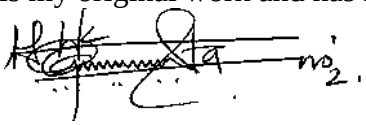
Clinical and Sub-Clinical Mastitis in Various Cattle Production Systems in Kenya: Molecular Analysis and Antibiotic-resistance of Associated Bacterial species

BY:
WALTER OKELLO OGUTA
Reg. No.: H56/6898/2017

A research proposal presented to the Department of Medical Microbiology at the School of Medicine University of Nairobi in partial fulfillment of the requirements for the award of the Degree of Masters of Science in Medical Microbiology

DECLARATION

This proposal is my original work and has not been presented to any University for any award.



8th December, 2022.

Signature: ……. Date: …….

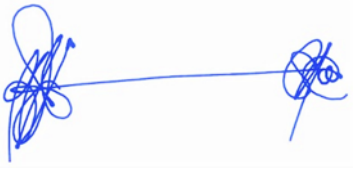
Walter Okello Oguta
Reg. No. H56/6898/2017
P. O. BOX 19676 – 00202 Nairobi, Kenya.
Mobile: +254711127631/+254737721649
Email: walteroguta@gmail.com

This proposal is submitted for examination with the approval of the following as University and Technical Supervisors:

8th December, 2022.

Signature: ……. Date: …….

Dr. Marianne Mureithi
Chairperson
Department of Medical Microbiology
University of Nairobi
P. O. BOX 19676 – 00202 Nairobi, Kenya.
Mobile: +254703704711
Email: marianne@uonbi.ac.ke



8th December, 2022.

Signature: ……. Date: …….

Dr. Moses Masika
Tutorial Fellow
Department of Medical Microbiology
University of Nairobi
P. O. BOX 19676 – 00202 Nairobi, Kenya.
Mobile: +254721770306.
Email: mosmasika@uonbi.ac.ke

Lillianwambua

8th December, 2022.

Signature: ……. Date: …….

Dr. Lillian Wambua
Diagnostic Assays (Post-Doctoral Fellow
International Livestock Research Institute
P. O. BOX 30709 – 00100 Nairobi, Kenya
Mobile: +254783505560
Email: wambua.lillian@gmail.com **or** L.Wambua@cgiar.org

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ABBREVIATIONS AND ACRONYMS USED

AST	**–**	Antimicrobial Susceptibility Testing
ATCC	–	American Type Culture Collection
CDC	**–**	Centre for Disease Control and Prevention
CGIAR	**–**	Consortium of International Agriculture Research Centres, formerly Consultative Group for International Agricultural Research
CMT	**–**	California Mastitis Test
CoNS	**–**	Coagulase-Negative *Staphylococci*
CRE	**–**	Carbapenems-resistant *Enterobacteriaceae*
CTX-M	**–**	Cefotaximase, Munich
EDTA	**–**	Ethylenediaminetetraacetic acid
ESBLs	**–**	Extended-spectrum β-lactamases
EUCAST	**–**	European Committee on Antimicrobial Susceptibility Testing
FLAIR	**–**	Future Leaders – African Independent Research (FLAIR) Fellowships
GES	**–**	Guiana-extended spectrum
hVISA	**–**	Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus*
ILRI	**–**	International Livestock Research Institute
IMI - 1	**–**	Imipenem-hydrolysing β-Lactamase-1
IMP	**–**	Imipenem-resistant *Pseudomonas*
KPC	**–**	*Klebsiella Pneumoniae* Carbapenemase
MALDI TOF MS		Matrix-Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry
MBL	**–**	Metallo-β-lactamases
MCR - 1	**–**	Mobilizable Colistin Resistance -1 gene
MIC	**–**	Minimal Inhibitory Concentration
MRSA	**–**	Methicillin-Resistant *Staphylococcus aureus*
NCTC	**–**	National Collection of Type Cultures
NDM - 1	**–**	New Delhi metallo-β-lactamase 1
OXA	**–**	Oxacillinases
PBP2a	**–**	Penicillin-binding Protein 2a
PCR – HRM	**–**	Polymerase Chain Reaction – High Resolution Melt
PER	**–**	Pseudomonas Extended resistance
SCCs	**–**	Somatic Cell Counts
SHV	**–**	Sulphydryl variable
STARD	**–**	Standards for Reporting Diagnostic Accuracy Studies
TEM	**–**	Temoniera
UoN	**–**	University of Nairobi
VIM	**–**	Verona Integrons-encoded metallo-β-lactamase

OPERATIONAL DEFINITION OF TERMS USED

Antimicrobial resistance: Ability of a microorganism (such as bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals and antimalarials respectively) from working against them. It's the increase in insensitivity of pathogens to drugs and as a result the standard treatments become ineffective, infections persist and may spread to others.

Antimicrobial sensitivity testing: refers to laboratory tests that are used to determine the antimicrobials that a particular microorganism or groups of organisms are susceptible to or resistant to.

Antimicrobial stewardship: is a coordinated programme that promotes the appropriate use of antimicrobials to improve patient outcomes, reduce antimicrobial resistance and limit the spread of multidrug-resistant organisms.

ß-lactam antibiotics: A group of antibiotics with a beta-lactam ring in the chemical structure e.g., penicillin, cephalosporins and carbapenems.

ß-lactamases: Enzymes that hydrolyse the beta-lactam ring of β-lactam antibiotics and render these antibiotic ineffective.

Carbapenems: ß-lactam antibiotics with a broader spectrum of activity compared to other ß-lactams due to the five-member ring and fused ß-lactam ring. Examples include imipenem-cilastatin, ertapenem, meropenem and doripenem.

Carbapenemases: β-lactamases that hydrolyse most β-lactam antibiotics like penicillin, cephalosporins and carbapenems.

Carbapenem-resistant *Enterobacteriacea*e (CRE): a group of resistant bacteria capable of producing carbapenemases that render them resistant to carbapenems. Examples include *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumanii etc.*

Carbapenemase-producing *Enterobacteriacea*e (CPE): include only those Enterobacteriaceae with a confirmed carbapenemase producing gene.

Characterization: means to give defining features and nature with a view to classifying the resistant bacterial genes associated with bovine mastitis into their taxonomic units/groups, entails both phenotypic and molecular classification.

Diagnostic stewardship: coordinated guidance and interventions to improve appropriate use of microbiological diagnostics to guide therapeutic decisions.

Dry cow therapy: the administration of intramammary antibiotics at the beginning of the dry period for the treatment of bovine mastitis caused by contagious pathogens such as *S. aureus*; treatment of subclinical cases of mastitis due to environmental pathogens such as *S. uberis*, which are detected late in lactation, may be deferred until the dry period.

Enterobacteriaceae: Family of Gram-negative, facultatively anaerobic, lactose fermenting rod shaped bacteria that include several important pathogens including *E. coli, Klebsiella, Salmonella, Shigella, Serratia* and *Proteus* species.

Isolate: A pure microorganism obtained from a specimen such as blood or stool or milk.

LAME: Organisms in which Cephalosporins have no activity on: **L**isteria, **A**typicals **M**ycoplasma & chlamydia, **M**RSA and **E**nterococci

Molecular classification: Grouping based on genomic sequence resemblance

Multidrug resistance: A case in which microorganisms can withstand at least one antibiotic in three or more drug classes that they previous susceptible to.

Minimal Inhibitory Concentration (MIC): The lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test

One Health Approach: A multisectoral, collaborative and transdisciplinary approach – that works at the local, national, regional and global levels – with the aim of achieving optimal health outcomes, incognisant of the interdependence between human beings, animals, plants, and their shared environmental.

Phenotypic classification: Grouping based on similar inhibitor profiles/drug resistance and sensitivity patterns.

Resistome: All the resistance genes in an organism, how they are inherited, and how their transcription levels vary to defend against pathogens. Here resistome refers to all the antibiotic resistance genes in pathogenic and non-pathogenic bacteria or inherited set of genes used to resist drugs.

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ABSTRACT

Background and Rationale: The silent epidemic of antibiotic resistance poses a danger to global public health. Worldwide, zoonotic strains of *Staphylococcus aureus* and *Escherichia coli* have been linked to both clinical and subclinical cases of bovine mastitis, However, in Kenya, sparse data exist on the antibiotic-resistant mechanisms and the resistant gene profiles related to bovine mastitis on drugs of the pis aller under various cattle production methods, including intensive, semi-intensive and extensive systems.

Objectives: To produce an understanding of bovine mastitis and associated AMR within the context of cattle production systems, to tell on-farm control strategies for mastitis and mitigate dissemination of antibiotic-resistant pathogens because of bovine mastitis in Kenya.

Methods: Cross-sectional laboratory study design in which MALDI-TOFMS will be used to confirm potential bacterial isolates causing mastitis from counties of Machakos, Makueni and Narok. Kirby Bauer disk diffusion technique will be used to phenotypically ascertain the mechanism of resistance for MRSA, ESBLs, CRE, and colistin resistant genes. Where applicable Polymerase Chain Reactions (RT-PCR) and DNA Sequencing will be applied.

Analysis: Generated data will be analyzed using R v.4.2.1 and Graphpad Prism v.8.2.3. for descriptive and inferential statistics. Chi-square test will be used to compare categorical variables/risk factors against mastitis or resistance. Further, univariate and multivariate logistic regression analysis will be applied to calculate inferential statistics and assess the correlation between mastitis prevalence, resistance and various risk factors.

Expected Results: Characterization of *S. aureus*, non-aureus *Staphylococcus*, *E. coli* and *Pseudomonas* spp as causative organisms in clinical and sub-clinical mastitis under various production systems. Generate data on their phenotypic antibiotic resistance patterns and genotypic determinants, including associated risk factors and genetic relatedness.

Discussions: Baseline information on MRSA, ESBLs, carbapenems, and colistin resistance genes will be made available by this study. In order to manage and or eliminate livestock, zoonotic, food-borne infections and antibiotic resistance in a highly one-health approach way, it is important to highlight the role that livestock production methods and other risk factors play in mastitis and antibiotic resistance in Kenyan farms.

1.0 CHAPTER ONE: INTRODUCTION

1.1 Background

1.1.1 Introduction

Antimicrobial resistance (AMR) might be a noiseless plague - a worldwide public health risk to human and livestock, notably to the medicines of the pis aller, comparable as 3rd, 4th and 5th cephalosporins, vancomycin, carbapenems and colistin (Van den Honert et al., 2018). As a result, there are more cases of recent illnesses that ordinarily may not have developed and higher rates of common diseases (Logan & Weinstein, 2017). Due to the severity of these infections and treatment failures, they come with additional financial consequences thereby overwhelming the economic costs attached (Fischetti et al., 2019). It is further troubling since there is strong evidence connecting the rise of AMR in animal production systems to the emergence of AMR in human populations (Wall et al., 2016; Wernli et al., 2020).

Globally, cattle contribute substantially to the animal-supply protein (Enahoro et al., 2018); in fact, 40% of the proteins people eat every day come from animals (Smith et al., 2017). Additionally, cattle offer financial stability and are undeniably valuable assets, particularly to males and pastoralists as well as to the agricultural populace and traders (Herrero et al., 2013). Finally, they provide capitalized raw materials for industries (Upton, 2004).

According to the Malabo report from 2020, there are 310 million to 356 million cattle in Sub-Saharan Africa as of 2018 (Glatzel et al., 2020). There are 18.8 million cattle in Kenya, and they are raised using a variety of production methods, including as intensive, semi-intensive, and extensive (controlled/ranches, and uncontrolled/pastoral) systems (FAO, 2020). Breeds raised, biosecurity measures used, housing, animal movement, and accessibility to and quality of veterinary treatment are all factors that affect the production processes. Variations in these elements may affect how exposed cattle are to the bacteria that cause mastitis and the resulting antibiotic resistance (MOHK, 2017; Wall et al., 2016)

Mastitis, an infection of the cows' mammary glands and the udder tissues (Cheng & Han, 2020), is generally accompanied by the infiltration of leukocytes, mainly neutrophils and serum proteins, into the site of infection (Thompson-Crispi et al., 2014). Clinical mastitis is marked by the presence of blood in milk, inflammation of the teats and reduction in milk production. These signs are visible to eyes whereas in subclinical mastitis no signs are observed except reduced milk production. Mastitis in cows is the most commercially significant endemic illness resulting in large economic losses in cattle production systems globally (Mbindyo et al., 2020). The disease's origin is complex, and it can present as either an acute or subacute form. *Escherichia coli, Staphylococcus aureus, Streptococcus uberis, Mycoplasma bovis,* and *Pseudomonas aeruginosa* are among the microorganisms most commonly involved in monomicrobial or polymicrobial infections (Markey et al., 2013). Studies in Kenya have shown that mastitis infection rates are quite high, especially for subclinical infection, which has a prevalence of 73.1%. Recently, the most recent incidence of 80% was discovered within the counties of Embu and Kajiado (Mbindyo et al., 2020). Antimicrobial resistance rates for *Staphylococci* to fluoroquinolones and ampicillin, respectively, were found to be 66.1% and 3.5% in 2021. 25% of *S. aureus* isolates and 10.8% of Coagulase-Negative Staphylococci (CoNS) isolates contained methicillin-resistant *Staphylococci* (Mbindyo et al., 2021)

Calves and people may consume *E. coli* and *Staphylococcus* species through contaminated milk in cases of subclinical mastitis without being aware of the animal's infection status. Subsequently, the bacteria are then shed into the environment by calves, potentially presenting a zoonotic hazard to people and other domestic animals. Consumption of contaminated milk from infected lactating cattle may provide a much higher public health danger in developing nations where cow milk is the main diet or replacement for newborns and children (Owusu-Kwarteng et al., 2020).

