EPIDEMIOLOGY OF BOVINE MASTITIS, ANTIMICROBIAL RESISTANCE AND MANAGEMENT PRACTICES ON SMALL-HOLDER DAIRY FARMS IN MOIBEN AND KAPSERET SUB-COUNTIES - UASIN-GISHU, KENYA

DAVID AYAH OUNAH

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Epidemiology of Bovine Mastitis, Antimicrobial Resistance and Management Practices on Small-Holder Dairy Farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya

David Ayah Ounah

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

Signature…….Date……

David Ayah Ounah

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

Signature…….Date……

Prof. Gideon Kikuvi, PhD

JKUAT, Kenya

Signature…….Date……

Prof Peter M. Gatongi, PhD

Moi University, Kenya

DEDICATION

I dedicate this work to my mentors; Prof. Gideon Kikuvi PhD, Prof. Peter Gatongi PhD, Prof. Losenge Turoop PhD and Prof. J. Ngure Ph.D Dean SoPH to whom I owe this success.

May God Bless You Always

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ACRONYMS AND ABBREVIATION

AMDR	Antimicrobial Drug Residue
AMIR	Antibody-mediated immune responses
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection, **c***ommercially available standard micro-organisms*
CMIR	Cell-mediated immune responses
EMB	Ethyl Methylene Blue Agar
KDB	Kenya Dairy Board
MHA	Mueller-Hinton agar
MIC	Minimal Inhibition Concentration
MRSA	Multi-drug Resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
SCM	Sub-Clinical Mastitis
SPSS	Statistical Package for Social Scientists
SRS	Stratified Random Sampling technique
TSI	Triple Sugar Iron

DEFINITION OF OPERATIONAL TERMS

Aerobically Micro-organisms that utilize oxygen for respiration

Anaerobically Micro-organisms that that do not utilize oxygen for respiration

Antimicrobials A substance including antibiotics that kills micro-organisms such as bacteria or fungi, and stops them from growing and causing disease.

Antibiotics Chemical substance produced by a living organisms, generally a micro-organisms that is detrimental to other micro-organisms

Caretaker Animal attendant without formal/professional training in animal health and production

Cumulative The mean antimicrobial effect against all causative bacteria of mastitis

Environmental sanitation Clearing of the bushes, draining stagnant water and maintenance of clean environmental sanitation to control mastitis pre=disposing factors

Epidemiology The distribution, patterns and trend of disease in community including its prevention and control

Extraneous factors – These are the independent variables which may also affect dependent variables but which the researcher does not wish to investigate.

Farm Manager Caretaker with professional training in Animal Health and production

Mastitis	A disease characterized by inflammation of the mammary glands and swelling of the udder
Mastitis culture positive	Mastitis microbial pathoen isolated from the culture media
Resistant	Antimicrobials that are an unable to inhibit the growth of micro-organisms
Respondent	Owner, farm manager or caretaker of dairy cows under study
Susceptibility	Antimicrobials that are able to inhibit the growth of micro-organisms
Zoonosis	Transmission of disease from animals to humans

ABSTRCT

Mastitis is multi-etiologic disease characterized by the swelling of the udder and inflammation of the udder tissues of dairy animals globally. The following prevalence of the disease was reported worldwide; **i**n United States of America at 67.9%, in China, at 53.8%, in Ethiopia at 62.6% and in Kenya at 58.7%. Food security and nutrition are key components of the economic priorities of the Kenya Government. Milk and its products formed significant components of human food chain. However, mastitis infection of dairy cows posed a major threat to this source of livelihood. This study therefore endeavored to inform and influence policy direction on mitigating the above adverse effects of mastitis. The study aimed to identify the determinants of bovine mastitis, antimicrobial resistance and management practices on small-holder dairy farms in Uasin-Gishu, with specific objectives to n determining the incidence of mastitis, its microbial profiles, determine susceptibility of mastitis causing pathogens against antimicrobial agents, determine presence of antimicrobial drug residues in raw milk and determine dairy cow characteristics and management practices. To achieve these specific objectives, a prospective cohort study using multi-stage sampling at administrative Ward level was conducted between January and October, 2021. Mastitis free dairy cows which were not on antimicrobial therapy seven days prior to commencement of the study were recruited. Study respondents were mainly males (63.0%,), with regard to marital status more than 95.1% of the were married and 42.0% had at least tertiary level of educationon. A sample size of (n=216) lactating cows on 81 farms were randomly selected and studied. The Principal Investigator obtained ethical approval from Masinde Muliro University and NACOSTI before commencing the study. Data was analyzed using descriptive and inferential statistics to give results as follows. The overall mastitis incidence in study area was (48.2%). *Staphylococcus* species were found to be most predominant micro-organisms occurring at (30.6%) followed by *Escherichia coli* at (5.1%). High cumulative antimicrobial resistance was demonstrated against ampicillin (81.7%) while low resistance was shown against Kanamycin (33.7%) and Gentamycin (5.8%) ($P<0.05$). The occurrence of Antimicrobial Residue was (6.9%) with Penicillin, Tetracycline and Streptomycin determined above safe Maximum Residue Levels. Management practices revealed that high mastitis infection was reported on intensive farming system at (71.9%) than (58.3%) on extensive farming system. Mastitis was high among farmers who did not take samples to laboratory (71.9%) compared to (52.9%) who utilized laboratory services. Those farmers who disinfected cow premises experienced less infection of mastitis at (50.0%) while there was no significant difference in infection between milking with hand (67.6%) and machine (70.0%) respectively. Mastitis infection on the basis of cattle breed indicated decreasing occurrence of (58.6%) for Friesian, followed by (29.8%) for Ayrshire and crossbreeds at (9.6%) ($P<0.05$). Primiparous cows were less vulnerable at (15.4%) than multiparous-2 at (24.0%) and multiparous-3 at (39.4%) ($P<0.05$). *Staphylococcal*-mastitis was a major burden, *c*onsequently, intervention strategies by way of rational treatment regimens should be put in place targeting predominant *Staphylococcal* species. Kanamycin and Gentamycin were found to be more efficacious and hence antimicrobials of choice ($P<0.05$). Strict observation of withdrawal period before using raw milk for

human consumption is recommended to reduce adverse effects of antimicrobial Drug residue. Primiparous Ayrshire and crossbreed cows demonstrated low vulnerability against mastitis and are recommended breeds ($P<0.05$).

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Mastitis is a multi-etiologic disease of the mammary glands and the udder tissues of an animal; with a high-risk potential for human infection - zoonosis. In these tissues the disease causes inflammation of the mammary glands and swelling of the udder which lead to reduced milk production and eventual culling of the infected animals (Bradley *et al*., 2007). Therefore, the disease is of paramount importance to researchers and all stakeholders in dairy sub-sector because not only does the disease impair the bovine body but the very product from the animal - milk, rendering it unfit for human consumption and harmful to human health. Bacteria and Fungi were the most significant and predominant microbes that were implicated in the etiology of bovine mastitis (Zadoks *et al*., 2011). These microbes included *Staphylococcus* species, *Streptococcus* species, *Klebsiella* species, *Escherichia coli*, *Proteus* species, *Pseudomonas aeruginosa*, *Corynebacterium* species, *Candida albicans*, *Aerobacter* species, *Pasteurella multocida*, *Pasteurella hymolytica*, *Mycobacterium* species, *Bacillus cereus*, *Serratia marcescens*, *Brucella* species, *Enterobacter* species, *Citrobacter* species, *Micrococcus* species, *Salmonella* species, *Shigella* species and many others (Zadoks *et al*., 2011). Generally, over 135 different types of microbial pathogens were implicated in the causation of mastitis (Mbindyo *et al*., 2020; Hawari *et al*., 2014)

Most scholars worldwide were in agreement that mastitis is one of the most expensive to treat diseases affecting cows in the dairy sub-sector globally. It was estimated that on average an infected quarter suffered 30.0% reduction in milk production while an infected cow decreased milk production by 15.0% for the whole lactation period (Radostits *et al*., 2006). Bovine mastitis had high economic implications which were derived from the high costs of antimicrobial therapy and diagnosis, decrease of milk production and early culling of infected cows. A larger proportion of mastitis may not only be diagnosed by clinical

examination of the udder and milk but also screened by confirmatory laboratory diagnostic methods. More often the microbial contamination of milk from mastitis infected cows did render it unsuitable for human consumption due to potential risks of zoonosis and food poisoning resulting from antimicrobial drug residues & bacteria (Sarba & Tola, 2017)

Staphylococcus aureus was the most occurring facultative bacterial microbe that was a major challenge in mastitis infection. A study conducted by Uhlemann described occurrence of Multi-drug Resistant *Staphylococcus aureus* (MRSA) in United Kingdom in 1961. Since then, the MRSA has been a major cause of wide spread mastitis infection worldwide (Uhlemann *et al.*, 2014). It is well documented that approximately one-third of all cases of occurring mastitis were due to Multi-drug Resistant *Staphylococcus aureus* (Bradley *et al.*, 2007; Botrel *et al.*, 2010)

Mastitis is worldwide and is most often transmitted by contact with the milking machines and through contaminated and materials (Uhlemann *et al*., 2014). The disease was reported in dairy farms in Europe with high incidence in countries like Britain, Denmark, Finland, Norway, New-Zealand, Australia, France and Sweden (Uhlemann *et al.*, 2014; Valde *et al.*, 2004).

Mastitis was also reported in the American continent where dairy sub-sector faced huge setback because of increased prevalence. The disease was documented in United States of America (USA), Brazil, Mexico, Canada. The physiological and biochemical gross changes in milk due to mastitis adversely affected the milk quality and quantity leading to high economic losses in the dairy sub-sector (Halasa *et al.*, 2007; Swinkels *et al.*, 2005).

The Asian continent also experienced high burden of mastitis. Countries like China, India, Bangladesh, Iran are part of the Asian nations that were worst hit by mastitis throughout the year with dairy sub-sector recording drastic reduced milk production. In both USA and India for example, the annual losses in dairy sub-sector due to mastitis were approximately recorded to be 2 billion US dollars and 526 million US dollars respectively

(Varshney *et al*., 2004). In Mexico, an annual loss due to reduced milk production was estimated at 140 to 300 US dollars (León-Galván *et al*., 2015).

African continent which forms a majority of the developing world also witnessed the brunt of mastitis, the disease was reported rampant in the West Africa, Northern Africa and the horn of Africa. The Food and Agriculture Organization of the United Nations (FAO, 2010), reported that small-holder farms were the most affected. In these herds on the farms, animal healthcare were haphazard; farmers neither practiced rational treatment of cows nor did they handled milk hygienically during milking (Jimnez-Jimnez *et al.*, 2011). The countries in the horn of Africa - Ethiopia, Djibouti and Eretria; also reported increasing prevalence of bovine mastitis**.** In Ethiopia, a country with highest cattle population in Africa (CSA., 2012), mastitis incidence using culture technique was reported to be 75.7% at herd level and 62.6% at cow level respectively. The sample size was 529 dairy cows (Abebe *et al.*, 2016).

The East and Central African region experienced as well many cases of mastitis. In Uganda the occurrence of mastitis was reported to be (61.3%) (Byarugaba *et al.*, 2008). While in Rwanda it was (50.4%) with *Staphylococcal* mastitis at 51.5% (Mpatswenumugabo *et al.*, 2017).

In Kenya, mastitis was rampant and described in many regions where dairy farming was practiced intensively, in particular in the former white highlands. Studies also documented predominant mastitis in Mount Kenya region, Nairobi County, Kiambu County, North and South Rift regions (Odongo *et al*., 2012; Gitau *et al.*, 2011) other regions also reported the presence of mastitis at varying levels. In the County of Kiambu the prevalence of mastitis was 93.0% and *Staphylococcal* mastitis was the predominat at 31.7% and fungal mastitis due to *Candida albicans* was low at 6.3% (Odongo *et al*., 2012). In Nakuru County mastitis occurrence was 58.7% of which *Staphylococcal* mastitis was high at 77.0% (Gitau *et al*., 2014).

Microbial pathogens pre-dominantly implicated in the etiology of mastitis in Kenya, included *Streptococcus* species, *Staphylococcus* species, *Escherichia coli*, *Trueperella* species and *Pseudomonas* species (Gitau *et al.,* 2014; Gitau *et al*., 2011)

Mastitis was a significant disease of dairy cattle in Uasin-Gishu County causing major economic losses for the dairy farmers. According to annual reports from the Regional Veterinary Investigation Laboratory (RVIL), Eldoret 2017; mastitis was estimated at 36.9%. The treatment failure of commonly used antimicrobials were also on the rise. There were no major studies to base the burden of mastitis in the County. Therefore, there was little known about mastitis and if any the information was scanty.

The following economic losses were reported in Uasin-Gishu County USD ~24 Millions (KDB, 2017; Annual report; CIDP – Uasin-Gishu County, 2017)

1.2 Statement of the problem

The burden of bovine mastitis was reported worldwide, this contributed to major economic loss in the dairy sub-sector due to upsurge in costly veterinary services, culling of sick cows and reduced milk quantity and quality (Park *et al.*, 2016). The following economic losses were reported globally: USA USD ~2 billion and India USD ~526 million (Varshney *et al.*, 2004). Mexico USD 140 – 300 million (León-Galván *et al*., 2015), Kenya USD ~210 and Uasin-Gishu County USD ~24 Millions (KDB 2017, Annual report; CIDP – Uasin-Gishu County, 2017). These necessitated need to establish the burden of mastitis and main causative micro-organisms in Moiben and Kapseret sub-counties.

Upsurge in mastitis infection was attributed to antimicrobial resistance against routinely used antimicrobial drugs due to increased and prolonged use of antimicrobials which was further complicated by growing counterfeit drugs in the market, irrational use, misdiagnosis and the emergence of antimicrobial-resistant bacterial genes which were a major burden in dairy sub-sector, Park, documented 68.3% AMR in humans which was directly linked to animal handlers who regularly consumed animal products (Park *et al.*,

2016). Moreover, AMR leads to prolonged hospitalization, increased medical costs, morbidity and mortality in over 97.1% of patients (Mahlangu *et al*., 2018). Hence it was necessary to establish antimicrobial susceptibility patterns of predominant causative microbial pathogens in this study area. The complexity of increased and prolonged use of antimicrobials led to elevated presence of antimicrobial residues (AMDR) in raw milk used for human consumption. A study by Asli reported 37.0% AMDR while Sarba and Tsola in Ethiopia reported slightly high AMDR at 56.2%. The antimicrobial drug residues have had adverse public health effects leading to upsurge of Non-Communicable Diseases (NCD) which included allergic reactions, interference with industrial processing using micro-organisms, rheumatoid heart fever and also contributed to secondary antimicrobial resistance in human populations (Asli *et a*l., 2017; Sarba & Tola, 2017). There was therefore need to determine the incidence levels of antimicrobial drug residues in raw milk in Uasin-Gishu County.

The effects of allergic reactions due to increased and prolonged use of antimicrobial agents in animals could lead to allergenic reactions in humans when man consumes milk and its products, which forms part of human food chain, this adverse effect is as a result of bacterial contamination of human food chain (Uhlemann *et al.*, 2014).

In the process of management of mastitis, there have been documented development of drug resistant bacterial pathogens, in Britain it was well documented that approximately one-third of all cases of occurring mastitis were due to Multi-drug Resistant *Staphylococcus aureus* (MRSA), (Bradley *et al.*, 2007; Botrel *et al.*, 2010) There is a possibility of this effect spilling over to humans especially in Uasin-Gishu where *Staphylococcus aureus* is thought to be the predominant pathogen causing mastitis (RVIL. Eldoret, 2018, Annual report)

1.3 Justification of the Study

Uasin-Gishu County is agriculturally rich and occasionally is referred to as the Kenya's "bread basket" particularly in Livestock production, maize and wheat; livestock vis-à-vis

dairy sub-sector is the single largest multi-billion sub-sector in the County with high potential and contributed 73.9% of GDP of the overall economy of the County (CIDP, 2017; Uasin-Gishu County). This high agricultural potential point to the pivotal role the sub-sector plays in addressing the food security and nutrition in the County and the Country as a whole. The findings of this study are aimed at benefitting the dairy farmers to realize increased production of quality milk and improved livestock productivity in the sub-sector.

Food security and nutrition is a key policy agenda for the National Government of Kenya. In light of this agenda, this study sought to achieve the pertinent issue of food security and nutrition and to the extend health. The livestock sector and especially the Dairy sub-sector play a significant role in bridging the gap of food insecurity. This study shall come up with strategies that shall achieve prevention and control of mastitis so that people do not consume mastitis infected milk, recommend interventions on reducing antimicrobial resistance so that this is nipped before it can be transferred to humans and reduction of antimicrobial drug residues in milk and food chain used for human consumption (MOALD, 2022; GOK., 2022).

The findings of this study shall inform prudent animal husbandry practices leading to improved productivity, thus addressing the Sustainable Development Goals (SDG) one (1) and two (2) on end poverty and hunger respectively (Mori *et al*., 2019; UN, 2015).

Children below ten (10) years stand to benefit from nutrition provided by improved milk productivity, thus preventing malnutrition in and associated childhood illnesses like aneamia in an estimated 79.2% of the children (FAO, 2012)

1.4 Objectives

1.4.1 General Objective

To determine the epidemiology of bovine mastitis, antimicrobial resistance and management practices on small-holder dairy farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya

1.4.2 Specific Objectives

1. To determine the incidence of bovine mastitis on small-holder dairy farms in Moiben and Kapseret Sub-counties - Uasin-Gishu, Kenya
2. To identify the microbial pathogens causing bovine mastitis on small-holder dairy farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya
3. To determine the susceptibility of microbial pathogens to antimicrobials commonly used for the treatment of bovine mastitis on small-holder dairy farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya
4. To determine antimicrobial drug residues in raw milk from small-holder dairy farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya
5. To identify dairy cow characteristics and management practices used by dairy farmers in prevention and control of bovine mastitis on small-holder dairy farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya

1.5 Research questions

1. What is the incidence of mastitis on small-holder dairy farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya?
2. Which are the microbial pathogens that cause mastitis on small-holder dairy farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya?
3. What are the susceptibility patterns of microbial pathogens to antimicrobials commonly used for the treatment of bovine mastitis on small-holder dairy farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya?

4. Which type of antimicrobial drug residues are present in raw milk from small-holder dairy farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya?
5. What are the dairy cow characteristics and management practices that are used by the dairy farmers in prevention and control of bovine mastitis on small-holder dairy farms in Moiben and Kapseret Sub-Counties - Uasin-Gishu, Kenya?

1.6 Conceptual Framework on Factors Associated with Management Practices of Bovine Mastitis by Farmers.

In conceptual framework depicted in Figures 1.1 below, factors associated with dairy cows characteristics and management practices of bovine mastitis were hypothesized to influence mastitis infections in dairy cows in Moiben and Kapseret Sub-Counties. Mastitis prevention and control included adherence to aseptic procedures during cow milking; rational utilization of antibiotics for treatment of mastitis; environmental sanitation through keeping cow crushes and shades clean and draining away stagnant water in animal crushes and shades. These frameworks, therefore, postulated that implementation of proper mastitis management practices by farmers directly reduced Antimicrobial Resistance (AMR), Antimicrobial Drug Residues (AMDR) and mastitis infections hence reduced adverse public health effects (Mbindyo *et al*., 2020).

1.7 Conceptual Framework

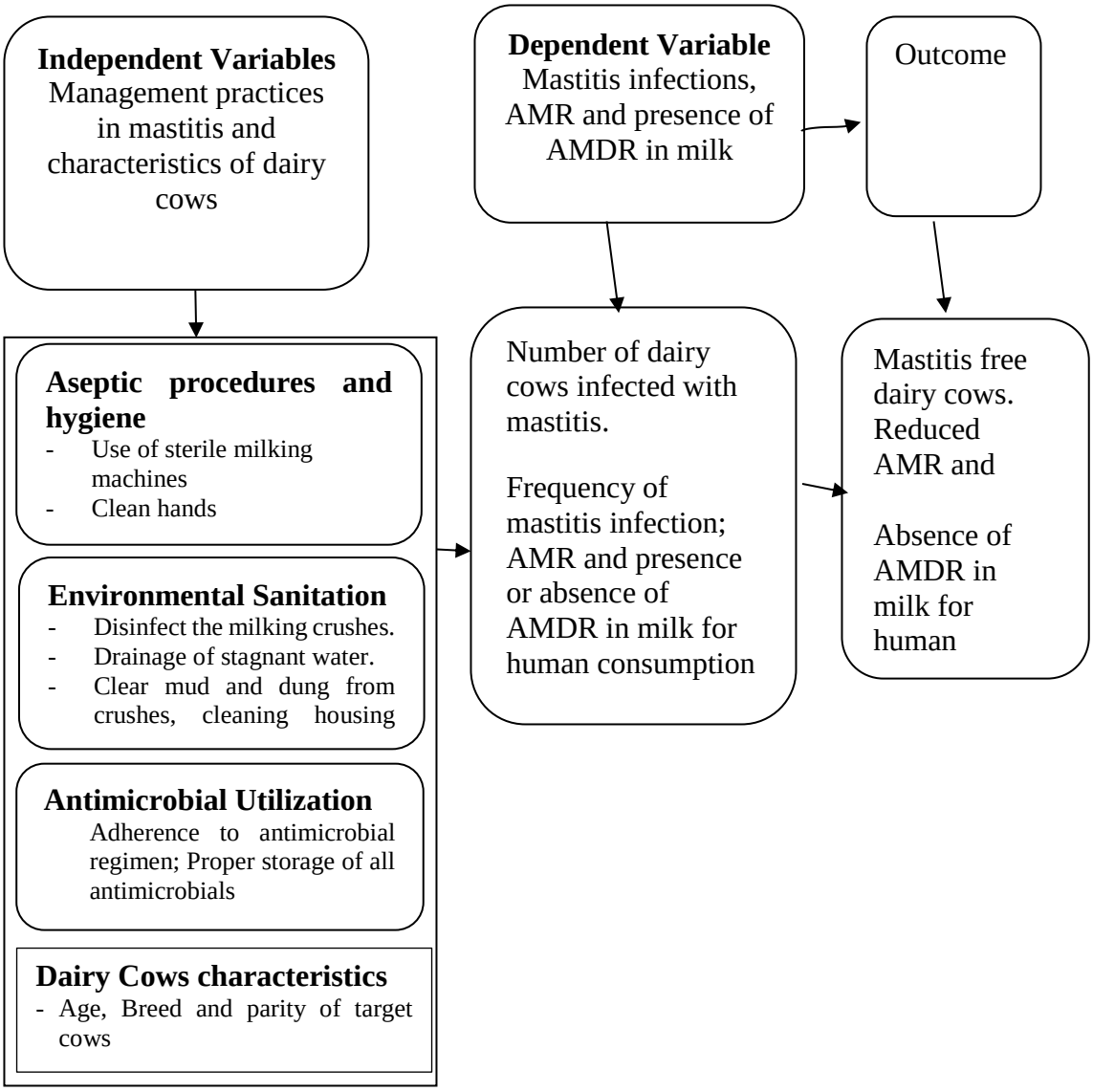

Figure 1.1: Conceptual Framework of the Relationship between Factors in the Management of Bovine Mastitis by Farmers (Independent Variables) and Bovine Mastitis Infection (Dependent Variables)

Source: (RVIL Eldoret, Annual report, 2017)

1.8 Scope of Study

This study broadly investigated dairy animal husbandry practices and effects of antimicrobial agents applied in therapeutic management of bovine mastitis in milk from small-holder dairy farms in Moiben and Kapseret Sub-Counties of Uasin-Gishu County. In particular it focused on; the incidence of bovine mastitis and microbial profile of mastitis causing pathogens, susceptibility effects of antimicrobials used in therapeutic management of bovine mastitis etiological pathogens and animal husbandry practices used to mitigate bovine mastitis in the study Sub-Counties. This scope captured fundamental issues in the containment of bovine mastitis.

The study design was a prospective cohort conducted between January and October, 2021. The principal investigator collected the data. The data was analyzed using Statistical Package for Social Scientists version 20, Chi-square, Fisher's exact test, regression model techniques and reported in percentages, texts, tables and figures

1.9 Limitation and Delimitations of the Study

The major limitation of this study design - cohort study - was bias associated with losses to follow up of study subjects that were likely to occur especially when they were followed up for several months. The losses were likely to affect the validity of the results especially if the study could have experienced high losses > (30) % (Kasiulevičius *et al*., 2006).

.To address the above limitations, the study sought to randomly select dairy cows with long post gestation period; farmers here value milk and they were unlikely to sell off the milking cows unless they had dried up. Further to this the study took a shorter time, ten (10) months, unlike longitudinal studies which take several years. The ten (10) months were reasonable and ensured that the cows completed a full cycle of lactation periods. Ten (10) months achieved quality results as expected and minimized greatly the bias associated with loss to follow-up.

Conventional culture, isolation and identification of microbial pathogens implicated in etiology of mastitis using biochemical testing was limiting unlike if we could have employed molecular sequencing and genotyping to identify bacterial isolates. Further, it could have been better if we could have used Analytical Profile Index (API) kits for biochemical testing and bio-typing sera for identification of *Streptococcus* species, but these were unavailable due to limited resources. These in itself might have limited microbial identification strategy in the current study.

The principal investigator could have undertaken random sampling of milk of approximately one third the sample size (72 samples) from the nearby commercial centers and other places where farmers market and hawk their milk, just to counter-check whether the farmers adhered to recommended withdrawal period following antimicrobial administration on their sick cows before they used milk for human consumption. The study was limited in confirming this and therefore the farmers could be selling milk laced with toxic drug levels.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Mastitis is a disease of the mammary and the udder tissues of animals caused by different types of micro-organisms. In these tissues the disease is characterized by inflammation and swelling of the udder which lead to reduced milk production and eventual culling of the infected animals (Bradley *et al*., 2007). Bacteria and fungi were the most significant and prevalent microbes implicated in the etiology of bovine mastitis (Zadoks *et al.*, 2011; Jones *et.al.*, 2010). The bacterial microbes included *Staphylococcus* species, *Streptococcus* species, *Klebsiella* species, *Escherichia coli*, *Proteus* species, *Pseudomonas aeruginosa*, *Corynebacterium* species, *Clostridium* species, *Aerobacter* species, *Pasteurella multocida, Pasteurella hymolytica*, *Mycobacterium* species, *Bacillus cereus, Serratia marcescens, Brucella* species, *Enterobacter* species, *Citrobacter* species, *Micrococcus* species, *Salmonella* species, *Shigella* species and fungi microbes - *Candida albicans* plus many others (Zadoks *et al.*, 2011). Generally, over 135 different types of microbial pathogens were implicated in the causation of mastitis (Mbindyo *et al.*, 2020*;* Hawari *et al*., 2014).

