Therefore, there is need for further characterization of the isolates using whole genome sequencing and *spa* typing to assess the clonal diversities of the isolates.

Streptococci: This study found moderate to low levels of antimicrobial resistance among *Streptococcus* spp. isolated from dairy cows in the study areas. The highest resistance was reported in tetracycline while the lowest was reported in gentamycin and fluoroquinolones. Resistance to vancomycin reported in this study is a public health concern and should be monitored. Further, continuous monitoring of the antimicrobial resistance in the region is needed to ensure optimal therapeutic results and prevent the further spread of AMR. Further studies incorporating a larger

sample size, molecular characterization of the *Streptococcus* isolates to species level and genotypic antimicrobial resistance profiles should be considered in future studies.

***E. coli* and *P. aeruginosa*:** This study reported high multidrug resistance among *E. coli and P. aeruginosa* isolates. This is indicative of the emergence of bovine mastitis that is caused by bacteria that are recalcitrant to antimicrobials commonly used for the treatment of mastitis in the regions. Urgent measures should be taken to address this issue because the two organisms are frequently found in the animal environment. The study provides the first report on the presence of imipenem-resistant *Pseudomonas aeruginosa* in mastitic milk and should be further investigated.

Study limitation: For the Gram negative and streptococcus the study analyzed fewer isolates and used only phenotypic antibiotic susceptibility testing methods. In addition, the antibiotics disc used were few and some of them especially in *P. aeruginosa* lacked approved clinical breakpoints. Future studies should target higher numbers of the isolates and consider using more antibiotics using minimum Inhibitory concentration (MIC) method. Determining the presence of antimicrobial-resistant genes conferring resistance to various antibiotics using whole-genome sequencing (WGS) is highly recommended. Such information will be key in deepening the understanding of *E. coli* and *P. aeruginosa* isolates, in terms of pathogens' dynamics and antimicrobial resistance (AMR) evolution. In addition, the information generated will be useful in the improvement of AMR stewardship and control of the further spread.

CHAPTER FIVE: RISK FACTORS ASSOCIATED WITH MASTITIS IN DAIRY COWS IN EMBU AND KAJIADO COUNTIES IN KENYA

5.1 INTRODUCTION

Mastitis is a multifactorial, widespread and costly disease of dairy cows in the world (Ruegg, 2017). Incidences of clinical and subclinical mastitis in cows per year range between 10%-40% and 19.2%-83.0% respectively in most of the countries in the world including Kenya (Jamali *et al.,* 2018, Bhakat *et al.,* 2020, Gitau *et al.,* 2014). Due to the multifactorial and multi etiological nature of bovine mastitis, treatment and control of the disease have remained one of the greatest challenges in dairy farming (Oliveira *et al.,* 2015). Host, pathogen and management factors have been shown to directly influence the occurrence and recurrence of mastitis in dairy cows (Ramírez *et al.,* 2014). Host factors such as parity (older cows) and early lactation periods have been described as key risk factors to the occurrence and recurrence of clinical mastitis in cows (Jamali *et al.,* 2018). On the other hand, cow breed, higher parity and late stage of lactation have been described as significant risk factors associated with subclinical mastitis (Ramírez *et al.,* 2014, Abebe *et al.,* 2016).

Several management risk factors associated with bovine mastitis have been described in many countries in the world (Neave *et al.,* 1969). However, in Kenya, only a limited number of studies have studied risk factors of mastitis in dairy cows despite the high prevalence of the disease (Mureithi & Njuguna, 2016). For instance, Mureithi & Njuguna, 2016, in their study in Kenya, found that multiparous cows, breed, mid-lactation, dirty udders and muddy/soil floor were significantly associated with subclinical mastitis in smallholder dairy farms in urban and peri-urban regions of the Thika, Kiambu County. The main bacteria isolated in their study was *S. aureus*.

Husbandry practices have shown to differ from one region to another and therefore control programs should be based on local specific risk factors within a setup (Ramírez *et al.,* 2014, Jamali *et al.*, 2018).

Moreover, due to the continued evolution of mastitis pathogens that are reported in many countries including Kenya, a review of the responsible risk factors will be useful in designing more efficient mastitis control programs (Ruegg, 2017). Therefore, the objective of this study was to establish and document farm management and cow level risk factors that are associated with dairy cow mastitis (clinical and subclinical) in Embu and Kajiado counties.

5.2 MATERIALS AND METHODS

5.2.1 Study area

As per section 3.2.1

5.2.2 Sample size determination

As per section 3.3.1

5.2.3 Case definition

A cow was defined to have subclinical mastitis based on two categories; CMT results and bacterial infection in this study. For subclinical mastitis based on CMT, a cow was positive for mastitis if one or more quarters had California Mastitis Test (CMT) score positive of +1 or higher. For, subclinical mastitis based on bacterial infection, a cow was positive for mastitis if one or more quarters had one or all the bacteria isolated from the milk sample. The bacteria isolated were; Coagulase-negative *Staphylococcus* (CNS), *Staphylococcus aureus, Escherichia coli, Bacillus* species, *Streptococcus agalactiae,* other non-*Streptococcus agalactiae*, species, *Micrococcus* species, *Enterobacter* species and *Klebsiella* species. Similarly, a farm was defined to be positive for subclinical mastitis if any milk samples from that farm had a CMT score positive of +1 or higher and if one or more quarters had one or all the bacteria isolated from the milk sample.

5.2.4 Collection of data on risk factors associated with dairy-cow mastitis

A pre-tested semi-structured questionnaire (Appendix 2) was used to collect data on farm biodata, farm level and cow level information. This was done through, interviewing the farmers and direct

observations by the investigator on the dairy cow's husbandry practices during the farm visits. Selected farm and cow level risk factors analyzed in this study are shown in Table 5.1 and Table 5.2 respectively. Three of the variables collected in this study were not analyzed. These included: milking techniques since all the farmers used hand milking, washing hands with clean water before milking, all milkers washed hands before milking their cows and cow housed in cow shed with a roof 99%(153) of the farmers housed their animals in a roofed cow shed.

5.2.5 Data entry and analysis

Data entry and management were done using Microsoft excel 2016, while data analysis was done using the STATA version15. Descriptive statistics were calculated for all variables of interest. All variables of interest were assessed for confounders, collinearity and interaction prior to analysis (Dohoo *et al*., 2003). In this study, variance linearization estimation procedures were used to account for standard errors that arise due to clustering and repeated measures structure of the data (multiple observation of cows clustered within farms) (Dohoo *et al.*, 2003). The farm was identified as the primary clustering unit (Dohoo *et al.*, 2003).

Multilevel mixed-effect logistic regression analysis was used to assess the association between the dependent variable (subclinical mastitis based on CMT score of +1 and above, and subclinical mastitis based on presence of bacteria isolated from milk samples) with each risk factor. All variables which had a p value <0.2 (20%) in the univariate analysis were fitted into a mixed effect multivariable model using backward elimination approach. In this analysis, the statistical

significance was set at $p<0.05$. The Output of the model was presented using Odds Ratio with 95% Confidence Interval (CI) of the Odds Ratio (Dohoo *et al*., 2003).

5.3 RESULTS

5.3.1 Descriptive statistics for mastitis risk factors

Farm-level management and cow-level factors collected and analyzed in this study are shown in Table 5.1 and 5.2. The prevalence of mastitis differed in this study between the two counties. Farms in Embu County reported a higher prevalence of mastitis at 78.8% (63/80) as compared to those in Kajiado County which had 74.3% (55/74). Based on the production system, a higher mastitis rate was reported in cows reared using an intensive production system at 79.5% (101/127) compared to those farms which used a semi-intensive system 63.0% (17/27).

This current study found that farms, where the cows were kept in cow sheds with earthen floors, had a higher mastitis rate at 83.8% (31/37) compared to those on houses with concrete floors 74.4% (87/117) (Figure 5.1). Farms, where dairy cows slept on bedding material (rubber mats) had less mastitis at 71.7% (33/46) compared to farms where cows had no bedding on their floor at 78.7% (85/108) in this study (Figure 5.2). Based on cleaning frequency of the floor, farms which cleaned the floors once a week had had higher prevalence of mastitis at 81.5% (31/38) compared to farms which did daily cleaning of the floor of the houses at 75.0% (87/116).

The prevalence of mastitis was higher in farms where the milkers did not use drying towel after washing the udder at 87.5% (7/8) as compared to farms where the milker used a drying towel 76.0% (111/146) in this study. The prevalence of mastitis was higher in farms where a single udder cloth

towel was shared among cows in the herd at 85.2% (75/88) as compared to farms where each cow had its own udder drying cloth after washing 65.2% (43/66) (Figure 5.3 A&B).

Farms, where teat dips were not used, had a higher prevalence of mastitis at 79.0% (109/138) compared to farms where teat dips were used either at the beginning or end of the milking at 56.2% (9/16). Farms that practiced dry therapy had lesser mastitis cases at 72.7% (24/33) as compared to farms where dry therapy was not practiced 77.7% (94/121). Farms, where the culling of mastitic cows was practiced in this study, had lesser cases of mastitis 69% (20/29) as compared to farms where culling was not practiced 78.4 % (97/ 125). Farms that never practiced routine screening of mastitis had a higher prevalence of mastitis 81.7% (47/58) as compared to farms that practiced routine screening for mastitis 74% (71/96) (Table 5.1).

Table 5.1: Farm management practices considered as risk factors for clinical and subclinical mastitis defined by culture positive in 154 smallholder dairy farms in Embu ad Kajiado Counties

Variable	Group	Description	Mastitis positive cases n (%)
County	Kajiado (n=74)		55 (74.3)
	Embu (n=80)		63 (78.8)
Production system	Semi-intensive (n=27)	Semi intensive farms cows were housed and allowed to graze on pastures and supplemented with concentrates	17 (63)
	Intensive (n=127)	Intensive farms cows were kept indoors and fed on concentrates	101(79.5)
Cleaning frequency of the floor	Daily (116)	Floor of the cow shed cleaned daily	87 (75)
	Weekly (38)	Floor of the cow shed cleaned weekly	31 (81.5)
Floor-type	Concrete (n=117)	Concrete floors made of cement blocks	87 (74.4)
	Earthen (n=37)	Earthen floors made of ground itself	31 (83.8)
Bedding material	Yes (n=46)	Presence of bedding material meant cows had rubber mats to sleep on	33 (71.7)
	No (n=108)		85 (78.7)
Milking mastitic cow the cows last	Yes (n=43)	Yes; milker milked cows with mastitis last No: milkers milked cows with mastitis with no order	25 (58.1)
	No (n=111)		93 (83.8)
Udder drying cloth towel	Yes (n=146)	Yes; teats were washed with water and dried with cloth towel during milking	111(76)
	No (n=8)	No: teat were washed with water and not dried	7(87.5)
Udder drying cloth towel for each cow	Yes (n=66)	Yes: each cow had its own udder drying cloth after washing	43(65.2)
	No (n=88)		75 (85.2)
	No (n=89)	No: A single udder cloth towel was shared in the herd	74 (83.1)
Use of Teat dips	Yes (n=16)	Yes; Farmers used a recommended chemical after milking	9 (56.2)
	No (n=138)	No; Farmers never used any recommended chemical after milking	109 (78.9)
Dry therapy	Yes (n=33)		24 (72.7)
	No (n=121)		94 (77.7)
Culling	Yes (n=29)	Yes; chronic mastitic cows were permanently removed from the herd	20 (69.0)
	No (n=125)	No; mastitis cows were not removed from the hers	97 (77.6)
Test for mastitis	Yes (n=96)	Yes: farms practiced routine screening for mastitis	71 (74.0)
	No (n=58)	No; farms never practiced routine screening of mastitis	47 (81.0)

Figure 5.1: A picture showing a cow in a shed with earthen floor type (indicated in blue arrow) and a soil (mud) bedding (indicated in blue arrow) in Embu County.

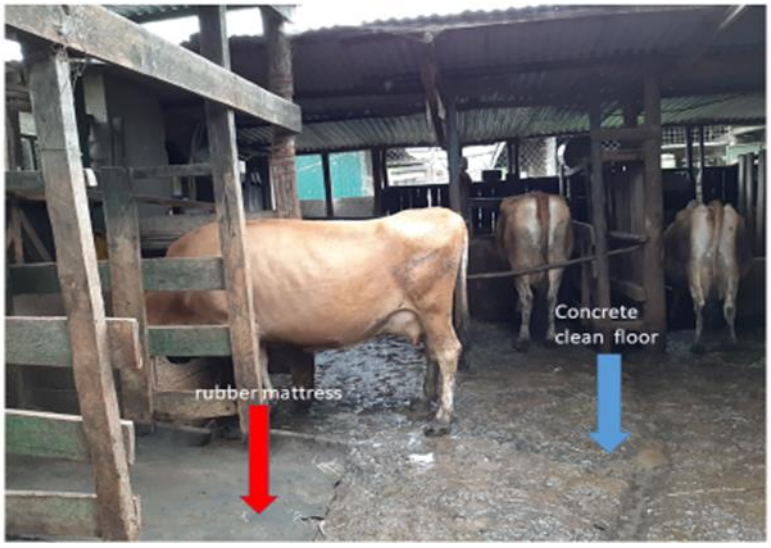


Figure 5.2: A picture showing a cow shed with a clean concrete floor (indicated by blue arrow) and a rubber cow mattress bedding (indicated by red arrow) in Kajiado County.

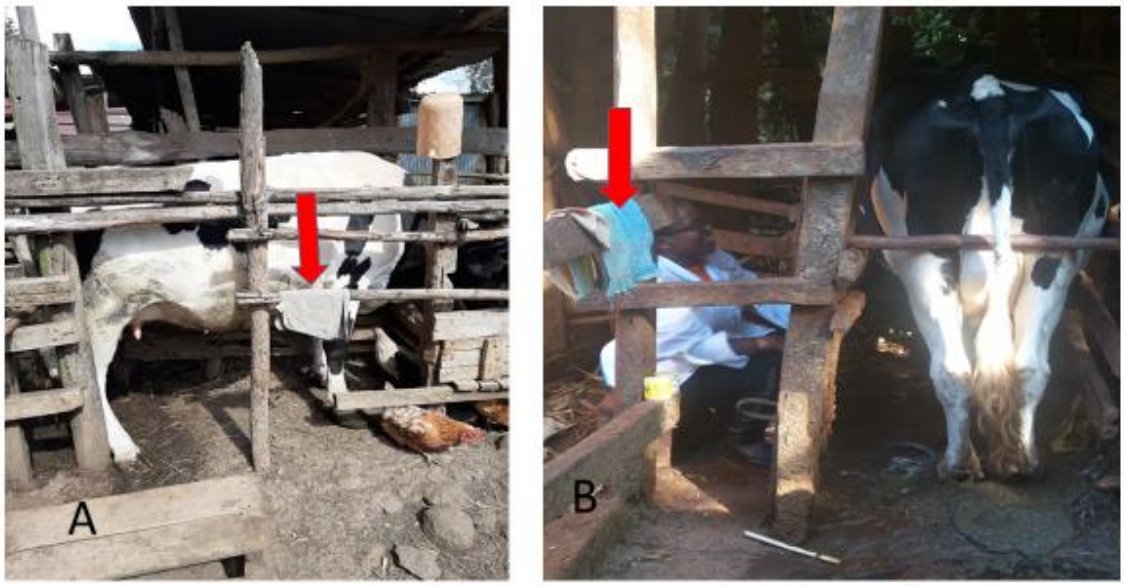


Figure 5.3 A&B: Picture showing reusable udder drying towel (pointed by red arrows) hang in the milking crushes in farms in Kajiado (image A) and Embu (image B) Counties

In this study, the prevalence of mastitis differed between the breeds. Exotic cows had a higher prevalence of mastitis at 75.3% (278/369) as compared to the crossbreed cows who had 61.5% (16/26). The prevalence of mastitis also varied based on parity level in this study. A high prevalence of mastitis was reported in cows in fourth parity or higher at 79% (79/100) while lower prevalence was reported in cows in the first parity 66.7% (68/102). Dairy cows in the early stages of lactation had a higher prevalence rate of mastitis at 80.2% (85/106) compared to cows in the mid and late stages of mastitis. The prevalence of mastitis also differed based on the history of mastitis. Dairy cows with a previous history of mastitis had a higher prevalence of mastitis at 80.4% (127/158) as compared to cows without a previous history of mastitis in this study (Table 5.2).

Table 5.2: Selected cow level factors considered as risk factors for clinical and subclinical mastitis defined by culture in 395 dairy cows in Embu and Kajiado Counties

Variable	Category	No. of observation (n)	Mastitis positive cases n (%)
Breed	Exotic [1]	369	278 (75.3)
	Cross[2]	26	16 (61.5)
Parity	1	102	68 (66.7)
	2	99	75 (75.8)
	3	94	72 (76.6)
	>4	100	79 (79)
Stage of lactation	Early(>1-2months)	106	85 (80.2)
	Mid (>3-6months)	134	94 (70.1)
	Late (>7 months	155	115 (74.2)
History of mastitis[3]	Yes	158	127 (80.4)
	No	237	167(70.5)

[1]Exotic cows included Jersey, Friesian, Ayrshire, Guernsey: [2]Crosses included Boran, Sahiwal, Zebu and several crosses of Friesian with Boran, Sahiwal and Zebu, [3]History of mastitis cows have had contracted mastitis during current lactation period or a previous one

5.3.2 Mixed effect univariate logistic regression of risk factors with the occurrence of subclinical mastitis

Several risk factors were considered for mixed effect univariate logistic regression for the presence of subclinical mastitis based California mastitis test (CMT) and culture (bacterial isolation) as shown in Table 5.3 and 5.4. Among the risk factors analyzed management factors such as frequency of cleaning the cow sheds, milking mastitic cow last, culling, use of udder drying towel in a farm, use of udder drying towel for each cow and routine testing for mastitis as well as cow factors such as parity and a previous history of mastitis were univariably associated with the presence of mastitis

at (p<0.2) in this study. However, the other risk factors were not significantly associated with mastitis as indicated in Table 5.3 &5.4.