The objectives of this project are to: establish a knowledge of mastitis and related AMR within the context of cattle production systems; provide on-farm management strategies for mastitis; to reduce the spread of antibiotic-resistant organisms as a result of bovine mastitis in cattle. The study will employ culture-based techniques, colony PCR, MALDI TOF MS and, when appropriate, PCR-HRM and nanopore DNA sequencing. The results of this study will provide information about/insights into how livestock production methods and other risk factors affect mastitis and antibiotic resistance in Kenyan farms. The development of diagnostic tools for efficient management of livestock, zoonotic, food-borne illnesses, and antibiotic resistance in an extremely one-health approach will be aided by the baseline data.

1.1.2 The Purpose of the study

Bovine mastitis infections in Kenya are headache for farmers to treat because of antimicrobial resistance, and as a result, the dairy sector continues to suffer significant financial losses as a result of the disease. Furthermore, because the involved bacteria, such as *E. coli* and *Staphylococcus aureus*, are zoonotic, bovine mastitis poses a risk to public health.

There is a lack of information on the bacterial resistance gene profiles associated with mastitis as it relates to the treatment options in Kenya's various cattle production systems. As a result, nothing is done to manage and control mastitis, which causes it to spread farther or acquire more virulent resistance genes, aggravating the situation. It's therefore essential to make precise diagnoses and profiles of the resistant clones using a quicker and less expensive way for efficient management.

The study will look at the bacterial markers (indicative organism) for bovine mastitis, their antibiotic resistance profiles, and the risk factors for bacterial infections in cow under various production systems. When using a one-health approach technique, the baseline data obtained will help in managing and/or eradicating livestock, zoonotic, food-borne illnesses, and antibiotic resistance.

1.1.3 Bacterial aetiology of Bovine Mastitis

Mastitis, an infection of the cows' mammary glands and the udder tissues (Cheng & Han, 2020), is generally accompanied by the infiltration of leukocytes, mainly neutrophils and serum proteins, into the site of infection (Thompson-Crispi et al., 2014). Physical, chemical, and pathogenic microorganisms like bacteria and viruses can all cause the disease but the majority of instances are brought on by bacteria (Markey et al., 2013).

According Motaung et al., 2017 and Hoque et al., 2020, bacterial, mycotic, algal, and viral mastitis are the four main aetiologies of bovine mastitis. The bacterial aetiology of bovine mastitis has been linked to more than 130 Gram-positive and Gram-negative pathogenic bacterial species, which can manifest as monomicrobial or polymicrobial infections (Markey et al., 2013). The predominant microorganisms that cause disease are located in the udder tissues and transmit from cow to cow from there, these are classified as contagious pathogens. Whereas, environmental pathogens are those that originate from the herd's surroundings such as bedding materials, dung, grass, and dirt. *Escherichia coli, Staphylococcus aureus,* and *Streptococcus uberis* are the main pathogens involved. The *Staphylococcus* and *Streptococci*, as well as *E. coli*, are also linked to mastitis infections in female humans (Markey et al., 2013).

1.1.3.1 Contagious pathogens

They persist on the cow's udder and teat skin, from whence they spread during manual or automatic milking from an infected cow (or quarter) to an uninfected cow (or quarter) (Markey et al., 2013). These pathogens stick to the cow's skin, colonize the end of the teat, proliferate, and then spread into the teat canal, thereby causing infection. Teat cleaning after milking and dry cow therapy are methods of control. The most contagious pathogens are *Staphylococcus aureus* and *Streptococcus agalactiae*, whereas *Mycoplasma bovis* and *Corynebacterium bovis* are less serious infections (MVB et al., 2011). *Staphylococcus* spp will be looked into in this study.

1.1.3.2 Environmental pathogens

These infections are found in the livestock's housing and bedding. They spread primarily when the cow is lazing around, eating, lying down, or when liner slippage happens during milking or in between milking sessions. Since these pathogens do not stick to and colonize the teat end, dry cow therapy is ineffective for controlling them. *Escherichia coli* and *Streptococcus uberis* are the key pathogens that are associated with this condition, whereas *Arcanobacterium pyogenes, Bacillus species, Enterobacter aerogenes, Enterococcus faecalis, Klebsiella pneumoniae, Leptospira serovars, Mannheimia haemolytica, Mycoplasma bovis, Peptoniphilus indolicus* and *Pseudomonas aeruginosa* are minor pathogens (MVB et al., 2011) During this study, *Escherichia coli* will be investigated.

1.1.4 Antimicrobial Resistance Association and the Cattle Production Systems in Kenya

Kenya offers many methods for raising cattle, including intensive, semi-intensive, and extensive methods with varying percentage distributions (FAO, 2020). These farms' varying practices for raising livestock may affect how exposed the cattle are to mastitis-causing bacteria and the related antibiotic resistance (MOHK, 2017; Wall et al., 2016). As livestock production becomes more intensive, it is predicted that the inappropriate use of antibiotics will increase globally (ILRI, 2021) this might result in an increase in antibiotic resistance. In this study, the Food and Agriculture Organization data (FAO, 2020) is used to categorize the cattle production systems in Kenya as shown in the table below:

Production system	**Intensive (Zero-grazing)**		**Semi –Intensive (Agropastoral)**	**Extensive**	
	Large scale	Small scale	Semi grazing	Controlled e.g., ranches	Uncontrolled e.g., Pastoralists
Proportion of farms (%)	5%	35%	45%	10%	5%

Table 1: Cattle Production Systems in Kenya (FAO, 2020)

1.1.4.1 Intensive zero grazing

This includes both small-scale and large-scale zero-grazing methods, which confine animals and require careful planning of feed resources and high levels of control. Farms may be found in peri-urban areas, urban areas, and rural areas. Small-scale farms can house 1 to 20 cows, while large-scale farms must have at least 20 cows. However, these numbers might vary depending on the individual's fiscal strength and stability (FAO, 2020).

Crop production is also carried out on the farms. Breeds such as Ayrshires, Friesian, Fleckvieh, Guernsey, and Jersey may be housed in modest shelters (FAO, 2020). Whether farmed or purchased, the feeds used to rear the animals are of the highest quality. Despite widespread use of high-quality medical treatments including immunizations, artificial insemination, and tick control, FAO reported in 2020 that mastitis prevalence remains high, ranging from 30 to 45 percent. Milk output ranges from 15 to 30 litres per day for market consumption, with little to no milk meant for domestic usage.

1.1.4.2 Semi-intensive agropastoral

The animals are partially contained here, allowed to graze freely or when paddocked, and are confined in the evening when they are receiving supplements. In addition to other domestic animals including chickens, lambs, goats, donkeys, the exotic breeds include Ayrshire, Friesian, Jersey and Guernsey and the indigenous Bos indicus, Zebu, Sahiwal, and Boran. Herd size ranges from 1 to 20.(FAO, 2020).

Natural grass, enhanced pasture/Napier grass, and post-harvest grazing/crop leftovers are used for feeding. Additionally, the farms also carry out crop production. Only the barest necessities are provided for rigorous animal care, and mating occurs naturally. To access the rivers or other sources of water, cattle must travel great distances. Simple structures for feeding and milking are offered. The majority of milk output is for domestic use and ranges from 5.9 litres for exotic breeds to 2 litres for native types.(FAO, 2020).

1.1.4.3 Extensive controlled/uncontrolled methods

Has both exotic and cross breeds. There is managed grazing on large farms, but uncontrolled grazing occurs on community and marginal grazing pastures. Additionally, this method is divided into extensive and controlled areas, such as ranches and conservancies, and extensive and uncontrolled areas, such as pastoralists and community grazers. Farm herds vary in size from 10 to over 50 animals, although some only have 1 to 5. As a result, the daily production of common milk ranges from 4 to 11 litres (FAO, 2020).

Animals are fed on natural and developed pastures such paddocks or strip grazing in the extensive controlled production systems, with the addition of mineral licks, premium feed, and/or commercial concentrates. Extensive uncontrolled grazing system is however characterized by unrestricted grazing and minimal supplementing. Additionally, the difference is shown in the management of parasites and immunization, both of which are more noticeable in controlled production systems than in uncontrolled production systems (FAO, 2020).

1.1.5 Cattle's Economic Contributions to the Global Economy

Cattle are a significant source of animal-derived protein (Enahoro et al., 2018), financial assets, and significant wealth for farmers and pastoralists across the world (Herrero et al., 2013). Livestock industry employs over 1.3 billion people worldwide (Kemi, 2016).

According to FAO, 2020 the dairy industry in Sub-Saharan Africa generated 35 billion kilos (77 billion pounds) of milk in 2019. The majority of the region's dairy consumption is milk, which in 2020 made up around 6% of global dairy commerce (measured in milk solids equivalent) (Hoogwegt Horizons, 2021). According to research by McDaniel et al.,2014 animals continue to be a source of animal traction, fertilizer for crops, and raw materials for industries.

Dairy production accounts for 4% of the country's GDP, 14% of agricultural GDP, and 44% of the GDP from livestock in Kenya, behind only tea production (Kibogy, 2019). There are 4.5 million dairy people and 18.8 million cattle, according to estimates. Annual production of whole milk is 5.28 billion litres. 600 million litres of milk are marketed annually from 1.82 million smallholder farmers, creating 1.2 million direct or indirect jobs (KDB, 2021). Nearly every homestead in Kenya raises animals, particularly cattle, goats, sheep, and chickens, and 100% of them are farmers. Due to the extreme scarcity of land, very few Kenyans practice agriculture within metropolitan areas.

1.1.6 Using the One Health Approach to manage Antibiotic Resistance

Our vulnerability to infectious illnesses and antibiotic resistance extends to animals as well. Antibiotic resistance in cattle production systems and human antimicrobial resistance are strongly correlated, according to the available research (Wernli et al., 2020; Wall et al., 2016). Handling and consuming fresh/uncooked or undercooked animal products, can indirectly lead to the acquisition of resistance genes known as "resistome" from animals (Cameron & McAllister, 2016; Dandachi et al., 2018).

Simply put, the risk of developing bovine zoonotic illnesses has increased due to increased contact to cattle and their products. Veterinarians, employees of abattoirs, meat inspectors, livestock handlers, lab professionals who handle biological samples from sick animals, and people who consume unpasteurized milk or other dairy products or inadequately cooked meat are the groups most at risk (McDaniel et al., 2014). Antibiotics are used and abused in cattle production systems to combat infections and infestations such as parasite hosts, which results in selection pressure and the creation of resistomes. Antibiotic spills also happen in the environment and in water sources, where they are absorbed by plants and other animals. Over time, under the influence of selection pressure, these animals and plants build resistomes that are subsequently acquired by humans.

Salvarsan 1908, Penicillin 1928, Cephalosporins 1948, Methicillin 1960, and Carbapenems 1976 are only a few of the antibiotics that have improved lives and increased both human and animal populations. AMR might, however, poses a health hazard on a worldwide scale right now. Effective

antimicrobial stewardship and diagnostic stewardship tools must be implemented in order to prevent medication spills into the environment, accelerate the control of AMR, improve the efficacy and sustainability of medications in animals, humans, and plants, and promote an all-inclusive and empowered one health approach (MOH, 2020; (CDC, 2021).