2.2 Epidemiology and Incidence of Bovine Mastitis

Mastitis is multi-etiologic disease with a global distribution (Bradley *et al*., 2007). On the American continent for instance, the dairy sub-sector suffered high incidence of mastitis (Swinkels *et al*., 2005)**. I**n United States of America for example, mastitis occurrence was estimated at 67.9% where annual losses due to mastitis was approximated to 526 million USD in 2003 (Varshney *et al.,* 2004). The Asian continent also encountered increased incidence of mastitis. In China, the disease incidence was reported at 53.8% (Wu *et al*., 2007). African countries also experienced high levels of of mastitis, the disease was reported in Mali, Chad, Sudan and the countries in the horn of Africa**; i**n Ethiopia, mastitis

incidence was 62.6% at cow level (Abebe *et al.,* 2016). The gross occurrence of bovine mastitis in the Inter-Governmental Authority on Development (IGAD) region showed increasing levels of bovine mastitis in different Districts of Ethiopia; 41.7%, 52.3%, 58% (Sarba & Tola, 2017). The high incidence of mastitis in the horn of Africa countries was because to low animal husbandry practices (Sarba & Tola, 2017; Abebe *et al.*, 2016).

The East and Central African countries experienced as well many cases of mastitis, Mpatswenumugabo in Rwanda reported an overall mastitis incidence of 50.4% where *Staphylococcal* - mastitis was 51.5%, the incidence was high on intensive farming systems. The proliferation of mastitis was faster on intensive farming system, this was attributed to poor cattle housing, poor milking practices and low udder hygiene (Mpatswenumugabo *et al*., 2017). In Zimbabwe, low mastitis occurrence was reported at 21.1% where *Staphylococcal*-mastitis reported at 43.9%, *Coli*-mastitis at 21.2%, *Streptococcal*-mastitis (1.6%) and *Klebsiella pneumoniae (Pneumococcal*-mastitis*)* 15.5% (Katsande *et al.,* 2009) in western Uganda mastitis occurrence was reported at 61.3% at herd-level and 64.0% at cow level respectively (Byarugaba *et al*. 2008).

In Kenya, bovine mastitis was rampant with an incidence of 93.0% in Kiambu County attributable to *Staphylococcal* mastitis at 31.7% (Odongo *et al.,* 2012), while in Nakuru County mastitis was reported at 58.7% (Gitau *et.al.,* 2014). In Thika Kenya, Mureithi & Njuguna, (2016) documented overall mastitis at 61.3%.

The trends, frequencies and distributions of the various microbial pathogens of mastitis on any given farm could determine the severity of on farm mastitis. In Kenya, the most commonly reported types of mastitis infections were *Staphylococcal* mastitis and coliform-mastitis (Gitau *et al*., 2014*;* Gitau *et al.*, 2011). However, the epidemiologic knowledge on infectivity of these types of mastitis in Kenya was at most lacking, scanty and was neither well documented nor clear (Kikuvi & Gatongi, 2021; Personal communication).

The epidemiology of both clinical mastitis (CLM) and sub-clinical mastitis (SCM) revealed the pattern where SCM was 3-4 times more frequently occurring than CLM probably because it was much easier to clinically diagnose CLM and treat than was for SCM. Radostits in one of the studies in Britain, conversely established higher incidence of CLM (31.8%) than SCM (9.9%) on small-holder farms of Holstein Friesian herds (Radostits *et al.,* 2006). Further to this, Abebe, based on Carlifonia Milk Test results and clinical examinations of the cows reported mastitis at cow-level in Ethiopia as (62.6%) (95% CI: 58.3, 66.7), of which (59.2%) was SCM and (3.4%) CLM (Abebe *et al*., 2016).

2.3 Microbial profiles of Pathogens Implicated in Causing Bovine Mastitis

Many studies documented the microbial pathogens implicated in the etiology of bovine mastitis. More than 135 different types of microbial pathogens were reported to cause mastitis (Mbindyo *et al.*, 2020; Hawari *et al*., 2014). Bacteria and fungi were the major causative pathogens of mastitis (Mbindyo *et al*., 2020; Uhlemann *et al*., 2014; Zadoks *et al.*, 2011). The bacterial micro-organisms included *Staphylococcus* species, *Streptococcus* species, *Klebsiella* species, *Escherichia coli, Proteus* species, *Pseudomonas aeruginosa, Corynebacterium* species, *Clostridium* species, *Aerobacter* species, *Pasteurella multocida, Pasteurella hymolytica, Mycobacterium* species, *Bacillus cereus, Serratia marcescens, Brucella* species, *Enterobacter* species, *Citrobacter* species, *Micrococcus* species, *Salmonella* species, *Shigella* species while fungal micro-organisms included *Candida albicans, Cryptococcus* species and many others (Mbindyo *et al.,* 2020; Zadoks *et al*., 2011).

In another study to establish bacterial profiles in Iowa state of USA, the following incidence were reported, *Staphylococcus aureus* (45.0%); environmental *Streptococcus* species (17.5%); *Escherichia coli* (17.5%); *Klebsiella* species (7.2%); Gram-negative non-coliform rods (2.6%); *Coliform* bacteria (2.1%); *Trueperella pyogenes* (3.1%), and *Corynebacterium bovis* (2.6%). *Serratia* species (1.0%); *Bacillus* species (1.0%); *yeast* (1.0%); *Pasteurella multocida* (1%); *Streptococcus* species. (1.0%); unidentified bacteria (0.5%) and gram-negative non-coliform bacteria (0.5%) (Kuehn *et al*., 2013). Multiple

growth of micro-organisms were detected in 25.0% of the cows. No significant growth was detected in 258 of the 601 samples that were tested (42.9%) (Kuehn *et al*., 2013).

In Finland Vakkamäki in his cohort study enumerated the most prevalent bacterial species involved in the etiology of mastitis as coagulase-negative *Staphylococcus* (43.0%), *Staphylococcus aureus* (21.0%) and *Streptococcus dysgalactiae* (8.0%). Other etiological agents were below the (0.1%) thresh-hold and were regarded as causing mild mastitis (Vakkamäki *et al*., 2017).

In West Littoral Region of Uruguay, one of the countries with high population of dairy cows in South America, a sub-sample of 1077 dairy cows from randomly selected farms were used to determine the incidence of bacteria implicated in etiology of sub-clinical mastitis (Ruegg *et al*., 2016). Mastitis infection was established as (52.4%) on a cow basis and (26.7%) on an udder quarter basis. The isolated microbial pathogens from sub-clinical cases and their relative frequencies were: *Staphylococcus aureus* (62.8%), *Streptococcus agalactiae* (11.3%), *Enterococcus* species (8.0%), coagulase-negative *staphylococci* (7.4%), *Streptococcus uberis* (6.4%), *Streptococcus dysgalactiae* (1.8%), *Escherichia coli* (1.5%) and *Staphylococcus hyicus* coagulase-positive (0.6%) (Ruegg *et al*., 2016). In Sweden, the frequency of microbial isolation was *Staphylococcus aureus* (37.0%), CNS (31.0%) and *Streptococcus uberis* (14.0%) (Schreiner & Ruegg, 2003), whereas in Finland CNS was most common at (53.5%) (Gianneechini *et al*., 2002).

In the horn of Africa region, countries struggled with the burden of mastitis too. Zeryehun in his epidemiological study to establish the burden of mastitis in Eastern Harrarghe zone of Ethiopia, predominantly isolated and identified in descending order the following contagious pathogenic micro-organisms as the cause of bovine mastitis in the zone; coagulase negative *Staphylococcus* species (CNS) at (34.2%) *Staphylococcus aureus* at (24.2%), *Streptococcus agalactiae* (17.1%), *Micrococcus species* at 2.1% and *Streptococcus faecalis* at (2.1%) (Zeryehun & Abera, 2017). In southern Ethiopia, a similar study by Adane. reported incidence of *Staphylococcus* species as 29.2%, *Streptococcus* species 12.5% and *Escherichia coli* 11.4% (Adane *et al*., 2012). Biffa in

Ethiopia also documented the influence of seasonality in the occurrence of bacterial isolates ($P < 0.001$), where the occurrence of bacterial pathogens were higher during rainy seasons (OR, 2.6; 95% CI, 2.0−3.4) than dry seasons (OR, 1.0) (Biffa *et al*., 2005).

In East and Central African Nations, studies documented varying occurrences of bacterial pathogens on farms, in Zimbabwe, high occurrence of *Staphylococcus* species at 43.9% was reported, this was followed by *Escherichia* c*oli* at 21.2%, *Klebsiella pneumoniae at* 15.5% and environmental *Streptococcus* species at 1.6% (Katsande *et al.*, 2009). In another study in Rwanda, *Staphylococcal* species was equally high at 51.5% and *Streptococcus* species at 10.3% (Mpatswenumugabo *et al.*, 2017)

In Kajiado Kenya, Mbindyo reported *Streptococcus* species 22.2% and *Pseudomonas aeruginosa* at 5.1% (Mbindyo *et al*., 2020). In another study in Kabete area of Kiambu County Kenya, Odongo in reported high occurrence of mastitis causing microbial pathogens as follows; *Staphylococcus* species (31.7%), *Escherichia coli* (17.2%), *Streptococcus* species (10.3%), *Klebsiella* species (9.7%), *Pseudomonas aeruginosa* (7.6%), *Candida albicans* (6.3%), *Bacillus* species (4.8%), *Pasteurella* species (0.4%), *Proteus* species (0.4%), *Clostridium* species (0.3%), and *Citrobacter* species (0.06%) (Odongo *et al.*, 2012). In another study in Thika Kenya, Mahlangu enumerated the occurrence of *Streptococcus* species at 1.2% and *Micrococcus* species as low as 1.0% (Mahlangu *et al*., 2018).

In majority of the incidences, *Staphylococcus aureus* remained the most predominat and persistent mastitis causing pathogen. This was partly attributable to the intracellular ability of this pathogen to form biofilms on natural body surfaces and prostheses which led to both acute and chronic infections. Biofilms are clusters of bacterial cells, extracellular matrix and water which are more tolerant to host immune defense mechanisms and antimicrobials hence facilitating and escalating chronic mastitis infections (Gogoi-Tiwari *et al*., 2017).

Generally, mastitis causing pathogens are categorized either as environmental or contagious. Environmental microbial pathogens include coliforms such as *Klebsiella* species, *Escherichia coli* which are a major cause of clinical mastitis. While contagious microbial pathogens include majorly *Staphylococcus* species (Thompson-Crispi *et al*., 2014).

2.3.1 Laboratory Isolation and Identification of Microbial Pathogens Implicated in Causing Bovine Mastitis

Fresh milk from lactating dairy cows were sampled aseptically into sterile cryovials and delivered to the laboratory for immediate microbiological culture within eight (8) hours. Alternatively, in case the milk sample was not processed immediately or reached the laboratory within eight hours of collection; the sample was stored in the refrigerator at (4-8)^{o}C for up to seven days or at (-20 - -80)^{o}C until required for processing (Cvetnić *et al*., 2021; Tianming *et al.*, 2017).

Milk samples and American Type Culture Collection (ATCC) standard micro-organisms were homogenized well by mixing firmly but gently and inoculated by streaking onto two primary media; (1). Two plates of 5.0% sheep blood agar, this was a general-purpose medium for growing fastidious micro-organisms and (2). One plate of MacConkey agar. Agar plates were inoculated with the sample and incubated. ATCC micro-organisms were incubated alongside these media plates appropriately and accordingly (Mureithi & Njuguna, 2016).

When fungal infection was suspected the milk sample was inoculated or sub-cultured on Sabouraud Dextrose Agar (SDA). SDA was the most suitable and preferred media for cultivation of fungal micro-organisms in the laboratory (Cvetnić *et al*., 2021).

In case *Salmonella* and *Shigella* species were suspected to be the causal agent of bovine mastitis, the microbiological culture was performed on *Salmonella Shigella* Agar (SSA).

This type of agar has special characteristics for isolating *Salmonella* and *Shigella* micro-organisms (Mbindyo *et al*., 2020).

Culture media results were read alongside the ATCC controls after incubation at 37^{O}C for their optimum respective times; those media plates that exhibited typical growth of micro-organisms were subjected to morphological colonial characterization, gram staining and biochemical tests for microbial isolation and identification. Conversely plates that exhibited no growth even after further incubation at 37^{O}C for another 18-24 hours were discarded and results scored as no growth obtained (Cheesbrough, 2006).

Alternatively, those plates that produced normal flora of milk were scored as non-significant growth or simply treated as no growth for this study (Cheesbrough, 2006).

Table 2.1: Biochemical Reactions of Enterobacteriaceae and Other Enteric Micro-Orgams

BIOCHEMICAL REACTIONS OF SOME ENTEROBACTERIA AND OTHER ENTERIC MICRO-ORGANISMS

	Most other biochemical tests												TSI Medium			
SPECIES	**Urea**	**VP**	**ONPG**	**Lact**	**Man**	**Glu**	**Suc**	**Ox**	**Cit**	**Mot**	**Ind**	**LDC**	**Slope**	**Butt**	**H_2S**	**Gas**
Escherichia coli	N	N	P	P	P	P	D	N	N	P[5]	P[2]	P	Y[6]	Y	N	P[2]
Shigella species	N	N	N[7]	N	D	P	N[1]	N	N	N	D	N	R	Y	N	N[3]
Salmonella typhi	N	N	N	N	P	P	N	N	N	P	N	P	R	Y	P weak	N
Salmonella paratyphi A	N	N	N	N	P	P	N	N	N	P	N	N	R	Y	N	P
Most other Salmonella	N	N	N	N	P	P	N	N	P	P	N	P	R	Y	P[2]	D
Citrobacter freundii	D	N	P	P Late	P	P	D	N	P	P	N[3]	N	R or Y	Y	D	P
Klebsiella p. pneumoniae	P slow	P	P	p	P	P	P	N	P	N	N[3]	P	Y	Y	N	P
Enterobacter species	N	P	P	p	P	P	D	N	P[2]	P	N	D	Y	Y	N	P
Serratia marcescens	D	P	P	D	P	P	P	N	P	P	N	P	R or Y	Y	N	D
Proteus vulgaris	P	N	N	N	N	P	P	N	D	P	P	N	R	Y	P	D
Proteus mirabilis	P	D	N	N	N	P	D	N	P[2]	P	N	N	R	Y	P	P
Morganella morganii	P	N	N	N	N	P	N	N	N	P[5]	P	N	R	Y	N	D
Providencia species	D	N	N	N	D	P	D	N	P	P	P	N	R	Y	N	D
Yersinia enterocolitica [4]	P slow	N	P	N	P	P	P	N	N	P	D	N	R	Y	N	N
Vibrio cholerae	N	D	P	N 24hrs	P	P	P	P	D	P	P	P	R	Y	N	N
Vibrio parahaemolyticus	N[3]	N	P	N	P	P	N	P	D	P	P	P	R	Y	N	N

KEY: LDC = Lysine decarboxylase, **VP** = Voges-Proskauer, **ONPG** = beta-galactosidase, **Lact** = Lactose, **Man** = Mannitol **Glu** = Glucose, **Suc** = Sucrose, **Ox** =Oxidase, **Cit** = citrate test, **Mot** = Motility, **Ind** = Indole test, **Urea** = Urease, **H_2S** =Hydrogen sulphide (blackening), **R** = Red-pink (alkaline reaction), **Y** = Yellow (acid reaction), **D** = different strains give different results.

Notes

1. S. sonnei ferments sucrose slowly
2. A minority of strains give a negative results
3. A minority of strains give a positive results
4. Tests should be incubated at 20 -28^{o}C
5. A few strains are non motile
6. Afew strains give reactions similar to Shigella species
7. S. sonneiis ONPG positive

N – Negative P - Positive

Source: Adapted from laboratory practice by Monica Cheesbrough

2.4 Susceptibility of Microbial Pathogens to Antimicrobials Commonly Used for the Treatment of Mastitis

In relentless efforts to manage mastitis in cows, antimicrobials were intensively and extensively used (Tiwari *et al*., 2013). However, there has been documented haphazard and overuse of antimicrobials leading to increasing AMR to microbial pathogens implicated in mastitis, consequently rendering antimicrobial therapy ineffective. The

implications of the haphazard and overuse of antimicrobials on public health have been costly and immense. In essence this therefore underscored the importance of *in-vitro* susceptibility testing of commonly used antimicrobial agents before their applications *in-vivo* to guard against development of antimicrobial resistance (Thompson-Crispi *et al.*, 2012; Silva *et al*., 2005).

In a study in Brazil by Freitas, 30 *Staphylococcal* isolates from udder quarters infected with sub-clinical mastitis were subjected to an antibiogram panel. The profile of susceptibility using disc diffusion method was tested against - ampicillin**,** amoxicillin, gentamicin, neomycin, norfloxacin, penicillin G, tetracycline and trimethoprim. All the *Staphylococcal* isolates (100.0%) were resistant to trimethoprim and 96.7% to tetracycline and neomycin. 10.0% of the *Staphylococcal* isolates were resistant to 12 antimicrobials while 24 *Staphylococcal* isolates (80.0%) were resistant to at least eight (8) different antimicrobials. The implication of these results demonstrated therapeutic difficulty in management of mastitis, because of bacterial resistance especially *Staphylococcus aureus* which present as multi drug resistance – MRSA (Freitas *et al*., 2018). Further, on overall antimicrobial resistance to ampicillin was (100.0%), tetracycline (96.7%) and streptomycin (80.0%) (Freitas *et al.,* 2018).

In China, a study by Jiang-Ping Li on antimicrobial susceptibility tests showed that (90.7%) of the microbial isolates were resistant to at least one antimicrobial agent. Resistance to penicillin and ampicillin was recorded as (77.3%), tetracycline (60.0%) and erythromycin (48.0%). Furthermore, some microbial pathogens exhibited multiple antimicrobial resistance to commonly used drugs; penicillin, ampicillin, tetracycline and erythromycin (Jian-Ping *et al.,* 2009).

In controlling bovine mastitis, the effectiveness of the antimicrobial therapy was dependent upon the microbial pathogen implicated in the etiology of bovine mastitis. It has been documented that intra-mammary administration of antimicrobial therapy increases the rate of cure in cows infected with coagulase-negative *Staphylococcus* species and environmental *Streptococcus* species. While the use of intra-mammary antimicrobial

therapy in cows with *Escherichia coli* mastitis – an environmental microbial pathogen - are ineffective and not recommended (Ganda *et al*., 2016). The judicious application of antimicrobial agents was paramount in efforts to control bovine mastitis.

In the face of increased antimicrobials resistance and ineffective antiseptic substances, the application of bacteriocins, such as nisin and lacticin-3147, have recently been proposed as an alternative to antimicrobials for treatment and prevention of bovine mastitis. The efficacy of bacteriocin-based therapies when administered by intra-mammary infusion or by dipping the teat were successfully described as effective in treatment of clinical and sub-clinical bovine mastitis (Wu *et al.*, 2007).

Messele in Ethiopia, enumerated antimicrobial resistance against ampicillin as (68.7%), sulfamethoxazole-trimethoprim (50.0%) and streptomycin (25.0%), the low resistance was due to reported low prevalence of mastitis and judicious use of antimicrobial therapy (Messele *et al.*, 2019). He further established that most coliform bacteria which included *Escherichia coli* and *Klebsiella pneumoniae* were reported to show resistance to majority of antimicrobial agents. In another study he reported prevalence of *Escherichia coli* as (7.1%) (Messele *et al.*, 2019). Multidrug resistance traits were documented in (68.7%) of *Escherichia coli* isolates - this was notable against Tetracycline and chloramphenicol where resistance was most predominant (Messele *et al.*, 2019).

In Kenya a study by Omwenga in Marsabit, described *Staphylococcus aureus* as resistant to ampicillin 37.0%, tetracycline (51.0%) and Kanamycin (16.0%). in the same study in Isiolo, *Staphylococcus aureus* was resistant to ampicillin (64.0%), tetracycline (83.0%) and Kanamycin (5.0%). He further established that indigenous cows were hardy and resistant to mastitis unlike exotic breeds which were more vulnerable (Omwenga *et al*., 2021).

The elevated Antimicrobial Resistance, were as a result of indiscriminate use of antimicrobials resulting to high levels of Antimicrobial Resistance. Necessitating key role

of *in-vitro* susceptibility testing of antimicrobials before *in-vivo* utilization (Silva *et al*., 2005; Thompson-Crispi *et al.,* 2012).

The close interaction between humans and infected animals and/or animal products such as raw or undercooked animal products for example milk, beef, ghee, butter fat, hides and skins, were a potential source for human infections. Some of the bacteria implicated in mastitis were reported to cause mild to severe human infections due to shading in large quantities of their toxins in raw milk, which rendered milk harmful to human health when consumed (*Zadoks et al.,* 2011; Sharif & Muhammad, 2009; González & Wilson, 2003). The zoonotic infection of man as a result of mastitis included diseases like *tuberculosis, streptococcal* intoxication, colibacillosis, *streptococcal* sore throat and *brucellosis* (Zeryehun & Abera, 2017).

The association (between animals and humans) over time led to worrying development of antimicrobial resistance against routinely used antimicrobials. This necessitated an urgent need to monitor antimicrobial resistance to establish susceptibility trends, which were vital in generating data on whose to rely when making informed therapeutic decisions as recommended by World Organization for Animal Health - OIE (2001), the systematic and consistent record keeping of status of bovine mastitis, antimicrobial resistance and therapeutic patterns were also significant. These were not only significant in animal health but importantly in human health as well because antimicrobial resistance in animals has been documented to concurrently translate to antimicrobial resistance in human (Awandkar *et al*., 2013). This was possible through transmission of antimicrobial-resistant pathogens in animals to human through milk and its products as food borne microbes (Awandkar *et al.,* 2013; Odongo *et al*., 2012).

Awandkar in his cohort study established an increasing trend of antimicrobial resistance of routinely used antimicrobials. This increasing trend of antimicrobial resistance was observed against streptomycin (57.4% to 79.8%), chloramphenicol (10.9% to 51.4%) and ciprofloxacin (16.9% to 41.43%). However, a near constant sensitivity trends of microbial pathogens was recorded against gentamicin (77.1% to 79.5%) (Awandkar *et al.,* 2013). .

In present study, antimicrobial resistance to routinely used antimicrobial agents was referred to as poor when antimicrobial resistance increased above 75.0% and good when resistance remained as low as 25.0% (Moges *et al*., 2011). In the Moges study, microbial pathogens with increased resistance were documented against amoxicillin and ampicillin. Antimicrobial resistance was attributable to under dosing, prolonged and indiscriminate use of antimicrobials in management of mastitis.

2.4.1 Laboratory Determination of Antimicrobial Susceptibility

The *in-vitro* antimicrobial susceptibility testing was important in establishing the sensitivity patterns and trends of routinely used antimicrobial agents against microbial pathogens implicated in bovine mastitis and conversely the application of sensitive antibiotics *in-vivo* for therapeutic management of bovine mastitis. Many bacterial species like *Staphylococcus aureus* have developed antimicrobial drug resistance generally referred to as Multi-drug Resistant *Staphylococcus aureus* (MRSA) because of lack of appropriate and accurate susceptibility testing programs. Resistant antimicrobial agents have been applied unknowingly hence enhancing multiple resistance against antimicrobials (Uhlemann *et al.*, 2014).

Antimicrobial susceptibility of the microbial pathogens was performed either onto blood agar or Mueller-Hinton agar (MHA) media with the same results. The MHA method is adopted for this study from the publications of Mbindyo, Mureithi, Leon and Gitau as enumerated below (Mbindyo *et al*., 2020; Mureithi & Njuguna, 2016; León-Galván *et al.*, 2015; Gitau *et al*., 2014). Mueller-Hinton agar was among media recommended for susceptibility testing because of its ability to support both fastidious and non-fastidious micro-organisms implicated in bovine mastitis and hence was adopted for this study.

The Bauer-Kirby disc diffusion method of antimicrobial drug sensitivity testing as used by Byarugaba was also adopted for this study. The Oct-disc was placed in the center of the media plate using a sterile forceps, it was firmly and gently secured on the media (Mbindyo *et al*., 2020; Mureithi & Njuguna, 2016; Byarugaba *et al.*, 2008).

2.5 The presence of Antimicrobial Drug Residues in Milk Consumed in Uasin-Gishu County

The antimicrobial drug residues in milk were potential source of many public health complications including non-communicable diseases and food poisoning in humans when consumed. Studies in other locations similar to Uasin-Gishu County revealed many adverse complications arising from excessive consumption of milk containing antimicrobial drug residues (AMDR); these complications ranged from rheumatoid heart diseases, hypertension, antimicrobial drug resistance in humans and animals (Park *et al.*, 2016) and development of bacteria resistant genes to commonly used antimicrobials (Mohamed *et al.*, 2020).

In USA, the consumption of raw cow milk tremendously decreased because of contamination of milk withf high levels of antimicrobial residues. A study by Welsh reported presence of antimicrobial residues in milk of (26.0-60.0%; n=35). These antimicrobial residue levels exceeded recommended federal limits for amoxicillin by (3.0 %), in multiple samples for sulfamethazine by (37.0 %) and sulfathiazole (26.0) % (Welsh *et al*., 2019). While in Benadir Somalia, Mohamed reported overall incidence of antimicrobial drug residue of (24.0%) with (30.0%) in raw farm milk and (18.0%) in raw milk from the market (Mohamed *et al.*, 2020). In Ghana, Addo reported overall residue prevalence of (3.1%) with prevalence in central region at (5.3%), Volta at (2.1%) and Greater-Accra region at 3(3.3%) (Addo *et al*., 2011). In North Eastern Ethiopia, antimicrobial residues in raw milk at farm level was 23.0% (Worku *et al*., 2017). In Kenya Aboge enumerated prevalence of 9.4% antimicrobial drug residue in raw milk at the farm level and 5.7% in milk sampled from the markets, all these antimicrobial drug residues were above the European Union SMRL (Aboge *et al.,* 2000).

Mahmoudi in a study in Azerbaijan – Iran obtained results which depicted that (26.0%) of raw milk samples collected from industrial dairies and; (16.0%) of raw milk collected from market centers showed detectible antimicrobial drug residues - further, (30.0%) of pasteurized milk samples collected from market centers produced positive Delvotest®

results. In conclusion, the study recommended routine monitoring of antimicrobial drug residue in milk and dairy products used for human consumption due to their adverse side effects on human health (Mahmoudi *et al*., 2014)

In Korea, a study conducted to establish the presence of antimicrobial drug residues in milk reported that humans were the worst hit by the harmful effects of antimicrobial drug residues in milk. In order to mitigate this effect, the Food and Agriculture Organization (FAO), European Union (EU) and Korean Ministry of Food and Drug Safety set safe maximum residue levels (SMRLs) as 0.2 μg/mL for Streptomycin, 1.5 μg/mL for Neomycin, Tetracyline 3.1 μg/mL and 4.0 μg/mL Penicillin as safe for antimicrobial drug residues in milk (Park *et al*., 2016).

Chowdhury in Chittagong, Bangladesh, determined the mean concentrations of antimicrobial drug residues in raw local milk at 9.8 μg/ml; and 56.2 μg/ml in raw commercial milk for amoxicillin. In this study Chowdhury reported significantly higher levels above SMRLs ($p \leq 0.05$) for Tetracycline, ciprofloxacin and amoxicillin residues; he further observed the antimicrobial drug residues decreased with boiling of milk (Chowdhury *et al*., 2015).