Table 5.3: Mixed effect univariate logistic regression analysis for association between management and cow level factors subclinical mastitis defined by California Mastitis Test(CMT) in Embu and Kajiado Counties Kenya

Variable	95% CI		*P*- value
Production system	-0.32	1.56	0.20
Cleaning frequency	0.26	1.59	**0.16***
Milking the cows last	0.21	1.84	**0.01***
Use of udder drying towel	0.80	3.35	0.23
Use of udder drying cloth towel for each cow	0.34	1.34	0.24
Washing hands between milkings	0.50	1.15	0.44
Teat dips	0.58	1.15	0.36
Culling	-1.83	1.22	**0.12***
Test for mastitis	1.76	1.83	**0.03***
Parity	0.09	0.48	**0.19***
History of mastitis	1.26	1.38	**0.14***

*Factors with significance at (p<0.2), CI Confidence Interval

Table 5.4: Mixed effect univariate logistic regression analysis for association between management and cow level factors and subclinical mastitis defined by culture in Embu and Kajiado Counties Kenya

Variables	95%	CI	*P*- value
Production system	0.50	1.23	0.41
Cleaning frequency of the cow sheds	0.22	1.43	**0.15***
Milking the cows last	0.23	1.69	**0.01***
Use of udder drying towel in a farm	0.56	3.31	**0.16***
Use of udder drying cloth towel for each cow	0.26	1.25	**0.20***
Washing hands between milkings	0.24	1.24	**0.18***
Culling	-1.16	0.52	0.46
Test for mastitis	-1.28	0.19	**0.15***
Parity	-1.64	0.59	0.27
History of mastitis	0.11	0.71	**0.06***

*Factors with significance at (p<0.2), CI Confidence Interval

5.3.3 Multivariate analysis of the subclinical mastitis risk factors

For subclinical mastitis defined by CMT, all factors showing p<0.2 (20%) in the initial univariate mixed-effect logistic regression were considered for inclusion in a multivariate mixed-effect regression analysis as previously described by Dohoo *et al*., 2003. The variables included were cleaning frequency of the cow sheds, milking mastitic cow last, routine testing for mastitis, culling, parity and history of mastitis. The multivariate analysis of these variables revealed that milking the

mastitic cow last (p=0.02), routine testing for mastitis (p=0.01) and history of mastitis (p=0.05) were significantly associated with the occurrence of subclinical mastitis in this study. Accordingly, the odds of having mastitis were significantly lower (0.51) in cows with no previous history of mastitis compared to cows who had a previous history of mastitis (O. R=0.51, 95% CI, 0.25-1.05). The odds of mastitis were significantly lower (0.35) in farms where no routine testing of mastitis was done compare to those who did (O. R=0.35, 95% CI, 0.15-0.79). Further, the odd of the occurrence of mastitis increased in the farm which did not milk mastitic cows last (O. R=3.42, 95% CI, 1.58-7.42). However, the other variable remained non-significant in this study (Table 5.5).

Table 5.5: Mixed effects multivariable logistic regression models for the association between farm management and cow level factors and subclinical mastitis defined by CMT in Embu and Kajiado Counties

Variables	β coefficient	OR	95% CI	p-value
Milking mastitic cow last				
Yes	Reference			
No	0.84	3.42	1.58-7.42	**0.02***
Routine testing for mastitis				
Yes	Reference			
No	-0.74	0.35	0.15-0.79	**0.01***
History of mastitis				
Yes	Reference			
No	-0.55	0.51	0.25-1.05	**0.05***

*Factors statistically significant at (p≤0.05), SE- Standard error, OR-Odd Ratio, CI-confidence interval

Similarly, for subclinical mastitis defined by culture (bacterial infection) all factors showing p<0.2 (20%) in the initial univariate mixed-effect logistic regression were considered for inclusion in a multivariate mixed-effect regression analysis as previously described by Dohoo *et al*., 2003. The

variables included were cleaning frequency of the cow sheds, use of udder drying towel in farms, use of udder drying towel for each cow, milking mastitic cow last, routine testing for mastitis, washing hands between milkings and history of mastitis. The multivariate analysis of these variables revealed that milking the mastitic cow last (p=0.04) and history of mastitis (p=0.03) were significantly associated with the occurrence of subclinical mastitis this study. Accordingly, the odds of having mastitis were significantly lower (0.50) in cows with no previous history of mastitis compared to cows who had a previous history of mastitis (O. R=0.50, 95% CI, 0.26-0.95). The odd of the occurrence of mastitis increased in the farm which did not milk mastitic cows last (O. R=2.62, 95% CI, 0.26-0.95). However, the other variable remained non-significant in this study (Table 5.6).

Table 5.6: Mixed effects multivariable logistic regression models for the association between farm management and cow level factors and subclinical mastitis defined by culture in Embu and Kajiado Counties

Variables	β coefficient	OR	95% CI	p-value
Milking mastitic cow last				
Yes	Reference			
No	0.74	2.62	1.30-5.27	**0.04***
History of mastitis				
Yes	Reference			
No	-0.58	0.50	0.26-0.95	**0.03***
***Factors statistically significant at (p≤0.05), OR-Odd Ratio, CI-confidence interval**				

5.4 DISCUSSION

The current study explored management farm practices and cow level factors associated with clinical and subclinical mastitis in dairy cows in Kajiado and Embu counties.

Failure to milk mastitic cows last was significantly associated with mastitis in this study. These findings were in agreement with reports by Abebe *et al*. (2016), in Ethiopia and Nielsen & Emanuelson (2013), in Sweden. They reported that failure to milk mastitic cow last increased the spread of mastitis in farms from one cow to another during milking. These findings may explain the reason for high farm-level prevalence in this study since all farmers used hand milking. Farmers need to be educated on the importance of knowing the cow's udder health status and encouraged to milk mastitic cow last to prevent the spread of mastitis (Nielsen & Emanuelson, 2013).

In this study, cows with no previous history of mastitis were found to be 0.50 less likely to have mastitis, compared to cows with a previous history of mastitis. Similar findings have been reported in several studies in Bangladesh (Sarker *et al*., 2013), Ethiopia (Mekonnen *et al.,* 2017), India (Kumar *et al*., 2016) and Brazil (Oliveira *et al.*, 2015). However, Abebe *et al*., 2016, in Ethiopia, found no association between history of mastitis and recurrence of mastitis. High recurrence of mastitis especially in clinical mastitis has also been described (Zadoks, 2001, Jamali *et al*., 2018). Inadequate screening and treatment of subclinical mastitis, lack of correct and specific identification of the mastitis-causing pathogens in clinical cases may have led to the recurrence of mastitis in this study (Jamali *et al*., 2018). In addition, the indiscriminate use of antibiotics by farmers leading to the development of mastitis-causing antibiotic-resistant pathogens was believed

to be a critical contributor to the recurrence of mastitis in this study (Omwenga *et al.,* 2020). Certainly, such cows need to be culled to prevent further transmission of mastitis in the farms (Kumar *et al*., 2016; Jamali *et al*., 2018).

Continues monitoring of mastitis leads to improved udder health (Lam *et al*., 2009, Ruegg, 2017). It was noted that cows in farms that did not do routine monitoring of mastitis (0.35) had a lower odd of getting mastitis compared to farms that did. These findings may be due to the fact farmers in this study used CMT and alcohol test methods to routinely test for mastitis. Although the use of CMT in the detection of bovine is valuable to a certain extent, this method has remained questionable. This is because the results are usually difficult to interpret due to the other factors that influence high somatic cell counts and the method fails to identify the real causative agent of mastitis (Duarte *et al.,* 2015, Sharun *et al.,* 2021). Therefore, more reliable methods such as a combination of CMT, culture and molecular should be used in the routine diagnosis of mastitis in the study regions (Lam *et al.,* 2009).

Unlike in other studies, parity was not a significant cow level factor of mastitis in this study. Studies have shown that level of parity is a key predictor of mastitis in dairy cows (Ramírez *et al.,* 2014, Mureithi & Njuguna 2016, Abebe *et al.,* 2016). Although parity was not a statistically significant risk factor associated with mastitis in this study, cows with parity of four and more had a higher odd of getting mastitis compared to cows in lower parities. These results could be explained by the fact older cows have pendulous udders and teats which are more susceptible to injury and therefore increased the risk of mastitis (Abebe *et al.,* 2016). In addition, older cows also have a higher chance

of exposure to mastitis pathogens in previous lactations and therefore they are more likely to come down with mastitis again (Oliveira *et al.*, 2015, Jamali *et al.*, 2018).

5.5 CONCLUSIONS

Factors associated with subclinical mastitis risk factors in this study were the previous history of mastitis, routine testing for mastitis and failure to milking mastitic cow last. These results suggest that farms in the study regions should consider these important risk factors when developing subclinical mastitis control programs.

Study limitation: Due to low prevalence of clinical mastitis cases in this study the logistic regression analysis was not done. Future studies should consider use of case control design in order to analyze the risk factors associated with clinical mastitis in the study sites.

CHAPTER SIX: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

In Kenya, a few studies have investigated bovine mastitis, bacterial diversity, associated risk factors, and antimicrobial resistance profiles of the isolates (Gitau *et al.,* 2014, Muriethi & Njuguna 2016, Richards *et al.,* 2019). However, all these previous studies were limited because they only used classical culture methods. The culture method has been faced with limitations such as a delay of 24–48 hours to obtain results, and most bacterial organisms present in milk are not detected (Kuehn *et al.,* 2013, Oikonomou *et al.,* 2013). As a consequence, effective treatment and prevention of the disease have been hindered (Kuehn *et al.,* 2013). This is coupled with the increasing numbers of potentially zoonotic multidrug-resistant (MDR) bacterial strains, associated with mastitis in dairy cows (Sharma *et al.,* 2018). Therefore, this study investigated the prevalence, bacterial diversity of mastitic milk using culture and culture independent 16S rRNA metagenomics analysis, associated risk factors and Antibiotic susceptibility profile (AMR) of the isolates in dairy cows in Kajiado and Embu Counties, Kenya in order to improve mastitis therapy and control emergence and the spread of AMR Kenya.

This study reported a higher overall prevalence of clinical mastitis at (6.8%) and subclinical mastitis at (74%) in Kajiado and Embu Counties compared to previous mastitis studies in Kenya. A lower prevalence of clinical mastitis ranging between (0.5%-0.9 %) and subclinical (30%-50%) has been reported previously in different parts of Kenya (Gitau *et al.,* 2014). However, a more recent study in one farm by Ondiek & Kemboi, (2018) reported a higher prevalence of mastitis at (82%) in Njoro, Egerton, Kenya. These results suggest that changes over time, spatial sampling,

geographical and management practices differences might explain discrepancies in prevalence levels between regions (Taponen *et al.,* 2009). Moreover, the high prevalence of subclinical mastitis reported in this study could suggest limited farmer awareness of this form of mastitis since it lacks clinical signs. Inadequate mastitis control programs as noted in the study areas and as reported in other studies in Kenya and Africa could also be a key contributor (Abebe *et al.,* 2016, Adamu *et al.,* 2020).

The predominance of subclinical mastitis in the study sites is of great concern as it may lead to high usage of antibiotics leading to multidrug resistance (MDR) in mastitis pathogens (Adamu *et al.,* 2020). Moreover, subclinical mastitis is associated with higher economic losses since it is hardly diagnosed by farmers due to a lack of clinical signs (Abebe *et al.,* 2016, Ismael, 2018). Therefore, control measures of mastitis should be instituted in the study sites.

Embu County had a significantly higher farm, cow, and quarter-level prevalence of mastitis compared to Kajiado. Higher prevalence in Embu could be associated with poor hygiene, poorly designed cow shed, poor drainage and the extremely wet weather experienced during the sampling period. Season variation has shown to directly influence the occurrence of mastitis (Kurjogi & Kaliwal, 2014). The presence of high moisture in the environment during the wet weather supports the proliferation of pathogenic bacterial resulting in increased mastitis incidences in dairy cows (Kurjogi & Kaliwal, 2014).

Coagulase-negative *staphylococcus* (CNS) (47%) was the predominant mastitis-causing organisms diagnosed using the culture method. However, metagenomics analysis of the same milk samples showed the predominance of the genera *Pseudomonas* in the analyzed samples. These results suggest that *Staphylococcus* may not be the predominant species in these quarters and provide new insights into the microbial diversity of the cow mastitic milk in Kenya. However, epidemiological studies have shown that CNS are emerging bacteria increasingly being identified as a major causative agent of cow mastitis infections worldwide (Sender *et al.,* 2017, Vakkamäki e*t al.,* 2017, Yang *et al.,*2018). These shifts have largely been attributed to change in management practices, new breeds, changes in mastitis causative agent's virulence mechanisms of host-adaptation, and increasing societal and economic pressure (Zadoks *et al.,* 2011, Ndahetuye *et al.,* 2019). Therefore, investigating the epidemiology and the specific risk factors contributing to the emerging/evolution of mastitis pathogens is necessary (Gitau *et al.,* 2014, Motaung *et al.,* 2017).

Streptococcus agalactiae a key contagious mastitis pathogen was reported at very low level in this study. Similar to other previous findings in other studies in Kenya (Gitau *et al.,* 2014, Muriethi *et al.,* 2017), these results indicate that *S. agalactiae* is no longer a major problem in bovine mastitis in the study areas.

This study reported 20% culture-negative milk samples from mastitic cows. These findings were lower than reported by Richards *et al.* (2019) in Kenya who reported lack of growth in 37.5% of the mastitis cases. However, this study results were higher than reported by Gitau *et al.* (2014) who reported lack growths in 10% in all mastitic cases. Globally, about 10%-40% of mastitis cases

remain culture-negative in routine culture assays (Kuehn *et al.*, 2013, Bhanderi *et al.*, 2014). The lack of growth samples has remained a challenge in the diagnosis of mastitis worldwide (Oikonomou *et al.*, 2013). Limitations of the culture methods, low level of bacteria in milk, cow pretreated with antibiotics before sampling, and causative agents of mastitis, not bacteria have been described as key reasons for lack of growth in mastitic milk (Kuehn *et al.*, 2013).

Analysis of the risk factors associated with mastitis found that failure to milk mastitic cows last, routine mastitis testing and cow with a previous history of mastitis were significantly associated with the occurrence of mastitis in this study. Although there is no previous data from the study regions to compare with, similar findings have been reported in Ethiopia and Sweden (Abebe *et al.*, 2016, Nielsen & Emanuelson, 2013). Failure to milk mastitic cow lasts increased the spread of mastitis in farms from one cow to another during milking may explain the high prevalence of subclinical mastitis reported in this study (Nielsen & Emanuelson, 2013).

In this study, the alpha and beta diversity comparison showed that there were no significant differences in bacterial numbers and diversity based on study region, culture growth status and disease status. These results indicate that bacterial number and diversities in milk from quarters did not differ between the region, culture growth status and disease status in this study.

Similar to other studies using culture-independent 16S rRNA metagenomics analysis of the mastitic milk revealed that the phyla *Proteobacteria* and *Firmicutes* were most abundant in all mastitic samples (Pang *et al.*, 2018, Zhang *et al.*, 2019). The dominance of *Proteobacteria* and *Firmicutes*

in mammary glands has been linked to the existence of potential an endogenous entero-mammary pathway facilitating the migration of gut bacteria into the mammary gland (Hoque *et al.,* 2019). These results indicate that similar to other studies globally, *Proteobacteria* and *Firmicutes* were the main bacteria in Kajiado and Embu, Kenya across all the categories and provides confidence in the study.

An increased relative abundance of some phyla and genera known to cause mastitis which are hard to identify using standard culture methods such as *Chlamydia*e and *Mycoplasma* in culture-negative mastitic milk were reported. These results indicate that it would be useful to apply more sensitive mastitis detection methods, especially on culture-negative clinical samples. These findings confirm the usefulness of metagenomics over culture methods (Kuehn *et al.*, 2013).

Other genera with increased relative abundance in the culture-negative clinical samples included; *Pseudomonas, Acinetobacter,* and *Staphylococcus* both in Kajiado and Embu counties. These findings suggest a great degree of similarities of microbial diversities in the two geographical regions (Hoque *et al.*, 2019). Further the current study provides a baseline of microbial diversity of mastitic samples with no culture growth in the two regions.

In this study, three bacterial genera diagnosed by culture were also reported in 16S rRNA metagenomics. However, the results did not match well since a higher relative abundance of these organisms was reported in metagenomics. Furthermore, eight other topmost abundant genera were recovered using metagenomics analysis and not the culture method. This lack of direct comparison

between culture and metagenomics analysis has been described (Oikonomou *et al.,* 2012, 2014). These results demonstrate that classical culture methods fail to describe the true bacterial complexity of mastitic cow milk (Oikonomou *et al.,* 2012, 2014; Kuehn *et al.,* 2013). Therefore, the application of more sensitive mastitis detection methods especially in clinical and early subclinical stages are recommended.

Overall, this study showed a high proportion of resistance (50%-100%) to at least one antibiotic tested in all bacteria. The high resistance profiles reported in this study could be reflective of the high predominance of subclinical mastitis reported in the study sites resulting in the indiscriminate use of antimicrobial in the study areas (Omwenga *et al.,* 2020, Adamu *et al.* ,2020). This observation was consistent with other studies that have reported low susceptibilities amongst mastitis bacteria to almost all antimicrobial tested worldwide (Phopi *et al.,* 2019, Aklilu *et al.,* 2020).

This study reported much higher resistance rates to β- lactams (83%-31%) among mastitis pathogens than those reported previously in dairy cows in Kenya (Shitandi & Sternesjö, 2004, Gitau *et al.,* 2014). These results indicate an increase in the antibiotics resistance by the mastitis bacteria over the years. The higher resistance rates are likely due to widespread use of penicillin and other β- lactams in the treatment of mastitis in dairy cows as observed in the study farms and as evidenced by other previous studies in Kenya (Mitema *et al.,* 2001, Shitandi & Sternesjö, 2004). Continuous monitoring of the antimicrobial resistant bacteria (AMR) and awareness creation on AMR among farmers in the region is needed to ensure optimal therapeutic results and prevent the

further spread of AMR.

Overall, low resistant levels to gentamycin, quinolones, and chloramphenicol were reported in all isolates. Authors in Ethiopia (Kalayu *et al.,* 2020), South Africa (Phopi *et al.*, 2019), Canada (Nobrega *et al.*, 2018) reported similar findings. These critically important human medicine antibiotics are restricted for use in the treatment of animal diseases in many countries including Kenya (Phobi *et al*., 2019). However, even the low resistance rates reported are of public health significance, and continuous monitoring, control measures should be implemented to curb further spread.