1.2 Problem Statement

Antibiotic resistance may pose a threat to both human and animal health on a global scale. According to estimates, in the future, around half of the resistance genes will come from animals and the environment that human populations will be exposed to. According to estimates, by 2050, attempts to cure infections and/or regulate resistance genes would cost the global economy trillions of dollars and result in the annual death of 10 million people if AMR is not controlled (Wilson & Török, 2018). According to a recent World Bank forecast, the increase in AMR would cause a 7.5% decline in animal output by 2030 (ILRI, 2020).

The dairy industry in Kenya is plagued by a persistent issue with bovine mastitis infections that results in severe economic losses due to the lack of a quick, inexpensive, and accurate detection tool, high treatment costs, decreased milk production, the need to discard contaminated milk, and animal deaths. Mastitis, which had a frequency of 31% on East and Southern Africa smallholder farms in 2010, was the main cause of lactating cow fatalities in those farms (Phiri et al., 2010). According to a research conducted in Kenya's Nyeri and Nakuru Districts in 2013 (G. K. Gitau et al., 2013) the prevalence of mastitis was found to be 73% for *S. aureus*. As of 2017, the prevalence of mastitis was about 30% across African nations (Motaung et al., 2017). In Kenya, Counties of Embu and Kajiado had an estimated 80% incidence of bovine mastitis in 2020 (Mbindyo et al., 2020)

Additionally, mastitis poses a threat to public health due to the zoonotic nature of the involved bacteria like *E. coli* and *staphylococcus* species, which are consumed by both calves and people through contaminated milk in situations of subclinical mastitis. The pathogens may then be shed into the environment by calves, providing a potential zoonotic hazard to people and other domestic animals. Consuming contaminated milk from infected lactating cows might be considerably more dangerous for the public's health in underdeveloped nations where cow milk is the main source of nutrition or a replacement for formula for newborns and children.

The bacterial resistance mechanism and resistant gene profiles related to mastitis under various cattle production systems in Kenya, such as intensive, semi-intensive, and extensive (controlled/ranches, and uncontrolled/pastoral), have received scant attention, particularly with regard to the drugs of expedience. The study is crucial for making accurate diagnoses and for identifying the resistant bacterial genes connected to cows' mastitis. This study will further explore the risk factors, compare data generated between various variables such as production systems, breed types, stage of lactation/age versus the resistant genes, and identify nucleotide mutations (where possible) - this can help in providing baseline data that will assist in tracking down the spread of antimicrobial resistance genes that circulate in cows, and possibly in humans when the resistomes are subsequently acquired.

1.3 Research questions

1. What are the risk factors for clinical and subclinical bovine mastitis and associated antibiotic - resistance factors in various cattle production systems in the counties of Machakos, Makueni and Narok?
2. What non-aureus *Staphylococcus* spp and *Pseudomonas* spp are associated with bovine mastitis in the study sites?
3. What are the *in-vitro* antibiotic resistance mechanisms and profiles of *Staphylococcus aureus*, non-aureus Staphylococcus, *Escherichia coli* and *Pseudomonas* spp in clinical and sub-clinal of mastitis cases?

1.4 Aims and objectives

1.4.1 Main objective

To produce an understanding of bovine mastitis and associated AMR within the context of cattle production systems, to tell on-farm control strategies for mastitis and mitigate dissemination of antibiotic-resistant pathogens due to bovine mastitis in the counties of Machakos, Makueni and Narok.

1.4.2 Specific objectives

1. To evaluate production-related risk factors for mastitis - clinical and sub-clinical from the study sites.
2. To investigate the presence of *S. aureus,* non-aureus *Staphylococcus* spp, *E. coli* and *Pseudomonas* spp as causative organisms for clinical and subclinical mastitis in milk samples from cattle raised under various production methods.
3. To investigate phenotypic resistance mechanisms and profiles of isolates of *S. aureus,* non-aureus *Staphylococcus, E. coli* and *Pseudomonas* spp to the highest priority critically important antibiotics (HPCIAs) and last-resort antibiotics.

1.4.3 Secondary objectives

1 To assess the associations between antibiotic resistance profiles of *S. aureus*, non-aureus *Staphylococcus* spp, *E. coli* and *Pseudomonas* spp and cattle production-related methods.

2 To investigate the genotypic resistance mechanisms, virulence and genetic relatedness of representative isolates of *S. aureus,* non-aureus *Staphylococcus, E. coli* and *Pseudomonas* spp from the study sites.

1.5 Hypothesis

Null hypothesis (**H_0**)**:** MRSA, ESBLs, carbapenems, and colistin resistant clone distribution patterns and features are same across all cattle production systems

Alternative Hypothesis (**H_A**)**:** Different cattle production systems exhibit different distribution patterns for resistant clones.

1.6 Justification of the study

Kenyan farmers have long struggled with bovine mastitis resistance, and the illness continues to have a large negative economic impact on the nation's dairy industry. Furthermore, it raises serious concerns due to the zoonotic nature of the involved bacteria, particularly *E. coli* and *Staphylococcus aureus*. Comprehensive data on genetic profiles of these bacteria that are resistant to antibiotics, the mechanisms underlying that resistance, and the risk factors connecting them to lactating cattle under various production systems, such as semi-intensive/agropastoral, intensive-zero grazing, and extensive (controlled/ranches and uncontrolled/pastoral), are lacking.

The future transmission of antibiotic resistance genes from animals and the environment to human populations is predicted to occur in around half of cases. This is of great concern particularly resistance to high priority critically important antibiotics (HPCIAs) such as tetracyclines and fluoroquinolones, aminoglycosides and macrolides and last resort antibiotics such as 3rd and 4th cephalosporins, carbapenems and colistin drugs used in livestock production. By 2050, a silent pandemic that would kill 10 million people yearly if AMR is not managed, according to mathematical models, will exist, and the cost of treatment for both animal and human health will be in the trillions of dollars. It is crucial to track and analyze these antimicrobial resistance genes and their potential reservoirs since the information will help manage and reduce AMR globally.

For successful management, employing a cheap, efficient, and quick diagnostic tool, accurate identification and profiling of resistance genes relevant to bovine mastitis are required. This study will provide a baseline data that can be used in controlling and/or eradicating livestock, zoonotic, food-borne illnesses, and antibiotic resistance in an incredibly one-health approach technique.

2.0 CHAPTER TWO: LITERATURE REVIEW

2.1 Background information

Here, the terminologies, concepts and ideas are defined. The topic of the study is based on reviews of theoretical and empirical research, studies, and analyses that primarily focus on the epidemiology, risk factors, diagnostic procedures, available treatments, and emergence of antimicrobial resistance of clinical and subclinical bovine mastitis in lactating cows. It demonstrates how the study's design, data collection, laboratory investigations, data analysis, and information-sharing approaches will be impacted by the literature. Lastly, it describes the theoretical approach taken in the study.

2.2 Bovine mastitis Incidences and History in Kenya

Bovine mastitis infection is widespread, however the bacterial, viral, or mycotic strains and frequency vary widely depending on geographic locations, breed type, used production methods, and herd management techniques. While milking or while lying down, the cows' teat canal becomes infected, infecting their mammary glands in return (Markey et al., 2013). The infection with *Mycoplasma* species differs in that it occurs through the blood stream (MVB et al., 2011). Environmental pathogens are transmitted through bedding when lounging, eating, or lying down, whereas contagious pathogens are transported from quarter to quarter via the dirty/filthy machine or milkers' hands.

Escherichia coli, Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae and *Streptococcus agalactiae* are often isolated and described bacterial pathogenic species from lactating cows with clinical mastitis (MVB et al., 2011). The main reason for lactating cows' deaths in East and Southern Africa's smallholder farms in 2010 was mastitis, which occurred 31% of the time (Phiri et al., 2010). As of 2017, mastitis prevalence in African nations was estimated to be 30% (Motaung et al., 2017). The overall incidence in each country vary depending on the type and length of the research.

In the districts of Nyeri and Nakuru, *S. aureus* was shown to be the cause of 73% of all mastitis cases in 2013 (G. K. Gitau et al., 2013). According to FAO the overall prevalence of mastitis in Kenya remained between 30-45% in 2020. Christine and her colleagues demonstrated in 2020 that the counties of Kajiado and Embu had the highest overall prevalence of mastitis at 80%, with just 7% of cases symptomatic and 73% subclinical. *Coagulase-negative Staphylococcus* (CNS) 42.8%, followed by *Streptococcus* species (22.2%), *Staphylococcus aureus* (15.7%), *Pseudomonas aeruginosa* (5.1%), and *Enterobacter* species (0.7%) made up the bacteria present (Mbindyo et al., 2020). Furthermore, her 2021 research showed that 10.8% of the coagulase-negative Staphylococci (CoNS) and 25% *of S. aureus* were phenotypically resistant to methicillin, suggesting the documenting antimicrobial resistance (Mbindyo et al., 2021).

2.3 Bovine mastitis Risk Factors

The three groups of factors for the mastitis disease include host/cow, pathogen/microbial, and environmental determinants. Age, breed, lactation stage, previous mastitis history, immunological variables, and teat injury/lesion like bovine ulcerative mammilitis, are examples of cow's factors (Thompson-Crispi et al., 2014). A cow that has had more than four lactations/births/parity is known to be more susceptible to mastitis infection. The likelihood is also increased by the age of the calf and the existence of a lesion or damage to the teats (Markey et al., 2013).

The microbial or pathogen factors comprise of microbial adhesion abilities as shown in infectious pathogens like *S. aureus* vs environmental pathogens, presence of such pathogens predisposes cows to mastitis infection. The potential of certain pathogens, like *E. coli* to bind iron, which allows them to colonize the teat canal. The ability to produce endotoxin, which is particularly evident in Gram-negative organisms, and last but not least, the antiphagocytic capacity, which supports the pathogen survival in the host, thereby leading to chronic infections.

For the environmental variables, the likelihood of infection increases when there are multiple pathogenic bacteria in the animal's immediate habitat, whether it is in housing or a pasture. Poor

management methods, such as poor feeding habits, may raise the risk of infection, much as early postpartum may impair the immune response. Poor milking techniques, an unhygienic milking shed environment, external trauma from rough, muddy approaches to the milking shed or from the calves' vigorous sucking, as well as milking machine malfunction, all contribute to an increase in the likelihood and prevalence of mastitis infection in lactating cows (Markey et al., 2013). In this study, certain host-and environment-related risk variables will be examined.

2.4 Bovine Mastitis Diagnosis and Classification

Mastitis is a complicated illness that continues to plague the worldwide dairy industry due to the quantity of pathogenic organisms that cause it, wide range of responses from hosts and the emergence of antibiotic resistance (Thompson-Crispi et al., 2014). To find out whether mastitis infection is present and at what stage, many diagnostic methods are employed. These techniques can be used at the level of a single cow, a mass, or a quarter. Leukocyte cell counts in milk can be performed using the modified Breed's Smear Method, the California Mastitis Test (CMT), Bulk Tank Somatic Cell Counts (BTSCC), Coulter or FossomaticTm7 (Foss Electric), Nucleocounter Somatic Cell Counter, and direct microscopic counting (Afimilk, 2021).

The typical diagnostic procedure used to identify the bacterial aetiology of mastitis is microbiological culture. Blood agar, Edward's medium, MacConkey agar for lactose and lactose fermenters, and Edward's medium are all frequently used media cultures (MVB et al., 2011) SELMA plus media (selective Mastitis media) is utilized for multiple spp isolation from milk samples (VetBact, 2021). The primary methods for assuming the identity of bacterial species include colony shape, pigmentation, haemolysis patterns, and growth characteristics on these medium. However, applying biochemical assays tailored to that organism allows for the final identification of a presumptive pathogen. Modern technology enables the use of new tools like MALDI TOF MS, Vitek 2 systems, and Bactec to immediately identify species and their antimicrobial resistance profiles. Colony PCR/PCR-HRM is also utilized to identify spp. and determine the mechanisms of resistance.

In order to identify the host's antibodies against bacterial infections, serological techniques like ELISA are utilized. Quantitative qRT-PCR is one molecular technique for mastitis pathogen identification that distinguishes between real pathogens and a number of contaminants (Deb et al., 2013). The key Gram-positive and Gram-negative bacteria causing mastitis have been identified using multiplex PCR (Markey et al., 2013; MVB et al., 2011). In this work, we suggest using PCR to identify the molecular properties of the isolated pathogens both from the grown plates and directly from the samples, and to perform qRT-PCR High Resolution Melting Analysis, also known as PCR-HRM, and/or nanopore DNA sequencing where appropriate for sample to answer.