In Kenya, a study by Kosgey in Uasin-Gishu showed mastitis upsurge among small holder farmers is as high as the demand for locally produced raw milk is. The therapeutic utilization of antimicrobials for management of mastitis among these farmers were as well widespread and increased leading to high antimicrobial drug residue levels in milk above the recommended safe maximum residue levels. It was also been reported that multidrug-resistant *Staphylococcus aureus*- one of the leading causes of bovine *staphylococcal* mastitis - in milk from small holder farms (SHF) is two times high than in larger farms. Most of the milk from the SHF were marketed locally and directly to the consumers and popular milk vendor machines (MVMs) or simply milk automated teller machines (milk ATMs) respectively. The Kenya Government regulation of antimicrobial drug residues in milk, especially from SHF is not currently in force, however, the large-scale farmers who

export most of their milk adhere to East African Regulatory Standards (Kosgey *et al*., 2018).

The huge micro and macro-economic implications associated with bovine mastitis were of far-reaching consequences, for instance there were unwarranted low milk quality and reduced milk quantity (Halasa *et al*., 2007), reduced profitability because of high cost of treatment and discarded milk due to high quantities of antimicrobial drug residues (Asli *et al*., 2017).

2.5.1 Laboratory Determination of Antimicrobial Drug Residues in Raw Milk

A high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for quantitative determination of antimicrobial drug residues in raw milk were adopted from a study by Chowdhury *et al*. (2015). The method was proved convenient, sensitive and accurate for determination of antimicrobial drug residues in milk. μg/mL microbiological culture qualitative screening method was as well adopted for this study from the studies of (Mahmoudi *et al*., 2014; Manafi *et al*., 2011; Mohamed *et al*., 2020). HPLC MS/MS was successfully applied to determine; streptomycin, tetracycline, and the four penicillin (ampicillin, penicillin G, amoxicillin and penicillin V) plus their four major beta-lactamase enzymatic metabolites (ampilloic acid, penilloic acid G, amoxiilloic acid and penilloic acid V) respectively. The antimicrobial drug residues in milk were extracted from the milk samples using acetonitrile and water, cleaned with HLB solid-phase extraction cartridges, then antimicrobials detected by HPLC-MS/MS and quantified using external standard method (Mohamed *et al*., 2020).

2.6 Management Practices of Bovine Mastitis by Dairy Farmers in Uasin-Gishu County

Many factors have been known to immensely contribute to SCM and CLM occurrence of mastitis globally. These factors have epidemiologically been categorized in to two; contagious and environmental (Cervinkova *et al*., 2013). Pathogens involved in

contagious factors are those which infect the udders – the udder then serve as the major reservoir for transmission. These pathogens would then spread from quarter to quarter and cow to cow mainly during milking. Some of contagious pathogens of mastitis include: *Staphylococcus aureus*, *Streptococcus agalactiae, Mycoplasma* species and *Corynebacterium bovis* (Radostits *et al., 2006).* The environmental microbial pathogens of mastitis on the other hand include *Escherichia coli, Klebsiella* species, *Streptococcus dysgalactiae* and *Streptococcus uberis* and are basically defined as pathogens inhabiting the environment in which the cow inhabits. (Abebe *et al*., 2016)

Some of the predisposing risk factors influencing the occurrence of mastitis infection at cow level include age, breed, parity, udder and leg hygiene, stage of lactation and somatic cell count (Thompson-Crispi *et al*., 2014), while the factors significantly associated ($p < 0.05$) with the presence of mastitis at herd-level include herd size, bedding material type and milking mastitis infected cows last (Abebe *et al.*, 2016).

High prevalence of mastitis was mostly linked to several predisposing risk factors. A study in Oromia Ethiopia showed that cows with advanced parity were more productive, but the advance in parity and age increased cow vulnerability to mastitis. Early diagnosis and regular screening of cows for sub-clinical mastitis together with proper therapeutic management of clinical mastitis were significant measures in control of mastitis (Sarba & Tola, 2017). In Rwanda, Ndahetuye also established that primiparous cows were less vulnerable than multiparous cows, he reported (66.7%) in primiparous cows and (82.1%) in multiparous cows (Ndahetuye *et al.*, 2019).

Many epidemiological studies revealed that the prevalence of mastitis was significantly higher ($p<0.05$) in crossbreed cows (47.2%) as compared to indigenous cows (15.4%) (Sarba & Tola, 2017). In a study by Ndahetuye in Kigali Rwanda, the prevalence of mastitis in Friesian cows was higher (87.8%) than in crossbreed cows (76.6%) and indigenous cows (50.0%) (Ndahetuye *et al.*, 2019). While the prevalence of mastitis in relation to parity revealed that multiparous older cows had significantly higher infection (75.0%) than the primiparous young cows (28.0%) (Abunna *et al.*, 2013). The high

mastitis prevalence rate was as a result of antimicrobial overuse and resistance. In addition, most animal health practitioners could be an unqualified and inexperienced as well (Ndahetuye *et al.*, 2019; Abebe *et al*., 2016)

Management practices at farm and cow level influenced transmission of on farm mastitis world over. In Ethiopia, the prevalence of mastitis on the basis of semi-intensive farming and animal level management systems was (47.1%) and intensive farming system was (42.4%) while extensive farming system was (8.1%). This scenario was attributable to variation in factors associated with hygienic standards of dairy cows living environmental conditions. The environment of the dairy cows was marked dirty and wet, unavailability of veterinary services on majority of the dairy farms and lack of training on dairy farming for farm owners. All these factors majorly contributing immensely to faster proliferation and transmission of mastitis (Biffa *et al*., 2005).

Abebe in Ethiopia and Kumar in India, the duo reported high mastitis prevalence of about 80.0% and 79.0% respectively on farms that admitted to experience mastitis. This was mainly attributable to low udder hygiene (Abebe *et al.*, 2016; Kumar *et al*., 2016).

Mbindyo in Embu and Kajiado Kenya, reported an infection among the farmers who did not take samples to the laboratory for mastitis testing as (39.8%) but surprisingly, the infection was high among those farmers who normally tested the samples in the laboratory (60.2%) (Mbindyo *et al*., 2020). She also reported infection among farms with housing shades at (74.7%), further, she observed that disinfecting cow housing/crush, is vital in reducing the spread of mastitis in any herd (Mbindyo *et al*., 2020).

Abebe in Ethiopia and Mbindyo in Kenya, reported mastitis infection at approximately 80.8% and 84.1% respectively among the farmers who used one towel for all the cows and did not wash hands before and after milking the cows (Mbindyo *et al*., 2020; Abebe *et al*., 2016). Consequently, Kumar observed that this practice should be avoided as is known to propagate contagious mastitis from one cow to another and from teat to teat (Kumar *et al*., 2016).

Mbindyo in a study in Embu and Kajiado Counties reported high mastitis infection among farmers practicing intensive farming system (83.7%) than among those practicing semi-intensive farming system (16.3%), (Mbindyo *et al*., 2020).

The infection rate of mastitis among farmers with housing with concrete floor in Embu and Kajiado was as high as 76.2% while infection among farmers with housing with earth floor was as low as (23.8%) (Mbindyo *et al*., 2020). However, in a study by Mureithi and Njuguna in Thika Kenya, infection among farmers with housing with concrete floor was low at (55.5%) and high in earth floor (82.1 %) (Mureithi & Njuguna, 2016). Further, Mureithi and Njuguna in Thika again, documented mastitis infection in cows with good udder hygiene at 54.3% and for cows with low udder hygiene at (69.9%), the duo investigators noted that maintaining good udder hygiene is unsustainable during heavy rainy season (Mureithi & Njuguna, 2016).

Farmers, in a sustained efforts to control mastitis reported to apply frequently and commonly used antimicrobial agents for treatment of mastitis. In China and Brazil, the Principal Investigators found out that antimicrobial resistance to penicillin and ampicillin was (77.3%) and tetracycline (60.0%) (Jian-Ping *et al*., 2009); and ampicillin (100.0%), tetracycline (96.7%) and streptomycin (80.0%) (Freitas *et al.*, 2018) respectively. These high Antimicrobial Resistance, were due to haphazard and indiscriminate use of antimicrobial agents leading to high levels of Antimicrobial Resistance. In essence this underscores the importance of *in-vitro* susceptibility testing of frequently used antimicrobial medicines prior to their applications *in-vivo* to guard against development of antimicrobial resistance (Thompson-Crispi *et al*., 2012; Silva *et al.*, 2005). However, low antimicrobial resistance was reported by Messele in Ethiopia, against ampicillin (68.7%), sulfamethoxazole-trimethoprim (50.0%) and streptomycin (25.0%), the low resistance was due to reported low prevalence of mastitis and judicious utilization of antimicrobial therapy (Messele *et al*., 2019).

The best management practices in mastitis control included milking sick animals last, treatment of sick animals with the right regimen of antimicrobials, completion of

antimicrobial regimen, using trained veterinary or para-veterinary personnel, provision of clean crushes & animal housing, provision of warm bedding materials, maintenance of hygiene and sanitation, train the farmers on animal husbandry, good on farm drainage system.

Management strategies mainly focused on disease prevention by farm management which included udder hygiene, trained staff to monitor minor changes in the udder or milk, and better diagnostic and treatment methods. New technologies which have the potential to unravel this complicated disease include improved diagnostic tools, based on advanced genomics or proteomics, prevention, based on vaccines and immune modulators, and metabolic products of probiotics such as bacteriocins and gene therapy (Abuna *et al.*, 2013)

2.6.1 The Predisposing Risk Factors Associated with Transmission of Bovine Mastitis

Mastitis occurred whenever udder tissues were infected with contagious or environmental microbial pathogens. Some of the predisposing risk factors that aided in the transmission of mastitis included contaminated milking hands, dirty milking unit liners, poorly cleaned milking units, soiled bedding materials, contaminated teat dip, dirty water used to clean udders prior to milking, teat trauma, flies transmitting microbes mechanically (Mureithi & Njuguna, 2016).

The other factors that influenced the transmission of mastitis included season, age of animal, stage of lactation and previous episodes of infection (Mbindyo *et al.*, 2020; Biffa *et al*., 2005) Susceptibility to mastitis infection by different breeds of dairy cows

The susceptibility pattern of different types of bovine breeds to mastitis infection was of paramount importance in sustained efforts to manage transmission of mastitis in dairy farms in Uasin-Gishu County. The variations in the vulnerability based on different types of breeds of bovine (i.e Holstein Friesian, Guernsey, Jersey, Ayrshire, Crossbreed, Zebu

etc) to mastitis infection indicated that genetic factors play a significant role in the immune response to infection (Figure 2.1) below. Antibody-mediated immune responses (AMIR) and cell-mediated immune responses (CMIR) have been utilized as indicator traits of adaptive and innate immune system responses in different breeds of dairy cattle. The CMIR and AMIR responses are essential components of immune system upon which the host protection mechanisms against microbial pathogens implicated in mastitis are hinged, further exotic breeds are likely more vulnerable than indigenous breeds as well as older cows are more vulnerable than young adult cows (Thompson-Crispi *et al*., 2012).

In Ethiopia, higher prevalence of mastitis was recorded in crossbreed cattle (47.2%) than in indigenous cattle (15.4%). There was also higher mastitis prevalence in larger cattle herds (46.6%) than in smaller herds (24.2%) and among semi-intensive farming systems (47.1%), intensive farming systems (42.3%) and extensive (8.1%) farming systems, vulnerability in crossbreed cows and larger cattle herds were fundamentally high as the genomic traits exhibited are at variance (Sarba & Tola, 2017).

A cross-sectional study in Ontario Canada in a pure herd of Holstein Friesian dairy cows established that *Escherichia coli* was the most frequently isolated pathogen, comprising (29.9%) of the isolates, followed by *Staphylococcus aureus* (22.2%) and *Streptococcus* species (16.2%). Contagious pathogens were responsible for (17.2%) of the recorded mastitis cases, and environmental pathogens were responsible for (46.0%) (Thompson-Crispi *et al*., 2013). The majority of mastitis cases, (53.3%), had a severity score of 1 (abnormal milk only), followed by severity score of 2 (abnormal milk and swollen udder) for (36.4%) and severity of 3 (abnormal milk, swollen udder, and sick cow) constituting (10.3%) of the cases (Thompson-Crispi *et al*., 2013).

The parity and age of the cows also played a significant role in determining mastitis infection. Zeryehun exemplified this by demonstrating the following prevalence rates as per the parity and age; age group of young adult cows (31.4%), adult (66.7%) and old (58.3%). On the other hand, parity prevalence was recorded as; cows giving birth to three calves (69.8%) and cows giving birth to six calves (62.5%) (Zeryehun & Abera, 2017).

Figure 2.1: Different Phenotypic Species of Dairy Cows under Intensive Farming System (Holstein Friesian, Ayrshire, Guernsey and Jersey)

Source: Adapted from RVIL Laboratories Eldoret, 2018

2.6.2 Clinical Diagnosis and Manifestation of Mastitis

Clinically as described by Adkins, mastitis can be diagnosed under two broad based categories (clinical and sub-clinical mastitis). Sub-clinical mastitis infection involved one or all the four quarters of the udder with no apparent signs of local inflammation or systemic involvement, however, there is a short period of abnormal milk and udder inflammation that may lapse unnoticed if not keen. This type of mastitis may last for at least two months or for the entire lactation period of a cow. The gold standard of diagnosis of sub-clinical mastitis remains 'microbiological culture' method where the microbial pathogens are cultured, isolated and identified (Adkins & Middleton, 2018; Lam *et al*., 2009)

Clinical mastitis infection also referred to as acute severe mastitis, manifested as mild or severe. In mild case, the signs and symptoms typically occur in only one quarter at a given

time in point. Other symptoms include visibly abnormal milk Containing fibrin clots and milk discoloration, visible changes to the udder leading to swelling, heat, pain, redness and hardness. In severe clinical mastitis the infection includes systemic involvement indicated by shock, fever, and anorexia (Adkins & Middleton, 2018; Lam *et al*., 2009).

In summary, the literature review reveals numerous research gaps that the present study seeks to cure. For instance, whereas the magnitude of mastitis is known in some neighbouring counties little is known in Uasin-Gishu county. It was key to establish he predominant microbial pathogen causing mastitis, the antibiotic susceptibility pattern was missing, this was necessary to establish. In sustained efforts to manage mastitis – prolonged application of antibiotics is experienced, however, monitoring of antibiotics residue in milk used for human consumption is haphazardly done or not observed at all. Prudent cow management practice aimed at reducing the incidence of mastitis is low in the present study site which calls for interrogation of the animal husbandry practices. This study seeks to interrogate these aspects and propose strategies to address them.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted in Moiben (0.8238° N, 35.3764° E) and Kapseret (0.4309° N, 35.2272° E) sub-counties of Uasin-Gishu County. The two sub-counties were randomly selected because they have conducive environment and climate for Livestock production. Over (90.0%) of the dairy cows kept here were exotic breeds and crossbreeds (between exotic cows and indigenous zebu cows) comprising of pedigree high milk yielding dairy cows. This was contrary to majorly indigenous with low milk yielding dairy cows (67.0%) domesticated in the neighbouring Counties of Bungoma, Kakamega, West Pokot and Elgeiyo-Marakwet (CIDP, 2017; Uasin-Gishu County). The population of the indigenous dairy cows in the present study comprised of a minimal 10.0% only. The two sub-counties constitute part of the Eldoret Municipality and thus were treated as one large study site. The geographical set-up of the two sub-counties were similar and the weather patterns and conditions are also similar. The study area lies in the Western lower region of North Rift and on the eastern side of the Lake Victoria basin. Rainfall is bimodal ranging from 500 mm to 1500 mm with average temperature of 18.5^0C. In 2021, the sub-counties received long rains between the months of March to May. However, during the period for short rains, the sub-counties experienced heavy downpour that started in July extending to November 2021. Moiben and Kapseret are peri-urban suburbs and are densely populated. The proximity of the two sub-counties to Regional Veterinary Investigation Laboratory (RVIL) Eldoret, combined with the fact that the annual and quarterly RVIL reports reveal rampant mastitis prevalence in the area of over (36.0%), made this area ideal for this study. In addition, the data generated from this study shall helpful to RVIL in terms of putting in place epidemiological surveillance plans and strategies. This study area therefore, provided a better catchment for bovine mastitis study unlike the rest of the sub-counties and Counties in the neighbouring North Rift region according to (County Director of Animal Production Uasin-Gishu, personal communication, 2018).

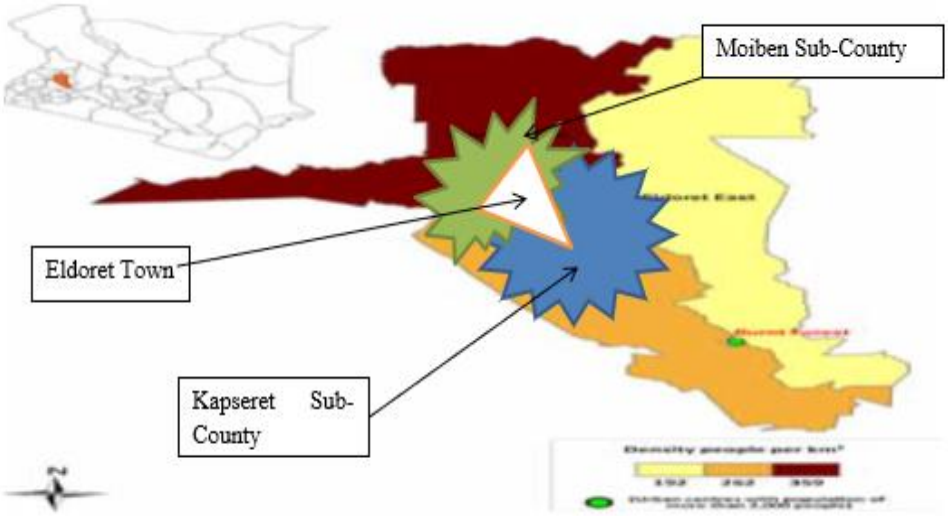

Figure 3.1: Map of Geographical Location of Moiben and Kapseret Sub-Counties in Uasin-Gishu County, Kenya.

Source: http://www.healthpolicyproject.com/

3.2 Study Design

This study employed prospective cohort design as its overall research strategy. This study design was adopted because of its suitability to in terms of follow up of lactating cows noting that the lactation period of majority of cows in this study area range from (10 – 14) months (Mbindyo *et al.*, 2020). The study cows were recruited at farm level to determine the baseline results. Only cows which tested negative for mastitis using Microbiological culture method were recruited into the study and followed up for ten (10) months equivalent to 303 days (January – October, 2021) to determine bovine mastitis endpoints. The sampling interval was 21 days or earlier should the farmer report a sick cow. A total of 14 follow-up visits in 303 days were done and a total of 2,770 cultures were performed as follows: Day of follow up visit (n=total number of microbiological cultures performed) 21(216), 42(215), 63(212), 84(209), 105(205), 126(202), 147(195),

168(192), 189(184), 210(179), 31(171), 252(162), 273(158), 294(143) and 303(127), n=2770. No cow was lost to follow-up visit (Appendix 14).

3.3 Study Variables

Mastitis infection was defined as growth of microbial pathogen on the primary culture media simply referred to as - culture positive results; hence mastitis infection and antimicrobial residues were dependent variables while the following variables were independent; aseptic handling procedures, environmental sanitation, cows characteristics and rational use of antimicrobials. The interaction of these variables determined the occurrence of disease in lactating dairy cows. These variables were conceptionalized as depicted in (Figure 1.1) above

3.4 Study Population

The total study population was 211,020 dairy cows of which 130,911 were in Moiben sub-county while 80,109 were in Kapseret sub-county. Out of these; 147,714 (70.0%) were pedigree exotic breeds, 42,204 (20.0%) crossbreeds and 21,102 (10.0%) Indigenous breeds. Each sub-county had five wards and the cow population was as follows: Karuna 53,732, Moiben 32,239, Tembelio 22,470, Sergoit 15,631, Kimumu 6,839, Kapseret 26,378, Ngeria 23,447, Kipkenyo 11,723, Langas 10,746 and Megun 7,816 cows. This information was as per the records of County Directorate of Livestock Production (CDLP 2018, Annual report). The cows were recruited during early lactating stage and at different parity status.

The annual production of milk was estimated at 219,489,100 litres and sold at 50 Kenya shillings per litre. This translated to 11 Billion Kenya shillings per annum (CDLP 2018, Annual report).

3.4.1 Inclusion Criteria

Small-holder Farms with (3-10) cows were selected for inclusion into study framework. Lactating dairy cows which were free from mastitis and whose milk was used for human consumption were recruited and sampled for study. Further, the cows were not on any antimicrobial treatment seven (7) days prior to commencement of the study and during the period of study.

3.4.2 Exclusion Criteria

The dairy cows on antimicrobial treatment immediately (less than seven (7) days) before the commencement of this study were excluded.

3.5 Sample Size Determination

Determination of sample size was adopted from the study by Kasiulevicius, on sample size estimation in epidemiology studies (Kasiulevičius *et al*., 2006).

To calculate optimal sample size using this formula, the following assumptions were made;

Power of the study = 90%, Significance level (P) = 0.05 and confidence interval of 95%.

$$n = \frac{(P_1(1-P_1) + P_2(1-P_2)}{(P_2-P_1)^2} \text{x } f(a, b)$$

Where:

n = sample size
f(a,b) = 10.5074 (Constant value derived from Table 3.1 below)
P_1 = prevalence effect in intensive population - Exposed
P_2 = prevalence effect in extensive population – Non-Exposed

$$n = \frac{0.613(1-0.613) + 0.387(1-0.387)}{(0.387 - 0.613)^2} x\ 10.5074$$

$$n = \frac{0.237 + 0.237\ x\ 10.5074}{0.051}$$

$$n = \frac{0.474}{0.051}\ x\ 10.5074$$

n = 9.294 x 10.5074

n = 97.657

n = ~ 98

Assuming attrition level is at 10%, then;

n = n + 10% attrition level = 98 + (10/100) x 98 = 108 Cows per population

Total sample size n = 216 cows

Table 3.1: Values for {f(a,b) = 10.5074}

POWER (1 – Beta)				
	0.05	0.1	0.2	0.5
0.1	10.8222	8.5638	6.1826	2.7055
0.05	12.9947	10.5074	7.8489	3.8415
0.02	15.7704	13.0169	10.0360	5.4119
0.01	17.8142	14.8794	11.6790	6.6349
Alpha (2 – sided)				

Source: Adopted from Kasiulevicius study on sample size calculation in epidemiological studies (Kasiulevičius et al., 2006).

Therefore, 216 lactating dairy cows on 81 farms were sampled and farmers interviewed using a semi-structured questionnaire. The 216 lactating dairy cows and farmers (respondents) were randomly selected from each ward; ensuring proportionate and

equitable distribution of the sample size across the two (2) Sub-Counties - Moiben and Kapseret and their respective administrative wards. This resulted in the following distribution; - Moiben sub-county 134 cows and Kapseret sub-county 82 cows and across the various wards as follows - Karuna 55, Moiben 33, Tembelio 23, Sergoit 16, Kimumu 7, Kapseret 27, Ngeria 24, Kipkenyo 12, Langas 11 and Megun 8.

3.6 Sampling Design

The study employed multi-stage sampling design. This type of sampling comprised stratified, cluster and simple random sampling methods. Uasin-Gishu County is sub-divided into five sub-counties and out of the five two sub-counties were randomly selected – Moiben and Kapseret. Then all wards were purposively selected. The following locations were then randomly selected; In Moiben sub-county, the following locations were selected, Kimumu ward – Chepkoilel, Hawaii and Kimumu locations; Tembelio ward – Kaptuktuk, Kabao, Marura, Tembelio, Tuiyonik, Naiberi, Kaptuly, Kimoning and Kapsoen locations; Sergoit ward – Kapchinga, Sergoit, Kapchesha, Keldy, Kiptugen and Kaborgei locations; Karuna ward – Maibeki, Karuna, Kimwarich, Suguti, Kapkoros, Kabomoi, Kapsonik, Kaplolwa, Cheweriweri, Kapsilliot, Kaprong, Sasitwo, Raifarm, Kapsegero and Chepkamai locations; Moiben ward – Toloita, Moiben, Karaji, Torochimoi, Kapngetunyi, Kapsubere, Mereweti, Kapitet, Ngoisa, Teluti, Lelaibei and Ranymoi locations.

In Kapseret sub-county we sampled Kapseret ward – Kapseret, Simat, Chepkatet, Kiwanja Ndege, Kaptendeti, Marben, Kapkaroi, Lemokwo and Mlango Kubwa locations; Kipkenyo ward – Kipkenyo, Tachasis, Samleti, Kiptendeni, Rivatex and Kapmoi locations; Ngeria ward – Ngeria, Cheplaskei, Cheptiret, Kimuri, Susibiko, Kipsamo, Chepkwai, Nairiri, Kiambaa, Outspan and Chepkongony locations; Megun ward – Megun, Kimwalu, Meguti, Kabongwa and Kaptum locations; and Langas ward – Mwiruti, Langas, Kisumu Ndogo, Racecourse and Soin locations.

Lastly study respondents were recruited at farm-level (household-level/herd-level) during baseline visit from each selected location using simple random sampling technique, with the help of local Animal Health Assistants. The follow-up sampling was accomplished as follows - Day of follow up visit (number of microbiological cultures performed) 21(216), 42(215), 63(212), 84(209), 105(205), 126(202), 147(195), 168(192), 189(184), 210(179), 31(171), 252(162), 273(158), 294(143) and 303(127).

A total of 2770 microbiological cultures were performed (Appendices 13 and 14). The study respondents included (either cow owners/farmers, Farm managers and/or cow caretakers). They were interviewed using semi-structured questionnaires as interview guides during the baseline visit to the farms; afterwards milk samples were aseptically obtained from each recruited cow in a sterile 2ml cryovial for microbiological culture – for microbial isolation and identification, antimicrobial susceptibility testing and antimicrobial drug residue testing.

The milk samples were obtained during normal milking hours; the farmer milked the first few drops of milk out then milked the mid-stream milk into the sterile 2ml cryovials.

The Milk samples that were not processed immediately within eight (8) hours were moved to the laboratory and stored at (4-8) ^{o}C for one week or frozen at −20^{o}C until they were required for analysis.

The response rate was 100.0% as there was no cow and/or respondent that was lost to follow up.

3.7 Data collection tools

3.7.1 Questionnaires

The study used semi-structured questionnaire to collect quantitative data on animal husbandry and management practices (Appendix 3). Cow owners/farmers, Farm managers

and/or cow caretakers acted as study respondents. The questionnaire was sub-divided into several sections which comprised sections on unique identification code, background/demographic factors of respondents and their cows and questions on management practices. The questionnaire was piloted in the neighbouring Mosoriot sub-county in Nandi County. This sub-county was similar to both Moiben and Kapseret sub-counties in Uasin-Gishu in all its aspects of livestock production activities.