This study reported the presence of some key World Health Organization (WHO) priority pathogens including methicillin-resistant staphylococci at 25% *S. aureus* and 10.8% CNS, vancomycin-resistant streptococcus species (13.6%), and 21% carbapenem (Imipenem) resistant *P. aeruginosa*. This is the first report, vancomycin resistant and Imipenem-resistant in mastitic isolates in Kenya. Although MRSA has been previously reported in Kenya by Omwenga *et al.* (2020) in raw milk in Isiolo Kenya, this study was the first to report methicillin resistance in Coagulase-negative *Staphylococcus* isolates from cow mastitic milk. Detection of these priority pathogens in mastitic milk is a public health concern and should be further investigated as most of these organisms are potentially zoonotic and multidrug-resistant and limits the role of therapy in the control of mastitis (Meng *et al.*, 2020).

Further, vancomycin and imipenem are highly restricted drugs for use in animals and are usually used as a last resort in the treatment of multidrug gram-positive and gram-negative bacterial infections in humans. The high resistance rates to these critically important antibiotics reported in this study are a clinical threat and public health concern (Ismail & Abutarbush, 2020). The finding from this study provides a baseline survey for further monitoring.

The presence of high prevalence resistance of genes encoding for the beta-lactamases *blaZ* gene recovered in *Staphylococcus* species was found in this study. This likely contributed to the high resistance level to beta-lactams recorded in this study. High *blaZ* genes might indicate an increased use and possible misuse of β lactams in the study farms in the treatment of staphylococcus mastitis (Qu *et al.*, 2017).

A high proportion of MDR in mastitis isolates was reported in this study with the highest being reported in gram-negative (66%-78.9%) and lowest among streptococci species at (13.6%). Higher MDR especially on gram negatives has been reported in bovine mastitis have been described (Ismail & Abutarbush, 2020). The high MDR in some bacteria like *P. aeruginosa* has been linked to innate resistance mechanisms such as impermeable cell walls, biofilm formation, and enzymes which have been shown to further limit therapeutic options (Meng *et al.,* 2020). However, for other organisms, overuse, and misuse of antibiotics in the dairy industry have shown to be the key contributors to the development of MDR based on other previous studies (Ameen *et al.,* 2017). The increasing emergence of mastitis-associated MDR isolates globally is alarming and a public health concern that requires urgent intervention (Ismail & Abutarbush, 2020). Molecular characterization

of the gram-negative bacterial isolates will be key in expanding the understanding of the *E. coli* and *P. aeruginosa* isolates, in terms of pathogens' dynamics and AMR evolution. Such information will be key in improving AMR stewardship and control of the further spread of AMR (Sharma *et al.*, 2018, Alawneh *et al*., 2020).

6.1 OVERALL CONCLUSIONS

1. Overall, there was a high prevalence (73%) of subclinical mastitis in Kajiado and Embu Counties with Coagulase Negative *Staphylococcus* (CNS) and *Pseudomonas* identified as the predominant bacteria in mastitis based on culture methods and 16S rRNA metagenomics analysis.
2. *Chlamydiae, Mycoplasma* and *Solibacillus* which are important causative agents of mastitis and are difficulty to culture were identified in culture-negative mastitic milk from Kajiado and Embu Counties
3. High antimicrobial resistance to penicillin and other Beta lactams antibiotics and a significant multidrug resistance (MDR) were observed in *Staphylococcus* species, *Escherichia coli*, and *Pseudomonas aeruginosa* in this study.
4. This is the first study to report the presence of phenotypic methicillin resistance (MRS) in Coagulase-negative *Staphylococcus*, carbapenem (Imipenem)-resistant *P. aeruginosa*, and vancomycin-resistant streptococci in mastitic milk isolates in Kenyan dairy farms.
5. This study identified a high presence of *blaZ* at (59%) and at *StrB* (35%) gene in *Staphylococcus* isolates thus signifying a public health concern and a challenge to bovine mastitis therapy.
6. The risk factors significantly associated with bovine mastitis in this study were failure to milk mastitic cow last, a previous history of mastitis and routine testing of mastitis.

6.2 RECOMMENDATIONS

1. Based on the high prevalence of subclinical mastitis dominated by CNS and *Pseudomonas* in the two counties there is a need to create awareness to the farmers regarding subclinical mastitis and the respective control measures such as milking mastitic cow last, using udder cloth for each cow, and culling mastitic cows adopted. Additionally, CNS and *Pseudomonas* species should be monitored routinely and further characterized to species level in order to improve mastitis management in the counties.
2. The presence of a high level of antimicrobial resistance among mastitis isolates including methicillin-resistant staphylococci (MRS), carbapenem resistant *P. aeruginosa* and Vancomycin-Resistant *Streptococcus* in the present study is a public health concern. Therefore, enhanced continuous surveillance of these priority bacterial pathogens is needed. In addition, effective antimicrobial stewardship especially to farmers and animal health practitioners in the study counties is recommended.
3. The use of more sensitive mastitis detection methods where possible such as 16S rRNA metagenomics analysis especially in culture-negative clinical and subclinical mastitic milk would be useful to improve diagnosis and therapeutic strategies of the disease in the study counties and Kenya at large.
4. Due to the high group of unassigned bacteria with the 16S rRNA metagenomics analysis future studies should consider the use of a shotgun or intermediate approaches to allow deeper microbial characterization of mastitic milk to species levels.

Study limitation and future studies

This study was carried out during the rainy season therefore the high prevalence of subclinical mastitis may be biased towards the wet weather season. The 66 milk samples used for the analysis of the microbial diversity in the 16S rRNA metagenomics analysis may not be sufficient to extrapolate the results to other counties. Therefore, extending metagenomics studies to other counties and increasing the number of milk samples would create robustness of the identified taxonomic profiles.

A major limitation of 16S rRNA studies is that they describe only bacterial communities at genus levels and higher taxonomies excluding diversity exploration at the species, strain levels. Moreover, these studies focus only on bacterial microorganisms and fail to identify archaeal, fungal, and viral communities which are also documented causative agents of mastitis. They fail to characterize microbial functions and resistomes. Therefore, future studies should consider the use of a shotgun or intermediate approaches to allow deeper microbial characterization.

Due to low prevalence of clinical mastitis cases in this study the logistic regression analysis was not done. Future studies should consider use of case control design in order to analyze the risk factors associated with clinical mastitis in the study sites.

REFERENCES

Abdel-rady, A. & Sayed, M. (2009): Epidemiological Studies on Subclinical Mastitis in Dairy cows in Assiut Governorate, Veterinary World, **2(10):**373-380

Abebe, R., Hatiya, H., Abera, M., Megersa, B., & Asmare, K. (2016): Bovine mastitis : prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. BMC Veterinary Research, **12**(1):270. https://doi.org/10.1186/s12917-016-0905-3

Abrahmsén, M., Persson, Y., Kanyima, B. M., & Båge, R. (2014): Prevalence of subclinical mastitis in dairy farms in urban and peri-urban areas of Kampala, Uganda. Tropical animal health and production, **46(1)**:99–105. https://doi.org/10.1007/s11250-013-0455-7

Adamu, H. O., Hussaini, R. O., Obasuyi, C., Anagha L.I, & Okoduwa G.O (2020): Prevalence of mastitis in Nigerian livestock : a Review. Journal of Open Veterinary **1**:20–29, https://doi.org/https://doi.org/10.1515/ovs-2020-0101.

Addis, M. F., Tanca, A., Uzzau, S., Oikonomou, G., Bicalho, R. C., & Moroni, P. (2016): The bovine milk microbiota: insights and perspectives from -omics studies. Journal of Molecular bioSystems, **12(8):** 2359–2372. https://doi.org/10.1039/c6mb00217j

Aguayo, P. R., Larenas, T. B., Godoy, C. A., Rivas, B. C., González-Casanova, J., Rojas-Gómez, D., & Caro Fuentes, N. (2020): Antimicrobial and Antibiofilm Capacity of Chitosan Nanoparticles against Wild Type Strain of Pseudomonas sp. Isolated from Milk of Cows Diagnosed with Bovine Mastitis. Antibiotics (Basel, Switzerland), **9**(**9):** 551. https://doi.org/10.3390/antibiotics9090551

Ahmadian, A., Ehn, M., & Hober, S. (2006): Pyrosequencing: history, biochemistry and future. Clinica chimica acta; international journal of clinical chemistry, **363(1-2):** 83–94. https://doi.org/10.1016/j.cccn.2005.04.038

Aklilu, E., Ying, H. C. (2020): First mecC and mecA Positive Livestock-Associated Methicillin Resistant Staphylococcus aureus (mecC MRSA/LA-MRSA) from Dairy Cattle in Malaysia. Microorganisms. **8(2):**147. doi: 10.3390/microorganisms8020147

Alawneh, J. I., Vezina, B., Ramay, H. R., Al-Harbi, H., James, A. S., Soust, M., Moore, R. J., & Olchowy, T. (2020): Survey and Sequence Characterization of Bovine Mastitis-Associated Escherichia coli in Dairy Herds. Frontiers in veterinary science, **7:** 582297. https://doi.org/10.3389/fvets.2020.582297

Ali, T., Ur Rahman, S., Zhang, L., Shahid, M., Zhang, S., Liu, G., Gao, J., & Han, B. (2016): ESBL-Producing Escherichia coli from Cows Suffering Mastitis in China Contain Clinical Class Integrons with CTX-M Linked to ISCR1. Frontiers in microbiology, **7**:1931. https://doi.org/10.3389/fmicb.2016.01931

Alnakip, M.A, Quintela-Baluja, M., Böhme, K., Fernández-No, I., Caamaño-Antelo, S., Calo-Mata, P., & Barros-Velázquez, J. (2014): The Immunology of Mammary Gland of Dairy Ruminants between Healthy and Inflammatory Conditions. Journal of veterinary medicine, **2014:**659801. https://doi.org/10.1155/2014/659801

Alonso, C. A., Zarazaga, M., Ben Sallem, R., Jouini, A., Ben Slama, K., & Torres, C. (2017): Antibiotic resistance in Escherichia coli in husbandry animals: The African perspective. Letters in applied microbiology, **64**(5): 318–334. https://doi.org/10.1111/lam.12724

Alves, L. F., Westmann, C. A., Lovate, G. L., de Siqueira, G., Borelli, T. C., & Guazzaroni, M. E. (2018): Metagenomic Approaches for Understanding New Concepts in Microbial Science. International journal of genomics, **2018**: 2312987. https://doi.org/10.1155/2018/2312987

Ameen, F., Reda, S. A., El-Shatoury, S. A., Riad, E. M., Enany, M. E., & Alarfaj, A. A. (2019): Prevalence of antibiotic resistant mastitis pathogens in dairy cows in Egypt and potential biological control agents produced from plant endophytic actinobacteria. Saudi Journal of biological sciences, **26(7):** 1492–1498. https://doi.org/10.1016/j.sjbs.2019.09.008

Amer, S., Gálvez, F. L. A., Fukuda, Y., Tada, C., Jimenez, I. L., Valle, W. F. M., & Nakai, Y. (2018): Prevalence and etiology of mastitis in dairy cattle in El Oro Province, Ecuador. Journal of Veterinary Medical Science, **80(6):**861–868. https://doi.org/10.1292/jvms.17-0504.

Amplicon (2013): 16S Metagenomic Sequencing Library Preparation. https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf Accessed on 20th May 2021

Anjum, M. F., Marco-Jimenez, F., Duncan, D., Marín, C., Smith, R. P., & Evans, S. J. (2019): Livestock-Associated Methicillin-Resistant Staphylococcus aureus From Animals and Animal Products in the UK. Frontiers in microbiology, **10:**2136. https://doi.org/10.3389/fmicb.2019.02136.

Ashraf, A., & Imran, M. (2018): Diagnosis of bovine mastitis: from laboratory to farm. Tropical animal health and production, **50(6):**1193–1202. https://doi.org/10.1007/s11250-018-1629-0

Asiimwe, B. B., Baldan, R., Trovato, A., & Cirillo, D. M. (2017): Prevalence and molecular characteristics of Staphylococcus aureus, including methicillin resistant strains, isolated from bulk can milk and raw milk products in pastoral communities of South-West Uganda. BMC infectious diseases, **17(1):**422. https://doi.org/10.1186/s12879-017-2524-4.

Ayele, Y., Gutema, F. D., Edao, B. M., Girma, R., Tufa, T. B., Beyene, T. J., Tadesse, F., Geloye, M., & Beyi, A. F. (2017): Assessment of Staphylococcus aureus along milk value chain and its public health importance in Sebeta, central Oromia, Ethiopia. BMC microbiology, **17(1):** 141. https://doi.org/10.1186/s12866-017-1048-9.

Barkema, H. W., Green, M. J., Bradley, A. J., & Zadoks, R. N. (2009): Invited review: The role of contagious disease in udder health. Journal of Dairy science, **92(10):** 4717–4729. https://doi.org/10.3168/jds.2009-2347

Bebe, B. O., Rademaker, C., Lee, J. Van Der, & Tonui, C. (2015): Executive summary Sustainable growth of the Kenyan dairy sector – A quick scan of robustness, reliability and resilience. http://dx.doi.org/10.18174/413390 accessed on 10th November, 2020.

Becker K. (2021): Methicillin-Resistant Staphylococci and Macrococci at the Interface of Human and Animal Health. Toxins, **13(1):**61. https://doi.org/10.3390/toxins13010061

Becker, K., Heilmann, C., & Peters, G. (2014): Coagulase-Negative Staphylococci. Clinical microbiology **27(4):**870–926. https://doi.org/10.1128/CMR.00109-13

Becker, K., van Alen, S., Idelevich, E. A., Schleimer, N., Seggewiß, J., Mellmann, A., Kaspar, U., & Peters, G. (2018): Plasmid-Encoded Transferable mecB-Mediated Methicillin Resistance in Staphylococcus aureus. Emerging infectious diseases, **24(2):** 242–248. https://doi.org/10.3201/eid2402.171074.

Bengtsson, B., H. E. Unnerstad, T. Ekman, K. Artursson, M. Nilsson- Ost, & K. P. Waller. (2009): Antimicrobial susceptibility of udder pathogens from cases of acute clinical mastitis in dairy cows. Veterinary Microbiology **136**:142–149.

Benić, M., Habrun, B., & Kompes, G. (2012): Clinical and epidemiological aspects of cow mastitis caused by Staphylococcus aureus and its methicillin-resistant strains. Medical Sciences, **37:** 113–122.

Bhakat, C., Mohammad, A., Mandal, D. K., Mandal, A., Rai, S., Chatterjee, A., Ghosh, M. K., & Dutta, T. K. (2020): Readily usable strategies to control mastitis for production augmentation in dairy cattle: A review. Veterinary world, **13(11):** 2364–2370. https://doi.org/10.14202/vetworld.2020.2364-2370

Bhanderi, B. B., Jhala, M. K., Ahir, V. B., Bhatt, V. D., & Joshi, C. G. (2014): Cultural and metagenomic based identifi cation of a microbiome from subclinical mastitis in cows. Veterinarski Arhiv, **84(3):** 215–228. Retrieved on 10th october, 2019 from http://www.vef.unizg.hr/vetarhiv/papers/2014-84-3-2.pdf

Bhatt, V. D., Ahir, V. B., Koringa, P. G., Jakhesara, S. J., Rank, D. N., Nauriyal, D. S., Kunjadia, A. P., & Joshi, C. G. (2012): Milk microbiome signatures of subclinical mastitis-affected cattle analysed by shotgun sequencing. Journal of applied microbiology, **112(4):** 639–650. https://doi.org/10.1111/j.1365-2672.2012.05244.x

Biesenkamp-Uhe, C., Li, Y., Hehnen, H. R., Sachse, K., & Kaltenboeck, B. (2007): Therapeutic Chlamydophila abortus and C. pecorum vaccination transiently reduces bovine mastitis associated with Chlamydophila infection. Infection and immunity, **75(2):** 870–877. https://doi.org/10.1128/IAI.00691-06

Bihon, A., Syoum, A., & Assefa, A. (2018): Assessment of risk factors and isolation of Staphylococcus aureus and Escherichia coli from bovine subclinical mastitic milk in and around Gondar , Northwest Ethiopia, Tropical animal health and production, **51(4):** 939–948. https://doi.org/10.1007/s11250-018-1777-2 .

Birhanu, M., Leta, S., Mamo, G., & Tesfaye, S. (2017): Prevalence of bovine subclinical mastitis and isolation of its major causes in Bishoftu Town, Ethiopia. BMC research notes, **10(1):** 767. https://doi.org/10.1186/s13104-017-3100-0

Björk, S., Båge, R., Kanyima, B. M., André, S., Nassuna-musoke, M. G., & Owiny, D. O. (2014). Characterization of coagulase negative staphylococci from cases of subclinical mastitis in dairy cattle in Kampala , Uganda, Irish veterinary journal, **67**(1):12. 4, 1–3.

Boireau, C., Cazeau, G., Jarrige, N., Calavas, D., & Madec, J. (2018): Antimicrobial resistance in bacteria isolated from mastitis in dairy cattle in France, 2006 – 2016. Journal of Dairy Science, **101(10):** 9451–9462. https://doi.org/10.3168/jds.2018-14835

Bradley, A. J. (2002): Bovine Mastitis : An Evolving Disease. Veterinary Journal (London, England), **164(2):**116–128. 1–13. https://doi.org/10.1053/tvjl.2002.0724.

Bradley, A. J., & Green, M. J. (2001): An investigation of the impact of intramammary antibiotic dry cow therapy on clinical coliform mastitis. Journal of dairy science, **84(7):** 1632–1639. https://doi.org/10.3168/jds.S0022-0302(01)74598-5

Bradley, A. J., Leach, K. A., Breen, J. E., Green, L. E., & Green, M. J. (2007): Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. The Veterinary record, **160(8):** 253–257. https://doi.org/10.1136/vr.160.8.253.

Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A., and Duchateau, L. (2003):

Severity of E. coli mastitis is mainly determined by cow factors. Journal of Veterinary Research **34(5):**521-564. doi: 10.1051/vetres:2003023.