Depending on the condition or stage of the illness, mastitis infections can be divided into several categories. When mastitis is subclinical, milk supply declines without obvious indications of illness, although somatic cell counts are increased and exceed 200,000 cells/mL (Mcfadden, 2011). If the infection lasts more than two months, it is termed chronic mastitis and only exhibit a few visible symptoms, such as a periodic aberrant production in the gland. A noticeable inflammatory reaction and abnormal milk are both signs of clinical mastitis. Visible udder changes in situations of acute or severe mastitis may include swelling, redness, fever, discomfort, aberrant secretion, anorexia, and shock. Peracute mastitis includes gangrenous mastitis in which changes in the mammary gland are similar to those in peracute instances in acute cases, although systemic symptoms are significantly less severe. The last class is subacute, when there are no systemic effects and less pronounced alterations to the gland and milk (Markey et al., 2013).

CMT and SCC Interpretation			
CMT score	**Interpretation**	**Visible reaction Milk**	**Somatic cell count (/mL) (SCC)**
0/N	Negative/Healthy	Normal Milk (no mammary infections SCC is less than 142,000 cells/mL)	0–200,000 0–25% neutrophils
T	Trace/very mild/Sub-clinical	Slight precipitation is noticed	200,001–500,000 30–40% neutrophils
1	Weak positive; mild/Sub-clinical	Distinct precipitation, but with no gel formation	500,001–1,500,000 40–60% neutrophils
2	Distinct positive; moderate/Clinical	The mixture thickens with an observed gel formation	1,500,001–5,000,000 60–70% neutrophils
3	Strong positive; heavy, almost solidifies/Clinical	Very high viscosity, strong cohesive gel with a convex surface	≥5,000,000 70–80% neutrophils

Table 2: Relations Interpretations of CMT & SCC (Mcfadden, 2011; Markey et al., 2013)

2.5 The burden and challenges in the control of bacterial bovine mastitis

Globally, bovine clinical and subclinical mastitis infections in cows cause huge losses in the dairy industry. These losses are caused by less milk production, huge amount of discarded milk, increased early culling, high cost in veterinary services and additional labour expenditure (Thompson-Crispi et al., 2014)

More than 130 Gram-positive and Gram-negative pathogenic bacterial species have been implicated, either as monomicrobial or polymicrobial infections, making the bacterial aetiology of the infection exceedingly complicated (Markey et al., 2013). Additionally, there are variances in host reactions that make treatment and management difficult (Thompson-Crispi et al., 2014; Ashraf & Imran, 2020); this is made worse by resistance, and therefore the disease continues to be a problem for the dairy sector.

It is known that the illness can be avoided by upholding udder hygiene, cleaning the cow barn, using clean food and water supplements, using a working milking machine, documenting everything properly, culling chronically ill cows, using dry cow therapy, and adhering to a milking schedule, among other advised practices (The Cattle Site, 2020). A successful bacterial therapy, on the other hand, depends on the clinical presentation history, the stage of the infection, the correct diagnosis of the particular pathological agent, the correct typing of the antimicrobial susceptibility, the proper selection and administration of drugs, and good animal husbandry. Amoxicillin, streptomycin, azithromycin, and ceftriaxone are examples of broad-spectrum antibiotics used in mastitis infection treatment. Supportive therapies are also employed, such as corticosteroids (prednisolone, dexamethasone) to control inflammation (G. Gitau et al., 2011). Similar to other infectious illnesses in people, controlling mastitis becomes difficult if adequate methods for diagnosis, treatment and prevention are not applied.

Since there are several processes involved in antimicrobial resistance, including as generation of β-lactamases, porin loss, and efflux pumps, there is no one diagnostic approach for antimicrobial resistance that is suitable in all circumstances. One pathogen like *E. coli*, can produce many carbapenemases, or numerous carbapenemase-producing *Enterobacteriaceae* can form one clone, which presents another problem in the identification of carbapenem resistance. A common example is blaKPC, which is generated by many *Enterobacteriaceae* that produce carbapenemase (Richter & Marchaim, 2017). Thus, only the enzymes interrogated by the assay's primers and probes are identified during screening, leaving out clones that were not included in the run or, if the resistance mechanism is through some other means other than the enzymes, leading to false negatives (Singh-Moodley & Perovic, 2018). Additionally, the clones come in a variety of forms. The lack of resources just makes

this situation worse. As a result, it is crucial to develop a pan-panel PCR-HRM that checks groups of resistance genes for potential management errors.

2.6 The emergence of Antimicrobial Resistance in the management of Bovine Mastitis

2.6.1 Methicillin-resistant *Staphylococcus aureus* (MRSA and hVISA) genes

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant nosocomial pathogen that causes community-acquired illnesses in people all over the world. It is also acknowledged as one of the causes of bovine mastitis in veterinary practice (Markey et al., 2013). When an isolate of *Staphylococcus aureus* possesses an altered penicillin-binding protein, known as PBP2a or PBP2c, which is encoded by the mecA or mecC genes, respectively, it develops resistance to oxacillin/methicillin. The PBP change results in β-lactam resistance because it hinders the medication from attaching to the bacterial cell (Markey et al., 2013) The benefits of immune evasion and transmission are helped by Staphylococcal protein A, which is encoded by the spa genes of all the of *Staphylococcus aureus*.

S. aureus may be identified via spa gene typing (spa typing) or PCR for the nuc whereas, MRSA confirmation is by detection of mecA or mecC genes. (Markey et al., 2013). The *Panton-Valentine leucocidin* gene, which encodes the synergohymenotropic toxin, is another gene that can be typed for the identification of MRSA. Finally, clones of MRSA are assigned to epidemic lineages based on their multilocus sequence typing (MLST) profile, SCCmec type, and antibiotic resistance profiles by typing of the Staphylococcal chromosomal cassette mecC (SCCmec) (Markey et al., 2013).

Heteroresistant Vancomycin-intermediate Staphylococcus aureus is another kind of MRSA (hVISA). When the peptide component of the peptidoglycan where the medication binds changes, vancomycin resistance develops. Vancomycin typically binds to D-alanyl-D-alanine, but binding is inhibited when it transforms to D-alanine-D-lactate. VanA is the most significant of four genes linked to vancomycin resistance (Levison, 2014). MRSA and hVISA genes investigations will be attempted.

2.6.2 Genes for Extended-Spectrum β-lactamase (ESBLs)

The majority of β-lactam antibiotics, including penicillin, third and fourth-generation cephalosporins like cefuroxime and oxyimino β-lactam, and monobactam compounds like aztreonam are hydrolyzed by the Extended-Spectrum β-lactamases (ESBLs). However, neither cephamycin nor carbapenems are hydrolyzed by these enzymes (EUCAST, 2017), as the class 1 and 2 integrons (chromosomal and plasmid-borne) of the ESBL-producing *Enterobacteriaceae* contain gene cassettes encoding resistance (Lim et al., 2009).

According to the amino acid sequence homology, there are many different forms of ESBLs made up of penicillases and cephalosporinases, including the TEM-types, SHV-types, CTX-M-types, OXA-types, CMY-types, and PER-types. There are several homologs or strains of these different categories. For instance, over 200 TEM-types and over 165 SHV-types, of which 86 and 43 are ESBLs, respectively, had been described. Several *Enterobacteriaceae* that produce ESBLs, including *E. coli, Klebsiella pneumoniae, Enterobacter spp, Salmonella spp, Morganella morganii, Serratia marscescens, Shigella dysenteriae, Proteus spp,* and *Citrobacter spp.,* produce the different homologs. *P. aeruginosa* and *A. baumanii* are two examples of non-Enterobacteriaceae that also generate ESBLs (Paterson & Bonomo, 2005).

While the SHV-type (sulphydryl variable) such as SHV-1 are chromosomally encoded and have primarily been documented in *K. pneumoniae* with some in *E. coli,* the TEM types (Temoniera) such as TEM-1 are plasmid-borne and have been described in *E. coli*. Additionally, chromosomally encoded like the CXT-M kinds are primarily found in *Salmonella* spp. and *Kluyvera* spp. with few plasmid-mediated forms also being reported in *E. coli* and *Klebsiella pneumoniae*. *P. aeruginosa* has been the predominant source of OXA-type ESBLs with some observed in *E. coli* and *K. pneumoniae*. The majority of the *Pseudomonas*-Extended resistance PER-types are from *P. aeruginosa*, while some have

also been found in *Salmonella spp.* and *E. coli* (Lim et al., 2009). We plan to look at the TEM-types, CXT-M-types, and SHV-types in this work.

2.6.3 Carbapenems Resistant genes

The carbapenems like imipenem, meropenem, ertapenem, doripenem, panipenem, and biapenem, are β-lactam drugs used frequently as expedients for metallo-lactamases (MBL) and ESBL producing organisms such as *K. pneumoniae, E. coli, Enterobacter aerogenes, Enterobacter cloacae, Serretia marcescens, Citrobacter freundii, A. baumannii, Pseudomonas* species and other *Enterobacteriaceae* which are resistant to cephalosporins, quinolones, aminoglycosides, trimethoprim–sulfamethoxazole (Codjoe & Donkor, 2018). They act by attaching to penicillin binding proteins, a transpeptidase (PBP). As a result, no peptide cross-linkages are created during the formation of peptidoglycans since this limits transpeptidation. Cells die as a result of autolytic processes (Codjoe & Donkor, 2018).

The majority of acquired carbapenem resistance is caused by enzymes that are encoded by genes on transposable elements that are found on plasmids. These carbapenemases, also known as β -lactamases, hydrolyze carbapenems, and their synthesis appears to be the most common reason for carbapenem resistance. The overexpression of certain AmpC β-lactamases, porin loss, changes in penicillin binding proteins, increased expression of efflux pumps, and combinations of these can also result in resistance. Class A carbapenemases, such as KPC and SME enzymes, class B metallo-lactamases, such as VIM, IMP, and NDM metallo-lactamases, and class D carbapenemases, such as OXA-23 and OXA-48, have recently proliferated throughout the world and are responsible for both nosocomial infections and community colonization, including animal infections (EUCAST, 2017).

Ambler classes A through D are the four molecular classes within which all carbapenemases fall. While class B enzymes are metallo-lactamases (MBLs) with zinc in their active site, class A, C, and D enzymes contain serine in the active catalytic site (Codjoe & Donkor, 2018). Ambler class A β-lactamases include those that are chromosomally encoded, such as NmcA (not metalloenzyme carbapenemase A), SME (Serratia marcescens enzyme), IMI-1 (Imipenem-hydrolysing -lactamase-1), and SFC-1 (Serratia fonticola carbapenemase-1). However, KPC (KPC-2 to KPC-13) and GES (GES-1 to GES-20) (Guiana-extended spectrum) variants are encoded by plasmids. KPC is the most common kind and causes severe infections globally (Codjoe & Donkor, 2018). In order to determine their clinical importance, blaKPC and blaIMI would be explored in this study to learn more about their distribution patterns within the study locations.

The metallo-lactamases (MBLs), also referred to as Ambler class β-lactamases, include NDM, VIM, IMP, SPM, GIM, SIM, KHM, AIM, DIM, SMB, TMB, and FIM. Because the genes that encode them are found on transposable genetic elements, or transposons, which are mobile genetic elements, the IMP, VIM, and NDM are plasmid-mediated and present across the planet. The New Delhi metallo-lactamase 1 (NDM-1) family, imipenem-resistant *Pseudomonas*-type carbapenemases (IMP-types), VIM (Verona integron-encoded metallo-lactamase), GIM (German imipenemase), and SIM (Seoul imipenemase) are among the most prevalent metallo-lactamase families. Eight variations of the NDM-gene, which have been discovered, are prominent in isolates of *K. pneumoniae* and *E. coli*, but they have also been linked to *A. baumannii* and *P. aeruginosa* organisms. There are 18 known variants of the IMP-type, which are prevalent in *Acinetobacter* and *Pseudomonas* species.(Codjoe & Donkor, 2018). The 14 different variations of the VIM gene are uncommon but are usually present in *Pseudomonas putida* and *aeruginosa*. Here, blaNDM genes, blaVIM genes, blaIMP genes will be examined.