3.7.2 Interviews

The semi-structured questionnaires were used as interview guide to collate data about background factors, knowledge and management practices of bovine mastitis by farmers. The Principal Investigator conducted face to face interviews by asking consenting respondents questions systematically - one after another - and recorded their answers. The interview took a minimum of 25 minutes and a maximum of 30 minutes. This technique was also validated during pilot study in the neighboring Mosoriot sub-county in Nandi County

3.7.3 Observation

To complement the interview mode of data collection, the observation method of data collection was applied to concretize data on the variables under the study. The Principal Investigator could for example prefer to score the cow udder hygiene by observing other than asking the respondent.

3.7.4 Pre-Testing of Data Collection Tools

3.7.4.1 Quality Control

The data collection tools were pre-tested for accuracy, reliability and validity - during pilot study in Mosoriot sub-county in the neighboring Nandi County. The tools passed the test of admissibility and were successfully used to collect the data on the present study.

3.8 Data Collection

Data was compiled from both laboratory and field results of microbiological culture of milk. Isolation and identification of microbial pathogens implicated in causation of mastitis, antimicrobial susceptibility testing of bacterial isolates and antimicrobial drug residue testing of milk from the laboratory on one hand while field data was collected using semi-structured questionnaires, interviews and observation techniques from study farmers and caretakers of the lactating dairy cows who acted as respondents on the other hand. The primary data which was collected using these techniques was quantitative data on epidemiology of bovine mastitis and practices associated with management of bovine mastitis. Background data on demographics of the respondents and target lactating dairy cows were also collected in this spectrum for this study. In order to overcome bias often associated with quantitative data, we incorporated triangulation in data collection, which was basically using multi-pronged methods to collect required data.

The data on management practices of bovine mastitis in dairy cows was collected on the farm at both cow and herd levels using a semi-structured questionnaire. The factors that were assessed comprised herd management system, milking practices, frequency of milking per day, cleaning and drying of the udder, milking mastitis infected cows last and culling chronically ill cows. Other routine practiced factors considered included good drainage system on the dairy cow farm and daily cleaning of cow-dung (i.e. good hygiene), poor farm drainage practice and not cleaning cow-dung daily (i.e. poor hygienic status) were some of the components considered in data collection (Abebe *et al.*, 2016).

3.9 Laboratory analysis of raw milk

3.9.1 Procedure for microbiological culture of raw milk to determine mastitis incidence and microbial profile

Fresh milk from lactating dairy cows were sampled aseptically into sterile cryovials; and delivered to the laboratory for immediate microbiological culture within eight (8) hours

(Tianming *et al.*, 2017). Alternatively, in case the milk sample could not be processed immediately or reach the laboratory within eight (8) hours of collection; the sample was stored in the refrigerator until required for processing (Mbindyo *et al.*, 2020; Mureithi & Njuguna, 2016).

Once the samples reached the laboratory, they were received by writing them in the Research Laboratory Register and Research Laboratory Report Form. Each sample was then assigned a unique laboratory code to maintain anonymity and confidentiality of the target cows and respondents. The Research Laboratory Report Form accompanied the milk sample in the laboratory during its processing in the Microbiology section of the laboratory. It is on this form that the laboratory diagnostic results were recorded. The details in these forms comprised the unique laboratory code of the farmer and the cow and other related demographic information.

Once the uniquely coded samples were delivered to the Microbiology section of the laboratory, the microbial culture of the samples and American Type Culture Collection (ATCC) controls (Appendix 9) commenced immediately. The samples and ATCC controls were homogenized well by mixing firmly but gently. Using a sterile wire loop, a loopful of milk equivalent to - 4mm diameter standard inoculating loop - was taken and inoculated by streaking onto two primary media; (1). Two plates of blood agar, this is a general-purpose medium for growing fastidious micro-organisms and (2). One plate of MacConkey agar, this is a selective medium; on MacConkey Agar, lactose fermenting micro-organisms produce bright red colonies and non-lactose fermenting micro-organisms do not produce bright red colonies but maintain the colour of the media. This microbiological culture procedure was performed in a Biosafety cabinet level 2 (BSL II). ATCC micro-organisms were incubated alongside samples appropriately and accordingly (Figure 3.2).

The remainder of the sample for culture were stored in the refrigerator at (4-8)^{o}C until the final results of culture were obtained. In case of any necessity to repeat the culture, the

refrigerated sample was verified and brought to room temperature and re-cultured. The samples were archived after seven (7) days at -20^{o}C for as long as was necessary.

The inoculated plates were incubated in an incubator at 37^{o}C for 18 – 24 hours aerobically. Further incubation for up to 48 hours aerobically at 37^{o}C for non-fastidious micro-organisms was allowed. In case of anaerobes (for example *Clostridium perfringens*), the inoculated Blood Agar plates were incubated at 37^{o}C for 18–24 hours anaerobically, a further incubation for up to 72 hours allowed with daily observation after every 18-24 hours.

Culture media results were then read alongside the ATCC controls after incubation at 37^{O}C for their optimum respective time period; those media plates that exhibited typical growth of micro-organisms (Figure 3.2) were subjected to morphological colonial characterization, Gram staining and biochemical testing for microbial isolation and identification. Conversely plates that exhibited no growth even after further incubation at 37^{O}C for another 48/72 hours were discarded and results scored as no growth/isolate obtained. In the event that it was discovered that there was a technical hitch in the microbiological processing of the sample, the remaining milk of the same sample stored in the refrigerator at (4-8)^{o}C was re-cultured, depending on the discretion of the technical personnel performing the assay.

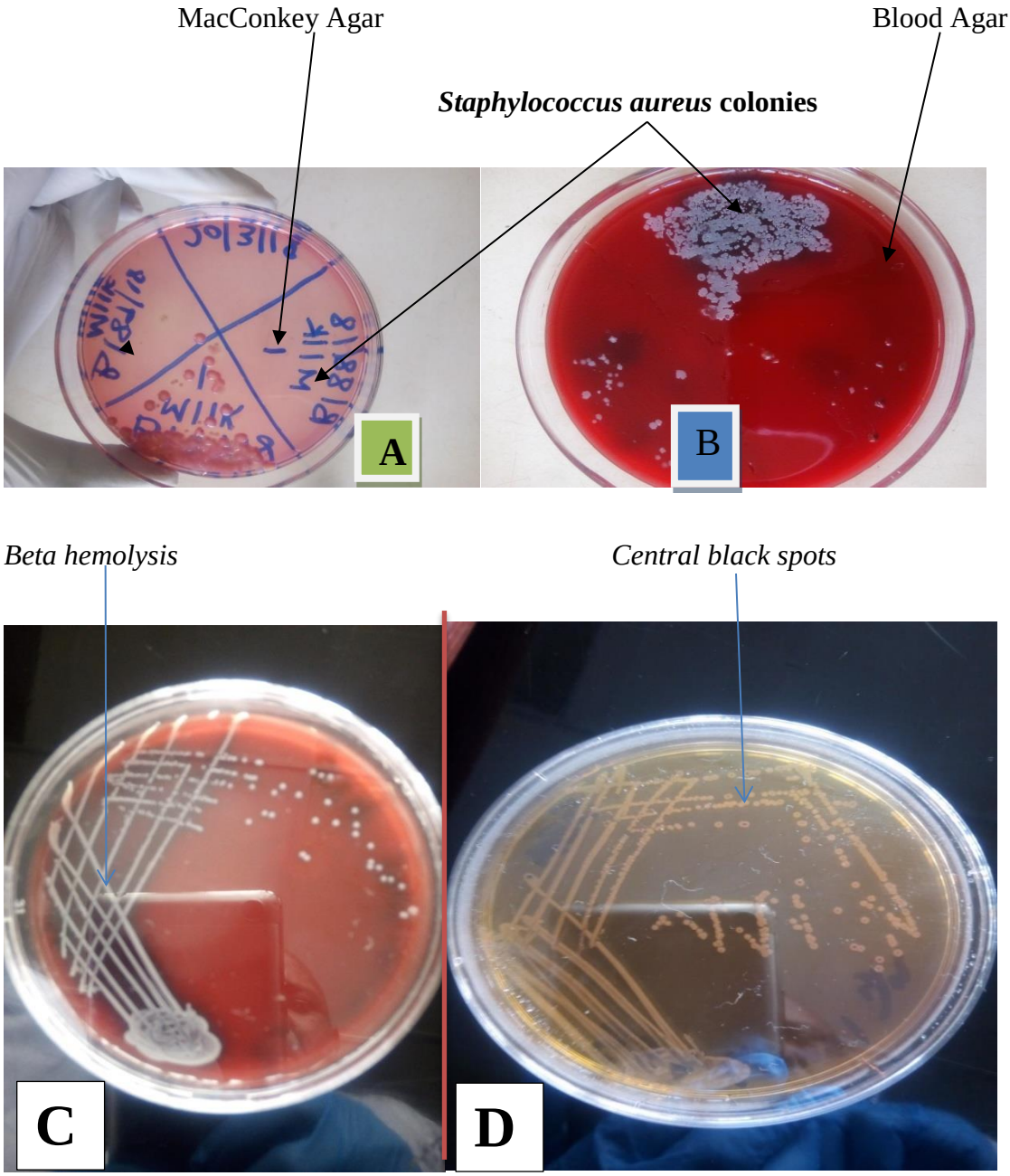

Figure 3.2: The Appearance of Colonial Morphology of *Staphylococcal* Bacteria on Primary Culture Media after 18-24 Hours at 37ºc

Key: (A). MacConkey agar (B) and 10% blood agar. (C) -The appearance of *beta hemolytic* bacteria on Blood Agar with hemolysis seen along lines of microbial streaking

and (D) -The appearance of Salmonella species on Salmonella Shigella Agar with distinct characteristic central black (H_2S) spots on isolated colonies

Adapted from: (RVIL Laboratories Eldoret, 2018)

3.9.2 Gram Status of Micro-Organisms Causing Mastitis in the Laboratory by Way of Gram Staining.

The culture media plates of samples and ATCC controls that exhibited typical growth of micro-organisms after 18-24 hours and 18-72 hours of incubation at $37^{O}C$ were described morphologically and stained for gram characterization of microbial pathogens (Mbindyo *et al*., 2020; Mureithi & Njuguna, 2016) (Appendix 15)

3.9.3 Identification of Different Types of Microbial Pathogens Causing Bovine Mastitis by Way of Biochemical Testing in the Laboratory

The aim of performing microbiological culture of micro-organisms is to eventually identify the different genera and species of microbial pathogens. The practical way to achieve complete identification of the microbial pathogens was to combine a variety of biochemical media and sugars, simply referred to as conventional testing and identification process. Several reagents and media that were used in these biochemical assays included - Simon's Citrate agar, Triple sugar iron Agar (TSI), Ethyl Methylene Blue agar (EMB), Indole agar, Urea agar, Oxidase reagent, Catalase reagent, Coagulase sera, Mannitol salt agar and many more as shown in Table 2.1 and Figure 3.4 (Cheesbrough, 2006; Quin, 2002).

The Gram-positive micro-organisms mainly implicated in causing mastitis belonged to several groups (1). *Staphylococcus* species and its related sub-genera – *Micrococcus* species (2). *Streptococcus* species and also its related sub-genera (3). *Corynebacterium* species and (4). *Bacillus* species. The groups comprise of cocci, coccobacili with few rods.

The gram-positive micro-organisms were first subjected to catalase and coagulase tests; catalase and coagulase positive micro-organisms were *Staphylococcus* species and its related sub-genera, though some *Staphylococcus* species were coagulase negative.

Catalase test was performed on a clean slide by putting a drop of catalase reagent (hydrogen peroxide) on the slide and introducing a discrete colony of suspect bacteria using non nichrome sterile wire loop on the H_2O_2 drop. If positive for *Staphylococcus* species*;* - bubbles formed (effervescence) almost immediately. To differentiate different species of *Staphylococcus* and related genera, coagulase slide test was performed and consequently colonies from blood agar were sub-cultured on to Manitol Salt Agar (MSA) and incubated at $37^{O}C$ for 18 – 24 hours aerobically. *Staphylococcus aureus* and *Staphylococcus saprophyticus* grew on MSA with fermentation, *Staphylococcus epidermidis* grew on MSA but without fermentation and *Micrococcus species* did not grow at all on MSA (Figure 3.4, D). To differentiate *Staphylococcus aureus* and *Staphylococcus saprophyticus*, the two were subjected to Novobiocin disc; S*taphylococcus aureus* was Novobiocin sensitive while *Staphylococcus saprophyticus* was Novobiocin resistant.

The catalase negative - gram positive micro-organisms were identified as members of the genus *Streptococcus or Enterococcus species*. *Streptococcus* species produced beta, and alpha hemolysis on Blood agar media unlike *Staphylococci* which are non-hemolytic. To identify *Streptococcus* species, they were subjected to pyrrolidonyl aminopeptidase (PYR) test; *Streptococcus pyogenes*, group C & G and *Enterococcus* species were PYR positive while *Streptococcus pneumonia*, *Viridan streptococcus* and other *Streptococci* were PYR negative. Further to differentiate between the PYR negative (*Streptococcus pneumonia and Viridan streptococcus* and other *Streptococci* optochin disc was used; *Streptococcus pneumonia* was optochin sensitive while *Viridan streptococcus* and other *Streptococci* were optochin resistant.

The most virulent catalase negative species implicated in bovine mastitis with Public Health implications were *Streptococcus agalactiae, Streptococcus uberis, Streptococcus*

lactis, Streptococcus fecalis and *Streptococcus dysgalactiae.* These led to many non-communicable diseases in humans (Mureithi and Njuguna, 2016).

Gram-negative bacterial rods were subjected to most other biochemical tests and Triple Sugar Iron (TSI) for identification. To identify *Escherichia coli* we performed Motility indole Test (MIT) – which was positive, *Escherichia coli* produced bright pink colonies on MacConkey agar – indicating lactose fermentation and it was ONPG positive. We sub-cultured *Escherichia coli* on EMB agar and incubated at 37^0C for 18-24 hours and it produced Green metallic sheen. On TSI *Escherichia coli* produced acidic (yellow) slope and butt with gas production indicated by cracks in TSI agar (Figure 3.4, A, B &C).

Citrobacter freundii was citrate and ONPG positive, Motility positive and fermented lactose though slowly by producing bright pink colonies on MacConkey agar. On TSI it was able to produce both acidic slope and butt with gas production. In identifying *Klebsiella pneumoniae* we observed that it was Voges Proskauer (VP), ONPG and Citrate positive but could utilize urea albeit slowly. It was lactose fermenter and produced both acidic slope and butt with gas production on TSI agar. *Serratia marcescens* colonies were VP, citrate, motility and ONPG positive. They were lactose fermenting colonies and produced alkaline (red) slope and acidic butt with little gas production.

Proteus vulgaris was successfully identified, it produced grey swarming colonies which were fast urease positive within 4 hours. Colonies also were positive on citrate agar and motility indole test (MIT) broth; however, they were non-lactose fermenter on MacConkey agar. They produced alkaline slant and acidic butt with both production of Hydrogen sulphide (H_2S) indicated by blackening in the media and gas on TSI agar. The study also isolated and identified *Pseudomonas aeruginosa* by subjecting colonies to oxidase test using filter papers dipped in oxidase reagent by smearing a discrete colony on it. *Pseudomonas aeruginosa* colonies produced strong purple to blue reaction, further the colonies were grey, medium to large and thick mucoid. They were non-lactose fermenting on MacConkey agar.

Incase micro-organisms were non-lactose-fermenters on MacConkey agar, had mucoid but discrete colonies on Blood agar and when they were sub-cultured on TSI agar they produced a reaction Acid/Acid/Gas/ H_2S (Figure 3.4, B), then *Salmonella* species were suspected. In this case, the colonies from the primary Blood agar were sub-cultured on *Salmonella-Shigella* Agar (SSA) and incubated at 37^0C for 18-24 hours for confirmation. *Salmonella* species grew on SSA with production of mucoid but discrete colonies with centric black spots (Figure 3.2, D), *Salmonella typhimurium* produced total blackening of TSI agar while *Salmonella paratyphi* produced partial blackening of TSI agar (Figure 3.4, B).

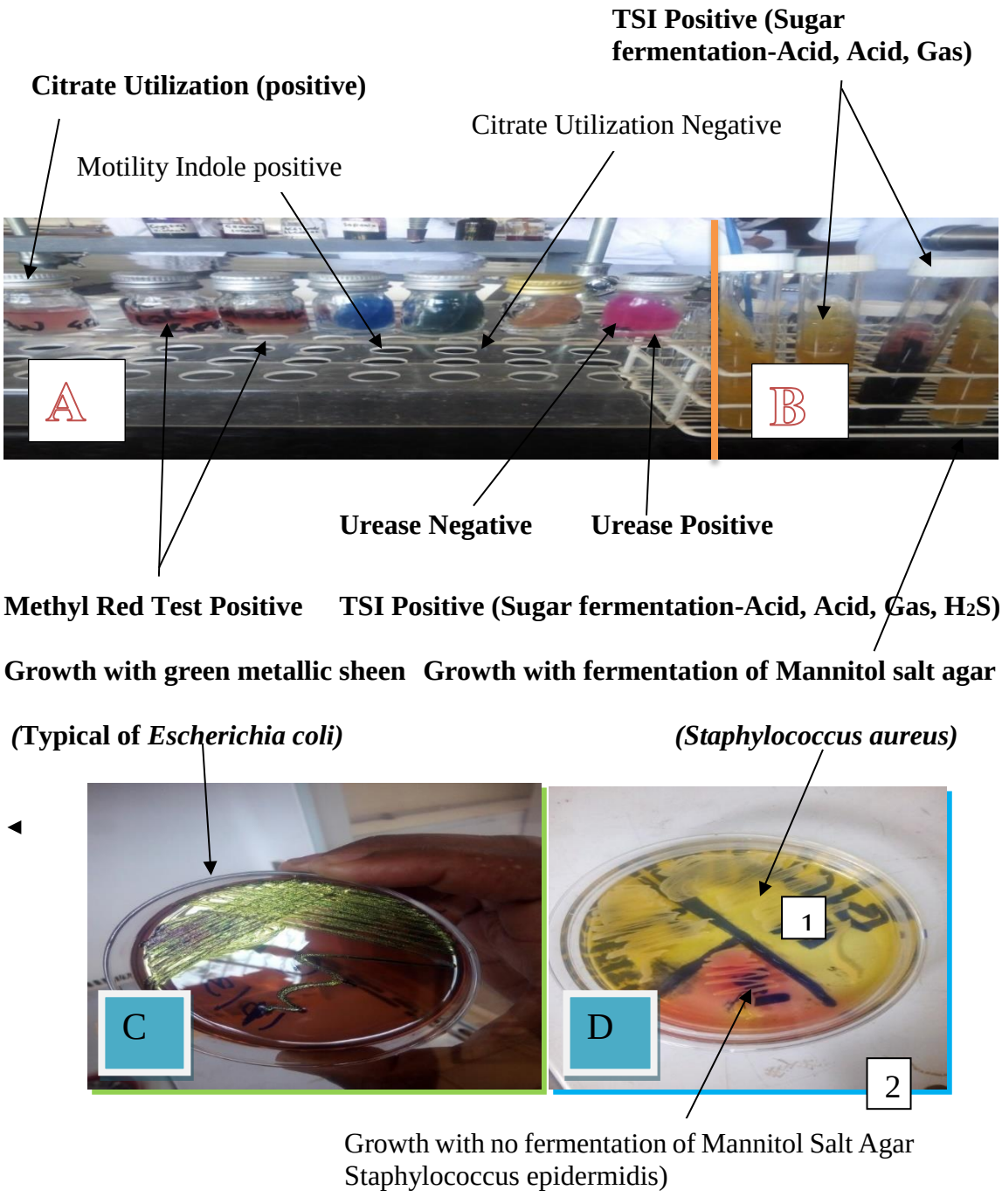

Figure 3.3: Biochemical Reactions of Microbial Pathogens on (A) Citrate Agar (B) TSI agar (C) Ethyl Methylene Blue (EMB) Agar and (D) Mannitol Salt Agar (MSA) D(1) Acid/Yellow - Fermentation D(2) Alkaline/Red - No Fermentation

Source: Adapted from RVIL Laboratories Eldoret, 2018

3.9.4 Determination of Susceptibility of the Microbial Pathogens to Antimicrobials Commonly Used for the Treatment of Mastitis in Dairy Cows

The Bauer-Kirby disc diffusion method of antimicrobial sensitivity testing as used by Mureithi and Byarugaba was adopted for this study (Mureithi & Njuguna, 2016; Byarugaba *et al.,* 2008). The octo-disc comprised of Ampicillin 25mcg, Tetracycline 25mcg, Cotrimoxazole 25mcg, Streptomycin 10mcg, Kanamycin 30mcg, Gentamicin 10mcg, Sulfamethoxazole 200mcg and Penicillin 30mcg (Himedia Laboratories Pvt. Ltd) (Figure 3.5; Appendix 10)

The test was performed on Mueller-Hinton agar culture plate taken from the refrigerator and let to acquire room temperature on the working bench. The Mueller-Hinton agar plate was labelled with a unique code of the sample and date. Using a sterile wire loop, distinct colonies from the primary culture plate were picked and emulsified in a 5ml test tube containing 3ml sterile physiological saline. They were gently shaked and mixed well to form a homogeneous turbidity of test micro-organisms that were equivalent to turbidity of 0.5 McFarland standard of micro-organisms (i.e. turbidity of 0.5 on a scale of McFarland). ATCC standard control micro-organisms were run alongside the test sample micro-organisms.

A sterile swab was dipped into test micro-organisms tube for 30 seconds, the swab was then withdrawn while pressing and rotating it against the wall of the test tube to tap off the excess micro-organism suspension; this avoided using excess inoculum. The surface of Mueller-Hinton agar was inoculated by gently swabbing the entire surface of the media; the plate was swabbed in three dimensions by rotating it at 60^{o} until it was uniformly covered. The Mueller-Hinton agar was left on working bench for a few seconds for the surface of the media to air dry. Once the surface was dry, the forceps was heat red hot in Bunsen burner flame to sterilize it and let to cool slightly, an impregnated antimicrobial susceptibility octo-disc was then picked and placed at the Centre of the plate. the disc was gently but firmly pressed against the media using the forceps to secure it so that it does not fall off during inverted incubation.

The susceptibility culture plates with sensitivity discs were incubated at 37^0C for 18-24 hours and the susceptibility test reactions read in accordance to Clinical and Laboratory Standards Institute standard criteria adapted from Himedia Laboratories Pvt. Ltd (Appendix X).

The zones of inhibition were measured using a Vanier-clipper and the interpretations were done in reference to the minimum inhibition zones (MIZs) diameters of Clinical and Laboratory Standards Institute (CLSI) standards illustrated in (Appendix 10).

The sensitive micro-organisms had a large clear zone of inhibition around the antimicrobial discs, resistant micro-organisms had a very small or no clear zone of inhibition around the antimicrobial disc further micro-organisms could grow up to the antimicrobial discs forming contact (Figure 3.5).

Table 3.2: Classes of Locally Available Antimicrobial Agents in Routine Use in this Study

S/No	Antibiotic disc	Class of antimicrobial agent
1	Ampicillin (AMP)	Beta-lactam
2	Tetracycline (TE)	Tetracycline
3	Co-trimoxazole (COT)	Potentiated sulphonamide
4	Streptomycin (S)	Aminoglycoside
5	Kanamycin (K)	Macrolide
6	Gentamycin (GEN)	Aminoglycoside
7	Sulfamethoxazole (SX)	Sulphonamide
8	Chloramphenicol (C)	Chloramphenicol

Source: Adopted from (RVIL Laboratories Eldoret, 2018)

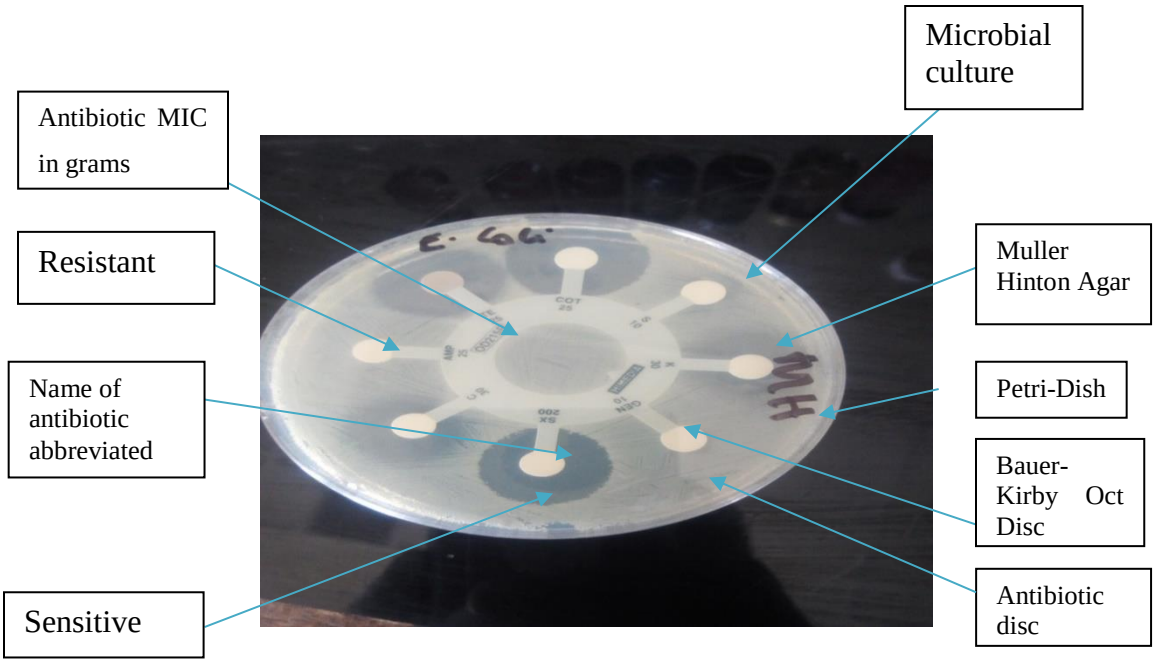

Figure 3.4: Microbial Pathogens Susceptibility against the Antimicrobials on Muller Hinton Agar

Source: Adapted from RVIL Laboratories Eldoret, 2018

3.9.5 Qualitative Determination of Presence of Antimicrobial Drug Residues in Raw Milk

A microbiological Modified culture method - Delvotest® was adopted for screening antimicrobial drug residues in milk in this study (Mohamed *et al*., 2020; Mahmoudi *et al*., 2014; Manafi & Rafat, 2011) The method is a qualitative broad-spectrum test for the detection of antimicrobial drug residues in raw milk. The test is based on growth inhibition of *Escherichia coli* (ATCC 25922). The principle behind tis is that living micro-organisms produce waste (excretory) products which result in pH changes of the environment in

which they inhabit. The pH change is indicated by colour change of bromocresol blue (pH indicator). Presence of antimicrobial agents will kill the *Escherichia coli* (ATCC 25922) hence no change in pH. *Escherichia coli* (ATCC 25922), produces an acid and causes bromocresol purple indicator to turn yellow. The presence of antimicrobial drug residues prevents acid formation and a purple or blue color persist. The minimum concentration of antimicrobials detected is in the range of 0.004 to 0.005 unit/ml. The test is AOAC approved at 0.007 unit/ml.