Bystroń, J., Podkowik, M., Korzekwa, K., Lis, E., Molenda, J., & Bania, J. (2010): Characterization of borderline oxacillin-resistant Staphylococcus aureus isolated from food of animal origin. Journal of food protection, **73(7):**1325–1327. https://doi.org/10.4315/0362-028x-73.7.1325.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Knight, R. (2010): QIIME allows analysis of high-throughput community sequencing data. Nature methods, **7(5):** 335–336. https://doi.org/10.1038/nmeth.f.303

Castanheira, M., Simner, P. J., & Bradford, P. A. (2021): Extended-spectrum β-lactamases: an update on their characteristics, epidemiology and detection. Journal of Antimicrobial Chemotherapy-antimicrobial resistance, **3**:3

Catozzi, C., Sanchez Bonastre, A., Francino, O., Lecchi, C., De Carlo, E., Vecchio, D., Martucciello, A., Fraulo, P., Bronzo, V., Cuscó, A., D'Andreano, S., & Ceciliani, F. (2017): The microbiota of water buffalo milk during mastitis. PloS one, **12:**9 e0184710. https://doi.org/10.1371/journal.pone.0184710.

Chapartegui-González, I., Lázaro-Díez, M., Redondo-Salvo, S., Navas, J., & Ramos-Vivas, J. (2021): Antimicrobial Resistance Determinants in Genomes and Plasmids from Acinetobacter baumannii Clinical Isolates. Antibiotics (Basel, Switzerland), **10(7):** 753.

https://doi.org/10.3390/antibiotics10070753

Cheng, J., Qu, W., Barkema, H. W., Nobrega, D. B., Gao, J., Liu, G., De Buck, J., Kastelic, J. P., Sun, H., & Han, B. (2019): Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. Journal of Dairy science, **102(3):** 2416–2426. https://doi.org/10.3168/jds.2018-15135

CLSI (2016): Performance Standards for Antimicrobial Susceptibility Testing: CLSI Supplement M100S; Clinical and Laboratory Standards Institute: Wayne, PA, USA. https://clsi.org/media/3481/m100ed30_sample.pdf accessed on 12th December, 2019

Cobirka, M., Tancin, V., & Slama, P. (2020): Epidemiology and Classification of Mastitis. Animals, MDPI, **10 (12):** 2212. https://doi.org/10.3390/ani10122212.

Cobo-Ángel, C., Jaramillo-Jaramillo, A. S., Lasso-Rojas, L.M., Aguilar-Marin, S.B., Sanchez, J., Rodriguez-Lecompte, J. C, (2018): Streptococcus agalactiae is not always an obligate intramammary pathogen: Molecular epidemiology of GBS from milk, feces and environment in Colombian dairy herds. PLoS ONE **13**:12 e0208990. https://doi.org/10.1371/journal.pone.0208990

Damm, M., Holm, C., Blaabjerg, M., Bro, M. N., & Schwarz, D. (2017): Differential somatic cell count-A novel method for routine mastitis screening in the frame of Dairy Herd Improvement testing programs. Journal of dairy science, 100(6), 4926–4940. https://doi.org/10.3168/jds.2016-12409

Derakhshani, H., Fehr, K. B., Sepehri, S., Francoz, D., De Buck, J., Barkema, H. W., Plaizier, J. C., & Khafipour, E. (2018): Invited review: Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility.

Journal of dairy science, **101(12):** 10605–10625. https://doi.org/10.3168/jds.2018-14860

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., & Andersen, G. L. (2006): Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and environmental microbiology, **72(7):**5069–5072. https://doi.org/10.1128/AEM.03006-05

Deurenberg, R. H., & Stobberingh, E. E. (2008). The evolution of Staphylococcus aureus. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases, **8**(6):747–763. https://doi.org/10.1016/j.meegid.2008.07.007

Devarajan, N., Köhler, T., Sivalingam, P., van Delden, C., Mulaji, C. K., Mpiana, P. T., Ibelings, B. W., & Poté, J. (2017): Antibiotic resistant Pseudomonas spp. in the aquatic environment: A prevalence study under tropical and temperate climate conditions. Journal of Water research, **115**:256–265. https://doi.org/10.1016/j.watres.2017.02.058

Dohoo, L., Martin, W. and Stryshn, H. (2003): Veterinary Epidemiologic Research. Charlottetown, Prince Edward Island, Canada: AVC Inc., 47- 49

Dominguez-salas, P., Alarcón, P., Häsler, B., Dohoo, I. R., Colverson, K., Alonso, S., E. Fèvre, M. Rushton J. and Grace, D. (2016): Nutritional characterisation of low-income households of Nairobi : socioeconomic, livestock and gender considerations and predictors of malnutrition from a cross- sectional survey. BMC Nutrition, **2**:47 https://doi.org/10.1186/s40795-016-0086-2

Dorneles, E., Fonseca, M., Abreu, J., Lage, A. P., Brito, M., Pereira, C. R., Brandão, H. M., Guimarães, A. S., & Heinemann, M. B. (2019): Genetic diversity and antimicrobial

resistance in Staphylococcus aureus and coagulase-negative Staphylococcus isolates from bovine mastitis in Minas Gerais, Brazil. Microbiology Open, **8**(**5):** e00736. https://doi.org/10.1002/mbo3.736

Du, F., Lv, X., Duan, D., Wang, L., & Huang, J. (2019): Characterization of a Linezolid- and Vancomycin-Resistant Streptococcus suis Isolate That Harbors optrA and vanG Operons. Frontiers in microbiology, **10**:2026. https://doi.org/10.3389/fmicb.2019.02026

Duarte, C. M., Freitas, P. P., & Bexiga, R. (2015): Technological advances in bovine mastitis diagnosis: an overview. Journal of Veterinary Diagnostic Investigation, **27(6):** 665–672. https://doi.org/10.1177/1040638715603087.

Dudek, K., Nicholas, R., Szacawa, E., & Bednarek, D. (2020): Mycoplasma bovis Infections-Occurrence, Diagnosis and Control. Pathogens (Basel, Switzerland), **9(8):** 640. https://doi.org/10.3390/pathogens9080640

Elemo, K. K., Sisay, T., Shiferaw, A., & Fato, A. (2017): Prevalence , risk factors and multidrug resistance profile of Staphylococcus aureus isolated from bovine mastitis in selected dairy farms in and around Asella town , Arsi Zone , South Eastern Ethiopia. African Journal of Microbiology **11**(**45):** 1632–1642. https://doi.org/10.5897/AJMR2017.8529

Elghafghuf, A., S. Dufour, K. Reyher, I. Dohoo, and H. Stryhn. (2014): Survival analysis of clinical mastitis data using a nested frailty Cox model fit as a mixed-effects Poisson model. Preventive Veterinary Medicine **117**:456–468 https://doi.org/10.1016/j.prevetmed.2014.09.013

Elhariri, M., Hamza, D., Elhelw, R., & Dorgham, S. M. (2017): Extended-spectrum beta-

lactamase-producing Pseudomonas aeruginosa in camel in Egypt: potential human hazard. Annals of clinical microbiology and antimicrobials, **16(1):** 21. https://doi.org/10.1186/s12941-017-0197-x

El-jakee, J. K., Aref, N. E., Gomaa, A., El-hariri, M. D., Galal, H. M., Omar, S. A., & Samir, A. (2013): Emerging of coagulase negative staphylococci as a cause of mastitis in dairy animals : An environmental hazard I. International Journal of Veterinary Science and Medicine, **1(2):** 74–78. https://doi.org/10.1016/j.ijvsm.2013.05.006.

Erkmen, O., (2021): Laboratory Practices in Microbiology 1st edition Edition Mosby, Elsevier Health Sciences

Escobar-Zepeda, A., Vera-Ponce de León, A., & Sanchez-Flores, A. (2015): The Road to Metagenomics: From Microbiology to DNA Sequencing Technologies and Bioinformatics. Frontiers in genetics, **6**:348. https://doi.org/10.3389/fgene.2015.00348

EUCAST (2021): The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version **11**. 2021. http://www.eucast.org.

Falentin, H., Rault, L., Nicolas, A., Bouchard, D. S., Lassalas, J., Lamberton, P., Aubry, J. M., Marnet, P. G., Le Loir, Y., & Even, S. (2016): Bovine Teat Microbiome Analysis Revealed Reduced Alpha Diversity and Significant Changes in Taxonomic Profiles in Quarters with a History of Mastitis. Frontiers in microbiology, **7:** 480. https://doi.org/10.3389/fmicb.2016.00480

Falodun, O. I, Musa, S. B. (2019). Pseudomonas species from cattle dung producing extended spectrum and metallo beta-lactamase European Journal of Biological Researchs: 10(1):

http://dx.doi.org/10.5281/zenodo.3630802

FAO (2021): Food and Agriculture Organization (FAO), Action Plan on Antimicrobial Resistance 2021-2025 Supporting innovation and resilience in food and agriculture sectors 10th March 2021. Retrieved from http://www.fao.org/3/ne859en/ne859en.pdf accessed in August 26th 2021

FAO (2018): Food and Agriculture Organization (FAO), World Livestock: Transforming the livestock sector through the Sustainable Development Goals. Rome. 222 pp. Retrieved from http://www.fao.org/3/CA1201EN/ca1201en.pdf accessed in 22nd January, 2021

FAO (2017): Food and Agriculture Organization (FAO), Africa sustainable Livestock 2050 Livestock and livelihoods spotlight Kenya cattle and Poutry sector. ASL 2050. Retrieved from http://www.fao.org/3/I8978EN/i8978en.pdf accessed in 13th May, 2020

FAO (2016): The Food and Agriculture Organization (FAO) action plan on antimicrobial resistance 2016-2020. Food and Agriculture Organization of the United Nations, 1–14. Retrieved from http://www.fao.org/3/a-i5996e.pdf on 20th May, 2020

FAO (2014): Food and Agriculture Organization (FAO),The State of Food and Agriculture http://www.fao.org/publications/sofa/en/ accessed on 15th April 2021

FAO (2011): Food and Agriculture Organization (FAO), Dairy Development in Kenya, by H.G. Muriuki. FAO, Rome. http://www.fao.org/3/a-al745e.pdf accessed on 15th, January, 2021

FAO (2004): Food and Agriculture Organization (FAO), Impact of mastitis in small-scale dairy production systems, Animal Production and Health Working Paper. No. 13. Rome, 2014. Retrieved from http://www.fao.org/3/i3377e/i3377e.pdf accessed on 20th January, 2021

Feng, Y., Qi, W., Xu-rong, W., Ling, W., Xin-pu, L. I., Jin-yin, L. U. O., Shi-dong, Z. (2016):

Genetic characterization of antimicrobial resistance in Staphylococcus aureus isolated from bovine mastitis cases in Northwest China. Journal of Integrative Agriculture :**15(12):**2842–2847. https://doi.org/10.1016/S2095-3119(16)61368-0

Fisseha, D., Tessema T. S. and Birhanu, B. (2020): Aerobic bacterial isolates , incidence rate and associated risk factors of heifer and cow mastitis in and around Debre-Libanos district , Oromia , Ethiopia, Journal of Veterinary Medicine And Health, **12**: 7–13.

Fouhy, F., Clooney, A. G., Stanton, C., Claesson, M. J., & Cotter, P. D. (2016): 16S rRNA gene sequencing of mock microbial populations- impact of DNA extraction method, primer choice and sequencing platform. BMC microbiology, **16(1):** 123. https://doi.org/10.1186/s12866-016-0738-z

Founou, L. L., Founou, R. C., & Essack, S. Y. (2016): Antibiotic Resistance in the Food Chain: A Developing Country-Perspective. Frontiers in microbiology, **7**: 1881. https://doi.org/10.3389/fmicb.2016.01881

Frey, Y., Rodriguez, J. P., Thomann, A., Schwendener, S., & Perreten, V. (2013): Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. Journal of Dairy Science, **96(4):** 2247–2257. https://doi.org/10.3168/jds.2012-6091.

Gagetti, P., Wattam, A. R., Giacoboni, G., De Paulis, A., Bertona, E., Corso, A., & Rosato, A. E. (2019): Identification and molecular epidemiology of methicillin resistant Staphylococcus pseudintermedius strains isolated from canine clinical samples in Argentina. BMC veterinary research, **15(1):** 264. https://doi.org/10.1186/s12917-019-1990-x.

Ganda, E. K., Bisinotto, R. S., Lima, S. F., Kronauer, K., Decter, D. H., Oikonomou, G., Schukken, Y. H., & Bicalho, R. C. (2016): Longitudinal metagenomic profiling of bovine milk to assess the impact of intramammary treatment using a third-generation cephalosporin. Scientific reports, **6**: 37565. https://doi.org/10.1038/srep37565

Gao, J., Ferreri, M., Liu, X. Q, Chen, L. B, Su, J. L, Han, B. (2011): Development of multiplex polymerase chain reaction assay for rapid detection of Staphylococcus aureus and selected antibiotic resistance genes in bovine mastitic milk samples. Journal of Veterinary Diagnostic Investigation, **23(5):**894-901. doi: 10.1177/1040638711416964

Gao, J., Barkema, H. W., Zhang, L., Liu, G., Deng, Z., Cai, L., Shan, R., Zhang, S., Zou, J., Kastelic, J. P., & Han, B. (2017): Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. Journal of dairy science, **100(6)**: 4797–4806. https://doi.org/10.3168/jds.2016-12334.

Garrido-Cardenas, J.A., & Manzano-Agugliaro, F. (2017): The metagenomics worldwide research. Journal of Current Genetics **63(5)**: 819–829 https://doi.org/10.1007/s00294-017-0693-8

Gentilini, E., Denamiel, G., & Betancor, A. (2002): Antimicrobial Susceptibility of Coagulase-Negative Staphylococci Isolated from Bovine Mastitis in Argentina. Journal of Dairy Science, **85(8)**:1913–1917. https://doi.org/10.3168/jds.S0022-0302(02)74267-7

Gitaka, J., Kamita, M., Mureithi, D., Ndegwa, D., Masika, M., Omuse, G., Ngari, M., Makokha, F., Mwaura, P., Mathai, R., Muregi, F., & Mwau, M. (2020): Combating antibiotic resistance using guidelines and enhanced stewardship in Kenya: a protocol for an implementation science approach. BMJ open, **10(3):**030823.

https://doi.org/10.1136/bmjopen-2019-030823

Gitau, G. K., Bundi, R. M., Vanleeuwen, J., & Mulei, C. M. (2014): Mastitogenic bacteria isolated from dairy cows in Kenya and their antimicrobial sensitivity. Journal of the South African Veterinary Association, **85(1):** 1–8. https://doi.org/10.4102/jsava.v85i1.950

Gitau, G., Wabacha, J., Mulei, C., Ndurumo, S., & Nduhiu, J. (2011): Isolation rates and antimicrobial sensitivity patterns of bovine mastitis pathogens in peri-urban area of Nairobi, Kabete, Kenya. Ethiopian Veterinary Journal, **15**: 1–13. https://doi.org/10.4314/evj.v15i1.67680

Gleeson, D., Flynn, J., & Brien, B. O. (2018): Effect of pre-milking teat disinfection on new mastitis infection rates of dairy cows. Irish veterinary journal, **71:**11 https://doi.org/10.1186/s13620-018-0122-4

Global Antibiotic Resistance Partnership (GARP) (2011): Situation Analysis and Recommendations: Antibiotic Use and Resistance in Kenya. Available online: https://cddep.org/wp-content/uploads/2017/08/garp-kenya_es.pdf (accessed on 2 March 2021).

Gomes, F., & Henriques, M. (2016): Control of Bovine Mastitis: Old and Recent Therapeutic Approaches. Current Microbiology, **72(4):** 377–382. https://doi.org/10.1007/s00284-015-0958-8.

Gordon, P. F., Borne, B. H. P. Van Den, Reist, M., Kohler, S., & Doherr, M. G. (2013): Questionnaire-based study to assess the association between management practices and mastitis within tie-stall and free-stall dairy housing systems in Switzerland. BMC Veterinary Research, **9(1):** 1. https://doi.org/10.1186/1746-6148-9-200

Grace, D. (2015): Review of Evidence on Antimicrobial Resistance and Animal Agriculture in Developing Countries; International Livestock Research Institute (ILRI) Nairobi, Kenya, https://amr.cgiar.org/case-study/review-evidence-antimicrobial-resistance-and-animal-agriculture-developing-countries Accessed on 13th June, 2019.

Gröhn, Y. T., D. J. Wilson, R. N. González, J. A. Hertl, H. Schulte, G. Bennett, and Y. H. Schukken (2004): Effect of pathogen-spe- cific clinical mastitis on milk yield in dairy cows. Jorunal of Dairy Science **87:**3358–3374. https://doi.org/10.3168/jds.S0022-0302(04)73472-4

Gunga, P. M. (2018): Antibiotic Resistance Phenotypes and Genotypes of Staphylococcus aureus Isolated From Milk Submitted To The Central Veterinary Laboratories, Master of science Thesis, in Biotechnology at the university of Nairobi, Kenya, 11 November, 2018.

Gussmann, M., Steeneveld, W., Kirkeby, C., Hogeveen, H., Nielen, M., & Farre, M. (2019): Economic and epidemiological impact of different intervention strategies for clinical contagious mastitis. Journal of Dairy Science, **102**(2): 1483–1493. https://doi.org/10.3168/jds.2018-14939

Hamel, M. J., Greene, C., Chiller, T., Ouma, P., Polyak, C., Otieno, K., Williamson, J., Shi, Y. P., Feikin, D. R., Marston, B., Brooks, J. T., Poe, A., Zhou, Z., Ochieng, B., Mintz, E., & Slutsker, L. (2008): Does cotrimoxazole prophylaxis for the prevention of HIV-associated opportunistic infections select for resistant pathogens in Kenyan adults?. The American journal of tropical medicine and hygiene, **79(3):** 320–330.

Hammer, J. F., J. M. Morton, and K. L. Kerrisk (2012): Quarter-milking-, quarter-, udder- and lactation-level risk factors and indicators for clinical mastitis during lactation in pasture-fed

dairy cows managed in an automatic milking system. Australian Veterinary Journal **90**:167–174.

Hand, K. J., Godkin, A., & Kelton, D. F. (2012): Milk production and somatic cell counts: a cow-level analysis. Journal of dairy science, **95(3):** 1358–1362. https://doi.org/10.3168/jds.2011-4927

Hansen, J. E., Larsen, A. R., Skov, R. L., Chriél, M., Larsen, G., Angen, Ø., Larsen, J., Lassen, D., & Pedersen, K. (2017): Livestock-associated methicillin-resistant Staphylococcus aureus is widespread in farmed mink (Neovison vison). Journal of Veterinary Microbiology, **207**: 44–49. https://doi.org/10.1016/j.vetmic.2017.05.027.