Although AmpC's class is unknown, it has some prolonged action toward carbapenems (Pitout et al., 2008). Class D β-lactamases (CHDLs) which include a variety of oxacillinases with hydrolytic activity against amino and carboxy penicillins, are poor against carbapenems (Ssekatawa et al., 2018). In *P. aeruginosa* and *A. baumannii*, the OXA-genes are predominating. Rarely had these clones been described in Africa, especially in Kenya. They also evolve more quickly than other classes, making them harder to detect and cure, which raises serious concerns around the world. The OXA-48 gene,

which is identified in *K. pneumoniae*, is the most prevalent OXA gene. While OXA-23 forms, which are disseminated globally, have been discovered in environmental *Acinetobacter* species. There have been multiple recorded outbreaks of the OXA-58 group worldwide (Pitout et al., 2008). This study will attempt to investigate AmpC, blaOXA-23, blaOXA-48, and blaOXA-58 genes, among others where possible.

2.6.4 Colistin Resistant genes

Colistin, commonly known as polymyxin E, is a limited-spectrum polycationic antibiotic that primarily affects Gram-negative bacteria, particularly those belonging to the *Enterobacteriaceae* family. Electrostatic interactions take place between the, α, γ-diamino butyric acid of colistin and the phosphate groups of the lipid A region of lipopolysaccharide when it binds to the lipopolysaccharides (LPS) on the outer membrane. As a result, Ca^{2+} and Mg^{2+} divalent cations are competitively displaced. When LPS is disrupted and the outer membrane's permeability increases due to this displacement, intracellular contents leak out and respiratory enzymes like NDH2 are inhibited, which results in cell death (Aghapour et al., 2019).

One of the final medications for Gram-negative bacteria that are resistant to carbapenem is thought to be colistin. However, there has been evidence of colistin resistance which is caused by a variety of processes, including the efflux pump, capsule loss on LPS, and LPS modification. *Proteus mirabilis* and *Serratia marcescens* naturally exhibit intrinsic resistance to colistin, which is caused by LPS modification with the addition of the 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine cationic groups. The primary method of colistin resistance in acquired resistance is likewise LPS modification, which requires the long-term alteration of lipid A by acquired plasmid genes known as mobilizable colistin resistance proteins (mcr-genes). The source of the acquisition is composite transposons from other bacteria with there's inborn resistance to colistin (Poirel, 2017). There are homologs of the Mcr-protein, a phosphoethalomine transferase, from mcr-1 to mcr-10 (WHO, 2018). Attempt to investigated a considerable number will be done.

2.7. Research gap

Little is known about the colistin (mcr-genes) and carbapenem-resistant that are prevalent in Kenya's cattle herds under various production methods. To the best of our knowledge, there is no information on carbapenem or colistin resistance in relation to bovine mastitis. Additionally, the Class D carbapenemases/oxacillinases in Kenya's cattle production system have not yet been defined. Additionally, there is a paucity of knowledge on the use of PCR-HRM as a diagnostic technique to the problem of antibiotic resistance in human and animal health. By providing detailed baseline data on the resistance profiles, prevalence, and carbapenemases and mcr genes implicated using PCR-HRM, the study seeks to close this knowledge gap.

This study will attempt to determine the presence and significance of risk factors for carbapenem and colistin resistance infection acquisition associated with bovine mastitis, as well as the genetic relatedness requiring the use of PCR-HRM and or DNA nanopore sequencing, where possible, and comparison between the various cattle production systems in Kenya.

2.8. Conceptual and analytical frameworks

These theoretical frameworks are intended to help the reader understand the suggested link between variables in the research through a graphical or diagrammatical portrayal of those relationships. Categorical variables such as age, breed type, cow production techniques and practices, antibiotic usage, teat injury/lesion, and mastitis history are the independent factors in the current study. The dependent variable includes infection with several pathogenic resistant clones linked to bovine mastitis, such as MRSA, hVISA, ESBLs, CREs, and mcr genes.

The microbial or pathogen risk factors linked to mastitis infection include microbial adherence as demonstrated in *S. aureus* infections, the capacity to colonize the teat canal, and the ability to bind iron, for instance in *E. coli* and investigations into endotoxin formation, particularly in Gram-negative

organisms with antiphagocytic potential that guarantees their survival in the host's immediate surroundings, will not be conducted.

The framework below demonstrates risk factors for bovine mastitis infection, which are the independent variables, and the outcome variable is clinical and subclinical mastitis, and presence of antibiotic resistance. The expected results include isolation and characterization of *Staphylococcus spp,* non-aureus *Staphylococcus* spp*, E. coli* and *Pseudomonas* spp. from milk of cows raised under various production systems; understanding of the phenotypic antibiotic resistance patterns and genotypic determinants of *Staphylococcus spp.* and *E. coli*.

Variables/data on antibiotic susceptibility patterns of *S. aureus*, non-aureus *Staphylococcus spp, E. coli* and *Pseudomonas* spp isolates, the source of isolates (counties, production systems, commercial vs. households etc), mastitis prevalence, antibiotic exposure and genotypic/sequence data will be collected. Association studies will be done, so predictor variables will be analyzed to help in coming up with control strategies for mastitis and AMR mitigations.

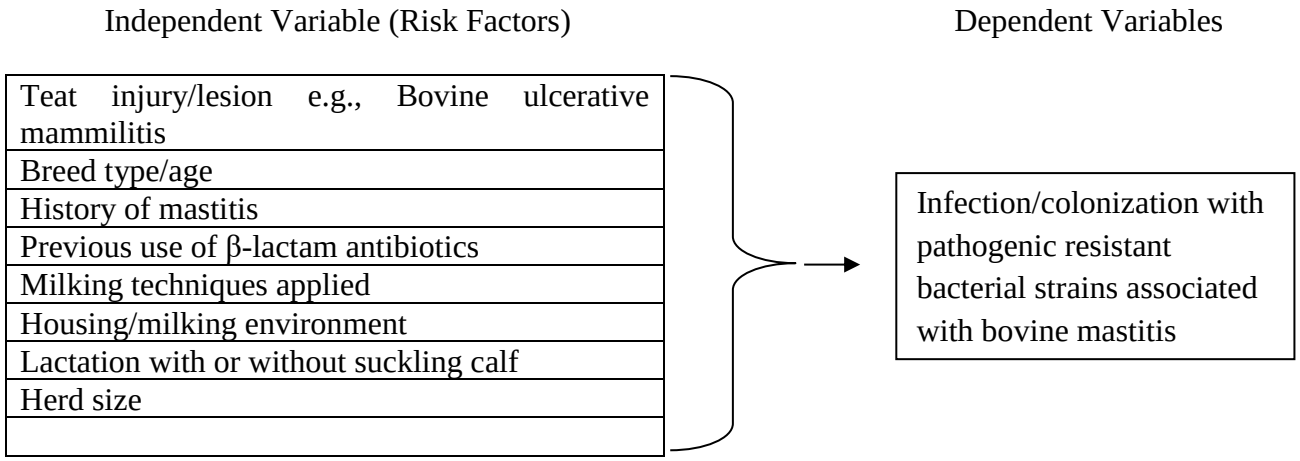

Figure 1: Conceptual framework

3.0 CHAPTER THREE: RESEARCH METHODOLOGY

3.1 Introduction

Here, the techniques for calculating sample size, gathering samples from the field, processing samples in the lab, managing data, analyzing data, presenting data, and discussing plans are presented.

3.2 Study design

This is a cross-sectional laboratory study in which archived mixed isolates from milk will be analyzed. Risk factors, bacteria linked to bovine mastitis, their antimicrobial resistance mechanisms and profiles, and their molecular characterization will be investigated. The study is a nested to the FLAIR AMR project titled, *"Diagnostics for Antimicrobial Resistance: Rapid and Low-Cost Tools to Support National Laboratory Networks in Surveillance of Antibiotic Resistance*" project from the ILRI. FLAIR project employed multistage sampling methods, including stratification and simple random sampling techniques during data collection. Variables necessary for this study will be data mined from FLAIR's main data, archived in FLAIR's ODK data servers.

3.3 Study area and population

In this cross-sectional laboratory study in which archived mixed isolates from milk samples will be investigated for microorganisms linked to bovine mastitis. Their antibiotic resistance and risk factors also will be examined. Milk samples were sourced from farms in three counties in Kenya that use various cow production methods. Machakos (6,043 km2), Makueni (8,009 km2), and Narok (17,921 km2). 31,973 km2 of area will be covered in total. Kenya is home to an estimated 18.8 million cattle, of which 14.3 million are beef cattle and 4.5 million are dairy cows. The nation also possesses an estimated 44.6 million chickens, 1.9 million donkeys, 26.7 million goats, 18.9 million sheep, 3.2 million camels, and 0.5 million pigs (Aggrey Omboki, 2021).

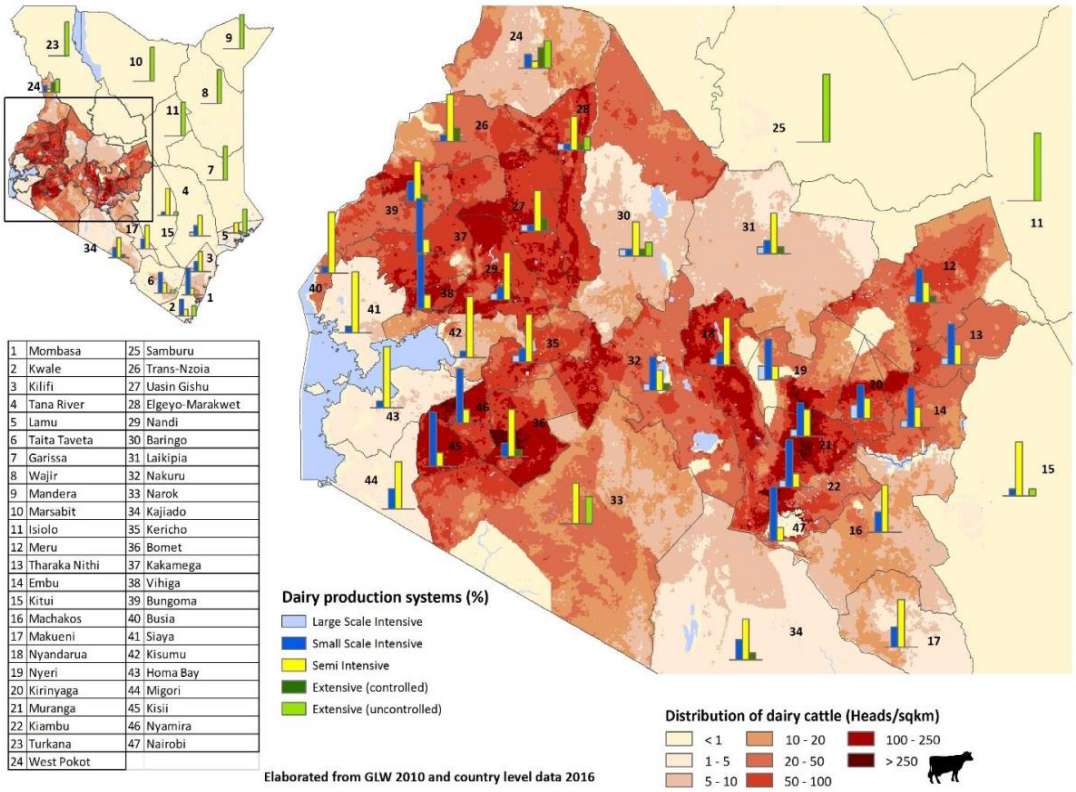

Figure 2: The Relative proportion of dairy cattle in the different production systems in Kenya (FAO, 2020).

The three counties were purposively chosen because they are accessible and have farms that are typical of those throughout Kenya - intensive, semi-intensive, and extensive farming practices (controlled/ranches and uncontrolled/pastoral) are among the distinctive and special characteristics of the farms. Additionally, the production methods differ according to the breeds raised, biosecurity, housing, animal movement, and amount of veterinarian access and care. Lastly, a good working connection already existed with the County Government's Department of Agricultural and Animal Services in those counties ensuring farmers' convenience. The International Livestock Research Institute (ILRI), situated in Nairobi, identified the Animal and Human Health/CGIAR AMR Hub laboratory as the location for studies including culturing, PCR-HRM, and DNA sequencing.

3.4 Inclusion criteria

In this study, all archived mixed isolates from milk samples obtained from randomly enrolled lactating cows with or without calves, and suffering from mastitis or not from Counties of Machakos, Makueni and Narok farms under various cattle production systems, such as intensive, semi-intensive, and extensive (controlled/ranches, and uncontrolled/pastoral) will be analyzed. Clinical mastitis is marked by the presence of blood in milk, inflammation of the teats or redness and reduction in milk production. These signs are visible to eyes, whereas in subclinical mastitis no signs are observed except reduction in milk. FLAIR AMR project collected samples from October 2021 through December 2022.