Escherichia coli (ATCC 25922) were picked using a sterile wire loop and streaked on culture media plate (preferably Blood agar or MacConkey agar plates), and incubated at 37^{o} c for 18-24 hours. Using a sterile wire loop, pure colony of the micro-organism was picked and inoculated in a trypticase soy broth and incubated at 37^{o}c for 18-24 hours. Using a sterile test tube and sterile droppers, equal volumes of the inoculum and milk sample were well mixed. 2-3 drops of bromocresol blue indicator were added and mix well using vortex mixer and incubated at 37^{o} c for up to 4 hours; the colour change was monitored and noted at intervals of 1 hour. Results: Purple/blue = Positive and

Yellow = Negative (Appendix 11)

3.9.6 Quantitative Determination of Presence of Antimicrobial Drug Residues In Raw Milk

A high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) methods for screening and determination of antimicrobial drug residues in raw milk were adopted from studies by Chowdhury *et al.* (2015) and Li *et.al*. (2013). The methods were sensitive and accurate for determination of antimicrobial drug residues in milk. HPLC MS/MS was successfully applied in the present study to determine; streptomycin, tetracycline, and the four penicillin (ampicillin, penicillin G, amoxicillin and penicillin V). The antimicrobial drug residues in milk were extracted from the milk samples using acetonitrile and water, cleaned with HLB solid-phase extraction cartridges, then

antimicrobial drug residuess detected by HPLC-MS/MS and quantified using external standard method.

HPLC MS/MS Procedure

The raw milk samples were prepared by thoroughly homogenizing them. Custom standard solution mixture with each chemical at 100µg/ml concentrations stored at – 20^{o}C in freezer was taken and allowed to thaw to room temperature (20^{o}C – 25^{o}C). Stock standard mixture of 1µg/ml per chemical was prepared by diluting to mark 200µl of custom standard of 20ml volumetric flask using acetonitrile. The prepared stock standard solution was then transferred into an amber-coloured vial with a teflon lined screw cap.

Sample Extraction and Cleanup

15g milk sample was weighed and put in a 50 ml centrifuge tube, 15ml solution of 1% acetic acid in acetonitrile weree added to each tube, capped and shaked (vortex) for 1 minute. Add 4g of sodium sulphate,1g of sodium chloride (Bond Elute extraction salt). Shake vigorously for 1 min and centrifuge at 5000rpm for 5 min at 4^{0}C. A 6ml aliquot of upper acetonitrile layer was transferred into 15ml PTFE centrifuge tube containing 50mg of PSA and 150mg C18CE and 900mg of anhydrous Na_2SO_4 (Bond Elute dispersive SPE>vet drugs in foods P/N5982-4950). Cap and vortex for 1 minute then centrifuge at 500 rpm for 5 min. 1ml extract was then transferred into another tube and dried by N_2 flow at 40^{0}C. Residues were redissolved into 1ml of MeOH/H_2O (1/9, V/V), filtered the residue through a 0.45µm membrane into a 2ml autosampler glass vial for LCMSMS analysis.

LC-MS/MS Analysis

By operating Agilent 1200 LCMSMS equipment using equipment's working instructions, the above extracts were consecutively subjected to calibration standards, quality control and matrix matched samples to qualitative and quantitative analysis as follows:

Vials were arranged in the autosampler and the batch developed sequentially starting with S0 for blanks followed by S1 - S5 calibration standards, QC1 quality control sample, test samples, QC2 quality control sample, and finally calibration standards S1 - S5. Then analysis method loaded (Appendix 12).

Calculations

The weighted linear regression of the calibration curve used for each targeted antimicrobial were to be greater or equal to 0.95 ($r^2 \geq 0.95$) for it to be used for the analysis. The concentration of each targeted antimicrobial drug residue was calculated from responses obtained from the calibration curve and scored in ug/ml.

3.10 Determination of Qualitative Data on Cow Characteristics and Management Practices Associated with Prevention and Control of Bovine mastitis

To collect qualitative data on cow characteristics and management practices associated with prevention and control of bovine mastitis, a semi-structured questionnaire was developed (Appendix 3). The questionnaire was used as an interview guide and was administered to respondents by principal investigator and/or research assistants in a face-to-face interview. The question was sub-divided into two sub-thematic parts (sections) as follows:

Part I: This part comprised the general information, background and demographic information of respondents and study subjects (cows). The general information captured included the County of resident, sub-county, administrative ward, location, sub-location and village. The respondent information that this part captured included - type of respondent, age, gender, marital status, education and occupation. The study subjects (cows) information included – age, breed, parity and lactation stage.

Part II: This part was meant to capture information on management practices adopted by farmers on their small-holder dairy farms. the information captured in this section

included – whether mastitis was a problem, knew signs and symptoms of mastitis, took samples to laboratory for mastitis diagnosis, had cow housing/milking crushes, frequency of cleaning cow housing/milking crushes, type of milking method, type of cow management system, type of floor of cow housing/milking crushes, cleaned of housing/milking crushes, duration of cleaning housing/milking crushes, udder hygiene and type of frequently used antimicrobial therapy.

The cow characteristics and type of management practices were analyzed in correlation with mastitis culture positive results to enable principal investigator draw conclusions (Table 4.10 and 4.11)

3.11 Data Management and Analysis

Data was analyzed using percentages, bivariate and multivariate regression models. The statistical analysis of Pearson Chi-square and Fisher's exact tests were performed; a p-value ($P< 0.05$) and at confidence interval of 95% was considered statistically significant. The results of the data analysis were presented using text, frequencies, percentages and tables

3.11.1 Data Processing

Data from the field on questionnaires were checked for validity and entered onto computer Microsoft excel spreadsheet by the principal investigator. Any inconsistences detected were corrected using the original data forms.

The data was then exported into statistical package for social scientists (SPSS) version 20 and analyzed. The results were presented using text, frequencies, percentages, tables and descriptive statistics.

3.12 Ethical Considerations

The study obtained requisite approvals before commencement of sample and data collection on small-holder dairy farms in Moiben and Kapseret sub-counties. Approval to collect, process and analyze animal samples in veterinary laboratories was obtained from the Ministry of Agriculture, Livestock, Fisheries and Co-operatives through Directorate of Veterinary Services Reference number (MOALF&I/SDL/DVS/GEN/VOL.1/57) (Appendix 5), Masinde Muliro University of Science and Technology ethics review committee approval number (MMUST/IERC/155/2021) (Appendix 6), National Commission for Science, Technology and Innovation (NACOSTI) research License number (NACOSTI/P/21/9459) (Appendix 8) and County Government of Uasin-Gishu approval of study Reference number (CDVS/UG/TRAINING/VOL.1/16) (Appendix 7).

The study incorporated rights of participants in all its research tools and protocols; right to privacy and confidentiality, right to voluntary participate or withdraw at will from the study without penalty. The study ensured coding of its participants and that their identities remained anonymous from the time of recruitment into the study up to the completion of the study. All information thus relating to study objects and participants remained anonymous and confidential. The Principal Investigator manipulated data to give meaning scientifically and professionally. Consequently, Principal Investigator determined findings and drew conclusions logically (Appendices 1 and 2).

The study also sought to collaborate with other stakeholders in the larger dairy sub-sector where necessary and practically applicable. These included the Central Veterinary Laboratories – Kabete, Kenya Dairy Board, the Regional Veterinary Investigation Laboratories (RVIL) Eldoret and Kericho, the County Director of Veterinary Services – Uasin-Gishu, the County Director of Livestock Production – Uasin-Gishu County, and all the farmers whose cows were sampled for study and diagnosis of bovine mastitis.

3.13 Dissemination and Utilization of Research Findings

The study findings are to be disseminated out for public consumption through public fora. These public fora are mainly to be organized by livestock and health sectors and both governmental and non-governmental organizations in collaboration with donors and well-wishers. The fora shall include seminars, workshops, conferences, public 'barazas', community field days and exhibitions. The findings have been published in relevant revered and peer reviewed scientific journals for wider coverage and sharing. We also hope to publish our findings in pamphlets and veterinary segment of local print media. Pamphlets shall be distributed free of charge to farmers during community field days and when farmers visit the RVIL Laboratories. We belief the sharing shall positively influence knowledge on the management and eventual control of bovine mastitis at the farm level in Uasin-Gishu County and similar areas.

CHAPTER FOUR

RESULTS

4.1 Background Characteristics of Study Respondents

The study collected demographic data on respondents as was and presented them in order to enable generalization of the findings of this study to other populations with similar background characteristics. This data included type of respondent, gender, age, marital status, level of education and occupation. Respondents were asked to indicate all these factors and they were summarized in a table (Table 4.1).

Table 4.1: Demographic Characteristics of Study Respondents

Characteristic	Category	Sub-county		Total Frequency	Percentage
		Moiben	Kapseret		
Type of respondent	Farmer/owner	39	23	62	76.5
	Farm Manager	4	7	11	13.6
	Caretaker	4	4	8	9.9
Gender	Male	36	15	51	63.0
	Female	11	19	30	37.0
Age (years)	21-30	1	4	5	6.2
	31-40	10	15	25	30.9
	41-50	18	6	24	29.6
	50+	18	9	27	33.3
Marital status	Married	46	31	77	95.1
	Single	1	3	4	4.9
Education	Informal	1	0	1	1.2
	Primary	7	2	9	1.2
	Secondary	14	8	22	27.2
	Tertiary	18	16	34	42.0
	University	7	8	15	18.5
Occupation	Farmer	31	12	43	53.1
	Business	4	0	4	4.9
	Formal job	2	5	7	8.6
	Caretaker	10	17	27	33.3

4.2 Background Characteristics of Dairy Cows

The dairy cows production on small-holder farms in Moiben and Kapseret sub-counties depicted that majority of farmers kept high milk producers - Friesian cows and Ayrshire

than they did for Jersey and indigenous Zebu which are known to produce least milk. Multiparous 2 and 3 cows aged between (5-8) and (>8) were most preferred by farmers as compared to primiparous young cows (Table 4.2).

Table 4.2: Characteristics of Dairy Cows

Characteristic	Category	Sub-county		Total frequency	Percentage
		Moiben	Kapseret		
Age (Years)	1-4	63	36	99	45.9
	5-8	60	42	102	47.2
	>8	11	4	15	6.9
Breed	Friesian	53	57	110	50.9
	Ayrshire	58	24	82	38.0
	Crossbreed	21	1	22	10.1
	Jersey	1	0	1	0.5
	Zebu	1	0	1	0.5
Parity	Primiparous 1	35	9	44	20.4
	Multiparous 2	34	21	55	25.5
	Multiparous 3	43	29	72	33.3
	Multiparous >3	22	23	45	20.8

4.3 The Incidence of Bovine Mastitis

4.3.1 Overall Incidence of Mastitis at Cow-Level in the Study Area

The overall incidence of mastitis was (48.2%), whereas the incidence was highest in Moiben sub-county than in Kapseret sub-county. Distribution of mastitis among the various wards in Moiben sub-county was less evenly spread with statistical significance of p-value ($p<0.005$) at 95% CI. While mastitis distribution in wards that comprised Kapseret sub-county depicted sporadic patterns and trends that showed no statistical significance (Table 4.3).

Table 4.3: The Incidence of Mastitis at Cow-Level in Moiben and Kapseret Sub-Counties

No.	Sub-county	Ward	Number Examined n=216	Mastitis culture results		Chi-square (x^2)	P-Value
				Positive (%)	Negative (%)		
1.	Moiben	Karuna	55	17(30.9)	38(69.1)	15.07	0.005
		Moiben	33	23(69.7)	10(30.3)		
		Tembelio	23	13(56.5)	10(43.5)		
		Sergoit	16	9(56.3)	7(43.8)		
		Kimumu	7	5(71.4)	2(28.6)		
Total			**134**	**67(31.0)**	**67(31.0)**		
2.	Kapseret	Kapseret	27	13(48.1)	14(51.9)	7.11	0.130
		Ngeria	24	12(50.0)	12(50.0)		
		Kipkenyo	12	8(66.7)	4(33.3)		
		Langas	11	2(18.2)	9(81.8)		
		Megun	8	2(25.0)	6(75.9)		
Total			**82**	**37(17.2)**	**45(20.8)**		
Grand Total			**216**	**104(48.2)**	**112(51.8)**		

4.3.2 Mastitis attributable to specific bacterial pathogens

Staphylococcal-mastitis was the predominant type of mastitis on small- holder dairy farms in this study area, this was followed by *Coli*-mastitis and *Citrobacter*-mastitis. The least common occurring form of mastitis was *Pseudomonal*-mastitis and *Pneumococcal*-mastitis determined at (0.9%) each (Table 4.4).

Table 4.4: Incidence of Mastitis Attributable to Specific Bacterial Pathogens at Cow-Level

Type of mastitis	Sub-county		Total number of mastitis isolates	Mastitis Positivity (%)
	Moiben isolates	Kapseret isolates		
Staphylococcal-mastitis	36	30	66	30.6
Streptococcal-mastitis	3	2	5	2.3
Micrococcal-mastitis	4	1	5	2.3
Coli-mastitis	9	2	11	5.1
Citrobacter-mastitis	5	1	6	2.8
Serratial-mastitis	4	0	4	1.9
Proteus-mastitis	3	0	3	1.4
Pseudomonal-mastitis	1	1	2	0.9
Pneumococcal-mastitis	2	0	2	0.9
Total	**67**	**37**	**104**	**48.2**

4.4 Profile of bacterial pathogens implicated in causing mastitis.

4.4.1 Microbial pathogens causing mastitis on small-holder farms

A total of ten bacterial pathogens were isolated; *Staphylococcus epidermidis* was the most predominant bacteria isolated followed by *Staphylococcus aureus* and *Escherichia coli*. The least isolated bacteria were *Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* Gram-positive bacteria attributable to causing contagious mastitis in this study area were predominant as compared to most gram-negative coliforms implicated in causation of environmental mastitis (Table 4.5).

Table 4.5: Microbial Pathogens Causing Mastitis in Moiben and Kapseret Sub-Counties

Bacterial isolate	Sub-counties		Total Bacterial isolate	Incidence (%)
	Moiben isolate	Kapseret isolate		
Gram positive				
Staphylococcus epidermidis	23	23	46	21.3
Staphylococcus aureus	15	5	20	9.3
Streptococcus species	4	1	5	2.3
Micrococcus species	3	2	5	2.3
Total gram-positive isolates	**45**	**31**	**76**	**35.2**
Gram negative				
Escherichia coli	9	2	11	5.1
Citrobacter freundii	6	0	6	2.8
Serratia marcescens	4	0	4	1.9
Proteus vulgaris	1	2	3	1.4
Pseudomonas aeruginosa	1	1	2	0.9
Klebsiella pneumoniae	2	0	2	0.9
Total gram-negative isolates	**23**	5	28	13.0
Total bacterial isolates	**68**	**36**	**104**	**48.2**
No Growth obtained	**66**	**46**	**112**	**51.8**
Grand Total	**134**	**82**	**216**	**100.0**

4.5 Antimicrobial susceptibility testing of the microbial pathogens causing mastitis.

On overall, the results obtained in this study showed that cumulative resistance of all bacterial isolates against antimicrobial agents was high against Ampicillin, Tetracycline, Cotrimoxazole, Streptomycin, Sulfamethoxazole and Penicillin. There was low resistance against both Kanamycin and Gentamicin. The findings on Kanamycin and Gentamicin were statistically significant ($P<0.0004$), meaning they were efficacious against majority of isolated bacterial pathogens. (Table 4.6).

Table 4.6: Antimicrobial Susceptibility Testing of Bacterial Isolates Causing Mastitis

Bacterial isolates	Total number isolated	Antimicrobial Resistance							
		Amp. (%)	Te. (%)	Cot. (%)	Strep. (%)	Kan. (%)	Gen. (%)	Sx. (%)	Pen. (%)
Gram positive									
Staphylococcus epidermidis	46	35 (76.1)	19 (41.3)	17 (37.0)	21 (45.7)	15 (32.6)	2 (4.4)	21 (45.7)	37 (80.4)
Staphylococcus aureus	20	16 (80.0)	11 (55.0)	18 (90.0)	14 (70.0)	7 (35.0)	1 (5.0)	16 (80.0)	18 (90.0)
Streptococcus species	5	4 (80.0)	3 (60.0)	3 (60.0)	3 (60.0)	2 (40.0)	0 (0.0)	4 (80.0)	5 (100.0)
Micrococcus species	5	2 (40.0)	0 (0.0)	1 (20.0)	1 (20.0	1 (20.0)	0 (0.0)	1 (20.0)	2 (40.0)
Gram negative									
Escherichia coli	11	11 (100.0)	10 (90.9)	11 (100.0)	8 (72.7)	3 (27.3)	1 (9.1)	9 (81.8)	7 (63.6)
Citrobacter freundii	6	6 (100.0)	3 (50.0)	2 (33.3)	5 (83.3)	3 (50.0)	1 (16.7)	4 (66.7)	4 (66.7)
Serratia marcescens	4	4 (100.0)	3 (75.0)	0 (0.0)	4 (100.0)	1 (25.0)	1 (25.0)	3 (75.0)	4 (100.0)
Proteus vulgaris	3	3 (100.0)	3 (100.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	3 (100.0)
Klebsiella pneumoniae	2	2 (100.0)	1 (50.0)	2 (100.0)	1 (50.0)	1 (50.0)	0 (0.0)	2 (100.0)	2 (100.0)
Pseudomonas aeruginosa	2	2 (100.0)	1 (50.0)	1 (50.0)	1 (50.0)	2 (100.0)	0 (0.0)	0 (0.0)	2 (100.0)
No Growth	112	0	0	0	0	0	0	0	0
Cumulative resistance		**85 (81.7)**	**54 (51.9)**	**55 (52.9)**	**61 (58.7)**	**35 (33.7)**	**6 (5.8)**	**63 (60.6)**	**84 (80.8)**
Cumulative sensitivity		**19 (18.3)**	**50 (48.1)**	**49 (47.1)**	**43 (41.3)**	**69 (66.3)**	**98 (94.2)**	**41 (39.4)**	**20 (19.2)**
Z-Score		**-6.4718**	**-0.3922**	**-0.5883**	**-1.7650**	**3.334**	**9.0213**	**-2.1573**	**-6.2757**
P-Value		**1.0000**	**0.6526**	**0.7219**	**0.9612**	**0.0004**	**0.0001**	**0.9845**	**1.0000**

Key: Amp. – Ampicillin, Te. – Tetracycline, Cot. -Cotrimoxazole, Strep. – Streptomycin, Kan. - Kanamycin, Gen. – Gentamicin, Sx. – Sulfamethoxazole and Pen. – Penicillin

4.6 Antimicrobial Drug Residues in Raw Milk Used for Human Consumption

4.6.1 Qualitative Presence of Antimicrobial Drug Residues in Raw Milk

The overall occurrence of antimicrobial drug residues in milk used for human consumption on dairy farms in Moiben and Kapseret was (6.9%) with high occurrence in Kapseret than in Moiben. Furthermore, the overall distribution of antimicrobial drug residues in all the wards in the present study area depicted sporadic trends and patterns where wards like Karuna, Moiben, Tembelio, Kimumu, Kapseret, Ngeria and Langas

reported positive antimicrobial drug residue results while wards which include Sergoit, Kipkenyo and Megun had negative AMDR results (Table 4.7).

Table 4.7: Qualitative Antimicrobial Drug Residues in Raw Milk

No.	Sub-county	Ward	Number Examined n=216	Antimicrobial drug residues	
				Positive (%)	Negative (%)
1.	Moiben	Karuna	55	2(3.6)	53(96.4)
		Moiben	33	1(3.0)	32(97.0)
		Tembelio	23	1(4.4)	22(95.6)
		Sergoit	16	0(0.0)	16(100.0)
		Kimumu	7	2(28.6)	5(71.4)
Total			**134**	**6(4.5)**	**128(95.5)**
2.	Kapseret	Kapseret	27	4(14.8)	23(85.2)
		Ngeria	24	1(4.2)	23(95.8)
		Kipkenyo	12	0(0.0)	12(100.0)
		Langas	11	4(36.4)	7(63.6)
		Megun	8	0(0.0)	8(100.0)
Total			**82**	**9(11.0)**	**73(89.0)**
Grand Total			**216**	**15(6.9)**	**201(93.1)**

4.6.2 Quantitative Presence of Antimicrobial Drug Residues in Raw Milk

Three classes of antimicrobial agents were identified and determined above Safe Maximum Residue Levels (SMRL), vis Beta-lactams (Penicillin), Tetracyclines (Tetracycline) and Aminoglycosides (Streptomycin). However, three penicillin from Kapseret and two from Moiben sub-counties were detected and identified but below SMRL (Table 4.8).

Table 4.8: Quantitative Presence of Antimicrobial Drug Residues in Raw Milk

Class of Antimicrobial agent	Type of Antimicrobial Residues	Occurrence of Antimicrobial Residues in Sub-counties		SMRLs Standard controls (µg/ml)	Quantity of residues in milk (µg/ml)
		Moiben	Kapseret		
Beta-lactam	Penicillin	0	1	4.0	15.9
	Penicillin	1	0	4.0	12.3
	Penicillin	0	1	4.0	3.7
	Penicillin	0	1	4.0	2.8
	Penicillin	1	0	4.0	2.5
	Penicillin	0	1	4.0	2.4
	Penicillin	1	0	4.0	2.1
Tetracycline	Tetracycline	1	0	3.1	21.8
	Tetracycline	1	0	3.1	2.9
	Tetracycline	0	1	3.1	1.6
	Tetracycline	0	1	3.1	7.3
Aminoglycoside	Streptomycin	1	0	0.2	11.0
	Streptomycin	0	1	0.2	9.6
	Streptomycin	0	1	0.2	9.2
	Streptomycin	0	1	0.2	4.9
Total	**15(6.9%)**	**6(2.8%)**	**9(4.1%)**	**-**	**-**

4.6.3 Correlation of Antimicrobial Drug Residues with Bovine Mastitis

Overally, antimicrobial drug residues were detected and determined in (6.9%) cows of which (1.0%) was determined in mastitis positive cow infected with gram-negative micro-organism - *Proteus vulgaris*. Most of AMDR were determined in mastitis negative cows. Penicillin was the most determined antimicrobial drug residue in raw cow milk on small-holder farms in Uasin-Gishu (Table 4.9).

Table 4.9: Correlation between Antimicrobial Drug Residues with Mastitis Culture Results and Microbial Pathogens Causing Mastitis

Parameter/variable	Cows in Sub-counties		Total cows in study area (%)	Antimicrobial residue		Fisher's exact test
	Moiben	Kapseret		Positive (%)	Negative (%)	(P-value)
Mastitis culture results						
Positive	68	36	104(48.2)	1(1.0)	103(99.0)	0.001
Negative	66	46	112(51.8)	14(12.5)	98(87.5)	
Total	**134**	**82**	**216(100.0)**	**15(6.9)**	**201(93.1)**	**-**
Microbial pathogens of mastitis						
Gram-positive	45	31	76(35.2)	0(0.0)	26(100.0)	0.999
Gram-negative	23	5	28(13.0)	1(1.3)	77(98.7)	
Total	**68**	**36**	**104(48.2)**	**1(0.5)**	**103(47.7)**	
Type of antimicrobial residue detected						
Penicillin	3	4	7(3.2)	7(46.6)	-	-
Tetracycline	2	2	4(1.9)	4(26.7)	-	
Streptomycin	1	3	4(1.9)	4(26.7)	-	
Total	**6(2.8)**	**9(4.1)**	**15(6.9)**	**15(6.9)**	**-**	**-**

4.6 Farm Management Practices

4.7.1 Management practices at herd-level

The results illustrated that majority of respondents knew presentation signs and symptoms of mastitis, similarly, most of respondents did not find mastitis as a problem on their farms. Equally, majority of farmers did not take samples of their sick cows to the laboratory for testing and confirmation of mastitis infection prior to treatment; no wonder, mastitis infection among these category of farmers remained high. Further, most of respondents had cow housing and milking crushes, but majority of them did not disinfect them regularly, surprisingly, this led to acute proliferation of mastitis (Table 4.10).