Harkinezhad, T., Geens, T., & Vanrompay, D. (2009): Chlamydophila psittaci infections in birds: a review with emphasis on zoonotic consequences. Veterinary microbiology, **135**(1-**2):** 68–77. https://doi.org/10.1016/j.vetmic.2008.09.046

Hennekinne, J. A., De Buyser, M. L., & Dragacci, S. (2012): Staphylococcus aureus and its food poisoning toxins: characterization and outbreak investigation. FEMS microbiology reviews, **36(4):** 815–836. https://doi.org/10.1111/j.1574-6976.2011.00311.x.

Halasa, T., & Kirkeby, C. (2020): Differential Somatic Cell Count: Value for Udder Health Management. Frontiers in veterinary science **7: 609055.** https://doi.org/10.3389/fvets.2020.609055

Hertl, J. A., Schukken, Y. H., Bar, D., Bennett, G. J., González, R. N., Rauch, B. J., Welcome, F. L., Tauer, L. W., & Gröhn, Y. T. (2011): The effect of recurrent episodes of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on mortality and culling in Holstein dairy cows. Journal of Dairy Science, **94(10):**4863–4877.

https://doi.org/10.3168/jds.2010-4000

Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., & Coukell, A. (2017): Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence?. BMC veterinary research, **13(1):** 211. https://doi.org/10.1186/s12917-017-1131-3

Hogan, J., & Smith, L. K. (2003): Coliform mastitis. Journal of Veterinary research, **34(5):** 507–519. https://doi.org/10.1051/vetres:2003022.

Holmes, M. A., & Zadoks, R. N. (2011): Methicillin resistant S. aureus in human and bovine mastitis. Journal of mammary gland biology and neoplasia, **16(4):** 373–382. https://doi.org/10.1007/s10911-011-9237-x.

Hoque, M. N., Istiaq, A., Clement, R. A., Sultana, M., Crandall, K. A., Siddiki, A. Z., & Hossain, M. A. (2019): Metagenomic deep sequencing reveals association of microbiome signature with functional biases in bovine mastitis. Scientific reports, **9(1):** 13536. https://doi.org/10.1038/s41598-019-49468-4

Hryniewicz, M. M., & Garbacz, K. (2017): Borderline oxacillin-resistant Staphylococcus aureus (BORSA) - a more common problem than expected?. Journal of medical microbiology, **66(10):** 1367–1373. https://doi.org/10.1099/jmm.0.000585

Idriss, S.E.; Foltys, V.; Tančin, V.; Kirchnerová, K.; Zaujec, K. (2013): Mastitis pathogens in milk of dairy cows in Slovakia. Slovak Journal of Animal Science **46**: 115–119.

Independent Electoral Boundaries Commission (IEBC) (2012): https://www.iebc.or.ke/uploads/resources/euzA6oIPCd.pdf. Accessed on 25th May, 2020.

Ismael, A. (2018): Epidemiology of Bovine Mastitis in Ethiopia, Journal of Veterinary Medicine

2(1): 1–7. https://www.omicsonline.org/open-access/epidemiology-of-bovine-mastitis-in-ethiopia-99539.html accessed on 25th June 2018

Ismail Z. B. (2017): Mastitis vaccines in dairy cows: Recent developments and recommendations of application. Veterinary world, **10(9):** 1057–1062. https://doi.org/10.14202/vetworld.2017.1057-1062

Ismail, Z. B., & Abutarbush, S. M. (2020): Molecular characterization of antimicrobial resistance and virulence genes of Escherichia coli isolates from bovine mastitis. Veterinary world, **13(8):** 1588–1593. https://doi.org/10.14202/vetworld.2020.1588-1593

Jamali, H., Barkema, H. W., Jacques, M., Lavallée-Bourget, E. M., Malouin, F., Saini, V., Stryhn, H., & Dufour, S. (2018): Invited review: Incidence, risk factors, and effects of clinical mastitis recurrence in dairy cows. Journal of dairy science, **101**(6):4729–4746. https://doi.org/10.3168/jds.2017-13730

Jisuvei, S. C., Osoti, A., & Njeri, M. A. (2020): Prevalence, antimicrobial susceptibility patterns, serotypes and risk factors for group B streptococcus rectovaginal isolates among pregnant women at Kenyatta National Hospital, Kenya; a cross-sectional study. BMC infectious diseases, **20(1):** 302. https://doi.org/10.1186/s12879-020-05035-1

Jost, B. H., & Billington, S. J. (2005): Arcanobacterium pyogenes: molecular pathogenesis of an animal opportunist. Antonie van Leeuwenhoek, **88(2):** 87–102. https://doi.org/10.1007/s10482-005-2316-5

Kaczorek, E., Małaczewska, J., Wójcik, R., Rękawek, W., & Siwicki, A. K. (2017): Phenotypic and genotypic antimicrobial susceptibility pattern of Streptococcus spp. isolated from cases

of clinical mastitis in dairy cattle in Poland. Journal of dairy science, **100(8):** 6442–6453. https://doi.org/10.3168/jds.2017-12660

Kalayu, A. A., Woldetsadik, D. A., Woldeamanuel, Y., Wang, S. H., Gebreyes, W. A., & Teferi, T. (2020): Burden and antimicrobial resistance of S. aureus in dairy farms in Mekelle, Northern Ethiopia. BMC veterinary research, **16**(1): 20. https://doi.org/10.1186/s12917-020-2235-8.

Kalińska, A., Gołębiewski, M., & Wójcik, A. (2017): Mastitis pathogens in dairy cattle – a review, World Scientific News **89:** 22-31 retreived from http://www.worldscientificnews.com/wp-content/uploads/2017/08/WSN-89-2017-22-31.pdf accessed on 12th February 2020

Käppeli, N., Morach, M., Zurfluh, K., Corti, S., Nüesch-inderbinen, M., & Stephan, R. (2019): Sequence Types and Antimicrobial Resistance Profiles of Streptococcus uberis Isolated From Bovine Mastitis, Frontiers in veterinary science, **6:** 234.. https://doi.org/10.3389/fvets.2019.00234

Kateete, D. P., Kabugo, U., Baluku, H., Nyakarahuka, L., Kyobe, S., Okee, M., Najjuka, C. F., & Joloba, M. L. (2013): Prevalence and antimicrobial susceptibility patterns of bacteria from milkmen and cows with clinical mastitis in and around Kampala, Uganda. PloS one, **8(5):** e63413. https://doi.org/10.1371/journal.pone.0063413.

Kavitha, K., Latha, R., Udayashankar, C., Jayanthi, K., & Oudeacoumar, P. (2010): Three cases of Arcanobacterium pyogenes-associated soft tissue infection. Journal of medical microbiology**, 59:6** 736–739. https://doi.org/10.1099/jmm.0.016485-0

Kawai, K., Kondo, Y., Shinozuka, Y., Kawata, R., Kaneko, S., Iwano, H., Enokidani, M.,

Watanabe, A., Yuliza-Purba, F., Isobe, N., & Kurumisawa, T. (2021): Immune response during the onset of coliform mastitis in dairy cows vaccinated with STARTVAC®. Animal science journal, **92(1):** e13502. https://doi.org/10.1111/asj.13502

KDB, (2020): Kenya Dairy Board (KDB) Website https://www.kdb.go.ke/dairyinformation/ accessed on 20th March, 2021.

KIHBS, (2018). Kenya Integrated Household Budget Survey (KIHBS),2015/ 2016 Basic report https://sun-connect-news.org/fileadmin/DATEIEN/Dateien/New/KNBS_-_Basic_Report.pdf accessed on 15 November 2020.

KIHBS, (2007). Kenya intergrated household budget survey (KHIBS) 2005/6 *https://catalog.ihsn.org/index.php/catalog/1472 accessed on 15 January 2021*.

Khorvash, F. A. (2008): Antimicrobial susceptibility pattern of microorganisms involved in the pathogenesis of surgical site infection (SSI); A 1 year of surveillance. Pakistan journal of biological sciences : Pakistan Journal of Biological sciences, **11(15):**1940–1944. https://doi.org/10.3923/pjbs.2008.1940.1944

Kiambi, S., Onono, J. O., Kang'ethe, E., Aboge, G. O., Murungi, M. K., Muinde, P., Akoko, J., Momanyi, K., Rushton, J., Fèvre, E. M., & Alarcon, P. (2020): Investigation of the governance structure of the Nairobi dairy value chain and its influence on food safety. Preventive Veterinary Medicine, **179:**105009. https://doi.org/10.1016/j.prevetmed.2020.105009.

Kibebew, K. (2017): Bovine Mastitis : A Review of Causes and Epidemiological Point of View. Journal of Biology, Agriculture and Healthcare**, 7(2):** 1–14. Accessed from https://www.iiste.org/Journals/index.php/JBAH/article/view/34975 on 25th January 2020

Kim, S. J., Moon, D. C., Park, S. C., Kang, H. Y., Na, S. H., & Lim, S. K. (2019): Antimicrobial resistance and genetic characterization of coagulase-negative staphylococci from bovine mastitis milk samples in Korea. Journal of Dairy science, **102(12):** 11439–11448. https://doi.org/10.3168/jds.2019-17028

Kirk, J. H., & Bartlett, P. C. (1984): Nonclinical Pseudomonas aeruginosa mastitis in a dairy herd. Journal of the American Veterinary Medical Association, **184(6):** 671–673.

Klaas, I. C. & Zadoks, R. N. (2018): An update on environmental mastitis: Challenging perceptions. Transboundary and emerging diseases, 65(1): 166–185. https://doi.org/10.1111/tbed.12704.

Klibi, A., Jouini, A., Boubaker El Andolsi, R., Kmiha, S., Ben Hamda, C., Ghedira, K., Hamrouni, S., Ghram, A., & Maaroufi, A. (2019): Epidemiology of *β*-Lactamase-Producing Staphylococci and Gram Negative Bacteria as Cause of Clinical Bovine Mastitis in Tunisia. BioMedical research international, **2019:** 2165316. https://doi.org/10.1155/2019/2165316

KNBS, (2019): Kenya National Bureau of Statistics (KNBS) Kenya Population and housing census*;* Volume 1 population by County and Subcounty *; http://www.knbs.or.ke* accessed on 5th January 2021

Koskinen, M. T., Holopainen, J., Pyörälä, S., Bredbacka, P., Pitkälä, A., Barkema, H. W., Bexiga, R., Roberson, J., Sølverød, L., Piccinini, R., Kelton, D., Lehmusto, H., Niskala, S., & Salmikivi, L. (2009): Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. Journal of dairy science, **92(3):** 952–959. https://doi.org/10.3168/jds.2008-1549

Kot, B., Wierzchowska, K., Piechota, M., & Grużewska, A. (2020): Antimicrobial Resistance Patterns in Methicillin-Resistant Staphylococcus aureus from Patients Hospitalized during 2015-2017 in Hospitals in Poland. Medical principles and practice : International Journal of the Kuwait University, Health Science Centre, **29(1):** 61–68. https://doi.org/10.1159/000501788

Kromker, V., Reinecke, F., Paduch J. and N.Grabowski, (2014): Bovine Streptococcus uberis Intramammary Infections and Mastitis, Clinical Microbiology, **3**: 4 https://doi.org/10.4172/2327-5073.1000157

Krupa, P. B., N, J. B. B., Podkowik, M., Empel, J., Mroczkowska, A., & Bania, J. (2015): Population Structure and Oxacillin Resistance of Staphylococcus aureus from Pigs and Pork Meat in South-West of Poland, Biomedical research international **2015:**141475. https://doi.org/10.1155/2015/141475

Kuehn, J. S., Gorden, P. J., Munro, D., Rong, R., Dong, Q., Plummer, P. J., Wang, C., Phillips, G. J. (2013): Bacterial Community Profiling of Milk Samples as a Means to Understand Culture-Negative Bovine Clinical Mastitis. *PLoS ONE*, **8**(4): e61959. https://doi.org/10.1371/journal.pone.0061959

Kumar, N., Manimaran, A., Kumaresan, A., Sreela, L., Patbandha, T. K., Tiwari, S., & Chandra, S. (2016): Episodes of clinical mastitis and its relationship with duration of treatment and seasonality in crossbred cows maintained in organized dairy farm. Veterinary world, **9(1):** 75–79. https://doi.org/10.14202/vetworld.2016.

Kumari, T., Bhakat, C., Choudhary R. K. (2018): A Review on Sub Clinical Mastitis in Dairy Cattle. International Journal of Pure and Applied Biosciences, **6 (2):** 1291-1299 (2018)

http://dx.doi.org/10.18782/2320-7051.6173.

Kurjogi, M. M., & Kaliwal, B. B. (2014): Epidemiology of Bovine Mastitis in Cows of Dharwad District. International scholarly research notices, 2014: 968076. https://doi.org/10.1155/2014/968076

Kusumawati A., Mustopa A Z, Wibawan I W T, Setiyono A. , Sudarwanto M B. (2021): Metagenomic analysis of pathogen mastitis in cow's milk from Cicurug, Sukabumi, West Java, Indonesia IOP Conference Series Earth and Environmental Science **762**(1):012064

Kyany'a, C., Nyasinga, J., Matano, D., Oundo, V., Wacira, S., Sang, W., & Musila, L. (2019): Phenotypic and genotypic characterization of clinical Staphylococcus aureus isolates from Kenya. BMC microbiology, **19(1):** 245. https://doi.org/10.1186/s12866-019-1597-1.

Lam, T., Olde Riekerink, R., Sampimon, O., & Smith, H. (2009): Mastitis diagnostics and performance monitoring: a practical approach. Irish veterinary journal, **62(4):** 34–39. https://doi.org/10.1186/2046-0481-62-S4-S34

Langata, L. M., Maingi, J. M., Musonye, H. A., Kiiru, J., & Nyamache, A. K. (2019): Antimicrobial resistance genes in Salmonella and Escherichia coli isolates from chicken droppings in Nairobi, Kenya. BMC research notes, **12(1):** 22. https://doi.org/10.1186/s13104-019-4068-8N

Lee J. H. (2003): Methicillin (Oxacillin)-resistant Staphylococcus aureus strains isolated from major food animals and their potential transmission to humans. Applied and environmental microbiology, **69(11):** 6489–6494. https://doi.org/10.1128/AEM.69.11.6489-6494.2003

Levison, L. J., Miller-Cushon, E. K., Tucker, A. L., Bergeron, R., Leslie, K. E., Barkema, H. W., & DeVries, T. J. (2016): Incidence rate of pathogen-specific clinical mastitis on

conventional and organic Canadian dairy farms. Journal of dairy science, **99(2):**1341–1350. https://doi.org/10.3168/jds.2015-9809

Liang, D., Arnold, L. M., Stowe, C. J., Harmon, R. J., & Bewley, J. M. (2017): Estimating USA dairy clinical disease costs with a stochastic simulation model. Journal of Dairy Science, **100(2):** 1472–1486. https://doi.org/10.3168/jds.2016-11565

Liu, J., Zhu, Y., Jay-Russell, M., Lemay, D. G., & Mills, D. A. (2020): Reservoirs of antimicrobial resistance genes in retail raw milk. Microbiome, **8(1):** 99. https://doi.org/10.1186/s40168-020-00861-6

Liu, G., Ding, L., Han, B., Piepers, S., Naqvi, S. A., Barkema, H. W., Ali, T., De Vliegher, S., Xu, S., & Gao, J. (2018): Characteristics of Escherichia coli Isolated from Bovine Mastitis Exposed to Subminimum Inhibitory Concentrations of Cefalotin or Ceftazidime. BioMedical research international, **2018:** 4301628. https://doi.org/10.1155/2018/4301628

Liu, H., Li, S., Meng, L., Dong, L., Zhao, S., Lan, X., & Wang, J.(2017): Prevalence , antimicrobial susceptibility , and molecular characterization of Staphylococcus aureus isolated from dairy herds in northern China. Journal of Dairy Science **100**(11): 8796–8803. https://doi.org/10.3168/jds.2017-13370

Thrusfield, M. (2005): Veterinary epidemiology, 3rd ed, Black Well Science Ltd., Oxford, UK

Mahato, S., Mistry, H. U., Chakraborty, S., Sharma, P., Saravanan, R., & Bhandari, V. (2017): Identification of Variable Traits among the Methicillin Resistant and Sensitive Coagulase Negative Staphylococci in Milk Samples from Mastitic Cows in India. Frontiers in microbiology, **8**: 1446. https://doi.org/10.3389/fmicb.2017.01446

Mahlangu, P., Maina, N., and **Kagira, J. (2018):** Prevalence, Risk Factors, and Antibiogram of

Bacteria Isolated from Milk of Goats with Subclinical Mastitis in Thika East Subcounty, Kenya. Journal of veterinary medicine, **2018:** 3801479. https://doi.org/10.1155/2018/3801479

Maina, F., Mburu, J., Gitau, G., and **Vanleeuwen, J. (2019):** Factors influencing economic efficiency of milk production among small-scale dairy farms in Mukurweini, Nyeri County, Kenya. Tropical animal health and production, **52**:533–539. https://doi.org/10.1007/s11250-019-02039-1

Majalija, S., Tumwine, G., &Kiguli, J. (2020): Pastoral community practices, microbial quality and associated health risks of raw milk in the milk value chain of Nakasongola District, Uganda. Pastoralism **10**: 3 https://doi.org/10.1186/s13570-020-0158-4

MALFI (2019): Ministry of agriculture, livestock, fisheries and irrigation (MALFI) State department for livestock, national livestock policy http://repository.kippra.or.ke/handle/123456789/483 accessed on 20th December 2020.

Man'ombe, E. (2014): Economic value and genetic prediction of clinical mastitis in South African Holstein cattle, Ph.D Thesis, Stellenbosch University South Africa (Available online at https://scholar.sun.ac.za/). Accessed on 20th June 2021

Marashifard, M., Karimi Aliabad, Z., Malek Hosseini, S., Darban-Sarokhalil, D., Mirzaii, M., & Khoramrooz, S. S. (2019): Determination of antibiotic resistance pattern and virulence genes in Escherichia coli isolated from bovine with subclinical mastitis in southwest of Iran. Tropical animal health and production, **51(3):** 575–580. https://doi.org/10.1007/s11250-018-1726-0

Markey, B.K., Leonard, F., Archambault, M., Cullinane, A. & Maguire, D., (2013): Clinical

Veterinary microbiology, Elsevier, Edinburgh.