3.5 Sample size

Using Fisher's method and the assumption that there is no general incidence of bovine mastitis in Kenya, an estimate of 50% at a 95% Confidence level is used; hence, a sample size of at least 385 cows will be needed.

Target population ≥10,000 and population size is given $N = \frac{z^2 PQ}{e^2}$

Where:

N = Target population
P = Estimate of prevalence 50% which is 0.5
Q = 1-P (difference with the prevalence) which is 0.5
Z = Value reflecting the confidence level 95% which is 1.96
e = Significance level/allowable error margin which is 0.05

Therefore, **the minimal sample size is 385 lactating cows, hence N = 385. However, the study will retrieve and analyze all archived mixed isolates obtained from collected milk samples of all lactating cows with or without suckling calf and are suffering or not from mastitis within the ongoing FLAIR AMR study project.**

3.6 Sampling procedure

3.6.1 Sampling frame and unit

Multistage sampling procedure comprising of stratification and simple random sampling techniques were employed during FLAIR AMR study data collection from October to December, 2021. Intensive, semi-intensive, and extensive farms (controlled/ranches, and uncontrolled/pastoral) were stratified/categorized, and farmers and cows were utilized as sample units. Randomization was done at each stage, starting with the cattle production systems, moving on to the farms, and finally, the subjects/lactating cows, to avoid bias. FLAIR AMR Study obtained verbal informed consent from the farmers or farm managers and administered questionnaires – both farm and individual lactating animals recruited, and collected aseptically milk samples thereafter. This study will retrieve and analyzed all archived mixed isolates obtained from milk samples.

3.6.2 Sample collection and transportation

Milk samples from randomly chosen lactating cows were collected from each farm in the following ways:

- On a restrained lactating cow, the teats will be cleaned aseptically with disinfectant wipes and milk will be collected on a clean labelled/barcoded container.
- All samples collected will be packed aseptically under - 4 ° C using large styrofoam boxes and or cooler boxes with ice packs and transported to the laboratory processing within 24 hours.

3.7. Data collection

The ongoing FLAIR AMR Study data will be mined for all the pertinent variables on cows and farms such as county, information about the cattle production system including feeding, housing, and health management, farm waste management, and the use of antibiotics. Basic demographic data like breed type, age, history of mastitis, parity, use of vaccine, and recent/ongoing treatment, herd size, presence of milk shed, manure storage, floor type etc gathered via the AMUSE tool v2 will be data mined too for analysis against presence of mastitis.

3.8. Laboratory methods

In this investigation, a laboratory cross-sectional study design will be utilized to investigate all archived mixed isolates of up to 385 milk samples or more. From these archived mixed isolate samples, screening for bacteria that causes mastitis will be done in accordance with the Standard Operating Procedures (SOPs) provided in this protocol. At the AHH/CGIAR AMR hub at the ILRI in Nairobi, laboratory work will be completed. The isolates will be put through the PCR and MALDI TOF MS technique to determine the species. Kirby Bauer disk diffusion method would be used to create the profiles of the AST/inhibitors. In order to characterize molecules based on gene sequence, PCR, PCR-HRM and DNA sequencing will be performed accordingly and where possible. Laboratory method is composed of: screening for mastitis-causing bacteria in milk samples, species inquiry, susceptibility pattern analysis, and finally molecular characterization where possible via PCR-HRM and DNA sequencing.

3.8.1 Tests for California Mastitis and Somatic Cell Counts

Using the Luna Automated Somatic Cell Counter/Nucleocounter and the ImmunCell California Mastitis Test Kit, were carried out in accordance with the manufacturer's recommendations. The findings will help to categorize mastitis as either clinically/acutely present or subclinically/subacutely present. Somatic cell counts test will be used to determine the overall prevalence of mastitis from the study sites and production types.

3.8.2 Revival, screening and subculture for bacteria-causing bovine mastitis

According to internal protocols and EUCAST 2017 and 2021 requirements, screening for microorganisms linked to mastitis will be conducted. Incubation to be done in air, at 36-37ºC for 18-24 hours (Hardy Diagnostics, 2015). For the purpose of screening *Staphylococcus aureus* and non-aureus *Staphylococcus* spp, Mannitol Salt Agar (MSA) will be the primary media, whereas MacConkey agar will be the primary media for screening lactose like *E. coli* and *Klebsiella* and non-lactose fermenting *Enterobacteriaceae* like *Pseudomonas* and *Acinetobacter* spp, and pathogenic Gram-positive cocci such as *Staphylococci* and *Enterococci* spp. Suspected *Pseudomonas* spp isolates will be sub-cultured in Pseudomonas Agar Base Media

Non-lactose fermenters like *Salmonella, Shigella,* and *Proteus* spp look colourless or translucent, lactose fermenters like *Citrobacter, E. coli, Enterobacter, Klebsiella* appear pink/red. *Pseudomonas* spp colonies have transparent, pale yellow-green, crinkled borders, and a distinctive metallic sheen. They may also have a slight iridescence. Staphylococcus spp appear yellow. For future usage, mixed or pure colonies will be kept in sterile 50% stock glycerol solution (100% glycerol combined with DEPC water in equal parts, then autoclaved).

3.8.3 Species identification

According to the manufacturer's identification kit instructions, species identification from the presumed resistant isolates will be carried out by colony PCR and or MALDI TOF MS into their genera and binomial nomenclatures.

3.8.4 Susceptibility patterns of the bacteria-causing bovine mastitis

Following screening, the Kirby Bauer disk diffusion technique will be used to identify resistant phenotypes in accordance with the EUCAST 2017 and 2021 criteria for detecting mechanisms of resistance (EUCAST, 2017, 2020). Cefoxitin is a very accurate and focused indicator of *S. aureus* strains with mecA/mecC-mediated methicillin resistance. Meropenem provides the best balance between sensitivity and specificity in terms of detection for carbapenemase production (EUCAST, 2017). According to the EUCAST recommendations, cefotaxime and colistin sulphate will be utilized for the phenotypic resistance screening of ESBLs and colistin resistance, respectively. The tables below have recommended medications to be used in full AST, and AMR research will follow internal and EUCAST requirements.

Antibiotic and abbreviations	**Class**	**Pathogen**	**Use**
Cefoxitin	2nd Cephamycin	*S. aureus*	*mecA & mecC & AST*
Gentamycin	Aminoglycoside	*S. aureus*	*AST*
Cefotaxime (CTX)	Cephalosporins (3rd Gen)	*S. aureus*	AST
Ceftaroline (CPF)	Cephalosporins (5th Gen)	*S. aureus*	AST
Ciprofloxacin CIP	Fluoroquinolone	*S. aureus*	AST
Vancomycin (VA)	Glycopeptide	*S. aureus*	*VanA* & AST
Tetracycline (TE)	Tetracyclines (1st Gen)	*S. aureus*	AST
Tigecycline (TGC)	Glycycline (Tetracycline 3rd Gen)	*S. aureus*	AST
Clindamycin (DA)	Lincosamide	*S. aureus*	AST
Erythromycin (E)	Macrolide	*S. aureus*	AST
Linezolid (LZD)	Oxazolidinone	*S. aureus*	AST
Amoxicillin (AM)	Penicillin	*S. aureus*	AST
Amoxicillin-Clavulanic Acid AMC	Penicillin	*S. aureus*	AST
Chloramphenicol (C)	Phenicols	*S. aureus*	AST
Quinupristin/Dalfopristin (QD)	Streptogramin	*S. aureus*	AST
Trimethoprim/sulphamethoxazole (SXT) (Co-trimoxazole)	Sulphonamides	*S. aureus*	AST

Table 2: Full AST for *S. aureus and* non-aureus *Staphylococcus* spp

Strain	Mechanism
S. aureus ATCC 29213	Methicillin & Glycopeptide susceptible
S. aureus NCTC12493	Methicillin resistant (mecA)
S. aureus NCTC 13552	Methicillin resistant (mecC)
S. aureus ATCC 700698	hVISA (Mu3)
S. aureus ATCC 700699	VISA (Mu50)

Table 3: Control strains for MRSA and VISA

Antibiotic and abbreviations	Class	Pathogen	Use
Chloramphenicol (C)	Phenicols	*E. coli*	AST
Ampicillin (AMP)	Penicillin	*E. coli*	AST
Amoxicillin + Clavulanic acid (AMC)	Penicillin	*E. coli*	AST
Trimethoprim/sulphamethoxazole (SXT) (Co-trimoxazole)	Sulphonamides	*E. coli*	AST
Nalidixic Acid (NA)	Quinolone	*E. coli*	AST
Ciprofloxacin (CIP)	Quinolone	*E. coli*	AST
Gentamycin (CN)	Aminoglycoside	*E. coli*	AST
Streptomycin (S)	Aminoglycoside	*E. coli*	AST
Kanamycin (K)	Aminoglycoside	*E. coli*	AST
Tetracycline (TE)	Tetracyclines (1st Gen)	*E. coli*	AST
Doxycycline (DOX)	Tetracyclines (2nd Gen)	*E. coli*	AST
Aztreonam (ATM)	Monobactam	*E. coli*	AST
Sulbactam (SB)		*E. coli*	AST
Tazobactam (TZ)		*E. coli*	AST
Avibactam (AV)	Diazabicyclooctanones	*E. coli*	AST
Cefalexin	Cephalosporins (1st Gen)	*E. coli*	AST
Cefazolin	Cephalosporins (1st Gen)	*E. coli*	AST
Cefoxitin	Cephalosporins (1st Gen)	*E. coli*	AST
Ceftazidime + Clavulanic acid (CZC)	Cephalosporins (3rd Gen)	*E. coli*	AST
Cefotaxime + Clavulanic acid (CTC)	Cephalosporins (3rd Gen)	*E. coli*	CTX-M-types
Cefotaxime (CTX)	Cephalosporins (3rd Gen)	*E. coli*	CTX-M-types
Ceftriaxone (CRO)	Cephalosporins (3rd Gen)	*E. coli*	AST
Cefpodoxime (CPD)	Cephalosporins (3rd Gen)	*E. coli*	AST
Ceftazidime (CZA)	Cephalosporins (3rd Gen)	*E. coli*	AST
Cefepime (FEP)	Cephalosporins (4th Gen)	*E. coli*	AST
Ceftaroline (CPM)	Cephalosporins (5th Gen)	*E. coli*	AST
Meropenem (MEM)	Carbapenem	*E. coli*	Carbapenemases
Meropenem + boronic acid	Carbapenem	*E. coli*	A carbapenemases
Meropenem + dipicolinic acid	Carbapenem	*E. coli*	B carbapenemases
Meropenem + Avibactam	Carbapenem	*E. coli*	Oxacillinases like OXA-48
Ertapenem (ERM) / Imipenem (IMP)	Carbapenem	*E. coli*	AST
Colistin sulphate (COS)	Polymyxins	*E. coli*	*Mcr*-types

Table 4: Full AST List for E. coli

Strain	Mechanism
K. pneumoniae ATCC 700603	SHV-18 ESBL
E. coli CCUG62975	CTX-M-1 group ESBL and acquired CMY AmpC
E. coli ATCC 25922	ESBL-negative
E. coli ATCC 25922	Colistin susceptible
E. coli NCTC 13846	Colistin resistant mcr-1 positive

Table 5: Control strains for ESBL, CRE and Colistin Resistance

3.8.7 Molecular Analysis

Any nucleotide sequence of interest inside a microbe may be detected using PCR-based approaches, including particular genes or mutations linked to virulence factors, antibiotic resistance, and other traits (Markey et al., 2013). The manufacturer's recommended methodology or internal rules like the boiling technique shall be followed for DNA extraction; PCR amplification, and probe detection test will be carried out according to the prevailing procedures for each gene of interest. Detection for the MRSA, ESBL, CRE, and mcr resistant genes of interest mentioned below will be attempted in certain cases or all together using direct samples and resistant isolates. The sequence of the primers may also be altered according to internal recommendations:

3.8.7.1 MRSA and VISA

***MRSA* Primer designs**		**Sequence 5' - >3'**	**Reference**
mecA	Forward	AAAATCGATGGTAAAGGTTGGC	(Hamid et al., 2017)
	Reverse	AGTTCTGCAGTACCGGATTTGC	
mecC	Forward	CAGCCAGATTCATTTGTACC	(Frey et al., 2013)
	Reverse	AACATCGTACGATGGGGTAC	
spa gene (X region)	Forward	CAA GCA CCA AAA GAG GAA	(Ali et al., 2018)
	Reverse	CAC CAG GTT TAA CGA CAT	
nuc gene	Forward	GCGATTGATGGTGATACGGTT	(Mbindyo et al., 2021)
	Reverse	CAAGCCTTGACGAACTAAAGC	
PVL	Forward	AATAACGTATGGCAGAAATATGGATGT	(Chamon et al., 2020)
	Reverse	CAAATGCGTTGTGTATTCTAGATCCT	
V*anA*	Forward	GGCAAGTCAGGTGAAGATG	(Maharjan et al., 2021)
	Reverse	ATCAAGCGG TCAATCAGTTC	

Table 6: MRSA Primer designs

3.8.7.2 ESBLs

***ESBL* Primer designs**		**Sequence 5' - >3'**	**Reference**
TEM-types	Forward	ATGAGTATTCAACATTTCCG	(Lim et al., 2009)
	Reverse	CTGACAGTTACCAATGCTTA	
SHV-types	Forward	GGTTATGCGTTATATTCGCC	
	Reverse	TTAGCGTTGCCAGTGCTC	
CTX – M types	Forward	CCGTCACGCTGTTGTTAGGA	(Ngaywa et al., 2019)
	Reverse	TTCATCGCCACGTTATCGCT	
OXA-types	Forward	ACACAATACATATCAACTTCGC	(Lim et al., 2009)
	Reverse	AGTGTGTTTAGAATGGTGATC	
PER-1	Forward	ATGAATGTCATTATAAAAGCT	(Celenza et al., 2006)
	Reverse	TTAATTTGGGCTTAGGG	
PER-2	Forward	ATGAATGTCATCACAAAATG	
	Reverse	TCAATCCGGACTCACT	
CMY-types	Forward	ATGATGAAA AAATCGTTATGCTGC	(Koga et al., 2019)
	Reverse	GCT TTT CAA GAA TGC GCC AGG	

Table 7: ESBL Primer designs

3.8.7.3 Carbapenemases

Class A: blaKPC, GES, and blaIMI; class B: blaNDM, blaVIM, and blaIMP-types; and class D: Carbapenemase blaOXA-23, blaOXA-24, blaOXA-48, and blaOXA-58. These are some of the genes and clones of interest. The following primer sequences for PCR are suggested for use:

Carbapenemase primers		Sequence (5'->3')
KPC 1/2	Forward (KPC-F)	TTA CTG CCC GTT GAC GCC CAA TCC
	Reverse (KPC-R)	TCG CTA AAC TCG AAC AGG
IMI	Forward (IMI-F)	TCT GCG ATT ACT TTA TCC TC
	Reverse (IMI-R)	ATA GCC ATC TTG TTT AGC TC
NDM	Forward (NDM-F)	GGT TTG GCG ATC TGG TTT TC
	Reverse (NDM-R)	CGG AAT GGC TCA TCA CGA TC
VIM 2	Forward (VIM-F)	CAG ATT GCC GAT GGT GTT TGG
	Reverse (VIM-R)	AGG TGG GCC ATT CAG CCA GA
IMP	Forward (IMP-F)	AAC CAG TTT TGC CTT ACC AT
	Reverse (IMP-R)	CTA CCG CAG CAG AGT CTT TG
IMP - 1	Forward (IMP-1-F)	CTA CCG CAG CAG AGT CTT TG
	Reverse (IMP-1-R)	AAC CAG TTT TGC CTT ACC AT
IMP - 2	Forward (IMP-2-F)	GTT TTA TGT GTA TGC TTC CAG C
	Reverse (IMP-2-R)	AGC CTG TTC CCA TGT AC
GES	Forward (GES-F)	CTG GCA GGG ATC GCT CAC TC
	Reverse (GES-R)	TTC CGA TCA GCC ACC TCT CA
OXA-1	Forward (OXA-1-F)	A TGA AAA ACA CAA TAC ATA TCA ACT TCG C
	Reverse (OXA-1-R)	GTG TGT TTA GAA TGG TGA TCG CAT T
OXA-2	Forward (OXA-2-F)	ACG ATA GTT GTG GCA GAC GAA C
	Reverse (OXA-2-R)	ATC TGT TTG GCG TAT CRA TAT TC
OXA-23	Forward (OXA-23-F)	G ATG TGT CAT AGT ATT CGT CG
	Reverse (OXA-23-R)	TCA CAA CAA CTA AAA GCA CTG
OXA-24	Forward (OXA-24-F)	TCC CCT AAC ATG AAT TTG T
	Reverse (OXA-24-R)	T ACT AAT CAA AGT TGT GAA
OXA-48	Forward (OXA-48-F)	TTG GTG GCA TCG ATT ATC GG
	Reverse (OXA-48-R)	GAG CAC TTC TTT TGT GAT GGC
OXA-58	Forward (OXA-58-F)	GGC ACG CAT TTA GAC CG
	Reverse (OXA-58-R)	AAC CCA CAT ACC AAC C
OXA-181	Forward (OXA-181-F)	GAG GCT TAT CGT GAA GAC AG
	Reverse (OXA-181-R)	GAA CGA CTT TGT CAA ACT CC

Table 8: Carbapenemase primer designs

3.8.7.4 Colistin resistance detection

All of the Mcr-variants—from protein's Mcr-1 to Mcr-10—will be attempted in this investigation.

Mcr-gene Primer designs		Sequence 5' - >3'	Reference
mgrB	Forward	AAG GCG TTC ATT CTA CCA CC	(Zafer et al., 2019)
	Reverse	TTA AGA AGG CCG TGC TAT CC	
Mcr-1	Forward	CGG TCA GTC CGT TTG TTC	
	Reverse	CTT GGT CGG TCT GTA GGG	
Mcr-2	Forward	ATG ACA TCA CAT CAC TCT TGG	
	Reverse	TTA CTG GAT AAA TGC CGC GC	
Mcr-3	Forward	AAATAAAAATTGTTCCGCTTATG	(Rebelo et al., 2018)
	Reverse	AATGGAGATCCCCGTTTTT	
Mcr-4	Forward	TCACTTTCATCACTGCGTTG	
	Reverse	TTGGTCCATGACTACCAATG	
Mcr-5	Forward	GGTTGGCCGAGAAGATAACA	(Liu et al., 2020)
	Reverse	ATGTTGCCAGAAGGTCCAAC	
Mcr-6	Forward	AGCTATGTCAATCCCGTGAT	(Borowiak et al., 2020)
	Reverse	ATTGGCTAGGTTGTCAATC	
Mcr-7	Forward	GCCCTTCTTTTCGTTGTT	
	Reverse	GGTTGGTCTCTTTCTCGT	
Mcr-8	Forward	TCAACAATTCTACAAAGCGTG	
	Reverse	AATGCTGCGCGAATGAAG	
Mcr-9	Forward	TTCCCTTTGTTCTGGTTG	
	Reverse	GCAGGTAATAAGTCGGTC	
Mcr-10	Forward	GGACCGACCTATTACCAGCG	(Lei et al., 2020)
	Reverse	GGCATTATGCTGCAGACACG	

Table 9: mcr gene Primer designs

NB: Avoid

Initiation Codon	ATG
Termination Codons	TAA, TAG &TGA

3.8.7.5 Whole Genome Nanopore DNA sequencing and PCR-HRM

The mechanism of resistance for MRSA, ESBLs, CRE, and colistin resistant genes will be identified utilizing direct samples and resistant isolates, and where possible, will be compared using nanopore DNA sequencing and the PCR-HRM. Both techniques shall be carried out in accordance with prevailing internal procedures and manufacturer's kit recommendations. The findings will serve as a roadmap for the creation of a "sample to answer" diagnostic tool for AMR genes linked to bovine mastitis.

3.8.7.6 Expected Results:

An understanding of phenotypic antibiotic resistance patterns and genotypic determinants of *Staphylococcus* spp. and *E. coli* implicated in clinical and sub-clinical mastitis, under various cattle production systems, will be provided by the study, along with baseline data on the isolation and characterization *of Staphylococcus aureus* and *E. coli* as marker organisms for bovine mastitis from milk of cattle raised under various production systems. PCR-HRM as a diagnostic tool for monitoring antibiotic resistance genes is likely to be evaluated. Aim is to give knowledge on bovine mastitis and associated AMR within the context of cattle production systems, to tell on-farm control strategies for mastitis and mitigate dissemination of antibiotic-resistant pathogens due to bovine mastitis.

3.9 Study approval, ethical considerations and informed consent

The study is an extension of FLAIR-AMR project which was approved by ILRI Institutional Research Ethics Committee (ILRI IREC) dealing with animal subjects - approval number ILRI-IREC2021-38, NACOSTI – approval number NACOSTI/P/21/13249 and the Ministry of Agriculture, Livestock, Fisheries and Cooperatives – State Department of Livestock approval ref: MOALF/SDL/DVS/DS/RES Vol.53/12.

The study is submitted for approval by KNH/UoN Ethical Review Committee at the university level as required for graduate studies within the Department of Medical Microbiology and Immunology of the University of Nairobi. No informed consent is required for the study for collection of data as the study is a laboratory cross-sectional study involving analyzing archived isolates. Relevant data will be data mined from FLAIR AMR study project which already had obtained consent from farmers/farm managers and relevant regulatory authorities.

3.10 Potential Confounders and Quality Assurance

Any potential confounding factors will be managed appropriately. For instance, the findings of the California Mastitis Test (CMT) and Somatic Cell Counts (SCC) will be used to track the effects of the presence of *Corynebacterium bovis*, a non-pathogenic bacterium, in the teat duct, which may increase the cell count. The maximum cell count in instances of chronic mastitis will be achieved by stripping at the end of milking. Cell counts will be performed within two hours after milk collection to account for the leukocytes/neutrophils in milk samples that degrade very quickly when stored without preservatives like bronopol. In order to prevent cross contamination, aseptic procedures will be used during milk collection and sample processing.

In order to guarantee the validity and correctness of the study results in terms of proper isolation, species identification, antimicrobial resistance detection, and molecular clone characterisation, quality control will be carried out during the duration of the study. Along with the specimens/isolates acquired throughout the investigation, standard panels of specified strains suggested by EUCAST or in-house will be cultured and put through the same processes as the samples. All laboratory operations shall be recorded, as well as the equipment and kit log that will be utilized. Before doing analyzing data and creating reports, the generated data will be verified for accuracy and completes for all data required. Any additional quality control measures that are suggested internally will be carried out.

3.11 Management and Analysis of Data

The accuracy and correspondence of all sample collection and processing worksheet files to the samples before to processing and following investigations will be verified. All during the study, privacy and confidentiality will be respected and protected. The principal researcher, any supervisor(s), and any other approved person(s) will be the only ones with access to the data, which will be kept in the CGIAR cloud area as password-protected documents. Data obtained from all laboratory research will be analyzed using the software R version 4.1.2 and GraphPad v.8.2.3 to provide descriptive and inferential statistics. When comparing categorical variables, such as breed type, age, and lactation stage, to mastitis prevalence, AMR phenotypic and genotypic trends, regression analysis, chi-square test (x^2 test), or Fisher's exact test will be used. Descriptive statistics will be provided as tables, charts, and graphs. Both univariate and multivariate logistic regression analysis will be applied to calculate inferential statistics and assess the correlation between mastitis prevalence and various risk factors, AMU and AMR, AMR pathogens, and different cattle production methods. Friedmann test will be utilized to examine the inter-rater and intra-rater reliability between the various testing methods. In accordance with the study goals, descriptive and inferential data will be translated into English, and typed for coding and analysis. Themes will be formed from the data generated. The outcomes and conclusions will be presented in accordance with the diagnostic stewardship best practices and Standards for Reporting Diagnostic Accuracy Studies (STARD) recommendations.

3.12 Application and Sharing of Data

The study's findings will be utilized to create a master's thesis and a publication to satisfy the University of Nairobi School of Medicine's criteria for the Master of Science Degree in Medical Microbiology. Additionally, this study aims to close the knowledge gap by providing detailed baseline data on antimicrobial resistance linked to bovine mastitis in various cattle production systems, including intensive, semi-intensive, and extensive (controlled/ranches, and uncontrolled/pastoral), and by developing a resistant gene profile diagnostic tool for bacteria that cause bovine mastitis. Principally. to produce an understanding of bovine mastitis and associated AMR within the context of cattle production systems, to tell on-farm control strategies for mastitis and mitigate dissemination of antibiotic-resistant pathogens due to bovine mastitis. The data produced will be copyrighted, shared with the ILRI and supervisors, presented in various scientific settings, published in print media, and shared online in veterinary or medical publications addressing antibiotic resistance.