Table 4.10: Correlation between Mastitis Positivity and Management Practices on Farms

Parameter/ Variable	Sub-county		Number of farms, n=81	Mastitis culture results		P-value (x^2)
	Moiben, n=47	Kapseret, n=34		Positive (%)	Negative (%)	
Is mastitis a problem?						
Yes	4	29	33(40.7%)	22(66.7)	11(33.3)	0.516
No	43	5	48(59.3%)	33(68.8)	15(31.2)	
Know signs and symptoms of mastitis?						
Yes	47	32	79(97.5%)	53(67.1)	26(32.9)	0.458
No	0	2	2(2.5%)	2(100.0)	0(0.0)	
Take samples to laboratory?						
Yes	15	2	17(21.0%)	9(52.9)	8(47.1)	0.117
No	32	32	64(79.0%)	46(71.9)	18(28.1)	
Action taken on suspecting mastitis?						
Call Vet. Officer	47	34	81(100.0%)	55(67.9)	26(32.1)	-
Treat myself	0	0	0(0.0%)	-	-	
Have cow housing/shade?						
Yes	39	34	73(90.1%)	49(67.1)	24(32.9)	0.495
No	8	0	8(9.9%)	6(75.0)	2(25.0)	
Do you have milking crush?						
Yes	39	34	73(90.1%)	48(65.8)	25(34.2)	0.202
No	8	0	8(9.9%)	7(87.5)	1(12.5)	
Disinfect premises?						
Yes	0	2	2(2.5%)	1(50.0)	1(50.0)	0.900
No	47	32	79(97.5%)	54(68.4)	25(31.3)	
Type of milking method?						
Hand	47	24	71(87.7%)	48(67.6)	23(32.4)	0.595
Machine	0	10	10(12.3%)	7(70.0)	3(30.0)	
Milking frequency per day?						
Once	0	0	0(0.0%)	-	-	-
Twice	47	34	81(100.0%)	55(67.9)	26(32.1)	
Clean udder with water before and after milking using one towel?						
Yes	45	34	79(97.5%)	53(67.1)	26(32.9)	0.458
No	2	0	2(2.5%)	2(100.0)	0(0.0)	
Type of farm management system?						
Extensive	8	16	24(29.6%)	14(58.3)	10(41.7)	0.125
Intensive	39	18	57(70.4%)	41(71.9)	16(28.1)	
Type of housing/crush floor?						
Earth	13	5	18(22.2%)	9(50.0)	9(50.0)	0.109
Concrete	34	29	63(77.8%)	46(73.0)	17(27.0)	
Maintain cleanliness of housing/crushes?						
Yes	20	24	44(54.3%)	21(63.6)	12(36.4)	0.112
No	27	10	37(45.7%)	28(75.7)	9(24.3)	
Duration of cleaning housing/crushes?						
Frequently	21	22	43(53.1%)	30(69.8)	13(30.2)	0.061
Infrequently	26	12	38(46.9 %)	25(65.7)	13(34.3)	
Maintain good udder hygiene?						
Yes - Satisfactory	15	21	36(44.4%)	25(69.4)	11(30.6)	0.847
No - unsatisfactory	32	13	45(55.6%)	30(66.7)	15(33.3)	

4.7.2 Type of management practices at cow-level based on age, breed and parity.

Majority of the farmers/cow owners preferred breeding cows aged between (3-4) and (4-5) years old. Mastitis infection was highest among older cows between (7-8) and (>8) years old. Majority of farmers embraced high milk producing exotic cows - Friesian and Ayrshire breeds of cows, however it was statistically significant ($p<0.05$) to keep crossbreed and Ayrshire cows than Friesian cows, very few farmers kept Jersey and indigenous zebu cows. More farmers reared multi-parous cows, the burden of mastitis was as well higher in the multi-parous cows (Table 4.11)

Table 4.11: Correlation between Mastitis Positivity and Age, Breed and Parity

Parameter	Sub-county		Total Number of cows (n=216)	Mastitis culture results		Chi-square (x^2)	P-Value
	Moiben (n=134)	Kapsere t (n=82)		Positive (%)	Negative (%)		
Age (Years)							
1-4	63	36	99(45.9)	34(34.3)	65(65.7)	5.895	0.05
5-8	60	42	102(47.2)	63(61.8)	39(38.2)		
>8	11	4	15(6.9)	9(60.0)	6(40.0)		
Breed							
Friesian	53	57	110(50.9)	61(55.5)	49(44.5)	5.915	0.05
Ayrshire	58	24	82(37.9)	33(40.2)	49(59.8)		
Crossbreed	21	1	22(10.2)	10(45.5)	12(54.5)		
Jersey	1	0	1(0.5)	0(0.0)	1(100.0)		
Zebu	1	0	1(0.5)	0(0.0)	1(100.0)		
Parity							
Primiparous 1	35	9	44(20.4)	16(36.4)	28(63.6)	3.074	0.08
Multiparous 2	34	21	55(25.5)	25(45.5)	30(54.5)		
Multiparous 3	43	29	72(33.3)	41(56.9)	31(43.1)		
Multiparous 3+	22	23	45(20.8)	22(48.9)	23(51.1)		

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Mastitis is a multi-etiologic disease that is caused by bacteria, fungai, mycoplasma and algae (Mbindyo *et al*., 2020; Zadoks *et al.*, 2011; Jones *et al.,* 2010). Over 135 microbial pathogens were reported to cause mastitis; (Hawari *et al.*, 2014), these pathogens comprise *Staphylococcus* species, *Streptococcus* species, *Klebsiella* species, *Escherichia coli, Proteus* species, *Pseudomonas aeruginosa, Corynebacterium* species, *Candida albicans, Aerobacter* species and many others (Mbindyo *et al*., 2020; Zadoks *et al*., 2011). In a sustained effort to manage the proliferation of the disease, antimicrobial medicines have been overwhelmingly and indiscriminately used. The overuse of antimicrobials led to adverse public health effects in humans (Awandkar *et al*., 2013) and economic losses in the dairy sub-sector globally (León-Galván *et al.,* 2015; KDB 2017; Annual report; Varshney *et al.,* 2004). These adverse public health effects included AMR rendering antimicrobial therapy less efficacious (Uhlemann *et al.*, 2014; Awandkar *et al*., 2013; Botrel *et al.*, 2010), AMDR compromising quality of human food chain especially milk and other animal products (Chowdhury *et al*., 2015; Halasa *et al*., 2007; Swinkels *et al*., 2005), and development of bacteria resistant genes to commonly used antimicrobials (Mohamed *et al.*, 2020). Therefore, the aim of this study was to determine incidence of bovine mastitis, identify bacterial pathogens implicated in causation of mastitis, determine Antimicrobial Resistance, establish AMDR in raw milk and determine cow management practices on small-holder dairy farms in Moiben and Kapseret Sub-counties - Uasin-Gishu, Kenya. The findings in the current study were discussed in relation to findings reported by other similar studies locally and globally.

In the present study, overall bovine mastitis incidence in Moiben and Kapseret study area was (48.2%) (Table 4.3). These results were in agreement with those documented in similar studies carried out in Rwanda (50.4%) and Ethiopia (52.3%) respectively

(Mpatswenumugabo *et al*., 2017; Sarba & Tola, 2017). These results however, sharply differed with the findings of (Mureithi & Njuguna, 2016; Abebe *et al*., 2016**;** Byarugaba *et al*., 2008) in Thika Kenya, Southern Herrhage District of Ethiopia and western Uganda respectively; - The trio reported elevated mastitis incidences of **(**62.6%), **(**61.3%) and (64.0%) at cow level respectively. The high incidence in the three East African countries could be attributable to poor animal husbandry practices and the (CMT) screening method that they used which is lower in specificity and sensitivity compared to microbiological culture method which we used and is superior (Abebe *et al*., 2016). Low mastitis, however, was reported in Zimbabwe at **(**21.1%) (Katsande *et al*., 2009). The implication of the findings in our current study is that the economic losses due to veterinary management costs were likely to increase among small-holder farmers.

The epidemiological distribution of mastitis in the two sub-counties was (31.0%) in Moiben and (17.2%) in Kapseret (Table 4.3). Majority of the farmers in Moiben practiced intensive farming system unlike majority of farmers in Kapseret who practiced extensive farming system. The incidence of mastitis was reported high among cows on intensive farming system. The high incidence was due to poor cattle housing, haphazard milking practices and low udder hygiene (Mpatswenumugabo *et al*., 2017). There was no significant difference in the distribution of mastitis between Moiben and Kapseret sub-counties ($P < 0.486$, CI = 95%). However, the difference in disease distribution among wards in Moiben sub-county were statistically significant ($P< 0.005$, CI = 95%) unlike the distribution in wards in Kapseret sub-county ($P< 0.130$, CI = 95%). In Moiben sub-county incidence was high in Kimumu ward (71.4%) and Moiben ward (69.7%) but low in Karuna ward (30.9%), while in Kapseret sub-county the frequency of distribution was almost ceven (Table 4.3).

Out of the overall mastitis incidence (48.2%) in Moiben and Kapseret study area, *Staphylococcal*-mastitis attributable to *Staphylococcus* species was found to have predominant occurrence (30.6%), this was followed by *Coli*-mastitis at (5.1%), *Citrobacter*-mastitis *at* (2.8%), *Streptococcal*-mastitis and *Micrococcal*-mastitis at (2.3%) each. Other different types of bacterial mastitis reported low incidence of less than (2.0%)

(Table 4.4). These results were comparable to similar studies in the region, for instance, in Kiambu County the incidence of *Staphylococcal*-mastitis was (31.7%) (Odongo *et al.*, 2012); in the two studies, microbiological culture method was used to determine incidence of mastitis. However, in Zimbabwe the incidence of *Staphylococcal*-mastitis was (43.9%), *Coli*-mastitis at (21.2%) and *Pneumococcal*-mastitis (15.5%) (Katsande *et al*., 2009). In Rwanda *Staphylococcal* -mastitis was equally high at (51.5%) (Mpatswenumugabo *et al.*, 2017), these results were higher than our findings because of the higher sample size, sampling during long rainy season, low udder hygiene, lack of teat therapy, in our study udder hygiene was moderately good. In sharp contrast, Katsande *et al*., (2009) recorded a low incidence of environmental *Streptococcal*-mastitis (1.6%) as compared to ours (2.3%), the difference in incidence being brought about by the variance in animal husbandry practices on the two farms. Further, contrasting findings were documented in Finland where contagious bovine mastitis attributable to *Staphylococcus* species was as low as (17.2%) and environmental bovine mastitis attributable to coli-forms was as high as (46.0%) (Thompson-Crispi *et al*., 2013). This was attributable to the fact that our study population comprised a mixture of exotic cows, crossbreeds and indigenous cows as opposed to Thompson-Crispi study which involved Holstein Friesian cows only, which are reported to be more vulnerable to environmental pathogens than other breeds of dairy cows (Thompson-Crispi *et al*., 2013).

Ten pathogenic bacteria known to cause mastitis were isolated and implicated in causation of mastitis in Moiben and Kapseret study area, *Staphylococcus epidermidis* was predominant at (21.3%), this was followed by *Staphylococcus aureus* at (9.3%), *Escherichia coli* at (5.1%), *Citrobacter freundii* at (2.8%), *Streptococcus* species and *Micrococcus* species at (2.3%) each. Other different types of bacterial pathogens reported low infection of less than (2.0%) (Table 4.5). The isolation of bacterial pathogens in the present study depicted seasonality with exponential increase during rainy season (Appendix 12 and 13) these findings were in tandem with results of Biffa in Ethiopia who also documented seasonality in the occurrence of bacterial isolates (Biffa *et al.*, 2005). Further, these results were comparable to similar studies by Vakkamäki in Finland who

enumerated the incidence of *Staphylococcus epidermidis* as (21.0%) and *Escherichia coli* 5.0%. (Vakkamäki *et al*., 2017). Zeryehun & Abera in an epidemiological study in Ethiopia, as well isolated *Micrococcus* species at (2.1%) (Zeryehun & Abera, 2017); in the three studies, the results were comparable due to similarity in cow environmental conditions and microbiological culture method used to determine occurrence of mastitis. However, high incidence of *Staphylococcus aureus* at (43.9%) was reported in Zimbabwe, this was followed by *Escherichia* c*oli* at (21.2%) and *Klebsiella pneumoniae at* (15.5%) (Katsande *et al*., 2009). In Rwanda *Staphylococcal* species was equally high at (51.5%) and *Streptococcus* species (10.3%) (Mpatswenumugabo *et al*., 2017), in southern Ethiopia *Staphylococcus aureus* was (29.2%), *Streptococcus* species was (12.5%) and *Escherichia* c*oli* (11.4%) (Adane *et al*., 2012). In Kajiado Kenya Mbindyo reported *Streptococcus* species at (22.2%) and *Pseudomonas aeruginosa* at (5.1%) (Mbindyo *et al.,* 2020) and in another study in Kabete area of Kiambu County Kenya, Odongo *et al.,* (2012) reported still high incidence of mastitis causing microbial pathogens as; *Staphylococcus aureus* (31.7%), *Escherichia coli* (17.2%), *Streptococcus* species (10.3%), *Klebsiella* species (9.7%), *Pseudomonas aeruginosa* (7.6%), (Odongo *et al.,* 2012). These results were higher than our findings because of low udder hygiene and lack of teat therapy, in present study udder hygiene was moderate. In sharp contrast, low incidence was recorded in Iowa state of USA, where *Serratia marcescens* and *Streptococcus* species were (1.0%) each (Kuehn *et al*., 2013). Another low occurrence was also documented by Katsande *et al*. (2009) in Zimbabwe where environmental *Streptococcus* species was (1.6%) and in Thika Kenya, a study by Mahlangu reported *Streptococcus* species *at* (1.2%) and *Micrococcus* species as low as (1.0%) (Mahlangu *et al*., 2018). The difference in occurrence being brought about by the variance in animal husbandry practices on dairy farms, our farms reported sporadically moderate hygiene and sanitation. In a study in Ontario Canada, *Escherichia coli* was the most predominant pathogen, isolated at (29.9%), followed by *Staphylococcus aureus* (22.2%) and *Streptococcus* species (16.2%) (Thompson-Crispi *et al.*, 2013). These results being due to low cow housing floor hygiene and sanitation which favoured environmental pathogens (Thompson-Crispi *et al.,* 2013) as compared to moderately good environmental hygiene and sanitation in our case. The findings in the

present study showed that contagious mastitis attributable to *Staphylococci* bacteria were predominantly the main cause of mastitis, strategies to manage the predominant pathogens should be instituted promptly.

Microbial pathogens demonstrated increased antimicrobial resistance to commonly used antimicrobials. The cumulative antimicrobial resistance for Ampicillin was scored at (81.7%), Tetracycline (51.9%), Cotrimoxazole (52.9%), Streptomycin (58.7%), Sulfamethoxazole (60.6%) and Penicillin (80.8%). Kanamycin and Gentamycin produced high cumulative sensitivity - (66.3%) and (94.2%) respectively;- these sensitivity results were statistically significant at P-value ($p<0.0004$) (Table 4.6). These findings were in concurrence with the findings of similar studies in China and Brazil. In both studies, the Principal Investigators found out that antimicrobial resistance to penicillin and ampicillin was (77.3%) and tetracycline (60.0%) (Jian-Ping *et al*., 2009); and ampicillin (100.0%), tetracycline (96.7%) and streptomycin (80.0%) (Freitas *et al.,* 2018). These high Antimicrobial Resistance, were due to indiscriminate and haphazard use of antimicrobial agents leading to reduced antimicrobial efficacy. In essence this underscored the importance of *in-vitro* antimicrobial susceptibility testing of frequently used antimicrobial medicines before their applications *in-vivo* to guard against development of antimicrobial resistance (Thompson-Crisp*i et al.*, 2012; Silva *et al*., 2005). Slightly low antimicrobial resistance was registered in a study by Messele in Ethiopia, antimicrobial resistance against ampicillin was (68.7%), sulfamethoxazole-trimethoprim (50.0%) and streptomycin (25.0%), the low resistance was due to reported low incidence of mastitis and judicious use of antimicrobial therapy (Messele *et al.*, 2019).

Specific bacterial isolates also registered high antimicrobial resistance, for instance resistance against ampicillin was (100.0%) for all gram-negative isolates vis *Citrobacter freundii, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris. Escherichia coli* and *Klebsiella pneumoniae* were (100.0%) resistant to cotrimoxazole while *Klebsiella pneumoniae* and *Proteus vulgaris* registered (100.0%) multiple drug resistance against sulfamethoxazole and penicillin. However, all microbial isolates showed low resistance of less than (<50.0%) against Kanamycin and Gentamicin, further interesting to note is

that *Micrococcus* species – produced a low antimicrobial resistance of <40.0% to all antimicrobial agents (Table 4.6). In contrast to current study, Omwenga in Marsabit, reported low *Staphylococcus aureus* resistance against ampicillin at (37.0%), tetracycline (51.0%) and Kanamycin (16.0%). in the same study in Isiolo, *Staphylococcus aureus* was resistant against ampicillin (64.0%) and Kanamycin (5.0%) (Omwenga *et al*., 2021). In our study high antimicrobial resistance was attributable to prolonged and haphazard use of antimicrobials and by the fact that over (90.0%) of study cows were exotic and crossbreed cows unlike in the Omwenga study where all cows were indigenous (boran and zebu) cows. Indigenous cows are known to be hardy and resistant to mastitis unlike exotic breeds which are more vulnerable (Omwenga *et al*., 2021). These findings actually imply that mastitis can be highly prevalent, unless farmers on small-holder dairy farms in Uasin-Gishu practice improved animal husbandry by maintaining good cow udder hygiene, judicious use of antimicrobials informed by laboratory results and regular teat dipping.

The overall occurrence of antimicrobial drug residues in milk used for human consumption on dairy farms in Moiben and Kapseret was (6.9%) with occurrence in Moiben at (4.5%) and in Kapseret at (11.0%). Furthermore, the overall distribution of antimicrobial drug residues in all the wards in the present study area depicted sporadic trends and patterns where wards like Karuna, Moiben, Tembelio, Kimumu, Kapseret, Ngeria and Langas reported positive antimicrobial drug residues results while wards like Sergoit, Kipkenyo and and Megun had negative results (Table 4.7). Results higher than in current study were reported by Manafi in a study in Azerbaijan – Iran where they obtained (26.0%) antimicrobial drug residue in raw milk samples collected from industrial dairies and (16.0%) of raw milk from market collection centers. Further, (30.0%) of pasteurized milk samples produced positive Delvotest® result (Manafi *et al*., 2011).

The antimicrobial drug residues determined in raw milk on small-holder dairy farms in Uasin-Gishu depicted a qualitative incidence of (4.2%) in Kapseret and (2.8%) in Moiben. Three classes of antimicrobial agents were identified and quantitatively determined above Safe Maximum Residue Limits (SMRL), vis Beta-lactams (Penicillin >4.0 μg/ml),

Tetracyclines (Tetracycline >3.1 µg/ml) and Aminoglycosides (Streptomycin >0.2 µg/ml). However, on overall five penicillin were detected below SMRL:- three penicillin from Kapseret sub-county detected at (3.7 µg/ml, 2.8 µg/ml and 2.5 µg/ml) respectively and two penicillin from Moiben sub-county detected at (2.5 µg/ml and 2.1 µg/ml) respectively. Two tetracyclines, one each from Moiben and Kapseret sub-counties were as well detected below SMRL (2.9 µg/ml and 1.6 µg/ml) respectively (Table 4.8). In a similar study by Chowdhury *et al*. (2015) in Chittagong, Bangladesh, the mean concentration of antimicrobial drug residue in raw local milk was 9.8 µg/ml and raw commercial milk was 56.2 µg/ml for amoxicillin. In this study, Chowdhury reported tetracycline, ciprofloxacin and amoxicillin residues at significantly higher levels above SMRL ($p \leq 0.05$) (Chowdhury *et al*., 2015). Welsh in another study reported slightly different results where presence of antimicrobial drug residues in milk was (26-60) % in 35 milk samples, the antimicrobial drug residue levels in this study exceeded recommended federal limits for amoxicillin by (3.0 %), sulfamethazine by (37.0 %) and sulfathiazole (26.0 %) (Welsh *et al*., 2019). The results in the current study were different because of utilization of Tetracycline, Penicillin and Streptomycin for treatment of bovine mastitis more frequently and routinely in Uasin-Gishu, Kenya. Furthermore, the antimicrobials were less costly, affordable and easily available for use by farmers without Animal Health Officers prescription. This was a dangerous trend that translated to use of antimicrobials indiscriminately; the net consequent of this was AMDR and AMR in both animal and human.

On overall, AMDR was detected in (6.9%) cows of which (1.0%) was a penicillin determined at 15.9 µg/ml in mastitis culture positive cows infected with gram-negative *Proteus vulgaris* bacterium from Kapseret sub-county. While (12.5%) AMDR was detected in cows free from mastitis. The variation of AMDR in association with mastitis status was statistically significant at P-value ($p<0.001$) (Table 4.9). *Proteus vulgaris*, as demonstrated (Table 4.6), produced a wide range of multi-drug resistance. In the same cohort study, we reported prolonged and overwhelming use of antimicrobial agents leading to prolonged AMDR effects in milk used for human consumption, the AMDR

effect in most cases persisted beyond the recommended drug manufacturers' withdrawal period. Penicillin was the predominant AMDR detected in cow milk at (46.6%) while tetracycline and streptomycin were detected at (26.7%) each.

Implementation of prudent cow management practices were found to be key tenets in prevention and control of proliferation of mastitis on small holder dairy farms in Uasin-Gishu County. In our current study, implementation of management practices were carried out at herd-level (also defined as farm-level in this study). Majority (59.3%) of farmers did admit that mastitis was a burden on their farms as opposed to few farmers (40.7%) who did confirm that mastitis was not a major problem, consequently, infection among these two groups was (68.8%) and (66.7%) respectively (Table 4.10). These findings were comparable to those by Abebe in Ethiopia and Kumar in India, the duo reported high mastitis occurrence of about (79.0%) and (80.0%) respectively on farms that admitted to experience mastitis (Abebe *et al.*, 2016; Kumar *et al*., 2016) This was mainly attributable to poor udder hygiene. In our study area, majority of farmers (79.0%) did not take samples of their sick cows to the laboratory for mastitis confirmation despite the existence of Regional Veterinary Investigation Laboratory (RVIL) in the nearby Eldoret town. Mastitis infection was high (67.1%) for this category of farmers. This culture point to the fact that farmers do not fully utilize the nearby RVIL Laboratory. No wonder the infection of mastitis was high (71.9%) among those farmers who admitted not to take samples for laboratory testing. Contrary results were produced in a study by Mbindyo *et al.* (2020) in Embu and Kajiado Kenya where infection among the farmers who did not take samples to the laboratory for mastitis testing was (39.8%) yet infection was high among those farmers who normally tested the samples in the laboratory (60.2%). This scenario could be attributed to irrational use of antimicrobials leading to antimicrobial resistance. Further to this, there was also no Veterinary laboratory services in Embu and Kajiado Counties, giving justification to failure to take samples to laboratory for testing.

All farmers on the 81 study farms confirmed that in case they suspected their cows to be sick of mastitis, they sought the services of a veterinary professional, however the infection among these farmers was on increase (67.9%). The high infection could be as a

result of antimicrobial overuse and resistance. In addition, the professionals could be an unqualified and inexperienced as well (Ndahetuye *et al.*, 2019; Abebe *et al.*, 2016).

The principal investigator confirmed by way of observation the existence of cow housing/shade and milking crushes on 73 farms. Among these farmers, (87.7%) admitted that they do not disinfect the cow housing and milking crushes. Nevertheless, mastitis infection on these farms was reported to be still high at (67.6%). These results were similar to those reported by Mbindyo *et al.* (2020) in Embu and Kajiado Counties where infection among farms with housing shades was (74.7%), further, she observed that disinfecting cow housing/crush, was vital in reducing the spread of mastitis in any herd.

All farmers on the 81 small holder dairy farms in Uasin-Gishu milked their lactating dairy cows twice a day. Almost all of them used hand milking technique (87.7%) as opposed to (12.3%) who used the machine, these farmers as well, confirmed that they cleaned the udder before and after milking (97.5%). However, they used a single towel and one bucket of water for all the cows. The mastitis infection occurrence among these groups was above (67.1%). These findings closely agreed with the results of Abebe *et al.* (2016) in Ethiopia and Mbindyo *et al.* (2020) in Kenya, where infection reported in two studies was (79.1%) and (80.8%) respectively among the farmers who use one towel for all the cows and don't wash hands in between milking one cow to another. This practice should be avoided as is known to propagate contagious mastitis from one cow to another and from teat to teat (Kumar *et al*., 201 6)

Farmers in Moiben and Kapseret sub-counties, embraced extensive and intensive type of farming systems in the following proportions: - extensive (29.6%) and intensive (70.4%) systems respectively. Mastitis infection on intensive farming system was high at (71.9%) than (58.3%) on extensive system (Table 4.9). Similar trend of results was documented by Mbindyo *et al.* (2020) in her cohort study in Embu and Kajiado Counties where infection on intensive farming system was higher (83.7%) than on extensive farming system (16.3%). The infection was high on intensive farming systems because of unsustainable housing and floor cleanliness especially during long rain seasons, this made

it difficult to maintain udder and teat hygiene. However, Sarba in her study in Ethiopia reported contrasting findings where mastitis infection on extensive farming systems was high at (47.1%) than in either intensive farming systems (42.3%). These findings were attributable to the fact that in Sarba's study, he used larger cattle herds which were fundamentally vulnerable as compared to our current study which used small-holder herds, that were less vulnerable (Sarba & Tola, 2017).

On (90.1%) farms which reported to have housing structure and milking crushes for cows, (77.8%) had concrete floor and (22.2%) had earth floor. On contrary, in spite of this, mastitis infection was high on those farms with housing with concrete floor (73.0%) and low on farms with housing with earth floor (50.0%). conventionally, the infection could be high on farms with housing with earth floor than concrete floor, however this could be attributable to low housing cleanliness by many farmers (45.7%), housing and floor cleanliness could have been hampered due to long rains and heavy down pour experienced during short rain season (Table 4.10). Comparable results were reported in similar research in Embu and Kajiado where infection among farmers with concrete floor housing was as high as (76.2%) while infection among farmers with earthen floor housing was as low as (23.8%) (Mbindyo *et al.*, 2020). Contrary results were reported in a study by Mureithi and Njuguna in Thika Kenya, where infection on farms with concrete floor was low (55.5 %) and high in earth floor (82.1 %) (Mureithi & Njuguna, 2016).

Though majority of farmers (53.1%) reported to cleaning the housing and milking crushes frequently, they could not maintain good cow udder hygiene which was coincidentally not satisfactory in (55.6%) of respondents. The infection among cows with low (not satisfactory) and good (satisfactory) udder hygiene was more-less similar, (66.7%) and (69.4%) respectively. The results in our current study were similar albeit with slight difference with the findings by Mureithi and Njuguna in Thika Kenya, where mastitis infection in cows with good udder hygiene was reported at (54.3%) and (69.9%) for cows with low udder hygiene (Mureithi & Njuguna, 2016), these results were so because in the two studies farmers were unable to maintain and sustain cow housings and floor in clean conditions due to long and heavy rains season.

Farmers, in a sustained efforts to control mastitis reported to apply frequently and commonly used antimicrobial agents for treatment of mastitis. They used antimicrobials in the following proportions; Penistrep (40.7%), Ampicillin (24.7%) and Tetracycline (13.6%). Despite this, the infection remained high in those farmers that reported to use Tetracycline at (90.9%) and Ampicillin at (70.0%), however infection was low in farmers who used Penistrep (36.4%) (Table 4.10). The high infection could be comparable to high antimicrobial resistance reported in many other similar studies. For example, in China and Brazil, the Principal Investigators found out that antimicrobial resistance to penicillin and ampicillin was (77.3%) and tetracycline (60.0%) (Jian-Ping *et al.,* 2009); and ampicillin (100.0%), tetracycline (96.7%) and streptomycin (80.0%) (Freitas *et al*., 2018) respectively. This high antimicrobial resistance, were due to haphazard and indiscriminate use of antimicrobial agents. In essence this underscores the importance of *in-vitro* susceptibility testing of frequently used antimicrobial medicines prior to their applications *in-vivo* to guard against development of antimicrobial resistance (Thompson-Crispi *et al.,* 2012; Silva *et al*., 2005). However, low antimicrobial resistance was reported by Messele in Ethiopia, against ampicillin 68.7%, sulfamethoxazole-trimethoprim 50% and streptomycin 25.0%, the low resistance was due to reported low incidence of mastitis and judicious utilization of antimicrobial therapy (Messele *et al.,* 2019).

In the current study, the young cows showed low mastitis infection as compared to older cows. We reported the following infection; 1-4 years (34.3%), 5-8 years (61.8%), and >8 years 9(60.0%) (Table 4.11). A similar trend in mastitis infection was documented by Zeryehun in Eastern Harrarghe Zone of Ethiopia, where young adult cows reported (31.4%) infection, adult cows (66.7%) and older cows reported (58.3%). (Zeryehun & Abera, 2017). Further, Biffa in Southern Ethiopia documented mastitis infection as follows;- young adult cows (23.6%), adult cows (38.1%) and older cows (44.6%) (Biffa *et al*., 2005). The results in the current study were attributed to the fact that the AMIR and CMIR of young cows was higher than aging older cows and that the teat canals of aging older cows were wider and thus more susceptible to microbial infection than in young

adult cows (Thompson-Crispi *et al*., 2012). The implication of this was that older cows were more vulnerable to mastitis than young adult cows.