Martineau F., Picard F. J., Grenier, L., Roy, P.H., Ouellette, M., Bergeron, M.G. (2000): Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. Journal of Antimicrob Chemotherapy **46(4):**527-34. doi: 10.1093/jac/46.4.527

Mburu, G. N. (2016): Factors affecting production of dairy products in kenya: a case of co-operative societies in Kiambu County. Master thesis, KCA university, Nairobi, Kenya.

McDougall, S., Penry, J., & Dymock, D. (2021): Antimicrobial susceptibilities in dairy herds that differ in dry cow therapy usage. Journal of dairy science, **104**(8), 9142–9163. https://doi.org/10.3168/jds.2020-19925

McMillan, K., Moore, S. C., McAuley, C. M., Fegan, N., & Fox, E. M. (2016): Characterization of Staphylococcus aureus isolates from raw milk sources in Victoria, Australia. BMC microbiology, **16(1):** 169. https://doi.org/10.1186/s12866-016-0789-1.

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS ONE, **8(4):**e61217. https://doi.org/10.1371/journal.pone.0061217.

Mdegela, R. H., Ryoba, R., Karimuribo, E. D., Phiri, E. J., Løken, T., Reksen, O., Mtengeti, E., & Urio, N. A. (2009): Prevalence of clinical and subclinical mastitis and quality of milk on smallholder dairy farms in Tanzania. Journal of the South African Veterinary Association, **80(3):** 163–168. https://doi.org/10.4102/jsava.v80i3.195

Mekonnen, S. A., Koop, G., Melkie, S. T., Getahun, C. D., Hogeveen, H., & Lam, T. (2017): Prevalence of subclinical mastitis and associated risk factors at cow and herd level in dairy

farms in North-West Ethiopia. Preventive veterinary medicine, **145**:23–31. https://doi.org/10.1016/j.prevetmed.2017.06.009

Meng, L., Liu, H., Lan, T., Dong, L., Hu, H., Zhao, S., Zhang, Y., Zheng, N., & Wang, J. (2020): Antibiotic Resistance Patterns of Pseudomonas spp. Isolated From Raw Milk Revealed by Whole Genome Sequencing. Frontiers in microbiology, **11**: 1005. https://doi.org/10.3389/fmicb.2020.01005

Metzger, S. A., & Hogan, J. S. (2013): Short communication: Antimicrobial susceptibility and frequency of resistance genes in Escherichia coli isolated from bovine mastitis. Journal of Dairy Science, **96(5):**3044–3049. https://doi.org/10.3168/jds.2012-6402

Middleton, J. R., Hardin, D., Steevens, B., Randle, R., & Tyler, J. W. (2004): Use of somatic cell counts and California mastitis test results from individual quarter milk samples to detect subclinical intramammary infection in dairy cattle from a herd with a high bulk tank somatic cell count. Journal of the American Veterinary Medical Association, **224(3):** 419–423. https://doi.org/10.2460/javma.2004.224.419

Milheiriço, C., Portelinha, A., Krippahl, L., de Lencastre, H., & Oliveira, D. C. (2011): Evidence for a purifying selection acting on the β-lactamase locus in epidemic clones of methicillin-resistant Staphylococcus aureus. BMC microbiology, **11**: 76. https://doi.org/10.1186/1471-2180-11-76

Minst, K., Märtlbauer, E., Miller, T., & Meyer, C. (2012): Short communication: Streptococcus species isolated from mastitis milk samples in Germany and their resistance to antimicrobial agents. Journal of dairy science **95(12):** 6957–6962. https://doi.org/10.3168/jds.2012-5852

Mišić, M., Čukić, J., Vidanović, D., Šekler, M., Matić, S., Vukašinović, M., & Baskić, D.

(2017): Prevalence of Genotypes That Determine Resistance of Staphylococci to Macrolides and Lincosamides in Serbia. Frontiers in public health, **5**: 200. https://doi.org/10.3389/fpubh.2017.00200

Mistry, H., Sharma, P., Mahato, S., Saravanan, R., Kumar, P. A., & Bhandari, V. (2020): Prevalence and Characterization of Oxacillin Susceptible mecA-Positive Clinical Isolates of Staphylococcus aureus Causing Bovine Mastitis in India. PloS one, **15**(4): e0232348. https://doi.org/10.1371/journal.pone.0232348

Mitema, E. S., Kikuvi, G. M., Wegener, H. C., & Stohr, K. (2001): An assessment of antimicrobial consumption in food producing animals in Kenya. Journal of veterinary pharmacology and therapeutics, **24(6):** 385–390. https://doi.org/10.1046/j.1365-2885.2001.00360.x

Moon, J. S, Lee, A. R, Kang, H. M, Lee, E. S, Kim, M. N, Paik, Y. H, Park, Y. H, Joo, Y. S, Koo, H. C.(2007): Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. Journal of Dairy Science **90**:1176–1185

Moosavi, M., Mirzaei, A., Ghavami A., Tamadon, A. (2014): Relationship between season, lactation number and incidence of clinical mastitis in different stages of lactation in a Holstein dairy farm. Journal of Veterinary Research Forum, **5** (1): 13 – 19

Motaung, T. E., Petrovski, K. R., Petzer, I. M., Thekisoe, O., & Tsilo, T. J. (2017): Importance of bovine mastitis in Africa. Animal Health Research Reviews, **18**(**1):**58–69. https://doi.org/10.1017/S1466252317000032

Mpatswenumugabo, J. P., Bebora, L. C., Gitao, G. C., Mobegi, V. A., Iraguha, B., Kamana,

O., & Shumbusho, B. (2017): Prevalence of Subclinical Mastitis and Distribution of Pathogens in Dairy Farms of Rubavu and Nyabihu Districts , Rwanda, Journal of Veterinary Medicine, **2017:** 8456713.

Muloi, D., Fèvre, E. M., Bettridge, J., Rono, R., Ong'are, D., Hassell, J. M., Karani, M. K., Muinde, P., van Bunnik, B., Street, A., Chase-Topping, M., Pedersen, A. B., Ward, M. J., & Woolhouse, M. (2019): A cross-sectional survey of practices and knowledge among antibiotic retailers in Nairobi, Kenya. Journal of global health, **9(2):** 010412. https://doi.org/10.7189/jogh.09.020412

Muloi, D., Kiiru, J., Ward, M. J., Hassell, J. M., Bettridge, J. M., Robinson, T. P., van Bunnik, B., Chase-Topping, M., Robertson, G., Pedersen, A. B., Fèvre, E. M., Woolhouse, M., Kang'ethe, E. K., & Kariuki, S. (2019): Epidemiology of antimicrobial-resistant Escherichia coli carriage in sympatric humans and livestock in a rapidly urbanizing city. International journal of antimicrobial agents, **54(5):** 531–537. https://doi.org/10.1016/j.ijantimicag.2019.08.014

Mungube, E. O., Tenhagen, B. A., Regassa, F., Kyule, M. N., Shiferaw, Y., Kassa, T., & Baumann, M. P. (2005): Reduced milk production in udder quarters with subclinical mastitis and associated economic losses in crossbred dairy cows in Ethiopia. Tropical animal health and production, **37(6):** 503–512. https://doi.org/10.1007/s11250-005-7049-y

Munoz, M. A., Welcome, F. L., Schukken, Y. H., & Zadoks, R. N. (2007): Molecular epidemiology of two Klebsiella pneumoniae mastitis outbreaks on a dairy farm in New

York State. Journal of clinical microbiology, **45**(12): 3964–3971. https://doi.org/10.1128/JCM.00795-07

Mureithi, D. K. & Njuguna, M. N., (2016): Prevalence of subclinical mastitis and associated risk factors in dairy farms in urban and peri-urban areas of Thika Sub County, Kenya. Journal of Livestock Research for Rural Development, **28:** 13 Retrieved from http://www.lrrd.org/lrrd28/2/mure28013.html accessed on 5th May 2019

Mureithi, D., Khang C., & Kamau, M. N. (2017): Antimicrobial resistance profile in bacterial isolates from subclinical mastitic milk samples in dairy herds in Kenya. Bull Animal Health Production African **65:** 167-173

Musila, L., Kyany'a, C., Maybank, R., Stam, J., Oundo, V., & Sang, W. (2021): Detection of diverse carbapenem and multidrug resistance genes and high-risk strain types among carbapenem non-susceptible clinical isolates of target gram-negative bacteria in Kenya. PloS one, **16(2):** e0246937. https://doi.org/10.1371/journal.pone.0246937

Mutua, F., Sharma, G., Grace, D., Bandyopadhyay, S., Shome, B., & Lindahl, J. (2020): A review of animal health and drug use practices in India, and their possible link to antimicrobial resistance. Antimicrobial resistance and infection control, **9(1):** 103. https://doi.org/10.1186/s13756-020-00760-3

Nagib, S., Rau, J., Sammra, O., Lämmler, C., Schlez, K., Zschöck, M., Prenger-Berninghoff, E., Klein, G., & Abdulmawjood, A. (2014): Identification of Trueperella pyogenes isolated from bovine mastitis by Fourier transform infrared spectroscopy. PloS one, **9(8),** e104654. https://doi.org/10.1371/journal.pone.0104654

Nam, H. M., Lim, S. K., Kang, H. M., Kim, J. M., Moon, J. S., Jang, K. C., Kim, J. M., Joo,

Y. S., & Jung, S. C. (2009): Prevalence and antimicrobial susceptibility of gram-negative bacteria isolated from bovine mastitis between 2003 and 2008 in Korea. Journal of dairy science, **92(5):** 2020–2026. https://doi.org/10.3168/jds.2008-1739.

National Dairy Master Plan, (2015): Kenya National Dairy. Volume II action plan and implementation Strategy http://kilimo.go.ke/wp-content/uploads/2021/06/dmp-vol-ll-strategies-and-action-plans.pdf accessed in 24th June, 2020

NMC (2017): National Mastitis Council (NMC) Laboratory Handbook on Bovine Mastitis; Revised Edition; National Mastitis Council Inc.: New Prague, MN, USA, 2017.

Navas-Molina, J. A., Peralta-Sánchez, J. M., González, A., McMurdie, P. J., Vázquez-Baeza, Y., Xu, Z., Ursell, L. K., Lauber, C., Zhou, H., Song, S. J., Huntley, J., Ackermann, G. L., Berg-Lyons, D., Holmes, S., Caporaso, J. G., & Knight, R. (2013): Advancing our understanding of the human microbiome using QIIME. Methods in enzymology, **531:** 371–444. https://doi.org/10.1016/B978-0-12-407863-5.00019-8

Ndahetuye, J. B., Persson, Y., Nyman, A. K., Tukei, M., Ongol, M. P., & Båge, R. (2019): Aetiology and prevalence of subclinical mastitis in dairy herds in peri-urban areas of Kigali in Rwanda. Tropical animal health and production, **51(7):** 2037–2044. https://doi.org/10.1007/s11250-019-01905-2.

Ndirangu, P. N., Siamba, D., Wesonga, H. O, Mungube, E. O., Maichomo, M. W., Mugambi, J. M. (2017): Prevalence of bovine mastitis and multi-antibiotic resistant Staphylococcus and Streptococcus species in a research centre farm at Naivasha, Kenya, The Bulletin of Animal Health and Production in Africa **65:**2

Neave, F. K., Dodd, F. H., Kingwill, R. G.& Westgarth, D. R. (1969): Control of mastitis in the

dairy herd by hygiene and management. Journal of Dairy Science 52, 696-707. https://doi.org/10.3168/jds.S0022-0302(69)86632-4

Ngaywa, C., Aboge, G.O., Obiero, G., Omwenga, I., Ngwili, N., Wamwere, G. & Bett, B. (2019): Antimicrobial resistant Escherichia coli isolates detected in raw milk of livestock in pastoral areas of northern Kenya. Food Control, **102**:173–178.

Nielsen, C. (2009): Economic Impact of Mastitis in Dairy Cows. PhD Thesis. Swedish University of Agricultural Sciences, Uppsala, Sweden. https://pub.epsilon.slu.se/1968/1/Christel_Nielsen_kappa.pdf accessed on 27th May 2020

Nielsen, C., & Emanuelson, U. (2013): Mastitis control in Swedish dairy herds. Journal of dairy science, **96(11):** 6883–6893. https://doi.org/10.3168/jds.2012-6026

Njage, P.M., Dolci, S., Jans C.,Wangoh, J., Lacroix, C. (2012): Ampicillin resistance and extended spectrum β-lactamases in enterobacteriaceae isolated from raw and spontaneously fermented camel milk, African Journal of Microbiology Research **6**: 1446–1452 .

Njarui, D. M. G., Gatheru, M., Wambua, J. M., Nguluu, S. N., Mwangi, D. M., & Keya, G. A. (2011): Consumption patterns and preference of milk and milk products among rural and urban consumers in semi-arid Kenya. Ecology of Food and Nutrition, **50(3):**240–262. https://doi.org/10.1080/03670244.2011.568908

Nobrega, D. B., Buck, J. De, & Barkema, H. W. (2018): Antimicrobial resistance in non- aureus

staphylococci isolated from milk is associated with systemic but not intramammary administration of antimicrobials in dairy cattle. Journal of Dairy Science **101(8):**7425–7436. https://doi.org/10.3168/jds.2018-14540

Nüesch-Inderbinen, M., Kindle, P., Baschera, M., Liljander, A., Jores, J., Corman, V. M., & Stephan, R. (2020): Antimicrobial resistant and extended-spectrum ß-lactamase (ESBL) producing Escherichia coli isolated from fecal samples of African dromedary camels. Scientific African, **7:** e00274. https://doi.org/10.1016/j.sciaf.2020.e00274.

Odero-Waitituh J. A. (2017): Smallholder dairy production in Kenya; a review. Journal of Livestock Research for Rural Development. **29:**7 Retrieved from http://www.lrrd.org/lrrd29/7/atiw29139.html 29, #139.

Odongo, M. O, Ndung'u, T.N, Mulei, C.M, Macharia J, Nduhiu J. (2012): Prevalence of microbial causes of bovine mastitis in the Kabete area of Kiambu County and its environs (2001-2010). Kenya Veterinarian **36:1**

Ohnishi, M., Sawada, T., Hirose, K., Sato, R., Hayashimoto, M., Hata, E., Yonezawa, C., & Kato, H. (2011): Antimicrobial susceptibilities and bacteriological characteristics of bovine Pseudomonas aeruginosa and Serratia marcescens isolates from mastitis. Veterinary microbiology, **154(1):** 202–207.

Oikonomou, G., Addis, M. F., Chassard, C., Nader-Macias, M., Grant, I., Delbès, C., Bogni, C. I., Le Loir, Y., & Even, S. (2020): Milk Microbiota: What Are We Exactly Talking About?. Frontiers in microbiology, **11**: 60. https://doi.org/10.3389/fmicb.2020.00060

Oikonomou, G., Bicalho, M. L., Meira, E., Rossi, R. E., Foditsch, C., Machado, V. S., Teixeira,

A. G., Santisteban, C., Schukken, Y. H., & Bicalho, R. C. (2014): Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. PloS one**, 9(1)**: 85904. https://doi.org/10.1371/journal.pone.0085904

Oikonomou, G., Machado, V. S., Santisteban, C., Schukken, Y. H., & Bicalho, R. C. (2012): Microbial Diversity of Bovine Mastitic Milk as Described by Pyrosequencing of Metagenomic 16s rDNA. PLoS ONE, ***7*****(10):** 1371 https://doi.org/10.1371/journal.pone.0047671

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., & Suggests, M. (2007): The vegan package. Community Ecology Package **10**: 631–637.

Oliveira, C. S. F., Hogeveen, H., Botelho, A. M., Maia, P. V, Coelho, S. G., & Haddad, J. P. A. (2015): Cow-specific risk factors for clinical mastitis in Brazilian dairy cattle. Preventive Veterinary Medicine, **121(3–4):** 297–305. https://doi.org/10.1016/j.prevetmed.2015.08.001

Oliveira, L., Hulland, C., & Ruegg, P. L. (2013): Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. Journal of Dairy Science, **96(12):** 7538–7549. https://doi.org/10.3168/jds.2012-6078

Oliver, S. P., & Murinda, S. E. (2012): Antimicrobial resistance of mastitis pathogens. The Veterinary clinics of North America. Food animal practice, **28(2):**165–185. https://doi.org/10.1016/j.cvfa.2012.03.005

Oliver, S. P., Murinda, S. E., & Jayarao, B. M. (2011): Impact of antibiotic use in adult dairy

cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. Foodborne Pathogens and Disease, **8(3):** 337–355. https://doi.org/10.1089/fpd.2010.0730.

Omore, A. O., McDermott, J. J., Arimi, S. M., & Kyule, M. N. (1999): Impact of Mastitis Control Measures on Milk Production and Mastitis Indicators in Smallholder Dairy Farms in Kiambu District, Kenya. Tropical Animal Health and Production, **31**: 347–361. https://doi.org/10.1023/a:1005269007104

Omwenga, I., Aboge, G. O., Mitema, E. S., Obiero, G., Ngaywa, C., Ngwili, N., Wamwere, G., Wainaina, M., & Bett, B. (2020): Antimicrobial Usage and Detection of Multidrug-Resistant Staphylococcus aureus, Including Methicillin-Resistant Strains in Raw Milk of Livestock from Northern Kenya. Microbial drug resistance (Larchmont, N.Y.), **27(6):** 843–854. https://doi.org/10.1089/mdr.2020.0252

Ondiek, J. O. & Kemboi, F. (2018): Bovine Mastitis Prevalence , Aetiology , Therapeutics and Control in Tatton Agriculture Park , Egerton University, International Journal of Current Microbiology and Applied Science, **7(9):** 3417-3426

Orucho, V.O, Ochoi, C.O, Nyangau, M.K, (2020): Antibiotic resistance of Streptococcus pneumonia serotypes in Kisii, Kenya Research Square https://doi.org/10.21203/rs.2.13786/v2 accessed on 20th January 2021

Paduch, J. H., & Krömker, V. (2011): Colonization of the teat skin and the teat canal of lactating dairy cattle by mastitis pathogens. Tierarztliche Praxis. Ausgabe G, Grosstiere/Nutztiere, **39(2):** 71–76.