3.13 Study's Limitations and their Mitigations

Although there may be carriers involved in the spread of the multi-drug resistant organism (MDRO) within the farms, this study emphasizes that it will not sample bulls or heifers or calves or other farm animals. A suitable method or study should be developed to look into these areas independently. The study also notes that it will not investigate the microbial or pathogen risk factors linked to mastitis infections in cows and that only few selected pathogens like *E. coli* and *S. aureus* and only a few associated AMR clones of interest will be investigated where possible depending on the availability of resources.

3.14 Conflict of interests

Regarding the research that is the subject of this publication, there is no conflict of interest.

4.0 TIMEFRAME

ACTIVITIES	TIME (YEAR)	
	2021-22	2023
Concept and proposal development	X	
Ethical review	X	X
Setting up	X	X
Project report	X	X
Specimen collection	X	X
Specimen analysis	X	X
Data analysis	X	X
Report preparation and submission		X
Thesis defence and dissemination		X
Manuscript submission and publication		X

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6.0 APPENDIXES

Appendix 1: Budget estimate

ITEM	2021	2022	TOTAL (KES)
Ethical approval	2,000	-	**2,000**
Laboratory supplies	700,000	-	**700,000**
Stationery and Publication	10,000	60,000	**70,000**
Miscellaneous	-	30,000	**30,000**
TOTAL (KES)	**712,000**	**90,000**	**802,000**

Budget justification:

- Laboratory Supplies and reagents include:
  - Different culture media
  - DNA extraction kit
  - TaqMan Universal master with EvaGreen dyes
  - SYBR safe dye
  - *MRSA, hVISA, bla for ESBLs and CRE* and *mcr-1* gene primer designs
  - Laboratory materials/apparatus e.g., pipettes, PCR reaction tubes, gloves, ethanol, DNAses, de-ionized water, masks etc.
- Stationery includes books, pens, printing, photocopying, binding.
- Publication includes those of print media and sharing online in medical journals dealing with antibiotic resistance.
- Miscellaneous and contingencies will cater for unforeseen items.

Appendix 2: Questionnaire - Enrolment of the Lactating Cows for Mastitis Investigation and Milk Sample Collection

Study Title: **Molecular Analysis and Antibiotic-resistance in Bacterial species Associated with Clinical and Sub-Clinical Mastitis in Different Cattle Production Systems in Kenya**

A. Socio-demographic data:

I. Farm ID: (Scan barcode/Autogenerated)
II. Lactating cow's ID: (Scan barcode)
III. What is the age of the lactating cow? Typing Years
IV. What's the breed type? List the breed types

B. Antibiotics and Vaccine Use:

I. Have you treated this lactating cow for the last 12 months? Y/N, if Y
    i. What diseases were this lactating cow suffering from? List; option for typing
    ii. Who carried the diagnosis test? Vet, paravet, Animal Health officer, the farmer
    iii. Which antibiotics did you use/were used? List; Option for typing
    iv. Did you finish the dosage? Y/N
II. Have you used a mastitis vaccine on this lactating cow recently? Y/N, If Y
    a. Which vaccine have you applied? Lysigin (*S. aureus* only); Startvac (for *E. coli* and *S. aureus*)

C. Risk factors Associated with Bovine Mastitis:

I. Has this lactating cow suffered from mastitis before? Y/N, IF Y
    i. How was the diagnosis done? Clinical, SCC, CMT, others
    ii. Which drugs did you use to treat mastitis? List, option for typing
    iii. Which treatment method was used? Dry cow therapy, others
    iv. Did you finish the dosage? Y/N
    v. How many days do you take before milking the cow after mastitis treatment?
II. Do you have a cow shed/pen for this lactating cow? Y/N, if Y
    i. Do you clean the cows pen? Y/N, IF Y
        1. How often do you clean the pen?
III. Do you have a milkshed for this lactating cow? Y/N, IF Y
    i. How often do you clean the milkshed?
IV. Do you use milk machine during milking session for this cow? Y/N, IF Y
    i. Do you clean your milk machine before and after milking this lactating cow? Y/N
    ii. How often to do you service your milk machine?
V. How many calves has this lactating cow had before? Option for typing
VI. What's the age of the calf? 0-6 moths (suckles less vigorously); 7-12 months (Suckles vigorously)
VII. Is there any observable teat injury? Y/N
VIII. Is there any observable tick infestation of the lactating cow's udder? Y/N

D. Sample type collected:

I. Milk (Scan Barcode)

Appendix 3: Ethical Approvals

REPUBLIC OF KENYA

MINISTRY OF AGRICULTURE, LIVESTOCK, FISHERIES & COOPERATIVES
STATE DEPARTMENT OF LIVESTOCK
Office of the Director of Veterinary Services

Telephone: 020 – 8043441
E-mail: infodvs@kilimo.go.ke

Veterinary Research Laboratories,
P. O. Private Bag, Kabete,
00625 – Kangemi, Nairobi

When replying, please quote:
Ref: **MOALF/SDL/DVS/DS/RES Vol.53/12**
All correspondences should be addressed to:
The Director of Veterinary Services

Date: 21st September,2021

Dr. Dieter Schillinger
Deputy Director General, Research Development and -Biosciences
ILRI, Nairobi.

Permission to conduct project on antimicrobial resistance in cattle production systems

Reference is hereby made to your letter dated 15th September, 2021 on the above subject matter.

This Director of Veterinary Services (DVS) takes note of your interest in conducting the above project and will appreciate the evidence produced by the study as it will increase the knowledge of *on-farm* AMR profiles and also contribute towards shaping the national policy on antimicrobials stewardship in the country.

The DVS has therefore no objection for you to implement the project in eight (8) counties, including Nakuru, Kericho, Narok, Laikipia, Kiambu, Machakos, Makueni and Kajiado. By a copy of this letter the DVS requests County Director of Veterinary Services and Regional Veterinary Laboratories to collaborate for both field work and laboratory analyses respectively.

The Directorate appreciates the ongoing partnership with International Livestock Research Institute (ILRI) in supporting research activities on livestock.

ILRI - KENYA
DDG - BIOSCIENCE OFFICE
NAIROBI
Date 21/9/2021
TO EOHS
ATTN ALLAN
C.C.

Dr. David M. Mwangangi
For: Director Veterinary Services

Cc: Dr. Allan Azegele - AMR Laboratories Coordinator.
CDVS: Nakuru, Kericho, Narok, Laikipia, Kiambu, Machakos, Mariakani.
OIC RVIL: Nakuru, Kericho, Karatina, CVL.

ILRI
INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE

CGIAR

26th August 2021

Our Ref: ILRI-IREC2021-31

International Livestock Research Institute
P.O. Box 30709 00100
Nairobi, Kenya.

Dear Lilian Wambua,

Ref: Diagnostics for antimicrobial resistance: Rapid and low-cost tools to support national laboratory networks in surveillance of antibiotic resistance

Thank you for submitting your request for ethical approval to the International Livestock Research Institute (ILRI) Institutional Research Ethics Committee (IREC). ILRI IREC is accredited by the National Commission for Science, Technology and Innovation (NACOSTI) in Kenya, and the Federalwide Assurance (FWA) for the Protection of Human Subjects in the United States of America.

This is to inform you that ILRI IREC has reviewed and approved your proposal titled *'Diagnostics for antimicrobial resistance: Rapid and low-cost tools to support national laboratory networks in surveillance of antibiotic resistance'*. Your approval reference number is ILRI-IREC2021-38 and the approval period is 26th August 2021 to 25th August 2022, and is subject to the following requirements:

- Only approved documents including (informed consents, study instruments) will be used.
- All changes including (amendments, deviations, and violations) are submitted for review and approval by ILRI IREC.
- Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to ILRI IREC within 72 hours of notification.
- Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to ILRI IREC within 72 hours.

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **803721**

Date of Issue: **01/October/2021**

RESEARCH LICENSE

This is to Certify that Dr.. Lillian Wambua of International Livestock Research Institute, has been licensed to conduct research in Kajiado, Kericho, Kiambu, Laikipia, Makueni, Nairobi, Nakuru, Narok on the topic: Diagnostics for antimicrobial resistance: Rapid and low-cost tools to support national laboratory networks in surveillance of antibiotic resistance for the period ending : 01/October/2022.

License No: **NACOSTI/P/21/13249**

803721

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

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Appendix 4: Research Personnel Information (Roles and responsibilities in the research project).

Study Title: Clinical and Sub-Clinical Mastitis in Different Cattle Production Systems in Kenya: Molecular Analysis and Antibiotic-resistance of Associated Bacterial species

RESEARCH CONTRIBUTIONS AND AGREEMENT

The agreement aims to facilitate a productive working relationship and consensus between the student (PI), Co-investigators, Collaborators, Research Administrators and External Supervisors.

PRINCIPAL INVESTIGATOR/ STUDENT Details:

- Name: **Mr. Walter Oguta**
- Organization/University: **International Livestock Research Institute (ILRI) – Animal and Human Health (AHH)/CGIAR AMR Hub/University of Nairobi**
- Email address: **walteroguta@gmail.com**
- Telephone: **+254711127631 OR +254737721649**
- Hosting Organization/Department/Program: **International Livestock Research Institute (ILRI) – Animal and Human Health (AHH)/CGIAR AMR Hub/University of Nairobi**

CO-INVESTIGATORS/COLLABORATORS/ADMINISTRATORS/ EXTERNAL SUPERVISORS:

University Supervisor 1 details:

- Name: **Dr. Marianne Mureithi**
- Institution: **University of Nairobi**
- Email address: **marianne@uonbi.ac.ke**
- Telephone: **+254703704711**
- Organization/Department/Program: **Department of Medical Microbiology**

University Supervisor 2 details:

- Name: **Dr. Moses Masika**
- Institution: **University of Nairobi**
- Email address: **mosmasika@uonbi.ac.ke**
- Telephone: **+254721770306**
- Organization/Department/Program: **Department of Medical Microbiology**

ILRI Research Administrator/Supervisor 1 details:

- Name: **Dr. Lillian Wambua**
- Email address: **wambua.lillian@gmail.com** or **L.wambua@cgiar.org**
- Telephone: **+254783505560**
  Organization/Department/Program: **International Livestock Research Institute (ILRI) – Animal and Human Health (AHH)/CGIAR AMR Hub**

Part A: Key ground rules:

- **Communication:** *We shall communicate amongst ourselves to ensure everyone in the team is updated about our work and what's happening. Regular email and webinars communication*
- **Teamwork:** *We shall build and maintain teamwork spirit and respect*

Part B: Key Roles and responsibilities:

- **Principal Investigator/ Student: Mr. Walter Oguta**
  - Conceptualization
  - Methodology
  - Investigations
  - Data curation
  - Formal analysis
  - Writing - original draft and editing

- **University Supervisor 1: Dr. Marianne Mureithi**
  - Methodology
  - Data curation
  - Supervision
  - Writing - review and editing

- **University Supervisor 2: Dr. Moses Masika**
  - Methodology
  - Data curation
  - Supervision
  - Writing - review and editing

- **ILRI Research Administrator/Supervisor 1: Dr. Lillian Wambua**
  - Conceptualization
  - Methodology
  - Formal analysis
  - Acquisition of funds
  - Supervision
  - Writing - review and editing

We do hereby confirm that we **discussed and agreed** on the above content that forms this agreement:

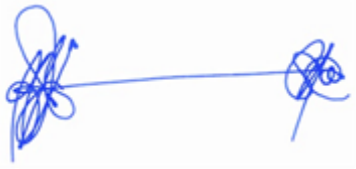
Principal Investigator/Student: Mr. Walter Oguta

Signature _____ Date: 8th December 2022.

University Supervisor 1: Dr. Marianne Mureithi

Signature _____ Date: 8th December 2022.

University Supervisor 2: Dr. Moses Masika



Signature _____ Date: 8th December, 2022.

ILRI Research Administrator/Supervisor 1: Dr. Lillian Wambua

Lillianwambua

Signature _____ Date: 8th December, 2002.

Appendix 5: Originality Report

Turnitin Originality Report

- Processed on: 27-Sep-2022 11:43 EAT
- ID: 1910263779
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Clinical and Sub-Clinical Mastitis in Various Cattle Production Systems in Kenya: Molecular Analysis and Antibiotic-resistance of Associated Bacterial species - WALTER OKELLO OGUTA (Reg. No.: H56/6898/2017)

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Dr. Moses Masika 27/09/2022