Mastitis infection due to different breeds depicted that Friesian cows were most vulnerable at (55.5%), followed by crossbreed cows at (45.5%) and Ayrshire at (40.2%) (Table 4.11). These vulnerabilities were similar to the findings of studies by Biffa in Southern Ethiopia who found out that Friesian cows were more susceptible at (56.5%) to mastitis infection followed by crossbreed cows at (28.2%) (Biffa *et al.*, 2005). Khasapane *et al*. (2022) in a review reported mastitis infection of Friesian at (64.8%), crossbreed at (53.4%) and indigenous cows at (37.8 %). A similar trend of mastitis infection in Friesian cows (87.8 %) as compared to crossbreed cows (76.6%) and other breeds which include indigenous cows at (50.0%) was reported in Rwanda (Ndahetuye *et al*., 2019), however the infection in this study was higher than that reported in our current study. These findings were probably as a result of the protection conferred by the innate immune response mechanisms attributable to antibody-mediated immune responses (AMIR) and cell-mediated immune responses (CMIR) which were reported to be higher in indigenous cows than the crossbreeds or exotic breeds of cows (Thompson-Crispi *et al*., 2012). Contrary findings to the results in our current research, were reported by Mbindyo *et al.* (2020) in a study in Embu and Kajiado counties, Kenya, Mbindyo documented interesting findings where crossbreed cows were most vulnerable to mastitis than exotic cows at (38.5%) and (24.7%) respectively. Similar results were reported in Thika sub-county Kenya by Mureithi and Njuguna where infection for Friesian cows was (65.6%) and Ayrshire (80.6%) (Mureithi & Njuguna, 2016). These contrary results could have been attributable to differences in the geographical conditions among the study sites. Mbindyo and Mureithi conducted their studies around Mount Kenya region which had long rains throughout the year and had low annual temperatures ranging from $10^{O}C – 19^{o}C$

Primiparous cows were found to be more resistant to mastitis infection as compared to multiparous cows. In this study, primiparous-1 cows reported a low infection of (36.4%), this was followed by multiparous-2 (45.5%), multiparous-3 (48.9%) and multiparous>3 (56.9%) (Table 4.11). Findings with a trend similar to our present study where multiparous

cows were more susceptible to mastitis infection than primiparous cows were reported in Ethiopia, where primiparous cows had mastitis infection of (40.7%) and multiparous cows (68.7%) (Adane *et al*., 2012). In Rwanda, Ndahetuye *et al*. (2019) established a similar infection trend - (66.7%) among primiparous-1 cows and (82.1%) among multiparous-2+. Biffa *et.al.* (2005) established an infection of (11.3%) in primiparous-1 cows, (31.7%) in multiparous-2 and (62.9%) multiparous-2+ in Ethiopia. In Thika Kenya, mastitis infection in primiparous cows was also similar (32.1%) and (70.1%) among multiparous cows (Mureithi & Njuguna, 2016). These results could be attributable to the fact that the teat canal of much older multiparous cows was more exposed as compared to young primiparous cows. In contrast to our findings, a study by Mbindyo *et al*. (2020) in Embu and Kajiado Counties, Kenya established that primiparous cows were most vulnerable to mastitis infection as compared to multiparous cows:- primiparous-1 (33.3%), multiparous-2 (24.2%), multiparous-3 (23.4%) and multiparous-4+ (21.0%). The difference in these findings can be based on the variance in cow management practices, which included timely prevention and control strategies employed on farms in Embu and Kajiado for instance rational administration of antimicrobial therapy, priority in management of high producing multiparous cows than primiparous ones, these strategies were found lacking in our case – leading to high incidences of mastitis.

This study has achieved a milestone through its findings as it contributes to the body of knowledge globally as far as bovine mastitis is concerned. The policy makers and farmers shall benefit in equal measure. Policy on withdrawal of resistant antimicrobials will benefit farmers as well as policy

In summary, the present study however experienced many limitations that ranged from difficulties to access farmers due to covd-19 pandemic, most farmers could not allow visitors on their farms for fear of covid-19 infection. Heavy rains were experienced during the sampling period making it difficult to access remote and some far flung areas – this implied that sampling was indeed very difficult to achieve 100.0% sample size for the day. Lastly on some few farms we experienced hostility and resistance, these farmers

demanded monetary benefits and free antibiotics before they could consent for their cows to be sampled

5.2 Conclusion

The overall incidence of bovine mastitis was high (48.2%), of which contagious-bovine-mastitis attributable to *Staphylococcus epidermidis* and *Staphylococcus aureus* was predominant (30.6%) on small-holder dairy farms in Moiben and Kapseret sub-counties. Further, results illustrate epidemiological distribution and trends where the burden of mastitis was evenly distributed in the two sub-counties and various wards. However, the difference in distribution patterns in the wards in Moiben sub-county was statistically significant ($P<0.05$).

Staphylococcus species and *Escherichia coli* were the main causative agents of mastitis in raw milk on small-holder dairy farms in Uasin-Gishu and exhibited varying degrees of resistance to frequently used antimicrobial agents. These pathogens were equitably distributed across the study area and demonstrated seasonality in occurrence with an exponential increase in their isolation thus isolation was increasing steadily during the long rain season (March-May, 2021) and during the heavy rain downpour that occurred between (July-November, 20221).

Antimicrobial resistance against bacterial pathogens isolated in raw milk from dairy farms in Moiben and Kapseret study area demonstrated that Ampicillin, Streptomycin, Cotrimoxazole, Tetracycline, Sulfamethoxazole and Penicillin produced high antimicrobial resistance and low sensitivity. Subsequently, bacterial pathogens implicated in the etiology of mastitis were sensitive against Gentamicin and Kanamycin - by exhibiting low antimicrobial resistance – the sensitivity was statistically significant ($P<0.004$), meaning they were effective in treatment of mastitis. AMR in animals leads to adverse public health effects in humans due to treatment failure.

On overall, AMDR of (6.9%) was established in Moiben and Kapseret study area, of this, three different types of AMDR were determined above SMRLs using HPLC MS/MS – Penicillin, Tetracycline and Streptomycin – consumption of these antimicrobial drug residues in raw milk is harmful and lead to public health complications ranging from rheumatoid heart diseases, hypertension and antimicrobial resistance in both humans and animals.

Older multiparous exotic cows were more vulnerable to mastitis infection than young primiparous Ayrshire and crossbreed cows. Results in this study indicated that mastitis infection was low in young primiparous, Ayrshire and crossbreed cows at ($P<0.05$). Low animal husbandry management practices contributed to upsurge of mastitis, though in small magnitude. Most of the farmers did not take samples of their sick cows to laboratory for antimicrobial susceptibility testing, only few farmers did disinfect the floor of cow housing and milking crushes regularly, the udder and teat hygiene and cleanliness of housing and milking crushes were observed to be low, it was almost evident that farmers did not engage qualified and certified animal health practitioners to manage mastitis on the farms and farmers reported to utilize frequently and commonly used antimicrobials indiscriminately; this led to AMR and AMDR which had adverse public health effects in human.

5.3 Recommendations

Prevention and control of contagious-bovine-mastitis attributable to *Staphylococcus* species by way of maintaining good udder and teat hygiene, rational administration of sensitive antimicrobial teat therapy and daily cleaning of cow housing and milking parlours was reliable to reduce disease incidence in study area and was therefore recommended.

Intervention strategies aimed at prevention and control of predominant *Staphylococcus* species and *Escherichia coli* pathogens are recommended. These strategies could include maintaining good hygiene and sanitation of cow housing and milking crushes, cleaning

cow teats and udder before and after milking by using single towel for each cow. These are sure strategies that could immensely minimise contagious transmission of mastitis on small-holder dairy farms.

Microbial pathogens implicated in etiology of mastitis were sensitive against Gentamicin and Kanamycin and are recommended for use by farmers for treatment of mastitis especially *Staphylococcal* and Coliform mastitis attributable to *Staphylococcus* species and *Escherichia coli*. Subsequently, Ampicillin, Streptomycin, Cotrimoxazole, Tetracycline, Sulfamethoxazole and Penicillin produced high antimicrobial resistance and we recommend their gradual withdrawal from the list of animal health essential medicines.

Maintaining and observing strict withdrawal period, active and regular surveillance of antimicrobial drug residue levels in raw milk and boiling of raw milk to decrease AMDR levels before using for human consumption are highly recommended to ensure safe public health standards are observed.

Adoption of prudent animal husbandry management practices which include (antimicrobial susceptibility testing of samples in the laboratory before administering treatment for sick cows, milking sick cows last, disinfecting the floor of cow housing and crushes regularly) are recommended to slightly mitigate against mastitis. Keeping primiparous young lactating Ayrshire and crossbreed cows of age (1-4) years old is highly recommended to drastically reduce mastitis to minimum levels ($P<0.05$).

The microbiological culture and HPLC MS/MS methods used in this study were appropriate however, we could recommend molecular sequencing method which is superior to be used for identification of bacterial isolates implicated in aetiology of mastitis, genotyping for antimicrobial resistant bacterial pathogens and antimicrobial drug residue testing.

Further research is recommended to find out multi-valent vaccines that can be used by farmers to prevent and control mastitis.

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APPENDICES

Appendix I: Informed Consent Agreement Form - English

Serial No. 2018/ICAF/01A

Form 1a **English**
version # 1.0

Consent to participate in the study

Date (day/month/year)……...

Participant Study unique code…….…….

Participant code…….

Principal investigator: David Ayah Ounah; P.O. Box 450 Eldoret, 30100

Main objective of study

To identify determinants for bovine mastitis, antimicrobial resistance and management practices on small-holder dairy farms in– Uasin-Gishu, Kenya

Duration of study: 17/09/2018 to 31/12/ 2023
Duration (Time) of participation: 25 – 30 minutes
Procedure and level of respondent participation
Consenting respondent
Interviewing respondent using semi-structured questionnaire
Sampling milk from the four quarters of the target lactating dairy cow

As respondent, you will be required to participate as a key informant in face-to-face Key informant interview.

Specific research objectives

This study will be guided by the following specific research objectives:

1. To determine the incidence of bovine mastitis

2. To identify the microbial pathogens causing bovine mastitis

3. To determine the susceptibility of microbial pathogens to antimicrobials commonly used for the treatment of bovine mastitis

4. To determine the presence of antimicrobial residues in milk

5. To identify dairy cow characteristics and management practices used by dairy farmers in control of bovine mastitis

Methods of data collection

The study will use quantitative methods of data collection. The semi-structured questionnaire will be administered face to face to collect data plus 'microbiological culture' of raw milk samples will also be used to collect data on variables under investigation of this study.

Benefits

There will be no immediate direct benefits from this study. Your participation therefore will play an integral part in this.

Compensation

There will be no form of any compensation at this point but in future we intend to design a minimal compensation package for all participants.

Risks

There are no known risks in participating in this study; milking is a routine process in cows, it has neither side effects nor risks.

Privacy and confidentiality

All information obtained in this study will remain anonymous and will be treated with confidentiality and will not be divulged to anyone. The participants' identity will be concealed, kept confidential and will not be used in any publication made from this study. Only the Principal Investigator will have access to all identifying information.

Rights to refuse or withdraw to particcipate

Consent to participate in this study is voluntary. There is freedom to take part or withdraw from the study at any time or stage.

Ethical clearance

The study has met all ethical requirements and has obtained all necessary ethical clearances to commence to collect and analyze data.

Feedback mechanism

The final outcome of the study will be published in one or more of the referred medical journals and also efforts will be made to have the findings published in local dailies. When this is done we will notify all participants in advance so they can obtain these copies for perusal. Public barazas, stakeholders' fora including any other probable dissemination methods will be used.

Important note

If you have any questions or need any clarifications, please feel free to ask the Principal Investigator Mr. David Ayah Ounah, 0735375280.

Consent by respondent

I have read/been explained to, all the above aspects of the study by Mr. Ounah. I confirm that I have fully understood all the aspects. I therefore, give authority for my verbatim to be used where necessary. I therefore agree to participate in the study.

Principal investigator's / research assistant's Signature: ----------Date------

Respondent's Signature: ----------Date----------

Respondent's contact address: ----------

Witness' signature: ----------Date----------

Witness' contact address: ----------

Appendix II: Makubaliano ya Kushiriki Katika Utafiti - Kiswahili

Serial No. 2018/ICAF/01B

Fomu 1a KISWAHILI
2.0

IDHINI YA KUSHIRIKI KATIKA UTAFITI

Tarehe (dd/mm/yyyy)……...

Nambari maalum ya utafiti…….

Nambari ya Mushirika…….

MADA: Upekusi wa ugojwa wa maziwa ya ng'ombe katika Kaunti ndogo ya Moiben an Kapseret ya kaunti ya Uasin-Gishu, Kenya

MTAFITI MKUU: David Ayah Ounah

SLP 450 Eldoret, 30100

MADUMUNI YA UTAFITI

Kupekua na kujua njinsi ugojwa wa maziwa umeenea na kuadhiri ng'ombe katika Kaunti ndogo ya Moiben an Kapseret ya kaunti ya Uasin-Gishu, Kenya

Muda wa utafiti: 17/09/2018 hadi 30/12/2023

MUDA WA KUSHIRIKI: Dakika 25 – 30

Namna ya kushiriki

Mshiriki atatakiwa kuhojiwa na mtafiti mkuu na kujibu maswali kwa njia ya moja kwa moja

Kukubali

Kuhojiwa na

Kuchukua sambuli

Maudhui ya utafiti

Kiwango cha Ugojwa wa maziwa katika Kaunti ndogo ya Moiben na Kapseret - Uasin-Gishu

Orodha ya viini vinavyosababisha ungojwa wa maziwa katika Kaunti ndogo ya Moiben na Kapseret - Uasin-Gishu

Madawa yanoyotumika kuthibiti virusi vya ugojwa wa maziwa katika mahabara katika Kaunti ndogo ya Moiben na Kapseret - Uasin-Gishu

Mabaki ya madawa yanayo patikana katika maziwa katika Kaunti ndogo ya Moiben na Kapseret - Uasin-Gishu

Mazingara yanayo changia kuweko kwa ugojwa wa maziwa katika Kaunti ndogo ya Moiben na Kapseret - Uasin-Gishu

MBINU ZA KUKUSANYA MAONI

Nakala ya maswali maalum itatumika katika mahojiano ya kukusanya maoni kutoka kwa muhuska kwa njia ya moja kwa moja ili kuthibitisha kinacho changia kuweko kwa ugojwa wa maziwa.

Manufa ya utafiti huu

> Japo hakutakuwa na manufa ya hapo kwa hapo, matokeo ya utafiti huu yatachangia kupuguza mzigo wa ugojwa wa maziwa katika Kaunti ya Uasin-Gishu kwa kiwago kikubwa muno.

Ridhaa

Hakuna ridhaa yotote kwa saasa japo siku za usoni kutakuweko ridhaa.

Maafa

Hakuna maafa yoyote yanayo julikana kwa kushiriki katika utafiti huu

Usiri

Hamna habari yoyote ya hujuma itakayo peyanua kwa mtu yeyote kuhusiana na utafiti huu. Habari zote zitahifathiwa kwa siri.

Haki ya kushiriki ama kutoshiriki

Muhusika ana haki ya kushiriki ama kutoshiriki katika utafiti huu wakati wowote

Kibali cha kufanya utafiti

Kabula ya kuanza utafiti, mtafiti mkuu ataomba kibali kutoka kwa bodi ya vibali ya chuo kikuu cha Jomo Kenyatta.

Majibu ya utafiti

Majibu ya mwisho ya utafiti yatazabazwa kupitia vyombo vya habari, majarida na mikutano mbali mbali ya washika dau.

Jambo la muhimu

Unapo kuwa na swali lolote tafadhali usisite kumuuliza mtafiti mkuu Bw. David Ayah Ounah, nambari ya simu ni 0735375280.

Kauli ya muhusika kukubali

Nimesomewa/nimesoma nakala hii ya utafiti na nimekubali kwa hiyari yangu mwenyewe kushirki katika utafiti huu.

Sahihi ya mtafiti mkuu/naibu mtafiti mkuu: ----------Tarehe----------

Sahihi ya muhusika: ----------Tarehe----------

Anwani ya muhusika: ----------

Sahihi ya mashahidi: ----------Tarehe----------

Anwani ya mashahidi: ----------

Appendix III: Questionnaire

Serial No. 2018/QS/01

Participant Study unique Code:……

I am a Doctoral student at Jomo Kenyatta University of Agriculture and Technology. I would like to thank you for consenting to participate in this study. The study aims to identify determinants of of bovine mastitis, antimicrobial resistance and management practices in Uasin-Gishu. welcome you to answer questions voluntarily.

PART I: GENERAL INFORMATION

Place of residence…….…….....

County……

Sub-County……

Ward……

Division……

Location……

Sub-location……

Village……..…….....

Dairy cow information (Circle appropriate option)

A). Age of dairy cow sampled (in completed years) 1. (1-4) years 2. (5-8) years 3. (>8) years 4. Others, specify_____

B). Breed of cow: 1. *Holstein Friesian 2. Ayrshire 3. Guernsey 4. Jersey* 5. Cross-breed 6. Indigenous 7. Other specify_____

C). Parity of cow:_1, 2, 3, >4,

D). Stage of lactation 1. < 3month 2. (3-6) month 3. >6 months

E). Soiling of teats 1. Clean 2. Slightly clean 4. Slightly dirt 4. Dirt 5. Other, specify_____

Respondent information (Circle appropriate option)

A). Type of respondent: 1). Mother 2). Father 3).Other (specify)…….

B). Sex of respondent: 1). Male 2). Female

C). Age of Respondent (years): 1. (< 20) 2. (21- 30) 3. (31 – 40) 4. (41 – 50) 5. (50

D). Marital status of respondent: 1. Single2. Married 3. Divorced 4. Widowed 5. Separated

E). Education: 1. Primary 2. Secondary 3. Tertiary 4. University 5. No formal education

F). Occupation: 1. Formal employment 2. Business 3. Farmer 4.Others specify…

Part II: Questions on mastitis management practices (circle the appropriate option)

Epidemiology of mastitis

D). i. Have you encountered any animal disease among any of your lactating cows? 1. Yes 2. No 3. Not applicable

ii. is mastitis a problem on your dairy farm? 1. Yes 2. No

iii. Do you know signs and symptoms of mastitis?

1. Yes 2. No 3. Do not know

iv. If yes, which symptoms and signs do you know? 1. Milk discolouration 2. Neusea/vomiting. 3. Hotness of body/Fever 4. Loss of appetite 5.Others, specify_____

v. Any time your cow is sick and you suspect it is mastitis do you take the appropriate sample to the Laboratory for diagnosis? 1. Yes 2. No 3. Not applicable

vi. if no why? 1. No money 2. Lab is far 3. Other specify_____

(vii). Is mastitis a major constraint on your farm? 1. Yes 2. No 3. Not applicable 4. Others specify__

(viii) If yes, how is it a major constraint? 1. High cost of treatment 2. Lack of veterinary services 3. Difficulty in diagnosis 4. Low income/poverty 5. Poor hygiene 6. Lack of supplies 7. Others specify_____

Management practices of bovine mastitis by farmers

B). i. Do you have a shade/housing for your cows during overnight sleeping and daytime resting? 1. Yes2. No 3. Not applicable

ii. Are the shades/housing clean? 1. Yes 2. No 3. Not applicable

iii. How often do you clean the shades/housing? 1. Frequently 2. Infrequently 3. Others specify_____

iv. Do you regularly and routinely disinfect the housing? 1. Yes 2. No 3. Not applicable

v. which type of floor do the shade of your dairy cows have? 1. Concrete 2. Earth 3. Other, specify___

vi. Do you provide bedding materials for your dairy cows? 1. Yes 2. No

vii). What type of farm animal management system do you practice? 1. Intensive 2. Semi-intensive 3. Extensive 4. Other, specify_____

C). i. Do you have holding and milking crushes? 1. Yes 2. No 3. Not applicable

ii. Are the crushes maintained and kept clean? 1. Yes 2. No 3. Not applicable

iii. How often do you clean the crushes? 1. Frequently 2. Infrequently 3. Others specify_____

iv. Do you regularly and routinely disinfect the crushes? 1. Yes 2. No 3. Not applicable

(ix). Which method do you use to prevent and control mastitis? 1. Treat with antimicrobials 2. Practice good hygiene 3. Cull sick cows 4. Dip teats 5. Other, specify_____

(x). which order do you follow when milking your lactating dairy cows when some among them are Mastitic? 1. Milk sick cows first 2. Milk sick cows last 3. No order 4. Other, specify_____

F). How long do you take to seek treatment for your sick cow once you notice suspect signs and symptoms of mastitis? 1. Immediately 2. One day 3. Two days 4. Three days 5. Others, specify…

G). If not immediate, what are the reason(s) for delay to seek treatment?

1. Lack of funds/money 2. Long distance to Veterinary offices 3. No means of transport 4. No time 5. Others specify

H). Do you use the whole dose of antimicrobial drugs when your cow has recovered?

1. Yes 2. No 3. Sometimes

I). Where do you keep and store antimicrobial drugs?

1. On table 2. Bed-side stool 3. On floor at corner 4. In a raised/lockable shelf 5.Others,
specify_____

J). How have you been administering your antimicrobial therapy? 1. Intramammary infusion 2.Intramuscular injection 3.Intraverscular injection 4.Others, specify_____

K). (i). Is the udder of your lactating dairy cows in good hygiene standards (observe)? 1. Yes – Satisfactory 2. No - Unsatisfactory 3. Other Specify_____

(ii). Can we have a look at your lactating dairy cow and asses the hygiene level of the udder and the hind limbs? 1. Yes 2. No 3. Other specify_____

(iii) Observe and score the UHS and LHS 1. No contamination of the skin of the udder and rear limbs 2. Slightly dirt udder and hind limbs 3. Moderately dirt 4. Highly dirt

L) (i). Which milking method do you use? 1. Machine 2. Hand

(ii) How frequent do you milk? 1. Once a day 2. Twice a day 3. Thrice a day 4. Other specify_____

(iii). Do you clean the udder before and after milking the cow? 1. Yes 2. No 3. Other specify_____

(ii). Observe and score the udder and leg hygiene of each lactating cow from the herd of each respondent 1. '1' will mean no contamination of the skin of the udder, teat or the hind limbs; 2. '2' slightly dirty (2–10% of the udder, teat or the hind limbs are dirt); 3. '3' moderately dirty (10–30% of the udder, teat or the hind limbs are dirt); 4. '4' dirt (>30% of the udder, teat or the hind limbs are completely covered in dirt) 5. '5' Other specify_____

(iii) Score the physical appearance of milk to the scale of 1-4; 1. '1' (abnormal milk only), 2. '2' (abnormal milk and swollen udder) 3. '3' (abnormal milk, swollen udder, and sick cow) 4. '4' (swollen udder only) 5. '5' (sick cow only) 6. '6' Other specify_____

N). (i). Who treats your cows when they are sick? 1. Veterinary officer 2. Self 3. Others specify_____

O). i. Do you administer treatment to your sick cow as per the prescription of your Veterinary personnel? 1. Yes 2. No 3. Others specify_____

ii. If no why?_____

(ii). How long do you take to treat a cow with mastitis? 1. 3 days 2. 7 days 3. 14 days 4. 21 days 5. Others specify_____

(vii) Which antimicrobial agent do you administer routinely and most frequently when you encounter mastitis on your farm? 1. Ampicillin 2. Tetracycline 3. Contrimoxazole 4. Streptomycin 5. Kanamycin 6. Gentamycin 7. Sulphamethoxazole 8. Chloramphenicol 9.

Others
pecify_____

vii. Do you complete the dose of medication? 1. Yes 2. No 3. Others specify_____

(viii). After antimicrobial treatment of the cow, How long do you take before you start using the milk? 1. 3 days 2. 7 days 3. 14 days 4. 21 days 5. Others specify_____

THAT QUESTION MARKS THE END OF OUR INTERVIEW.

THANK YOU FOR YOUR PARTICIPATION

Appendix IV: Research Laboratory Report Form

Serial No. 2018/RLRF/01

RESEARCH LABORATORY REPORT FORM

CONFIDENTIAL

FOR LABORATORY USE ONLY Specimen Serial No…….	Sample Received by……... Date Received……. Time Received……. Date Processed……	Research/Study Unique Code……

Sample submitted by:…… …… Sample processed by…… …… Place of residence of respondent…… County:…… …… Sub-county…… ……	Ward…… …….. Division…… …….. Location…… …….. Sub-location…… …… Village…… ……

Animal Species ……Date milk sample collected…….

Date milk dispatched to the lab……Time milk collected……..

Number of milk samples submitted……
……..

Lab Test required……
……

Herd history

Number of Animals: In herd……..infected……
……

Duration of infection……
……

Clinical signs:……
……

Animal History

Age……..sex……
…..Breed……

Parity…… Stage of lactation……
……..

Farm Management system:……
……..

Vaccination History……..Treatment History……...

Remarks……
……

LABORATORY RESULTS

Lab. Diagnosis Performed by:…….Signature:……Date:…… ……..

Appendix V: Directorate of Veterinary Services Approval

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

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

Veterinary Research Laboratories
Private Bag, Kabete, Kangemi 00625
Nairobi

When replying, please quote:
MOALF&I/SDL/DVS/GEN/VOL.I/57
All correspondences should be addressed to:
The Director of Veterinary Services

Date: 23rd September,2019

David Ayah Ounah
P/NO. 2010048513
CVL Private Bag Kangemi
Mobile: **0715880051**

Ethical Approval

In reference to your application for Ethical Approval dated 2nd Sept, 2019.The Director approves your request to carry out a research on `Determinants of Management Practices of Bovine Mastitis and Antibiotic Residues in Milk from Dairy Farms in Moiben and Kapseret Sub Counties – Uasin-Gishu, Kenya.

You are therefore allowed to collect, process and analyze Bovine milk samples as per your request.

Dr.Obadiah Njagi, PhD,OGW
Director of Veterinary Services

Appendix VI: Institutional Ethical Approval of Research Proposal

Tel: 056-31375
Fax: 056-30153
E-mail: ierc@mmust.ac.ke
Website: www.mmust.ac.ke

MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY

P. O. Box 190-50100
Kakamega, Kenya

Institutional Ethics Review Committee (IERC)

Ref: MMU/COR: 403012 Vol 3 (01)

Date: 12th January, 2021

David Ayah Ounah,
Jomo Kenyatta University of Agriculture and Technology
P.O. Box **62000 – 00200,**
Nairobi.

Dear Mr. Ouna

RE: Determinants of Management practises of Bovine Mastitis and antibiotics residues in milk from dairy farms in Moiben and Kapsaret sub counties in Uasin Gishu, Kenya.- *MMUST/IERC/155/2021*

Thank you for submitting your proposal entitled as above for initial review. This is to inform you that the committee conducted the initial review and approved (with no further revisions) the above Referenced application for one year.

This approval is valid from **12th January, 2021** through to **12th January, 2022**. Please note that authorization to conduct this study will automatically expire on **12th January, 2022.** If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the MMUST IERC by **12th January, 2022.**

Approval for continuation of the study will be subject to submission and review of an annual report that must reach the MMUST IERC Secretariat by **12th January, 2022.** You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to MMUST IERC prior to implementation.