Panchal, V. V., Griffiths, C., Mosaei, H., Bilyk, B., Sutton, J.AF., Carnell, O. T, Hornby, D.P.,

Green, J., Hobbs, J. K, Kelley, W. L, Zenkin, N., Foster, S. J. (2020): Evolving MRSA: High-level β-lactam resistance in Staphylococcus aureus is associated with RNA Polymerase alterations and fine tuning of gene expression. PLoS Pathogen **16(7):**e1008672. doi: 10.1371/journal.ppat.1008672

Pang, M., Xie, X., Bao, H., Sun, L., He, T., Zhao, H., Zhou, Y., Zhang, L., Zhang, H., Wei, R., Xie, K., & Wang, R. (2018): Insights Into the Bovine Milk Microbiota in Dairy Farms With Different Incidence Rates of Subclinical Mastitis. Frontiers in microbiology, **9**:2379. https://doi.org/10.3389/fmicb.2018.02379

Park, A. J., Surette, M. D., & Khursigara, C. M. (2014): Antimicrobial targets localize to the extracellular vesicle-associated proteome of Pseudomonas aeruginosa grown in a biofilm. Frontiers in microbiology, **5**: 464. https://doi.org/10.3389/fmicb.2014.004.

Patel, R., Kunjadia, P., Koringa, Joshi, P. C., & Kunjadiya, A. (2019): Microbiological profiles in clinical and subclinical cases of mastitis in milking Jafarabadi buffalo. Research in veterinary science, **125**: 94–99. https://doi.org/10.1016/j.rvsc.2019.05.012

Patel, J. R., Pandit, R. J., Bhatt, D. V., Kunjadia, D. P., Nauriyal, S. D., Koringa, G. P., Joshi, G.C., Kunjadia, P. A. (2017): Metagenomic approach to study the bacterial community in clinical and subclinical mastitis in buffalo, Meta Gene, **12:**4-7 https://doi.org/10.1016/j.mgene.2016.12.014.

Pekana, A., & Green, E. (2018): Antimicrobial Resistance Profiles of Staphylococcus aureus Isolated from Meat Carcasses and Bovine Milk in Abattoirs and Dairy Farms of the Eastern Cape, South Africa. International journal of environmental research and public health, **15(10):** 2223. https://doi.org/10.3390/ijerph15102223

Pérez-Serrano, R. M., Domínguez-Pérez, R.A., Ayala-Herrera, J.L., Luna-Jaramillo, A.E., Zaldivar-Lelo de Larrea, G., Solís-Sainz, J.C., García-Solís, P., Loyola-Rodríguez, J.P. (2020): Dental plaque microbiota of pet owners and their dogs as a shared source and reservoir of antimicrobial resistance genes. Journal of Global Antimicrobial Resistance **21:**285-290.https://doi: 10.1016/j.jgar.2020.03.025

Persson, Y., & Olofsson, I. (2011): Direct and indirect measurement of somatic cell count as indicator of intramammary infection in dairy goats. Acta veterinaria Scandinavica, **53(1):** 15. https://doi.org/10.1186/1751-0147-53-15

Phophi, L., Petzer, I. M, & Qekwana, D. N. (2019): Antimicrobial resistance patterns and biofilm formation of coagulase-negative Staphylococcus species isolated from subclinical mastitis cow milk samples submitted to the Onderstepoort Milk Laboratory. BMC Vet Res. 2019 Nov 26;15(1):420. doi: 10.1186/s12917-019-2175-3.

Piepers, S., Peeters, K., Opsomer, G., Barkema, H. W., Frankena, K., & De Vliegher, S. (2011): Pathogen group specific risk factors at herd, heifer and quarter levels for intramammary infections in early lactating dairy heifers. Preventive veterinary medicine, **99(2-4):** 91–101. https://doi.org/10.1016/j.prevetmed.2011.02.010

Piessens, V., Van Coillie, E., Verbist, B., Supré, K., Braem, G., Van Nuffel, A., De Vuyst, L., Heyndrickx, M., & De Vliegher, S. (2011): Distribution of coagulase-negative Staphylococcus species from milk and environment of dairy cows differs between herds. Journal of Dairy science, **94(6):**2933–2944. https://doi.org/10.3168/jds.2010-3956.

Podder, M. P., Rogers, L., Daley, P. K., Keefe, G. P., Whitney, H. G., & Tahlan, K. (2014): Klebsiella species associated with bovine mastitis in Newfoundland. PloS one, **9(9):**

e106518. https://doi.org/10.1371/journal.pone.0106518

Pol, M., & Ruegg, P. L. (2007): Relationship Between Antimicrobial Drug Usage and Antimicrobial Susceptibility of Gram-Positive Mastitis Pathogens. Journal of Dairy Science, **90(1):**262–273. https://doi.org/10.3168/jds.S0022-0302(07)72627-9.

Pyatov, V., Vrtková, I., & Knoll, A. (2017): Detection of selected antibiotic resistance genes using multiplex PCR assay in mastitis pathogens in the Czech Republic. Acta Veterinaria Brno, **86(2):** 167–174. https://doi.org/10.2754/avb201786020167

Qu, Y., Zhao, H., Nobrega, D.B., Cobo, E.R., Han, B., Zhao, Z., Li, S., Li, M., Barkema, H.W., Gao, J. (2019): Molecular epidemiology and distribution of antimicrobial resistance genes of Staphylococcus species isolated from Chinese dairy cows with clinical mastitis. Journal of Dairy Science: **102(2):**1571-1583. doi: 10.3168/jds.2018-15136.

Rademaker, C. J., Omedo B. B., Van der Lee, J., Kilelu, C., Tanui C. (2016): Sustainable growth of the Kenyan dairy sector: A quick scan of robustness, reliability and resilience. (No. WLR. 2016;979) https://edepot.wur.nl/391018. Acccessed on 15th December 2020

Radostits, O., C. Gay, D. Blood, Hinchcliff, K., Constable P. D. (2007): Veterinary Medicine. A Text Book of the Disease of Cattle, Sheep, Pigs, Goats and Horses, 9th edition, W.B. Sounders Company Ltd, New York, NY, USA, 2007 pp 430-432

Rahil, I. (2019): Coagulase-Negative Staphylococci (CNS) as Emerging Mastitis Pathogens Journal of Dairy and Veterinary Sciences, **13**(4): 2–3. https://juniperpublishers.com/jdvs/pdf/JDVS.MS.ID.555868.pdf accessed on 12th December, 2020

Ramírez, N. F., Keefe, G., Dohoo, I., Sánchez, J., Arroyave, O., Cerón, J., Jaramillo, M., &

Palacio, L. G. (2014): Herd- and cow-level risk factors associated with subclinical mastitis in dairy farms from the High Plains of the northern Antioquia, Colombia. Journal of dairy science, **97(7):** 4141–4150. https://doi.org/10.3168/jds.2013-6815

Reinhold, P., Sachse, K., & Kaltenboeck, B. (2011): Chlamydiaceae in cattle: commensals, trigger organisms, or pathogens?. Veterinary journal (London, England : 1997), **189(3):** 257–267. https://doi.org/10.1016/j.tvjl.2010.09.003

Richards, S., Vanleeuwen, J. Peter, S. G., Wichtel, J. Kamunde, C., Uehlinger F. & Gitau, G. (2019): Impact of mineral feeding on reproductive efficiency on smallholder dairy farms in Kenya Livestock Research for Rural Development, **31**:80 Retrieved December 9, 2020, from http://www.lrrd.org/lrrd31/6/srich31080.html

Rivas, A. L., Quimby, F. W., Blue, J., & Coksaygan, O. (2001): Longitudinal evaluation of bovine mammary gland health status by somatic cell counting, flow cytometry, and cytology. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, **13(5):**399–407. https://doi.org/10.1177/104063870101300506

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016):VSEARCH: a versatile open source tool for metagenomics. PeerJ, 4, e2584. https://doi.org/10.7717/peerj.2584

Romero, J., Benavides, E., & Meza, C. (2018): Assessing Financial Impacts of Subclinical Mastitis on Colombian Dairy Farms. Frontiers in Veterinary Science, **5:** 273 https://doi.org/10.3389/fvets.2018.00273

Rowe, S. M., Godden, S. M., Royster, E., Timmerman, J., & Boyle, M. (2019): Cross-sectional study of the relationship between cloth udder towel management, towel bacteria counts, and

intramammary infection in late-lactation dairy cows. Journal of dairy science, **102(12):**11401–11413. https://doi.org/10.3168/jds.2019-17075

Ruegg P. L. (2017): A 100-Year Review: Mastitis detection, management, and prevention. Journal of dairy science, **100(12):** 10381–10397. https://doi.org/10.3168/jds.2017-13023

Sadashiv, O. S. and Kaliwal, B. B. (2014): Isolation , characterization and antibiotic resistance of Bacillus sps . from bovine mastitis in the region of north Karnataka, India. International Journal of Current Microbiology Applied Science **3**: 360-373.

Saed, H., & Ibrahim, H. (2020): Antimicrobial profile of multidrug-resistant *Streptococcus* spp. isolated from dairy cows with clinical mastitis. Journal of advanced veterinary and animal research, **7(2):** 186–197. https://doi.org/10.5455/javar.2020.g409

Saini, V., McClure, J. T., Léger, D., Keefe, G. P., Scholl, D. T., Morck, D. W., & Barkema, H. W. (2012): Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. Journal of Dairy Science, **95(8):** 4319–4332 https://doi.org/10.3168/jds.2012-5373.

Santos, A. S., Lima, D., Abad, A., Oliveira, P., Silva, J., Moura, G. S., Silva, A., Amorim, V., Costa, M., & Mota, R. A. (2020): Antimicrobial resistance profile of non-aureus *Staphylococci* isolates from buffalo, goat and sheep mastitis in the Northeast region of Brazil. The Journal of dairy research, **87(3):** 290–294. https://doi.org/10.1017/S0022029920000771.

Sarba, E. J., & Tola, G. K. (2017): Cross-sectional study on bovine mastitis and its associated risk factors in Ambo district of West Shewa zone, Oromia, Ethiopia. Veterinary world **10(4):** 398–402. https://doi.org/10.14202/vetworld.2017.398-402

Scaccabarozzi, L., Leoni, L., Ballarini, A., Barberio, A., Locatelli, C., Casula, A., Bronzo, V., Pisoni, G., Jousson, O., Morandi, S., Rapetti, L., García-Fernández, A., & Moroni, P. (2015): Pseudomonas aeruginosa in Dairy Goats: Genotypic and Phenotypic Comparison of Intramammary and Environmental Isolates. PloS one, **10(11):** e0142973. https://doi.org/10.1371/journal.pone.0142973

Schalm, O. W., Coroll E. J. and Jain, N. C. (1971): Bovine Mastitis, Lea and Febiger, Philadelphia, PA, USA, 1971.

Schauer, B., Wald, R., Urbantke, V., Loncaric, I., & Baumgartner, M. (2021): Tracing Mastitis Pathogens-Epidemiological Investigations of a Pseudomonas aeruginosa Mastitis Outbreak in an Austrian Dairy Herd. Animals : an open access journal from MDPI, **11(2):** 279. https://doi.org/10.3390/ani11020279

Schaumburg, F., Alabi, A. S., Peters, G., & Becker, K. (2014): New epidemiology of Staphylococcus au-reus infection in Africa. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, **20(7):** 589–596. https://doi.org/10.1111/1469-0691.12690

Schmidt, T., Kock, M. M., & Ehlers, M. M. (2015): Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. Journal of Dairy Science, **98(9):** 6256–6269. https://doi.org/10.3168/jds.2015-9715

Schnitt, A., & Tenhagen, B. A. (2020): Risk Factors for the Occurrence of Methicillin-Resistant Staphylococcus aureus in Dairy Herds: An Update. Foodborne pathogens and disease, **17(10):** 585–596. https://doi.org/10.1089/fpd.2019.2638

Scholtzek, A. D., Hanke, D., Walther, B., Eichhorn, I., Stöckle, S. D., Klein, K. S., Gehlen, H.,

Lübke-Becker, A., Schwarz, S., & Feßler, A. T. (2019): Molecular Characterization of Equine Staphylococcus aureus Isolates Exhibiting Reduced Oxacillin Susceptibility. Toxins, **11(9):**535. https://doi.org/10.3390/toxins11090535

Schukken, Y. H., González, R. N., Tikofsky, L. L., Schulte, H. F., Santisteban, C. G., Welcome, F. L., Bennett, G. J., Zurakowski, M. J., & Zadoks, R. N. (2009): CNS mastitis: nothing to worry about?. Veterinary microbiology, **134(1):** 9–14. https://doi.org/10.1016/j.vetmic.2008.09.014.

Schukken, Y., Chuff, M., Moroni, P., Gurjar, A., Santisteban, C., Welcome, F., & Zadoks, R. (2012): The "other" gram-negative bacteria in mastitis: Klebsiella, serratia, and more. The Veterinary clinics of North America. Food animal practice, **28(2):** 239–256. https://doi.org/10.1016/j.cvfa.2012.04.001

Sender, G., Pawlik, A., & Korwin-kossakowska, A. (2017): Current concepts on the impact of coagulase-negative staphylococci causing bovine mastitis as a threat to human and animal health – a review. Animals science paper and reports **35(2):** 123–135. http://yadda.icm.edu.pl/yadda/element/bwmeta1.element.agro-da479bc9-fa1a-476f-b244-d6755a105b08 accessed on 12th February, 2020

Sharma, C., Rokana, N., Chandra, M., Singh, B. P., Gulhane, R. D., Gill, J., Ray, P., Puniya, A. K., & Panwar, H. (2018): Antimicrobial Resistance: Its Surveillance, Impact, and Alternative Management Strategies in Dairy Animals. Frontiers in veterinary science, **4**: 237. https://doi.org/10.3389/fvets.2017.00237

Sharun, K., Dhama, K., Tiwari, R., Gugjoo, M. B., Iqbal Yatoo, M., Patel, S. K., Pathak, M., Karthik, K., Khurana, S. K., Singh, R., Puvvala, B., Amarpal, Singh, R., Singh, K. P.,

& Chaicumpa, W. (2021): Advances in therapeutic and managemental approaches of bovine mastitis: a comprehensive review. The veterinary quarterly, **41(1):** 107–136. https://doi.org/10.1080/01652176.2021.1882713

Shitandi, A., & Sternesjö, Å. (2004): Prevalence of Multidrug Resistant Staphylococcus aureus in Milk from Large- and Small-Scale Producers in Kenya. Journal of Dairy Science, **87(12):** 4145–4149. https://doi.org/10.3168/jds.S0022-0302(04)73557-2

Sieber, S., Gerber, V., Jandova, V., Rossano, A., Evison, J. M., & Perreten, V. (2011): Evolution of multidrug-resistant Staphylococcus aureus infections in horses and colonized personnel in an equine clinic between 2005 and 2010. Microbial drug resistance (Larchmont, N.Y.), **17(3):** 471–478. https://doi.org/10.1089/mdr.2010.0188

Smith, T. C. (2015): Livestock-Associated Staphylococcus aureus : The United States Experience. PLoS Pathogen **11(2)**: 1–8. https://doi.org/10.1371/journal.ppat.1004564

SnapGene® software (from Insightful Science; available at https://www.snapgene.com/) (accessed on 1st December, 2020).

Sokolov, S., Fursova, K., Shulcheva, I., Nikanova, D., Artyemieva, O., Kolodina, E., Sorokin, A., Dzhelyadin, T., Shchannikova, M., Shepelyakovskaya, A., Zinovieva, N., & Brovko, F. (2021): Comparative Analysis of Milk Microbiomes and Their Association with Bovine Mastitis in Two Farms in Central Russia. Animals: an open access journal from MDPI, **11(5):**1401. https://doi.org/10.3390/ani11051401

Srinivasan, V., Gillespie, B. E., Lewis, M. J., Nguyen, L. T., Headrick, S. I., Schukken, Y. H., & Oliver, S. P. (2007): Phenotypic and genotypic antimicrobial resistance patterns of Escherichia coli isolated from dairy cows with mastitis. Veterinary microbiology, **124**(3-

4): 319–328. https://doi.org/10.1016/j.vetmic.2007.04.040

Sriram A., Kalanxhi E., Kapoor G., Craig J., Balasubramanian R., Brar S., Criscuolo N., Hamilton A., Klein E., Tseng K., Boeckel T. V & Laxminarayan R. (2021): State of the world's antibiotics 2021: A global analysis of antimicrobial resistance and its drivers. Center for Disease Dynamics, Economics & Policy, Washington DC. Retrieved from https://www.lshtm.ac.uk/research/centres/amr/news/227421/state-worlds-antibiotics-2021 Accessed on 26th August 2021

Ssajjakambwe, P., Bahizi, G., Setumba, C., Kisaka, S. M. B., Vudriko, P., Atuheire, C., Kabasa, J. D & Kaneene, J. B. (2017): Milk Hygiene in Rural Southwestern Uganda: Prevalence of Mastitis and Antimicrobial Resistance Profiles of Bacterial Contaminants of Milk and Milk Products. Veterinary medicine international, **2017**:8710758. https://doi.org/10.1155/2017/8710758

Stevens, M., Piepers, S., & Vliegher, S. De. (2016): Mastitis prevention and control practices and mastitis treatment strategies associated with the consumption of (critically important) antimicrobials on dairy herds in Flanders, Belgium. Journal of Dairy Science**, 99(4)**: 2896–2903. https://doi.org/10.3168/jds.2015-10496.

Suleiman, T. S., Karimuribo, E. D., & Mdegela, R. H. (2018): Prevalence of bovine subclinical mastitis and antibiotic susceptibility patterns of major mastitis pathogens isolated in Unguja island of Zanzibar, Tanzania. Tropical animal health and production, **50(2):** 259–266. https://doi.org/10.1007/s11250-017-1424-3.

Sundquist, A., Bigdeli, S., Jalili, R., Druzin, M. L., Waller, S., Pullen, K. M., El-Sayed, Y. Y., Taslimi, M. M., Batzoglou, S., & Ronaghi, M. (2007): Bacterial flora-typing with

targeted, chip-based Pyrosequencing. BMC microbiology, **7:** 108. https://doi.org/10.1186/1471-2180-7-108.