Please note that any unanticipated problems or adverse effects/event resulting from the conduct of this study must be reported to MMUST IERC. Also note that you are required to seek for research permit from NACOSTI prior to the initiation of the study.

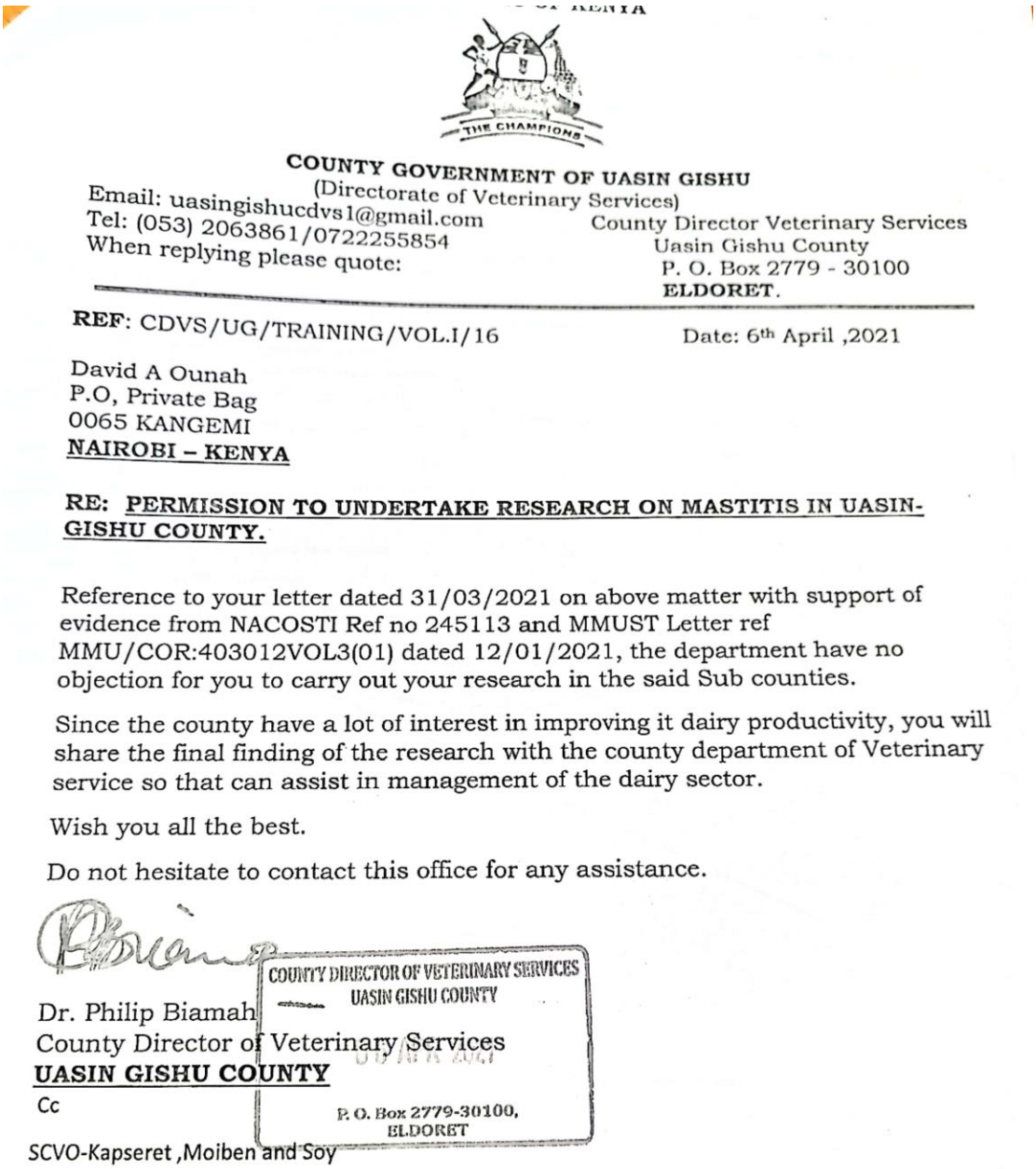
OFFICE OF THE DIRECTOR RESEARCH
RECEIVED
15 JAN 2020
MMUST

Yours faithfully,

Dr. Gordon Nguka (PhD)
Chairman, Institutional Ethics Review Committee

Copy to:
- The Secretary, National Bio-Ethics Committee
- Vice Chancellor
- DVC (PR&I)

Appendix VII: County Government of Uasin-Gishu Approval



COUNTY GOVERNMENT OF UASIN GISHU
(Directorate of Veterinary Services)

Email: uasingishucdvs1@gmail.com
Tel: (053) 2063861/0722255854
When replying please quote:

County Director Veterinary Services
Uasin Gishu County
P. O. Box 2779 - 30100
ELDORET.

REF: CDVS/UG/TRAINING/VOL.I/16 Date: 6th April ,2021

David A Ounah
P.O, Private Bag
0065 KANGEMI
NAIROBI – KENYA

RE: PERMISSION TO UNDERTAKE RESEARCH ON MASTITIS IN UASIN-GISHU COUNTY.

Reference to your letter dated 31/03/2021 on above matter with support of evidence from NACOSTI Ref no 245113 and MMUST Letter ref MMU/COR:403012VOL3(01) dated 12/01/2021, the department have no objection for you to carry out your research in the said Sub counties.

Since the county have a lot of interest in improving it dairy productivity, you will share the final finding of the research with the county department of Veterinary service so that can assist in management of the dairy sector.

Wish you all the best.

Do not hesitate to contact this office for any assistance.

COUNTY DIRECTOR OF VETERINARY SERVICES
UASIN GISHU COUNTY
P. O. Box 2779-30100,
ELDORET

Dr. Philip Biamah
County Director of Veterinary Services
UASIN GISHU COUNTY

Cc
SCVO-Kapseret ,Moiben and Soy

Appendix VIII: NACOSTI Approval of Research

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR
SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 245113

Date of Issue: 19/March/2021

RESEARCH LICENSE

This is to Certify that Mr.. David Ayah Ounah of Jomo Kenyatta University of Agriculture and Technology, has been licensed to conduct research in Uasin-Gishu on the topic: Determinants of management practices of bovine mastitis and antibiotics residues in milk from dairy farms in Molben and Kapseret Sub-Counties, Uasin-Gishu Kenya for the period ending : 19/March/2022.

License No: **NACOSTI/P/21/9459**

245113

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR
SCIENCE,TECHNOLOGY &
INNOVATION

Verification QR Code

OTE: This is a computer generated License. To verify the authenticity of this document,
Scan the QR Code using QR scanner application.

Appendix IX: List of ATCC Standard Organisms Used In The Study

REPUBLIC OF KENYA

MINISTRY OF AGRICULTURE LIVESTOCK
FISHERIES AND COOPERATIVES
STATE DEPARTMENT FOR LIVESTOCK
DIRECTORATE OF VETERINARY SERVICES

DVS LABORATORIES
CONTROLLED COPY

Document Title	**Culture Worksheet**
Document Number	LTPS/ NVL/FM/B/002
Version	01
Effective Date	1/10/2021
Laboratory	Bacteriology Laboratory

Lab No: QC	**Received by**	**Date/time Received& analyzed**	**Tested by:**	**Date finalized**	**Checked by**

	Sample type	**Culture media**	**Colony characteristics**	**Gram stain**
1.	ATCC 25922	BA	Medium grey NH colonies	Gram –ve rods
		MAC	Medium Lactose fermentor colonies	
2.	ATCC 21853	BA	Large grey shiny colonies	Gram –ve rods
		MAC	No growth	
3.	ATCC 29213	BA	Small cream NH colonies	Gram +ve cocci in clusters
		MAC	No growth	
4.	ATCC 70063	BA	Medium grey raised NH colonies	Gram –ve rods
		MAC	Medium Lactose fermentor colonies	

Appendix X: Antimicrobial Interpretation Chart

Table showing the antimicrobial interpretive chart displaying different sizes of minimum inhibitory zones (MIZs) of antimicrobial spectra against microbial pathogens of mastitis**.**

S/NO	ANTIMICROBIAL AGENT	ANTIMICROBIAL CODE	ANTIMICROBIAL CONC. in mcg	*CLSI SUSCEPTIBILITY DIAMENTERS IN MM		
				Resistant	Intermediate	Sensitive
1.	Ampicillin	AMP	25	≤20	21-28	≥29
2.	Tetracycline	TE	25	≤14	15-18	≥19
3.	Cotrimoxazole	COT	25	≤10	11-15	≥16
4.	Streptomycin	S	10	≤11	12-14	≥15
5.	Kanamycin	K	30	≤13	14-17	≥18
6.	Gentamycin	GEN	10	≤12	-	≥13
7.	Sulphamethoxazole	SX	200	≤12	13-16	≥17
8.	Penicillin	P	30	≤12	13-17	≥18

*CLSI Performance Standards for disc diffusion susceptibility testing KGL 2/4

Source: Adapted from Himedia Laboratories Pvt. Ltd.

Appendix XI: Microbiological Screening of Antimicrobial Drug Residues in Raw Milk Using Modified Delvotest® Method

PROTOCOL FOR DETECTION OF ANTIMICROBIAL RESIDUE IN RAW MILK USING MODIFIED DELVOTEST®

THE PRINCIPLE OF THE TEST

Modified Delvotest® is a qualitative broad-spectrum test for the detection of Antimicrobial drug residues in raw milk. The test is based on growth inhibition of *Escherichia coli* (ATCC 25922)

Living microorganisms produce waste (excretory) products which result in pH changes of the environment in which they are in. The pH change in indicated by colour change of bromocresol blue (pH indicator).

Presence of antimicrobial agents will kill the *Escherichia coli* (ATCC 25922) hence no change in pH.

Escherichia coli (ATCC 25922), produces an acid and causes bromocresol blue/purple to turn yellow. The presence of an Antimicrobial drug residues prevents acid formation and a purple or blue color is observed. The minimum concentration of antimicrobials detected is .004 to .005 unit/ml. The test is AOAC approved at .007 unit/ml.

METHOD: Modified DelvoTest

REQUIREMENTS:

- Culture Broth
- Reference organism *Escherichia coli* (ATCC 25922)
- Bromocresol blue indicator
- Milk sample
- Droppers
- Sterile Test tubes
- Vortex mixer
- Incubator
- Sterile wire loop
- Culture media plates (Blood agar and MacConkey)

PROCEDURE

1. Using a sterile wire loop, pick a pellet of *Escherichia coli* (ATCC 25922) and streak on culture media plate (preferably Blood agar and MacConkey plates).
2. Incubate at 37^{o} c for 18-24 hours.
3. Using a sterile wire loop, pick pure colony of the organism and inoculate in a broth.
4. Incubate at 37^{o}c for 18-24 hours.
5. Using a test tube and sterile droppers, Mix equal volumes (2ml) of the inoculum and milk sample.
6. Add 2-3 drops of bromocresol blue indicator.
7. Mix well using vortex mixer.
8. Incubate at 37^{o} c for up to 4 hours
9. Note the colour change at intervals of 1 hour.

RESULTS:

Purple/blue = Positive

Yellow = Negative

References.

Antimicrobial drug residue standard operating procedures (2021), Regional Veterinary Investigation Laboratory, Kericho.

Joneserin G. M. and Seymour H. (1985). Cowside Antimicrobial drug residue Testing. *J Dairy Sci* 71:1691-1699 1691

Manafi M., Hesari J., Rafat Seyed Abbas (2011). Monitoring of Antimicrobial drug residue in raw and pasteurised milk In East Azerbaijan Of Iran By Delvotest Method. Journal of food research (university of tabriz) fall 2010-winter 2011, volume 20/3 , number 2; page(s) 125 to 131.

Mohamed Abdelrahman Mohamed[1,&], Ahmed Ali Sheikh Elmi[1], Abdirahman Bare Dubad[1], Yasin Hussein Sheikh Hassan[1], Abdirahman Mohamed Osman[2], Asinamai Athliamai Bitrus (2020). Antibiotic residue in raw milk collected from dairy farms and markets in Benadir, Somalia. *PAMJ-One Health. 2020;2:19.* [doi. 10. 11604/pamj-oh.2020.2.19.248 14]

Appendix XII: Quantitative Determination of Antimicrobial Drug Residue in Raw Milk Using HPLC MS/MS Procedure

Samples Processing.

Prepare the samples by thoroughly homogenizing.

Reagents and Kits

1) Acetonitrile: HPLC grade
2) Isooctane: HPLC grade
3) Methanol HPLC grade
4) QuEChERS Extraction kit
5) QuEChERS Dispersive (DSPE) Kit

Auxiliary equipment

1) Nitrogen concentrator.
2) Blender.
3) Vortex Mixer (3000rpm).
4) Gases of 99.99% purity; Helium, Argon, Nitrogen and Methane.
5) Centrifuge 5000 rpm.
6) 2ml amber coloured and clear glass autosampler vials, caps, teflon-lined septa.
7) 50ml peak-bottomed centrifuge tubes.
8) Stainless steel kitchen grater.
9) Analytical balance—Capable of measuring to 0.1 mg.
10) 15ml glass measuring cylinder.
11) Water bath 25^{o}C – 100^{o}C.
12) 10ml test tubes.
13) Micropipette (100-1000ul).
14) Syringe filters: 13mm, 0.2um.
15) 5ml syringes.
16) 10ml, 20ml and 25ml volumetric flasks.

Veterinary Drugs Standards

1) Obtained custom standard solution mixture with each chemical at 100μg/ml concentrations stored at – 20^{o}C in freezer.
2) Allow the custom standard solution to thaw to room temperature (20^{o}C – 25^{o}C).

3) Prepare stock standard mixture of 1µg/ml per chemical by diluting to mark 200µl of custom standard of 20ml volumetric flask using acetonitrile.
4) Transfer the prepared stock standard solution into an amber coloured vial with a teflon lined screw cap.
5) Store the samples at – 20±5^{o}C if the extraction does not begin immediately.

Sample extraction and cleanup.

1) Weigh 15 g milk sample in a 50 ml centrifuge tube.
2) Add 15ml solution of 1% acetic acid in acetonitrile to each tube.
3) Cap and shake (vortex) for 1 minute.
4) Add 4g of sodium sulphate,1g of sodium chloride (Bond Elute extraction salt).
5) Shake vigorously for 1 min and centrifuge at 5000rpm for 5 min at 4^{0}C.
6) Transfer a 6ml aliquot of upper acetonitrile layer into 15 ml PTFE centrifuge tube containing 50mg of PSA and 150mg C18CE and 900mg of anhydrous Na_2SO_4 (Bond Elute dispersive SPE>vet drugs in foods P/N5982-4950).
7) Cap and vortex for 1 minute then centrifuge at 500 rpm for 5min.
8) Transfer 1ml extract into another tube and dry by N_2 flow at 40^{0}C.
9) Redissolve residues into 1ml of MeOH/H_2O (1/9, V/V).
10) Filter the residue through a 0.45µm membrane into a 2ml autosampler glass vial for LCMSMS analysis.

LC-MS/MS analysis

By operating Agilent 1200 LCMSMS equipment using equipment's working instructions ref:----attached annex ----, subject the above extracts consecutively with calibration standards, quality control and matrix matched samples to qualitative and quantitative analysis as follows.

1) Arrange the vials in the autosampler and develop the batch sequentially starting with S0 for blanks followed by S1 - S5 calibration standards, QC1 quality control sample, test samples, QC2 quality control sample, and finally calibration standards S1 - S5.
2) Load the analysis method as outline in table 4 below.

Table 4: Agilent 1200 LCMSMS parameters for the analysis (qualitative and quantitative) of antibiotics and anthelmintic Veterinary Drugs chemical groups.

Instrument	**Agilent LC-MS/MS(1200)**

Software	MassHunter solution
Sample introduction	Agilent
Injection port temperature	250^0C
Column	Zobrax Eclipse XDB-C18,size 100 x 2.1mm
Column temperature	40^0C
Run time	25 mins
Mobile Phase flow rate	0.300ml/min.
Injection volume	10 µl
LC-MS/MS (Agilent 6460) parameters	
Mode	ESI Positive mode
Gas temperature	300^0C
Gas Flow	7 l/min
Nebulizer	45psi
Sheath gas temperature	350^0C
Sheath gas flow	10 l/min

Calculation

1) The weighted linear regression of the calibration curve use for each targeted chemical should be greater or equal to 0.95 ($r^2 \geq 0.95$) for it to be use for the analysis.
2) Calculate the concentration of each chemical targeted from responses obtained from the calibration curve.

Reference

AOAC: International Guidelines for Laboratories Performing Microbiological and Chemical Analysis of Food, Dietary Supplements, and Pharmaceuticals.

AOAC Official 2007.01: Method Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate [4]

ISO/IEC 17025:2017: General requirements for the competence of testing and calibration laboratories.

SANTE 11945/2015: Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed

Chowdhury Suchayan, Mohammad Mahmudul Hassan, Mahabub Alam, Sarmina Sattar, Md. Saiful Bari, A. K. M. Saifuddin, and Md. Ahasanul Hoque (2015). Antibiotic residues in milk and eggs of commercial and local farms at Chittagong, Bangladesh. *Vet World*. 2015 Apr; 8(4): 467–471.Published online 2015 Apr 10. doi: 10.14202/vetworld.2015.467-471. PMCID: PMC4774793. PMID: 27047116

Appendix XIII: Isolation and Identification of Bacterial Pathogens Causing Mastitis

Isolation and identification of bacterial pathogens causing mastitis on dairy farms

The results below demonstrate sampling design used to achieve the study, bacterial culture, isolation and identification strategies. Sampling was done at the interval of 21 days; on day 21 - 216 cultures were performed and only one bacterial pathogen was isolated and identified (*Staphylococcus epidermidis*); on day 42, 215 cultures were processed and three bacterial isolates were identified while the highest isolates were identified on day 294 and day 303 where 16 and 15 bacteria were isolated and identified respectively. A total of 76 gram-positive bacteria were identified as compared to 28 gram-negative (Table 4.5/7.1).

Table 7.1: Showing isolation of bacterial pathogens causing mastitis on dairy farms

Bacterial isolate	Follow-up visits in days (number of cultures)															Total
	21(216)	42(215)	63(212)	84(209)	105(205)	126(202)	147(195)	168(192)	189(184)	210(179)	31(171)	252(162)	273 (158)	294 (143)	303 (127)	n= 2770
Gram positive																
S. epi	1	3	0	0	1	5	0	4	1	6	8	2	4	5	6	46
S. aur	0	0	0	2	0	1	0	1	3	1	0	0	3	5	4	20
Str. Sp	0	0	1	0	0	0	0	1	0	0	0	1	0	2	0	5
Mic sp	0	0	1	0	0	0	0	0	0	0	1	0	2	1	0	5
Total	1	3	2	2	1	6	0	6	4	7	9	3	9	13	10	76
Gram negative																
E. coli	0	0	0	1	0	1	1	2	0	1	0	0	3	0	2	11
Cit.. fr	0	0	0	0	0	0	2	0	0	0	0	0	0	1	3	6
S. mar	0	0	0	1	0	0	0	0	1	0	0	0	2	0	0	4
Pr. vu.	0	0	0	0	2	0	0	0	0	0	0	0	0	1	0	3
Ps. Ae	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	2
Kl. Pn	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	2
Total	0	0	1	2	2	1	3	2	1	1	0	1	6	3	5	28
Grand Total(%)	**1 (0.9)**	**3 (2.9)**	**3 (2.9)**	**4 (3.8)**	**3 (2.9)**	**7 (6.7)**	**3 (2.9)**	**8 (7.7)**	**5 (4.8)**	**8 (7.7)**	**9 (8.7)**	**4 (3.8)**	**15 (14.4)**	**16 (15.4)**	**15 (14.4)**	**104(48.2)**

Key: *S. epi – Staphylococcus epidermidis; S. aur – Staphylococcus aureus; Str. Sp – Streptococcus species; Mic. Sp – Micrococcus species; E. coli – Escherichia coli; Cit. Fr – Citrobacter freundii; S. mar – Serratia marcescens; Pr. vu. – Proteus vulgaris; Ps. ae – Pseudomonas aeruginosa; Kl. pn – Klebsiella pneumoniae* **Calendar Days** – Day 21 – January; Day 42 – February; Day 63 – March; Day – March; Day 105 – April; Day 126 – May; Day 147 – May; Day 168 – June; Day 189 – July; Day 210 – July; Day 2 31 – August; Day 252 – September; Day 273 – October, Day 294 – October and Day 303 – October.

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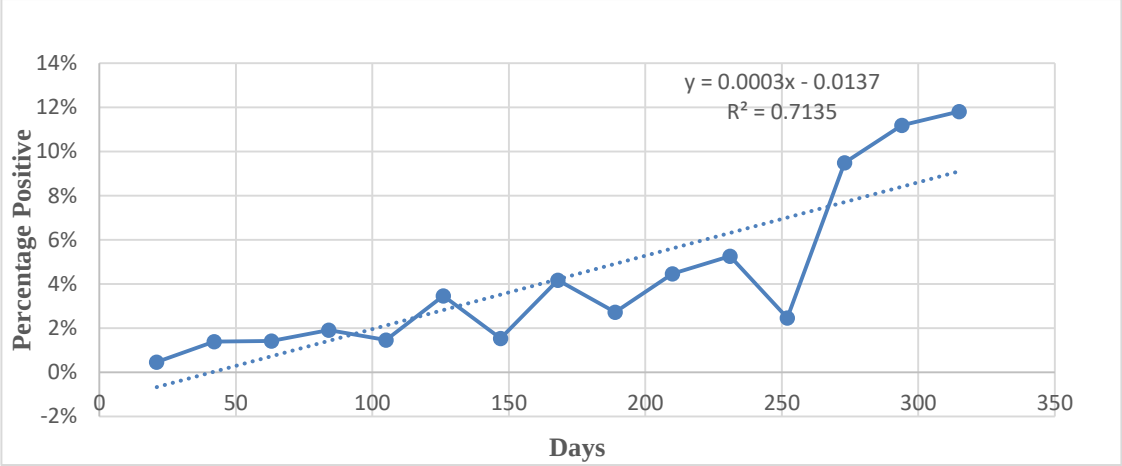
Appendix XIV: Trend Analysis of Isolated and Identified Bacterial Pathogens

Trend analysis of isolated and identified bacterial pathogens causing mastitis on dairy farms

The trend analysis depicted seasonality where there was an exponential increase in recovery of bacterial pathogens during long rain season from March – May and during July – November 2021 heavy rain down-pour period. This was statistically significant given the value of $R^2 = 0.7135$ (Figure 7.1).



Appendix XV: Gram Stain

Gram status of micro-organisms causing mastitis in the laboratory by way of gram staining.

The culture media plates of samples and ATCC controls that exhibited typical growth of micro-organisms after 18-24 hours and 18-72 hours of incubation at $37^{O}C$ were described morphologically and stained for gram characterization of microbial pathogens (Mbindyo *et al*., 2020; Mureithi & Njuguna, 2016) as follows:

Using a labeled grease free microscope glass slide; a drop of Normal saline was picked using a sterile standard wire loop and placed at the center third of the slide. A discrete colony from the culture media plate was picked using a sterilized wire loop then emulsified in the normal saline drop on the microscope glass slide to make a thin smear. The smear was heat-fixed onto the microscope glass slide by passing the slide through a Bunsen flame (just above the inner blue core of the flame) three times and let to dry. The smear on microscope glass slide was not to be overheated; over-heating the smear could have compromised its integrity. The fixed smear on slide was then placed onto the staining rack over a laboratory water sink then stained with brilliant crystal violet stain for 30 seconds. This was then gently rinsed with running tap water.

The smear was then flooded with gram's iodine for 30 seconds then gently rinsed with tap water. The slide lifted on one end and decolourized using acetone for about 5 seconds until no stain was coming out, then it was gently rinsed in running tap water. The smear was flooded again with the counterstain (1/10 dilute carbol Fuchsin or Safranin) for 30 seconds then gently rinsed in running tap water.

The smear on slide was allowed to air dry or dried using a blotting paper. The slide was then examined under the microscope, using X100 oil immersion objective.

Gram-positive results appeared purple, the colour of primary stain (brilliant crystal violet) while the Gram-negative micro-organisms took the colour of counterstain (1/10 carbol fuchsin) and appeared red (Figure 3.3).

Incase microbial colonies were non-lactose-fermenters on MacConkey agar and had poor growth with medium to large gram-positive colonies on blood agar but did not produce a typical gram-positive morphology (cocci/coccobacilli/rods), fungal infection was suspected. In this case, the colonies from primary Blood agar were sub-cultured on Sabouroud Dextrose Agar (SDA) and subjected to germ-tube test. *Candida alibicans* produced gram-positive twin budding cells.

Gram-stained *Staphylococcus* species appeared in clusters when observed under x100 oil objective power of a microscope (Figure 3.3A) while *Streptococcus* species appeared in chains.

This technique was useful as it categorized the micro-organisms into two major significant groups; the Gram positive and Gram-negative micro-organisms. Each of these two categories were indicative of the type of biochemical tests to be performed.

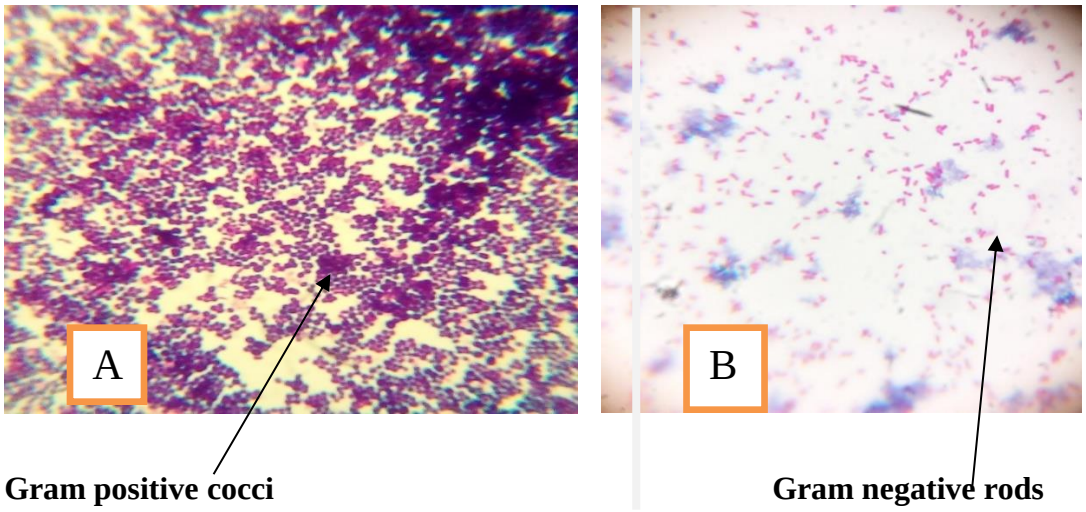


Figure 3.3: Showing gram reactions of microbial pathogens implicated in the etiology of bovine Mastitis using X100 oil immersion objectives of a microscope (A) Gram positive cocci (purple colour) and (B) Gram negative rods (red colour)

Source: Adapted from RVIL Laboratories Eldoret, 2018