Tančin, V.; Ipema, A.H.; Hogewerf, P. (2007): Interaction of Somatic Cell Count and Quarter Milk Flow Patterns. Journal Dairy Science, **90**: 2223–2228. https://doi.org/10.3168/jds.2006-666

Taponen S., Mcguinness, D., Hiitiö, H., Simojoki, H., Zadoks, R., and Pyörälä, S. (2019): Bovine milk microbiome : a more complex issue than expected. Journal of Veterinary Research, **50**: 44 https://doi.org/10.1186/s13567-019-0662-y

Taponen, S., Liski, E., Heikkilä, A.-M., & Pyörälä, S. (2017): Factors associated with intramammary infection in dairy cows caused by coagulase-negative staphylococci, Staphylococcus aureus , Streptococcus uberis , Streptococcus dysgalactiae , Corynebacterium bovis , or Escherichia coli. Journal of Dairy Science, **100(1)**: 493–503. https://doi.org/10.3168/jds.2016-11465

Thorberg, B. M., Danielsson-Tham, M. L., Emanuelson, U., & Persson Waller, K. (2009): Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. Journal of Dairy science, **92(10)**: 4962–4970. https://doi.org/10.3168/jds.2009-2184

Thrushfield, M. (2005): Veterinary epidemiology, 3rd edition, Black Well Science Ltd., Oxford, UK.

Tilahun, A., & Aylate, A. (2015): Prevalence of Bovine Mastitis in Lactating Cows and its Public Health Implications in Selected Commercial Dairy Farms of Addis Ababa. Global Journal of Medical Research, G. Veterinary Science and Veterinary Medicine **15**(2): 17-23

Tolosa, T., Verbeke, J., Ayana, Z., Piepers, S., Supré, K., & De Vliegher, S. (2015): Pathogen

group specific risk factors for clinical mastitis, intramammary infection and blind quarters at the herd, cow and quarter level in smallholder dairy farms in Jimma, Ethiopia. Preventive veterinary medicine, **120(3-4):** 306–312. https://doi.org/10.1016/j.prevetmed.2015.05.001

Tomazi, T., Freu, G., Alves, B. G., de Souza Filho, A. F., Heinemann, M. B., & Veiga Dos Santos, M. (2019): Genotyping and antimicrobial resistance of Streptococcus uberis isolated from bovine clinical mastitis. PloS one, **14(10):** e0223719. https://doi.org/10.1371/journal.pone.0223719

Tzouvelekis, L. S., Markogiannakis, A., Piperaki, E., Souli, M., & Daikos, G. L. (2014): Treating infections caused by carbapenemase-producing Enterobacteriaceae. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, **20(9):** 862–872. https://doi.org/10.1111/1469-0691.12697

UNFPA, (2020): United Nations Population Fund (UNFPA) State of the World population https://www.unfpa.org/sites/default/files/pubpdf/UNFPA_PUB_2020_EN_State_of_World_Population.pdf accessed on May 26th 2020

Vakkamäki, J., Taponen, S., Heikkilä, A. M., & Pyörälä, S. (2017): Bacteriological etiology and treatment of mastitis in Finnish dairy herds. Acta Veterinaria Scandinavica, **59:** 33 (2017). https://doi.org/10.1186/s13028-017-0301-4

Vali, L., Dashti, A. A., Mathew, F., & Udo, E. E. (2017): Characterization of Heterogeneous MRSA and MSSA with Reduced Susceptibility to Chlorhexidine in Kuwaiti Hospitals. Frontiers in microbiology, **8:**1359. https://doi.org/10.3389/fmicb.2017.01359.

Van, T., Yidana, Z., Smooker, P. M., & Coloe, P. J. (2020): Antibiotic use in food animals

worldwide, with a focus on Africa: Pluses and minuses. Journal of global antimicrobial resistance, **20**:170–177. https://doi.org/10.1016/j.jgar.2019.07.031

Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermans, K., & Butaye, P. (2010): Methicillin-resistant Staphylococcus aureus (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Veterinary Microbiology, **144(1):** 166–171. https://doi.org/10.1016/j.vetmic.2009.12.044

Verbeke, J., Piepers, S., Supré, K., & Vliegher, S. De. (2014): Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds , severity , and association with herd hygiene. Journal of Dairy Science, 97(11): 6926–6934. https://doi.org/10.3168/jds.2014-8173

Verraes, C., Claeys, W., Cardoen, S., Daube, G., De Zutter, L., Imberechts, H., Dierick, K. & Herman, L. (2014): A review of the microbiological hazards of raw milk from animal species other than cows. International Dairy Journal, **39**(1): 121–130. https://doi.org/10.1016/j.idairyj.2014.05.010

Wald, R., Hess, C., Urbantke, V., Wittek, T., & Baumgartner, M. (2019): Characterization of Staphylococcus Species Isolated from Bovine Quarter Milk Samples. Animals, MDPI, 9(5):200. https://doi.org/10.3390/ani9050200

Wang R .F., Cao WW, Cerniglia CE (1997): A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. Journal of Applied Microbiology **83(6):**727-36. doi: 10.1046/j.1365-2672.1997.00300.

Wang, D., Wang, Z., Yan, Z., Wu, J., Ali, T., Li, J., Lv, Y., & Han, B. (2015): Bovine mastitis Staphylococcus aureus: antibiotic susceptibility profile, resistance genes and molecular typing of methicillin-resistant and methicillin-sensitive strains in China. Infection, genetics

and evolution : Journal of molecular epidemiology and evolutionary genetics in infectious diseases, **31**: 9–16. https://doi.org/10.1016/j.meegid.2014.12.039.

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007): Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Journal of Applied and environmental microbiology, **73(16):** 5261–5267. https://doi.org/10.1128/AEM.00062-07

Wang, W., Lin, X., Jiang, T., Peng, Z., Xu, J., Yi, L., Li, F., Fanning, S., & Baloch, Z. (2018): Prevalence and Characterization of Staphylococcus aureus Cultured From Raw Milk Taken From Dairy Cows With Mastitis in Beijing, China. Frontiers in microbiology, **9**: 1123. https://doi.org/10.3389/fmicb.2018.01123

Wenning, M., & Scherer, S. (2013): Identification of microorganisms by FTIR spectroscopy: perspectives and limitations of the method. Applied microbiology and biotechnology, **97(16):** 7111–7120. https://doi.org/10.1007/s00253-013-5087-3

WHO, (2019): World Health Organization (WHO) Critically important antimicrobials for human medicine, 6th revision. Retrieved from https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf?ua=1) accessed on 3rd October, 2021

WHO, (2016): World Health Organization (WHO), Antimicrobial resistance National action plans, 2015(March), 1–16. Retrieved from http://www.who.int/antimicrobial-resistance/national-action-plans/en/Accessed on 26th February 2020.

WHO, (2015): World Health Organization (WHO),Worldwide country situation analysis: response to antimicrobial resistance. Retrieved from

https://apps.who.int/iris/bitstream/10665/163468/1/9789241564946_eng.pdf Accessed on 15th August 2021.

Yang, F., Zhang, S., Shang, X., Li, H., Zhang, H., Cui, D., Wang, X., Wang, L., Yan, Z., & Sun, Y. (2020): Short communication : Detection and molecular characterization of methicillin-resistant Staphylococcus aureus isolated from subclinical bovine mastitis cases in China. Journal of Dairy Science, **103(1):** 840–845. https://doi.org/10.3168/jds.2019-16317.

Zadoks, R. N. & Fitzpatrick, J. L. (2009): Changing trends in mastitis. Irish Veterinary Journal, **62(4):**59–70. https://doi.org/10.1186/2046-0481-62-S4-S59.

Zadoks, R. N., H. G. Allore, H. W. Barkema, O. C. Sampimon, G. J. Wellenberg, Y. T. Gröhn, and Y. H. Schukken. (2001): Cow- and quarter-level risk factors for Streptococcus uberis and Staphylococ- cus aureus mastitis. J. Dairy Sci. **84**:2649–2663.

Zadoks, R. N., Middleton, J. R., McDougall, S., Katholm, J., & Schukken, Y. H. (2011): Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. Journal of Mammary Gland Biology and Neoplasia, **16(4):** 357–372. https://doi.org/10.1007/s10911-011-9236-y.

Zeryehun, T. and Abera G. (2017): Prevalence and Bacterial Isolates of Mastitis in Dairy Farms in Selected Districts of Eastern Harrarghe Zone , Eastern Ethiopia," Journal of Veterinary Medicine, **2017:** 6498618 https://doi.org/10.1155/2017/6498618

Zhang, S., Piepers, S., Shan, R., Cai, L., Mao, S., Zou, J., Ali, T., De Vliegher, S., & Han, B. (2018): Phenotypic and genotypic characterization of antimicrobial resistance profiles in Streptococcus dysgalactiae isolated from bovine clinical mastitis in 5 provinces of China.

Journal of dairy science, **101(4)**:3344–3355. https://doi.org/10.3168/jds.2017-14031

Zhang, D., Zhang, Z., Huang, C., Gao, X., Wang, Z., Liu, Y., & Tian, C. (2018): The phylogenetic group , antimicrobial susceptibility , and virulence genes of Escherichia coli from clinical bovine mastitis. Journal of Dairy Science, **101(1):** 572–580. https://doi.org/10.3168/jds.2017-13159.

Zhong, X.Z., Ma, S.C., Wang, S.P. , Wang, T.T., Sun, Z.Y., Tang, Y.Q., Deng, Y., Kida, K.J.(2018): A comparative study of composting the solid fraction of dairy manure with or without bulking material: Performance and microbial community dynamics. Bioresour. Technol., **247** :443-452. https://doi.org/10.1016/j.biortech.2017.09.116

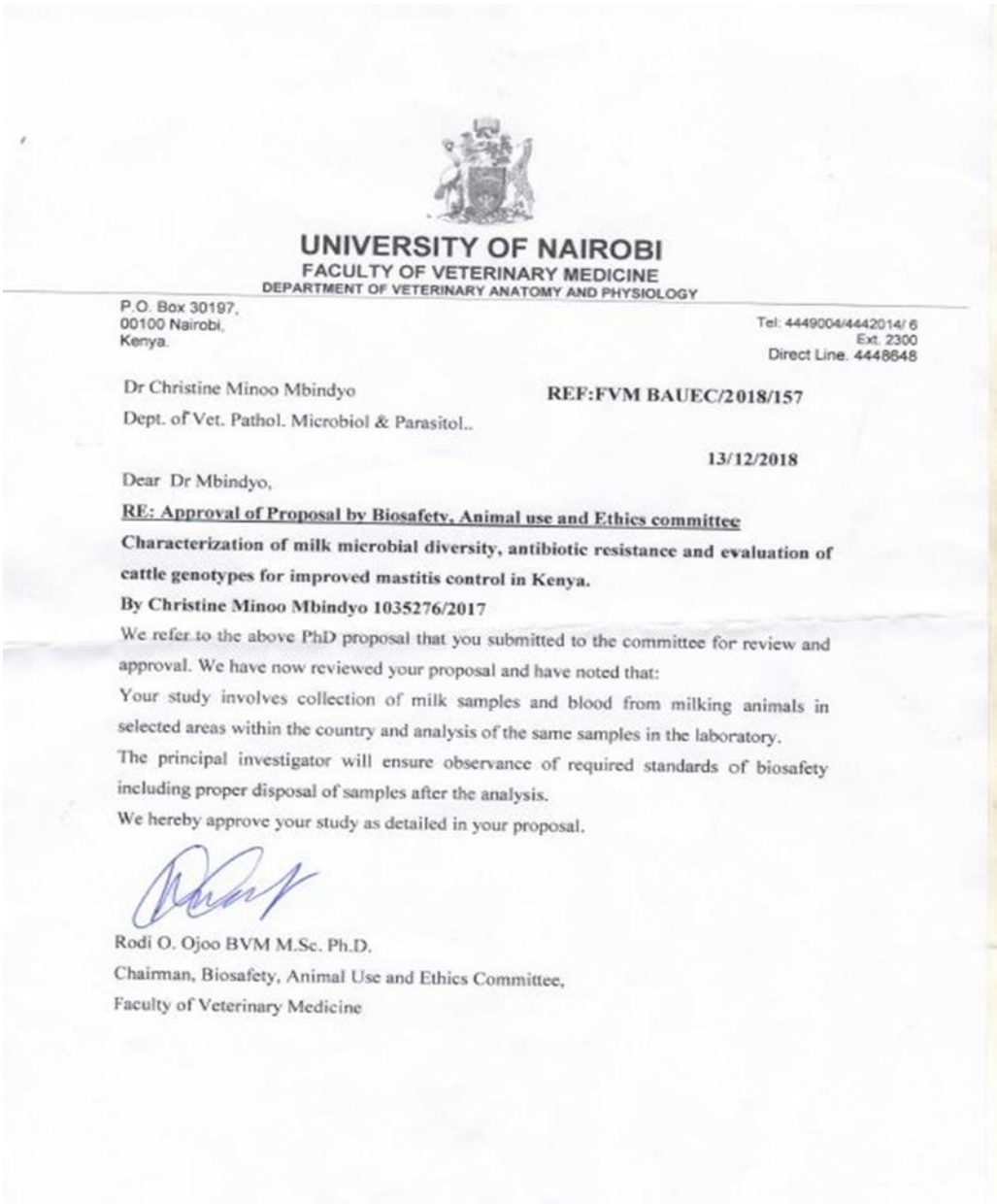
Zigo, F., Vasil, M., Ondrašovičová, S., Výrostková, J., Bujok, J., & Pecka-Kielb, E. (2021): Maintaining Optimal Mammary Gland Health and Prevention of Mastitis. Frontiers in veterinary science, **8**:607311. https://doi.org/10.3389/fvets.2021.607311

APPENDICES

APPENDIX 1: ETHICAL APPROVAL

The study was reviewed and approved by the Biosafety, Animal Use, Care, and Ethics Committee Faculty of Veterinary Medicine, University of Nairobi, before initiation of the study; reference: FVM/BAUEC/2018/157 (appendix 1.1). Informed consent was obtained verbally from farmers before participation in the study.

APPENDIX 1.1: SHOWING THE ETHICAL APPROVAL LETTER USED IN THIS STUDY



UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,
00100 Nairobi,
Kenya.

Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line. 4448648

Dr Christine Minoo Mbindyo
Dept. of Vet. Pathol. Microbiol & Parasitol..

REF:FVM BAUEC/2018/157

13/12/2018

Dear Dr Mbindyo,

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Characterization of milk microbial diversity, antibiotic resistance and evaluation of cattle genotypes for improved mastitis control in Kenya.

By Christine Minoo Mbindyo 1035276/2017

We refer to the above PhD proposal that you submitted to the committee for review and approval. We have now reviewed your proposal and have noted that:

Your study involves collection of milk samples and blood from milking animals in selected areas within the country and analysis of the same samples in the laboratory.

The principal investigator will ensure observance of required standards of biosafety including proper disposal of samples after the analysis.

We hereby approve your study as detailed in your proposal.

Rodi O. Ojoo BVM M.Sc. Ph.D.
Chairman, Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine

APPENDIX 2: FARMS SURVEY QUESTIONNAIRE FOR DAIRY FARMERS

To be filled once for each farm

Demographic information	
Farm no	
Name of the respondent	
Gender	
Telephone Number of respondent	
Age	
Date of the interview	
County	
Sub –County	
Division	
Location	
Village	
GPS coordinates	
Agro-ecological zone (AEZ)	

A. Farm Background

1. Who manages the farm?

   A) Owner B) Relative C) Employee

2. What is your level of education?

   A) Informal __B) Primary___C) Secondary _____ D) University _____

3. For how long have you been keeping dairy cattle

   A) <1 year _____ b)>1 year and <5 years _____ c) >5 year _____

4. Production system

   A) Intensive ___ B) Semi intensive____

5. How many dairy cows do you have? ____
6. What cattle breed do you keep a) Exotic____ b) Crosses___
7. Do you keep records? Yes _____ No_____

   If yes specify a) Production____ b) Breeding____ C) others (specify)

8. What feed are given to the cows a) concentrate ____ b) Pastures only ____(c) Both
9. Are the cattle housed with a roof? A) Yes ____ B) No ____
10. If housed, what is the nature of the floor where the milking cows lie down? A) Concrete____ B) Earthen ___
11. Presence of Bedding A) Yes ____ B) No ____ (Describe type of bedding)
12. How often is the floor cleaned?

A. Daily____ B) Weekly____ C) Monthly____ D) Others (specify)____

B. Mastitis and control practices

1. Have you ever experience cases of mastitis in the farm? A) Yes __ B) No ____
2. Do you milk mastitic cows last? A) Yes B) No
3. Is the cow milked using proper technique (observe)? A) Yes B) No
4. Do you wash hands before milking the cows? A) Yes _____ B) No _____
5. a) Do you wash the udder pre-milking? A) Yes B) No

b) Is the udder dried before milking with clean cloth/towel/paper? A) Yes B) No

c) Is a different clean drying cloth/towel/paper used for each milking cow? A) Yes __ B) No ____

d) if you have > 1 milking cow, do you wash your hands between milking cows? Yes __ No _

6. Do you use a teat dip post milking? A) Yes ___ B) No ____
7. Do you practice dry cow therapy? A) Yes __ B) No ____
8. Do you cull chronically infected cows? A) Yes ____B) No ____
9. Who treats your animals?

A) Vet Surgeon B) Animal Health Assistant C) Self D) Others (specify)

10. Do they perform any test to confirm mastitis?

A) CMT B) Alcohol test C) Culture D) Other (specify)

11. For how long did mastitis during the last case take to resolve (in general)?

A) One week
B) Above one week
C) Never resolved

C. Cow characteristics details

Cow ID	**Breed**	**Parity**	**Milk production per day in L**	**Stage of lactation (1-2Early (3-6) mid (>7)late**	**History of mastitis (yes/No)**	**CMT score results for each quarter**
Cow 1						FR HR FL HL
Cow 2						FR HR FL HL

APPENDIX 3: LIST OF PUBLICATION

1. **Mbindyo C.M**, Gitao GC, Plummer PJ, Kulohoma BW, Mulei CM, Bett R. Antimicrobial Resistance Profiles and Genes of Staphylococci Isolated from Mastitic Cow's Milk in Kenya. Antibiotics. 2021; 10(7):772. https://doi.org/10.3390/antibiotics10070772
2. **Mbindyo, C.M**; Gitao, C.; Mulei, C. Prevalence, Etiology, and Risk Factors of Mastitis in Dairy Cattle in Embu and Kajiado Counties, Kenya. Veterinary Medicine Internation 2020, 2020 https://doi.org/10.1155/2020/8831172